ORIGINAL SUBMISSION

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GRN 000597

August 24, 2015

Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Subject: GRAS Notification for *Bacillus coagulans* SNZ1969 spore preparation

Dear Sir/Madam:

Pursuant to proposed 21 CFR 170.36 (62 FR 18960; April 17, 1997), Sanzyme Limited, India, through Soni & Associates Inc. as its agent, hereby provides notice of a claim that the food ingredient *Bacillus coagulans* SNZ1969 spores preparation described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be Generally Recognized As Safe (GRAS), based on scientific procedures.

As required, please find enclosed three copies of the notification. If you have any questions or require additional information, please let me know by phone at 772-299-0746 or by email at msoni@soniassociates.net or sonim@bellsouth.net.

Sincerely,

(b) (6)

Madhu G. Soni, PhD, FACN, FATS
Agent for
Sanzyme Limited
INDIA

000002

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GRAS NOTIFICATION

I. Claim of GRAS Status

A. Claim of Exemption from the Requirement for Premarket Approval Requirements Pursuant to Proposed 21 CFR § 170.36(c)(1)

Sanzyme Limited (the notifier) has determined that *Bacillus coagulans* SNZ1969 spores preparation is Generally Recognized As Safe, consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures as described in the following sections, under the conditions of its intended use as a food ingredient. Therefore, the use of *B. coagulans* SNZ1969 spores preparation is exempt from the requirement of premarket approval.

Signed,

(b) (6)

Date August 24, 2015

Madhu G. Soni, Ph.D., FATS
Agent for
Sanzyme Limited
India

000003

RECEIVED
AUG 26 2015
OFFICE OF
FOOD ADDITIVE SAFETY

B. Name and Address of Notifier:

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C. Common or Usual Name of the Notified Substance:

The common name of the substance of this notification is *Bacillus coagulans* SNZ1969. The preparation contains spores.

D. Conditions of Intended Use in Food

Bacillus coagulans SNZ1969 spores preparation, is intended for use as a probiotic in the following food categories: 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 at a maximum level of approximately 2 x 10^9 colony forming units (CFU)/serving (reference amounts customarily consumed, 21CFR 101.12). *B. coagulans* SNZ1969 spores preparation is not proposed for uses in foods that are intended for infants and toddlers, such as infant formulas or foods formulated for babies or toddlers, as well as it is not intended for use in meat and poultry products that come under USDA jurisdictions. The intended use of *B. coagulans* SNZ1969 spores in the above mentioned food categories, is estimated to result in a maximum daily intake of 36.4 x 10^9 cfu/day.

E. Basis for GRAS Determination:

In accordance with 21 CFR 170.30, the intended use of *B. coagulans* SNZ1969 spores preparation has been determined to be Generally Recognized As Safe (GRAS) based on scientific procedures. The determination is supported by the opinion of the Expert Panel. A comprehensive search of the scientific literature was also utilized for this determination. There exists sufficient qualitative and quantitative scientific evidence, including human and animal data to determine safety-in-use for *B. coagulans* SNZ1969 spores preparation. Since 2011, *Bacillus coagulans* spore preparations has been the subject of two GRAS notifications (GRN 399; GRN 526) to FDA. In response to both these notices, FDA did not question the conclusions that the use of *Bacillus coagulans* spores preparation is GRAS under the conditions of use described in

these notices. The safety determination of *B. coagulans* SNZ1969 spores preparation is based on the totality of available evidence.

There are different strains of *Bacillus coagulans* that are used as probiotic to improve and maintain ecological balance of the intestinal microflora. In the published literature several experimental studies, including subchronic toxicity, chronic toxicity, reproduction toxicity, *in vitro* and *in vivo* genotoxicity and human clinical trials, have appeared. All these studies support the safety in use of *B. coagulans* at the intended use levels. Sanzyme Limited has conducted a series of phenotypic and genotypic studies of *B. coagulans* SNZ1969 strain. These studies further support the non-toxigenic and non-pathogenic nature of this strain. On the basis of scientific procedures[1], Sanzyme Limited considers the consumption of *B. coagulans* SNZ1969 spores preparation, as a food ingredient, is safe.

F. Availability of Information:

The data and information that forms the basis for this GRAS determination will be provided to Food and Drug Administration upon request or will be available for FDA review and copying at reasonable times at the above mentioned offices of the notifier (Section I, B) or in the US by contacting one of the Expert Panel members: Madhu G. Soni, PhD, FATS, Soni & Associates Inc., 749 46th Square, Vero Beach, FL 32068; Telephone: +1-772-299-0746; Email: sonim@bellsouth.net

II. Detailed Information About the Identity of the Notified Substance:

Bacillus coagulans SNZ1969 spores preparation is a standardized dark grayish white powder (5 x 10^{10} spores/g - 50 billion spores/g). It is a member of a subgroup of *Bacillus* spp. and the current strain is derived from *B. coagulans* isolated from green malt in 1949 by a Japanese physician, Dr. Nakamura.

A. Common or Usual Name:

Bacillus coagulans SNZ1969.

B. Identity of Microorganism:

B. coagulans SNZ1969 is a rod-shaped, slightly acidophilic, gram-positive, catalase-positive, spore forming, thermotolerant, aerobic to microaerophilic, highly resilient bacteria. This particular strain originated in Japan. In 1949, a Japanese physician, Dr. Nakamura isolated *B. coagulans* from green malt. This particular isolate was tested for its potential effects against diarrhea and constipation in adult as well as infants during 1964. In 1972, at the request of Sankyo Corporation, the Japanese Ministry of Health and Welfare approved the use of this particular *B. coagulans* (designated as strain SANK 70258). Subsequently, in 1973, Sankyo Corporation (currently known as Daiichi Sankyo Co. Ltd) offered formulation and fermentation technology to Sanzyme Limited (earlier known as Uni-Sankyo Ltd). Since then, it is marketed in India under the brand name Sporlac[2] and has been designated as strain SNZ1969.

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[1] 21 CFR §170.3 Definitions. (h) Scientific procedures include those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance.

[2] It should be noted that *B. coagulans* SNZ1969 is marketed in India under the name Sporlac. However, in the US another company (Sabinsa Corporation) has registered their *Bacillus coagulans* strain under the trade mark Sporlac®

The phenotypic and genotypic characteristics of *B. coagulans* SNZ1969 have been established. *B. coagulans* SNZ1969 strain has been deposited with the Microbial Type Culture Collection (MTCC) - assigned number MTCC 5724 and with Belgian Coordinated Collections of Microorganism (BCCM™/LGM) with the assigned number LMG S - 27484. Additionally, the partial gene sequencing of this strain can be found at the National Center for Biotechnology Information database (Swamy and Soman, 2013) as well as at DNA Data Bank of Japan (DDBJ). The phylogenetic characterization based on 16S rRNA and as compared to other related species and designates has been studied. The findings from this analysis indicate that *B. coagulans* SNZ1969 is closely related (>99% similar) to *B. coagulans* ATCC 7050.

C. Specifications

Food grade specifications of *Bacillus coagulans* SNZ1969 preparation have been established by Sanzyme Limited and are summarized in Table 5. Analytical results from five non-consecutive lots (Appendix II) demonstrate that *B. coagulans* SNZ1969 is consistently manufactured to meet these specifications.

Table II.C.1. Specifications of *Bacillus coagulans* SNZ1969 preparation

Parameter	**Characteristics (Sanzyme, 2013)***
Appearance	Dark grayish white powder
Identity	A. Aerobic, gram positive thermostable spores identified by blue colored rods and red colored spores as seen under microscope, when stained by basic fuchsin solution B. Producing Lactic Acid
Loss on drying (105° for 1 hour)	NMT 5%
Lactic acid producing capacity	Not Less Than 10 ml of 0.05 M NaOH is consumed
Viable spore	NLT 5 x 10^{10} spores/g
Lactic acid producing capacity	NLT 10 ml of 0.05 N NaOH consumed
Heat Resistant Ratio (At 85°C)	Not Less Than 70%
Heavy metals	
Arsenic	NMT 3 ppm
Lead	NMT 3 ppm
Mercury	NMT 0.1 ppm
Cadmium	NMT 1 ppm
Microbiological assays	
Total bacterial counts (other organisms)	NMT 0.1 million cfu/g
Yeast and Mold	NMT 10 cfu/g
Escherichia coli	Negative/10 g
Salmonella	Negative/10 g
Pseudomonas aeruginosa	Negative/1 g
Staphylococcus aureus	Negative/1 g
Bacillus cereus	Negative/1 g
Lysteria monocytogens	Negative/25 g

*Based on information provided by Sanzyme; NMT = Not more than; NLT = Not less than.

D. Manufacturing process

B. coagulans SNZ1969 is manufactured (Figure II.E.1.) according to current good manufacturing practices (GMP) at a ISO:9001:2008 certified facility. The processing aids, fermentation medium

and diluents used in the manufacturing are either approved as food additives or are GRAS substances. The manufacturing of *B. coagulans* SNZ1969 involves the following steps: In the first step, seed media is prepared. For this, accurately weighed media ingredients such as peptone as a nitrogen source, dextrose as carbohydrate source, Corn Steep Liquor as vitamins and trace elements are transferred carefully in to a vessel that contains water and dissolved thoroughly. The pH of the medium is adjusted and the media is transferred to flasks and autoclaved. In the second step, seed inoculation is carried out aseptically from stock culture and the seed flasks are kept on an Orbital Shaker with constant shaking for minimum 20 - 24 hours. The growth of seed culture is checked under Phase contrast microscope. In the third step, the seed culture from the seed flasks was used to inoculate the sterilized fermenter media under aseptic conditions. The microorganism growth is observed under microscope frequently and once the sporulation is obtained and pH reached above 8.0 and no further change is observed, the spores are allowed to mature for 3 hours. The matured spore media is added to separator and centrifuged. In the final step, the separated liquid is transferred to a spray dryer receiver tank and dried under standardized conditions. The blending and collecting process is repeated to obtain the desired material. In order to obtain desired final concentration of spores, the powder is mixed with food grade maltodextrin or lactose.

E. Manufacturing process diagram

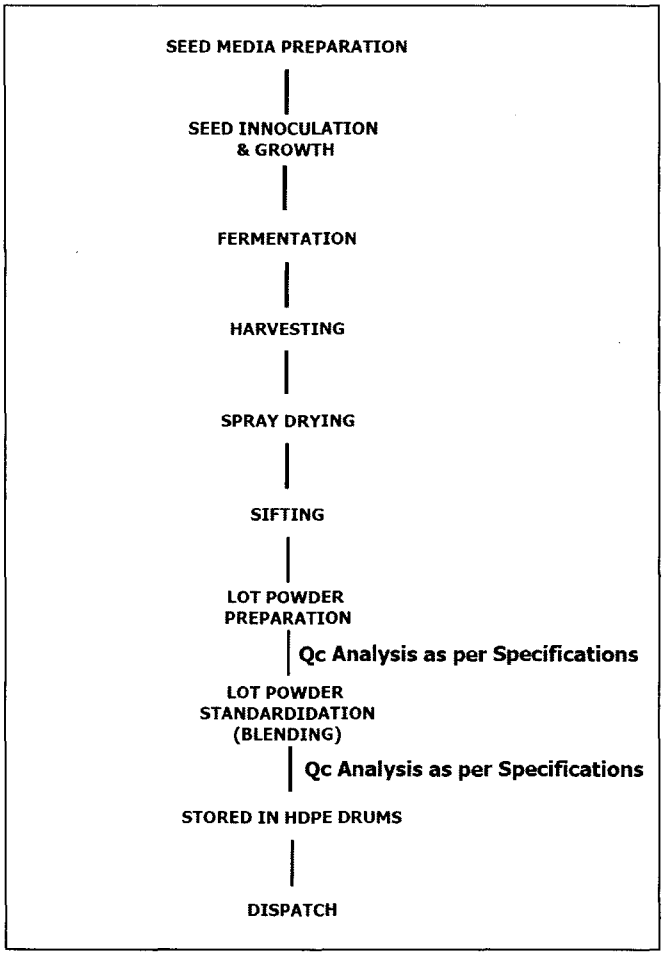

Figure II-E.1. Manufacturing process of *Bacillus coagulans* SNZ1969

III. Summary of the Basis for the Notifier's Determination that *Bacillus coagulans* SNZ1969 is GRAS

The determination that *B. cogulans* SNZ1969 spores preparation is GRAS is based on scientific procedures. A comprehensive search of the scientific literature for safety and toxicity information on *Bacillus cogulans* was conducted through June 2015[3] (updated from January 204) and was utilized for this assessment. Based on a critical evaluation of the pertinent data and information summarized here and employing scientific procedures, it is determined that the addition of *B. cogulans* SNZ1969 to the selected foods described in this notice and at a maximum use level of approximately 2 x 10^9 colony forming units (cfu)/serving (in accordance with established reference amounts customarily consumed, 21 CFR 101.12) meeting the specification cited above and manufactured according to current Good Manufacturing Practice, is GRAS under the conditions of intended use as specified herein.

The updated database search since the Expert Panel review revealed a randomized, double-blind, placebo-controlled, parallel group clinical trial on efficacy and safety of association of Simethicone and *B. coagulans* (Colinox®) in patients with irritable bowel syndrome (IBS) (Urgesi et al., 2014)[4]. In this study, adult subjects (n=52; 18 male and 34 female) suffering from IBS were enrolled, and bloating, discomfort, abdominal pain were assessed as primary end point. Subjects (aged 18-75 years) received the active treatment (n=26) or placebo (n=26) 3 times a day after each meal for 4 weeks of study period. All adverse events reported by patients at week 2 and four visits were recorded. A significant reduction of the bloating, discomfort and pain in Colinox® group compared to placebo group. No serious adverse effects were recorded in both groups. The investigators concluded that the results demonstrate the efficacy and safety of a combination of Simethicone and *B. coagulans* in treatment of IBS. Additionally, as indicated earlier, recently FDA received another GRAS notice for the use of *B. coagulans* as a food ingredient. The notifier concluded that *B. coagulans* Unique IS2 spore preparation is GRAS (GRN 526), through scientific procedures, for use as an ingredient in a variety of food categories (excluding meat, poultry, and infant formula products) at a maximum level of 2 x 10^9 cfu/serving (Unique, 2014)[5]. Following its review of the GRAS notice and other information available to FDA, the agency issued a "no questions" letter (FDA, 2015)[6].

In coming to this decision that *B. cogulans* SNZ1969 spores preparation is GRAS, Sanzyme Limited relied upon the conclusions that *B. cogulans* SNZ1969 does not pose any toxicological hazards or safety concerns at the intended use levels, as well as on published toxicology studies

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[3] The updated database searches performed subsequent to the Expert Panel review of the *B. coagulans* SNZ1969 GRAS assessment in January 2014 did not reveal any significant findings that will affect the panel conclusion. A few studies that were related to the safety are summarized.

[4] Urgesi, R., Casale, C., Pistelli, R., Rapaccini, G.L., de Vitis, I., 2014. A randomized double-blind placebo-controlled clinical trial on efficacy and safety of association of simethicone and *Bacillus coagulans* (Colinox®) in patients with irritable bowel syndrome. Eur. Rev. Med. Pharmacol. Sci. 18(9):1344-1353.

[5] Unique Biotech, 2014. Notice to US Food and Drug Administration that *Bacillus coagulans* strain Unique IS2 spores preparation is Generally Recognized as Safe for use in Foods. Available at: http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm443472

[6] FDA, 2015. *Bacillus coagulans* strain Unique IS2 spores preparation. Agency Response Letter GRAS Notice No. GRN 000526. Available at: http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm443472.

and other articles relating to the safety of the product. Other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion.

IV. Basis for a Conclusion that *Bacillus coagulans* SNZ1969 is GRAS for its Intended Use

An independent panel of recognized experts, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened by Sanzyme in January 2014 to determine the safety of *B. cogulans* SNZ1969 spores preparation used as a food ingredient. The Expert Panel consisted of the following individuals: Robert L. Martin, Ph.D. (Retired FDA Deputy Director); Professor Douglas L. Archer, Ph.D. (University of Florida; microbiology expert); and Madhusudan G. Soni, PhD, FACN, FATS (Food Ingredient Safety Consultant).

Based on a critical evaluation of the pertinent data and information summarized in the attached Panel statement, the Expert Panel members have individually and collectively determined by scientific procedures that the addition of *B. cogulans* SNZ1969 spore preparation to 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 at a maximum use level of approximately 2 x 10^9 colony forming units (cfu)/serving (reference amounts customarily consumed, 21CFR 101.12) when not otherwise precluded by a Standard of Identity as described here and resulting in the estimated daily intake of 36.4 x 10^9 cfu *B. coagulans* spores/day is GRAS. It is also their opinion that other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion. The published studies and information appeared subsequent to the Panel review and GRAS assessment did not reveal any new safety concerns.

Since 2011, *Bacillus coagulans* spores preparation has been the subject of two GRAS notifications (GRN 526 and GRN 399) to the FDA for its uses as a food ingredient. Both these notices received a "no questions" letter from the agency. The safety information and other relevant information described in these GRAS notices are hereby incorporated by reference into this document and were considered in evaluating the GRAS status of Sanzyme Limited's intended use of *Bacillus coagulans* SNZ1969 spores preparation. A synopsis of the pertinent information related to the safety of *Bacillus coagulans* is presented below (see attached Expert Panel Statement).

EXPERT PANEL STATEMENT

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF *BACILLUS COAGULANS* SNZ1969 FOR USE IN FOOD

Prepared for:

SANZYME LTD
Plot No. 13, Sagar Society
Banjara Hills Road No. 2
Hyderabad - 500 034, AP
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Prepared by:

Soni & Associates Inc.
749 46th Square
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CONFIDENTIAL

Panel Members

Douglas Archer, Ph.D.
Robert L. Martin, Ph.D.
Madhusudan G. Soni, Ph.D., F.A.T.S.

January, 2014

EXPERT PANEL STATEMENT

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF *Bacillus coagulans* SNZ1969 FOR USE IN FOOD

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DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF *Bacillus coagulans* SNZ1969 FOR USE IN FOOD

1. INTRODUCTION

The undersigned, an independent panel of recognized experts (hereinafter referred to as the Expert Panel)[1], qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened by Soni & Associates Inc., at the request of Sanzyme Limited, India (Sanzyme), to determine the Generally Recognized As Safe (GRAS) status of *Bacillus coagulans* SNZ1969 spores 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 at a maximum use level of approximately 2 x 10^9 colony forming units (cfu)/serving. A comprehensive search of the scientific literature for safety and toxicity information on *Bacillus coagulans* SNZ1969 was conducted through December 2013 and made available to the Expert Panel. The Expert Panel independently and critically evaluated materials submitted by Sanzyme and other information deemed appropriate or necessary. Following an independent, critical evaluation, the Expert Panel conferred on February 4, 2014 and unanimously agreed to the decision described herein.

1.1. Background

Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit to the host (Araya et al., 2002). *Bacillus* species have been used as probiotics for at least 50 years with the Italian product known as Enterogermina™ registered during 1958 in Italy as an over-the-counter (OTC) supplement. The scientific interest in *Bacillus* species as probiotics though, has only occurred in the last 20 years and several reviews have covered the field (Mazza, 1994; Sanders et al., 2003; Hong et al., 2005; Cutting, 2011). *Bacillus* species are spore forming bacteria that are widely distributed in nature. In recent years, bacterial spores are gaining significant attention on account of their stability in high acid and high temperature environments. Spores have a number of advantages over non-spore formers such as *Lactobacillus* species, namely, that the product can be stored at room temperature in a desiccated form without any deleterious effect on viability. A second advantage is that the spore is capable of surviving the low pH of the gastric barrier (Barbosa et al., 2005; Spinosa et al., 2000) which is not the case for all species of *Lactobacillus* (Tuohy et al., 2007), so in principle a specified dose of spores can be stored indefinitely without refrigeration and the entire dose of ingested bacteria can reach the small intestine intact.

Among the *Bacillus* species, *Bacillus coagulans* is a lactic-acid producing bacteria with typical characteristics of both *Lactobacillus* and *Bacillus* genera. *B. coagulans* was originally isolated and described in 1932 by Horowitz and Wlassowa and named *Lactobacillus sporogenes*

[1]Modeled after that described in section 201(s) of the Federal Food, Drug, and Cosmetic Act, As Amended. See also attachments (curriculum vitae) documenting the expertise of the Panel members.

(Bergey et al., 1939). In 1957, this microorganism was reclassified in Bergey's Manual of Determinative Bacteriology based on its biochemical properties, and the current correct nomenclature is *Bacillus coagulans.* Among different probiotcis, this microorganism is unique as it possesses a protective, spore-like protein coating (endospores) that allows it to survive the acidic conditions in stomach, reach the small intestine, germinate, and multiply. Unlike more labile lactobacilli, the spore forming ability of *B. coagulans* and resistance to high heat and acidic conditions, also allows spores to survive industrial manufacturing and ensures long term viability (Sanders et al., 2001). *B. coagulans* is also an economically important species that is frequently involved in the production of high concentrations of optically pure lactic acid, coagulin, and other thermostable enzymes (Su et al., 2012). For over four decades, *B. coagulans* has been marketed as a probiotic to maintain the ecological balance of the intestinal microflora and normal gut function. Given the potential benefits of *B. coagulans*, Sanzyme intends to market a specific strain of *B. coagulans* SNZ1969 for use as a food ingredient in selected foods as described in this dossier.

1.2. Identity and Description

1.2.1. Name and Source of GRAS Organism

The specific bacterial strain which is the subject of this Generally Recognized As Safe (GRAS) assessment is *Bacillus coagulans* SNZ1969. It is a member of a subgroup of *Bacillus* spp. and is isolated as a spore-forming bacterium from green malt.

1.2.2. Description of GRAS Organism

General descriptive parameters and properties of the *B. coagulans* SNZ1969 preparations manufactured by Sanzyme are summarized in Table 1. *B. coagulans* SNZ1969 is a unique strain of spore forming *Bacillus* species. It is a gram-positive, catalase-positive, spore forming, rod-shaped, slightly acidophilic, thermotolerant, aerobic to microaerophilic, highly resilient bacteria. *B. coagulans* strain (SNZ1969), the subject of present GRAS determination, has been deposited with the Microbial Type Culture Collection (MTCC) - assigned number MTCC 5724 and with Belgian Coordinated Collections of Microorganism (BCCM™/LGM) with the assigned number LMG S - 27484. Additionally, the partial gene sequencing of this strain can be found at the National Center for Biotechnology Information database (Swamy and Soman, 2013) as well as at DNA Data Bank of Japan (DDBJ). The spores of *B. coagulans* can withstand temperatures in excess of 100°C, while the vegetative cells can grow at temperatures as high as 65°C. *B. coagulans* is a highly resilient bacteria commonly found in the soil, air and dust. It can grow in a highly alkaline environment and the spores can also withstand the acidic environment of the stomach. The hierarchical classification of *B. coagulans* SNZ1969 is presented in Table 2.

Table 1. General Descriptive Characteristics of *Bacillus coagulans* SNZ1969

Parameter	Description *
Organism	*Bacillus coagulans* SNZ1969
Origin	Isolated from green malt
Physical characteristics	A dark grayish white powder
Taste	Slightly sweet in taste
Odor	Characteristic
Shelf life	36 months

*Based on information provided by Sanzyme

Table 2. Classification of *Bacillus coagulans* SNZ1969

Bacteria
Endospore-Forming Bacteria
Gram-Positive Endospore-Forming Bacteria
Firmicutes
Gram-Positive Endospore-Forming Rods
Bacillaceae
Bacillus
Bacillus coagulans
Bacillus coagulans SNZ1969

1.2.3. Identification and Characterization

B. coagulans is an annotated microorganism that has been well characterized. This strain originated in Japan. In 1949, a Japanese physician, Dr. Nakamura isolated *B. coagulans* from green malt. This particular isolate was tested for its potential effects against diarrhea and constipation in adult as well as infants during 1964. In 1972, at the request of Sankyo Corporation, the Japanese Ministry of Health and Welfare approved the use of this particular *B. coagulans* (designated as strain SANK 70258). Subsequently, in 1973, Sankyo Corporation (currently known as Daiichi Sankyo Co. Ltd) offered formulation and fermentation technology to Sanzyme (earlier known as Uni-Sankyo Ltd). Since then, it is marketed in India under the brand name Sporlac[2] and has been designated as strain SNZ1969. The phenotypic characteristics of *B. coagulans* SNZ1969 are summarized in Table 3.

Table 3. Phenotypic Characteristics of *Bacillus coagulans* strains SNZ1969

Test	*B. coagulans* SNZ1969
Gram staining	+
Catalase	+
Gelatin hydrolysis	-
Nitrate reduction	-
Oxidase	+
Lactose	-
Xylose	-
Maltose	+
Fructose	+
Dextrose	+
Galactose	w
Raffinose	-
Sucrose	-
L-Arabinose	-
Inulin	w
Sorbitol	-
Mannitol	-
Rhamnose	w

*Based on information provided by Sanzyme; + = 90% or more of strains are positive, - = 90% or more of strains are negative, w = weak positive reaction

[2] It should be noted that *B. coagulans* SNZ1969 is marketed in India under the name Sporlac. However, in the US Sabinsa Corporation has registered their *Bacillus coagulans* strain under the trade mark Sporlac®.

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

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

Characteristics	*Bacillus* sp.	*Bacillus coagulans*	*Lactobacillus* sp.	Sporolacto bacillus
Catalase	+	+	-	-
Benzidine	+	NA	-	-
Nitrate red	+	NA	-	-
Gram-reaction	+	+	+	+
Endospores	+	+	-	+
Motility	+	+	-[1]	+
Lactic acid	-[2]	+	+	+
m-A2-PM[3]	+	+	-[1]	+
Fatty acid	Bacillus type	Lactobacillus type	Bacillus type	

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

1.2.4. Genotypic Identification

In an attempt to further characterize *B. coagulans* SNZ1969, genotypic identification was carried out. The DNA Data Bank of Japan accession number for the 16S rRNA gene sequence of *B.coagulans* is KC146407. The phylogenetic characterization based on 16S rRNA and as compared to other related species and designates was studied (Swamy and Soman, 2013). To assign strains to bacterial species for each isolate the almost entire 16S rRNA gene was amplified. The 1491 bp amplicon was then sequenced using Sanger sequencing method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication. The partial sequence is presented in Appendix I.

1.2.4.1. Genomic DNA isolation and quality assessment

For genomic identification, *B. coagulans* strain SNZ1969 (referred for this analysis as BCUSS) and ATCC 7050[3] cultures were grown on were grown on Sterile PNY medium (HiMedia) for 24 hours at 37°C in a temperature controlled incubator. The cells were then resuspended in 0.9% saline and pelleted for further processing and isolation of genomic DNA. For nucleic acid isolation, STE buffer (0.1 M Tris-HCl, 0.1 M NaCl and 1 mM EDTA pH 8.5), Lysozyme and Proteinase K (10 µg/ml) were used. This method employed NaCl and SDS lysis

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

followed by phenol: chloroform: iso-amyl alcohol purification of nucleic acids. The DNA was then deproteinised thrice with Tris-saturated phenol (Phenol: $CHCl_3$: iso-amyl alcohol, 50:48:2), and then with $CHCl_3$: isoamyl alcohol (24:1). DNA was then precipitated with 2% sodium acetate and absolute ethanol. Dried DNA was dissolved in nuclease free water. Quality assessment of genomic DNA was performed by 1% agarose gel electrophoresis as well as DNA was quantified using QubitTM Fluorometer (Invitrogen, USA) for measurement of DNA concentration. Genomic DNA isolated from the cultures was high quality and was used for additional studies.

1.2.4.2. rRNA gene PCR and phylogenetic analysis

The phylogenetic characterization based on 16S rRNA and as compared to other related species and designates was studied. Polymerase chain reaction based amplification 16S rRNA gene was carried out using Microbial Identification kit. The kit is comprised of two different primer sets targeting 16S rDNA from bacteria. Two overlapping fragments are generated that spans across more than 1300 bases of the 16S rRNA gene of bacteria. Amplification was carried out in a Gene Amp PCR System (Applied Biosystems, USA). The amplified DNA fragments of approximately 780 bp and 890 BP separated on a 2% agarose gel and purified by using PCR purification Kit. Sequencing products were precipitated, cleaned and loaded onto an automatic DNA Sequencer (ABI Prism Model 3130, Applied Biosystems, California, USA) for sequence analysis using Sequencing Analysis software version 5.2. The amplification for both the sample was confirmed by agarose gel electrophoresis on 2% gel. The PCR products were purified using PCR purification kit to remove unused dNTPs and primers. The purified PCR products were again checked by gel electrophoresis and then used for DNA sequencing. The DNA sequence was trimmed and edited during quality assessment and then used for phylogenetic analysis. The finding from this analysis is presented in Figure 1. The findings from this analysis indicate that *B. coagulans* SNZ1969 (BCUSS) is closely related to *B. coagulans* ATCC 7050.

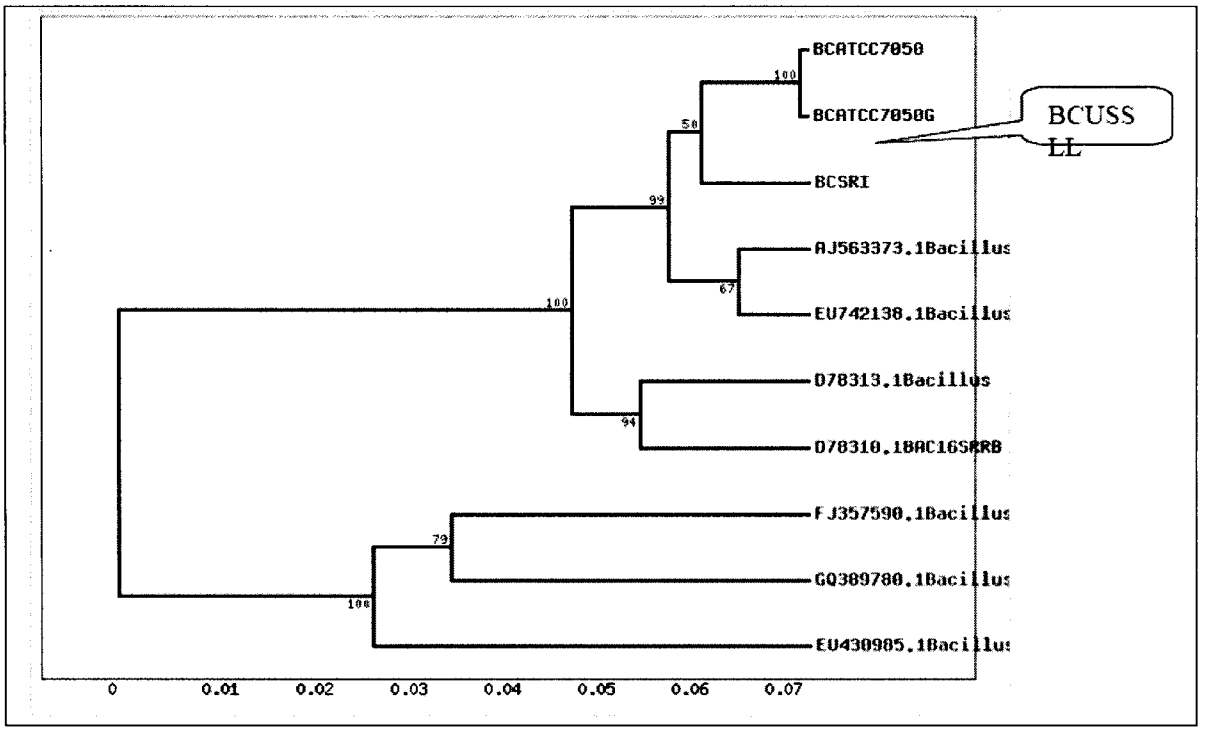

Figure 1. The phylogenetic tree of *B. coagulans* SNZ1969 (BCUSS) with the different *Bacillus* reference sequences.

The numbers at the node represent the percent bootstrap support for 1000 replicates. Bars at the base of the tree show genetic divergence. Description of Phylogeny: BCATCC7050: Bacillus coagulans ATCC 7050 strain reference sequence, BCATCC7050G: B coagulans strain ATCC 7050 sequence generated at our lab, BCUSS: Bacillus coagulans USS sequence generated at our lab, J563373.1Bacillus coagulans reference sequence, D78313.1Bacillus coagulans strain JCM2257 reference sequence, EU742138.1Bacillus coagulans strain SKU 12 reference sequence, D78310.1BAC16SRRB Bacillus badius strain ATCC14574 reference sequence, FJ357590.1Bacillus ginsengihumi strain BBN1R2-01 reference sequence, GQ389780.1Bacillus acidicola strain TSAS-1 reference sequence, EU430985.1Bacillus oleronius isolate 2 reference sequence. Note: All reference sequences used from NCBI database. Distance Matrix for these strains is presented below:

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

000018

1.3. Manufacturing Process

B. coagulans SNZ1969 is manufactured according to current good manufacturing practices (GMP), as summarized in Figure 2. The manufacturing procedure assures a consistent

and high-quality product that meets the specifications (Table 5). The processing aids, fermentation medium and diluents used in the manufacturing of *B. coagulans* SNZ1969 are either approved as food additives or are GRAS substances. The manufacturing facility is ISO:9001:2008 certified. The manufacturing of *B. coagulans* SNZ1969 involves the following steps:

1.3.1. Seed Media Preparation

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

1.3.2. Seed Inoculation and Culture Growth

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

1.3.3. Sterlization, Fermentation and Separation

The sterilization of the media in the fermenter is done at temperature 121°C for 45-60 min. Steam is used to attain this temperature. Initially steam is circulated in the jacket of the fermenter till the temperature of the media in the fermenter will reach up to 110°C, during this step steam doesn't come in contact with the media. After attaining media temperature to 110°C, steam will be injected to the media through the sparger line to attain the media temperature 121°C. During this step, steam will come in contact with the media. After reaching the fermenter media temperature it is maintained for 60 minutes. Following this, the fermenter temperature is allowed to cool to 37±1°C by circulating chilled water generated by a Chiller. Following completion of sterilization, the steam inlet valve is closed. The seed culture that was grown in the seed flasks on shakers was inoculated. After complete transfer of inoculums in to fermenter, the inoculation tank bottom valve is closed. The steam is passed through inoculation tank for 20 minutes. The pressure is maintained at 1.2±0.1 Kg/cm^2 and temperature 37±1°C. The air flow is maintained at 350-400 LPM, agitation kept at 125-150 RPM and agitation RPM is set based on pH and growth pattern. Every hour Batch Manufacturing Record reading is noted. The organism growth is observed under microscope every hour. Once the sporulation is obtained and pH is maintained with no further change. Chilled water is circulated through jacket and the temperature is brought down below 28°C. The matured spore media is added to separator and centrifuged at 7500 rpm until completed.

1.3.4. Drying, Shifting, Blending, Packaging

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

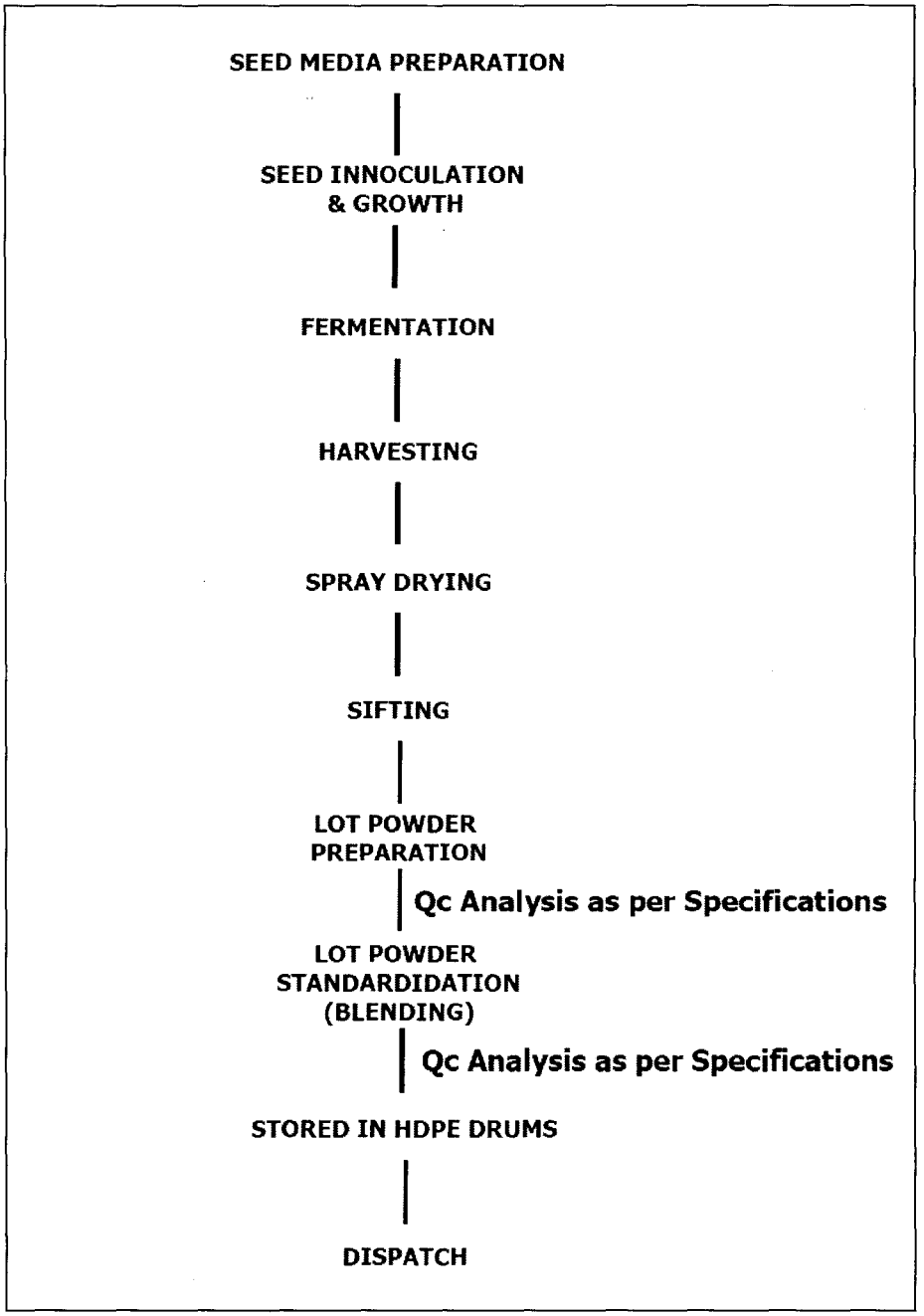

Figure 2. Manufacturing process of *Bacillus coagulans* SNZ1969 (Sanzyme, 2013)

1.4. Specifications

Food grade specifications of *Bacillus coagulans* SNZ1969 preparation have been established by Sanzyme and are summarized in Table 5. Analytical results from five non-consecutive lots (Appendix II) demonstrate that *B. coagulans* SNZ1969 is consistently manufactured to meet these specifications. The *B. coagulans* SNZ1969 strain used in this determination was found, through 16s rDNA Analysis to be over 99% similar to *B. coagulans* type strain ATCC 7050.

Table 5. Specifications of *Bacillus coagulans* SNZ1969 preparation

Parameter	**Characteristics (Sanzyme, 2013)***
Appearance	Dark grayish white powder
Identity	A. Aerobic, gram positive thermostable spores identified by blue colored rods and red colored spores as seen under microscope, when stained by basic fuchsin solution B. Producing Lactic Acid
Loss on drying (105° for 1 hour)	NMT 5%
Lactic acid producing capacity	Not Less Than 10 ml of 0.05 M NaOH is consumed
Viable spore	NLT 5 x 10^{10} spores/g
Lactic acid producing capacity	NLT 10 ml of 0.05 N NaOH consumed
Heat Resistant Ratio (At 85°C)	Not Less Than 70%
Heavy metals	
Arsenic	NMT 3 ppm
Lead	NMT 3 ppm
Mercury	NMT 0.1 ppm
Cadmium	NMT 1 ppm
Microbiological assays	
Total bacterial counts (other organisms)	NMT 0.1 million cfu/g
Yeast and Mold	NMT 10 cfu/g
Escherichia coli	Negative/10 g
Salmonella	Negative/10 g
Pseudomonas aeruginosa	Negative/1 g
Staphylococcus aureus	Negative/1 g
Bacillus cereus	Negative/1 g
Lysteria monocytogens	Negative/25 g

*Based on information provided by Sanzyme; NMT = Not more than; NLT = Not less than.

1.5. Current Uses and Regulatory Approvals

Spore-forming bacteria, such as *B. coagulans and B. subtilis,* are used as dietary supplement probiotics for human consumption (Sanders et al., 2003; Hong et al., 2008). At present, across the world, *B. coagulans* has been sold as a dietary supplement under different names such as GanedenBC3°, Nature's Plus, Fresh Start Bolus, Pit-Stop, Tarm-X Balans™, GutFlora (VSL-3), Sprolac®, Sanvita, Ampilac, Bactlyte, etc. Additionally, it is also marketed as a constituent with several other products. These formulations contain *B. coagulans* alone or in combination with lactobacilli or bifidobacteria, minerals, vitamins (particularly B complex), and prebiotics. The recommended dose of *B. coagulans* ranges from 3.6 x 10^8 – 1.5 x 10^9 cfu/capsule, two or three times *per* day for a healthy adult. Catanzaro and Green (1997) suggested a standard dose of *B. coagulans* at levels of 1.5 x 10^9 cfu once or twice per day.

In European countries, the European Food Safety Authority granted Qualified Presumption of Safety (QPS) status for *B. coagulans* since 2008 (EFSA, 2012). In Japan, the Japanese Ministry of Health and Welfare has approved the use of *B. coagulans* product (Lacbon) for improvement in symptoms caused by abnormalities in the intestinal flora or in dysbiosis (Majeed and Prakash, 1998). In Japan, Sankyo Corporation marketed the *B. coagulans* (SANK 70258) product under trade name Lacbon. In India, *B. coagulans* is approved and has been marketed for the past four decades under the brand name Sporlac[4]. In 1969, Uni Sankyo Ltd (now known as Sanzyme Ltd) was incorporated as an Indo Japanese joint venture in collaboration with Sankyo Co. In 1973, Uni-Sankyo Ltd received the *B. coagulans* (SANK 70258) strain from Sankyo along with its manufacturing technology. In the past three years (2011-2013), Sanzyme has manufactured 13,78,986 kg of *B. coagulans* SNZ1969 (5×10^9 spores/g) and has marketed it in various countries, including India, European countries, USA, Korea, Indonesia, etc., without reports of any significant adverse reports.

In the USA, FDA (2001) has approved *B. coagulans* for use in the preparation of enzymes used for food production. As per 21 CFR 184.1372, *B. coagulans* (a nonpathogenic and nontoxicogenic microorganism) is recognized as GRAS in the production of insoluble glucose isomerase enzyme. Additionally, FDA's Center for Veterinary Medicine has approved use of *B. coagulans* as GRAS for veterinary purposes. Similar, Health Canada has permitted the use of *B. coagulans* in the production of glucose isomerase enzyme. In August 2011, FDA received a GRAS notice (GRN 399) for the use of *B. coagulans* strain in conventional foods (Ganedan, 2011). In this notification, *B. coagulans* was proposed for addition to a wide variety of foods at levels up to approximately 2×10^9 cfu/serving and the acceptable daily intake (ADI) was determined as 93.8×10^9 cfu/person/day. Following its review, on July 31, 2012, FDA issued a "no questions" letter for GRN 399 (FDA, 2012).

The ATCC has classified *B. coagulans* as Biosafety Level 1, indicting that this bacteria is not known to cause disease in healthy humans. In Nigeria, *B. coagulans* has been used as part of the fermenting process for the production of a protein-rich food known as ugba that is commonly consumed (Isu and Njoku, 1997).

1.6. Intended Uses and Food Categories

Sanzyme intends to use a *B. coagulans* SNZ1969 preparation as a food ingredient, in multiple food categories. The intended use of *B. coagulans* SNZ1969 preparation by Sanzyme is in the same foods and at the same levels (2×10^9 cfu/serving) to those described in the GRN 399 (Ganeden, 2011). There are no new food uses proposed for *B. coagulans* SNZ1969. The intended uses are as a food ingredient in foods such as 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 (excluding meat and poultry products).

[4] As mentioned earlier, *B. coagulans* SNZ1969 is marketed in India under the name Sporlac. In the US, Sabinsa Corporation has registered their *Bacillus coagulans* strain under the trade mark Sporlac®.

The application of *B. coagulans* SNZ1969 to the same foods and at the same levels (2 x 10^9 cfu/serving) as those described in GRN 399 is unlikely to affect the intake of *B. coagulans* SNZ1969 in the diet of the public from introduction into the market by another supplier who will have to compete in essentially the same market and foods. In determining the estimated daily intake, Ganeden (2011) used the assumption that males aged 51 and older consume the largest number of servings of food a day at 18.2 servings/day. Using this estimate of the number of servings/day at a level of 2 x 10^9 cfu/serving, Ganeden estimated the daily intake of *B. coagulans* spores at 36.4 x 10^9 cfu/day. The US FDA did not question the proposed use levels and the resulting intake.

1.7. Traditional Uses

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

Historically, various lactic acid producing bacteria have been consumed in the diet. For the past several decades, the role of lactic acid bacteria has been extensively studied in the intestinal microecology. These bacteria play an important role in maintaining the healthy digestive tract (Adams, 1999; Soomro et al., 2002; Ouwehand et al., 2004). One such bacteria that has a long history of use for its potential health benefits is *B. coagulans*. In a review article, Sanders et al. (2003) reported that among the 77 recognized *Bacillus* species, *B. coagulans* has been evaluated for probiotic functionality and sold worldwide for both human and animal uses. For human health, *B. coagulans* is considered as beneficial bacteria (Catanzaro and Green, 1997). *B. coagulans* was first isolated in 1932 (Horowitz-Wlassowa and Nowotelnow, 1932) and has been used in the production of food products. In a series of investigations from Portugal between 1958 and 1959, the potential gastrointestinal benefits of *B. coagulans* and other spore-forming bacteria have been studied (Guida et al., 1958; Guida and Guida, 1959). These articles were published in Portuguese and are cited in databases such as PubMed. The available information indicate that *B. coagulans* has been in use for over 50 years.

1.7.1. Uses in Africa

The dietary consumption of fermented foods has a long history in Africa (Okonko et al., 2006). In the Ibo ethnic group of Nigeria, *ugba* is a popular protein-rich solid, flavorful alkaline food, among other fermented foods. *B. coagulans* is one of the species identified in the preparation of *ugba*. It is produced by fermentation of African oil bean with *B. coagulans*. Consumption of *ugba* is known to result in the intake of *B. coagulans* and its spores (Isu and Njoku, 1997). Onofiok et al. (1996) reported that a large proportion of the population (76%) has been reported to consume *ugba* as a snack. The presence of bacillus cells in *ugba* supports the intake of *B. coagulans*. The level of bacteria (*B. coagulans*) present in the *ugba* indicates that

consumption of *B. coagulans* is greater than 1 x 10^9 cfu/day. This provides support for the traditional use and consumption of *B. coagulans*.

2. SAFETY RELATED DATA

B. coagulans has been found to be susceptible to a majority of the commonly used antibiotics. The acute and long-term effects of *B. coagulans* have been investigated in a number of pre-clinical studies. In a majority of the animal studies, efficacy of *B. coagulans* has been studied. In addition to efficacy, relevant safety endpoints were also included in some of these studies. For the present GRAS assessment, these studies are reviewed as part of the safety evaluation. In addition to animal studies, in the published literature several human studies have appeared in which safety and efficacy of *B. coagulans* has been investigated. The assessment of efficacy studies is limited to a review of the results related to safety and tolerability. In a majority of the studies the form of *B. coagulans* used was not clear. However, it is likely in these studies *B. coagulans* in endospore form has been used.

2.1. Antibiotic Susceptibility

In two separate experiments, antibiotic sensitivity of *B. coagulans* SNZ1969 was investigated (Ashtekar, 2012) using the agar disk diffusion method developed by Charteris et al. (1998). In addition to SNZ1969, sensitivity to *B. coaguans* ATCC 7050 culture was also studied. In the first experiment, the antibiotic resistance of *B. coagulans* was tested according to protocols of the Clinical and Laboratory Standards Institute (CLSI) for 20 antibiotics. The zone of inhibition around antibiotic discs was measured and the sensitivity level was determined using CLSI for antimicrobial disc susceptibility. The findings from these studies are summarized in Table 6. The results of these investigations suggest that *B. coagulans* SNZ1969 varies from ATCC 7050 in some antibiotic sensitivity tests. *B. coagulans* SNZ1969 was sensitive to Ciprofloxacin and with intermediate response to Cefaclor and Trimethoprim.

Table 6. Antibiotics Resistance Pattern for *B. coagulans* strains SNZ1969 and ATCC 7050

Antibiotic Disk	Concentration (µg)	ATCC7050	SNZ1969
Cefaclor	30	S	R
Chloramphenicol	30	S	S
Cefoxitin	30	R	R
Clindamycin	2	I	I
Ciprofloxacin	5	I	S
Colistin (Methane Sulphonate)	10	R	R
Doxycyclin Hydrocloride	30	S	S
Erythromycin	15	I	I
Gentamicin	10	I	I
Kanamycin	30	I	I
Nalidixic Acid	30	I	I
Pencilin-G	10 units	S	S
Polymyxin-B	300 units	I	I
Rifampicin	5	S	S
Streptomycin	10	S	S
Tetracycline	30	S	S

Table 6. Antibiotics Resistance Pattern for *B. coagulans* strains SNZ1969 and ATCC 7050

Antibiotic Disk	Concentration (µg)	ATCC7050	SNZ1969
Bacitracin	8 units	S	S
Trimethoprim	5	S	I
Novobiocin	5	R	R
Metronidazole	4	R	R

S: Sensitive; **R:** Resistant and **I:** Intermediate

In addition to the above described antibiotic sensitivity using CLSI standards, Hexa Discs method was used to determine antibiotic sensitivity (Ashtekar, 2012) as per agar disk diffusion method of Charteris et al. (1998). In these experiments, both *B. cogulans* strains SNZ1969 and ATCC 7050 cultures were tested against 46 antibiotics and the zone of inhibition around antibiotic discs was measured. The findings from these experiments are presented in Table 7. The results of these investigations show that *B. coagulans* SNZ1969 is sensitive to majority of the antibiotics except, Metranidaloze and Cefuroxine.

Table 7. Antibiotics Resistance Pattern for *B. coagulans* strains SNZ1969 and ATCC 7050 using HexaDiscs

Antibiotic Disk	Concen. (µg)	ATCC7050	SNZ1969	Antibiotic Disk	Concen. (µg)	ATCC7050	SNZ1969
Penicillin G	10	LZNR	17	Cefotaxine	30	LZNR	15
Oxacillin	1	LZNR	14	Chloromphenicol	20	19	20
Cephalothin	30	LZNR	LZNR	Streptomycin	25	15	13
Clindamycin	2	18	LZNR	Sufatriad	30	15	14
Erythromycin	15	14	15	Methicillin	5	LZNR	LZNR
Amoxyclav	30	13	15	Fusic acid	30	LZNR	LZNR
Co-Trimoxazole	25	15	15	Cefepime	30	LZNR	LZNR
Vancomycin	10	14	12	Augmentin	30	LZNR	LZNR
Ampicillin	10	10	10	Benzyl penicillin	2	LZNR	LZNR
Sulbactam	10	LZNR	LZNR	Imipenem	10	LZNR	LZNR
Ofloxacin	5	15	LZNR	Metronidazole	5	R	R
Teicoplanin	30	LZNR	LZNR	Ceftriaxone	30	LZNR	13
Ceftazidin	30	R	18	Levofloxacin	5	LZNR	LZNR
Gentamycin	50	12	10	Fosfomycin	200	LZNR	LZNR
Cefoxitin	30	LZNR	LZNR	Nitrofurantoin	10	12	12
Piperaciilin	100	LZNR	16	Norfloxacin	10	LZNR	11
Linezolid	30	LNZR	LNZR	Doxycycline	30	LZNR	LZNR
Ciprofloxacin	5	16	LNZR	Rifampicin	5	LZNR	LZNR
Tetracycline	30	23	25	Doxycycline hydrochloride	30	LZNR	LZNR
Cloxacillin	1	17	15	Penicillin	10	LZNR	20
Lincomycin	2	LZNR	LZNR	Amikacin	30	12	14
Cefuroxine	30	LZNR	R	Netillin	30	13	12

Zone mentioned in mm; R: Resistant; LZNR = Large zone not readable

In a published study, Moldenhauer et al. (1996) reported the antimicrobial resistance potentials of three *Bacillus* species, including *B. coagulans* to thirty antimicrobial agents. In this study, Trypticase Soy Agar (TSA) plates were swabbed with *B coagulans* ATCC 51232 spores,

and then individual discs were impregnated with an antimicrobial agent dispensed onto the surface to determine a zone of inhibition of the growth of *B. coagulans* by the antibiotic. *B. coagulans* was found to be susceptible to 28 antibiotics tested in this study. The results of this study suggest that *B. coagulans* is susceptible to commonly used antibiotics.

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

2.2. Antimicrobial Action

In an *in vitro* study, antimicrobial potentials of *B. coagulans* SNZ1969 against some pathogenic bacteria such as *Salmonella abony, Streptococcus faecalis* and *Escherichia coli* were investigated (Ashtekar, 2012). *B. coagulans* SNZ1969 did not inhibit any of these pathogenic strains, indicating that SNZ1969 cultures do not produce detectable levels of inhibitory substances against the test cultures. In another *in vitro* study, antimicrobial action of *B. coagulans* SNZ1969 was investigated against some of the gastrointestinal resident microorganisms such as *Lactobacillus panis*, *L. fermentum* and *L. plantarum*. In this study, none of the test cultures were inhibited, indicating that *B. coagulans* SNZ1969 cultures do not produce detectable levels of inhibitory substances against the test cultures. Similar results were noted with *B. coagulans* ATCC 7050.

The available information indicates that *B. coagulans* does not produce antibiotics. FDA has listed the use of *B. coagulans* in the production of glucose isomerase enzyme. In its list of enzyme preparations used in food, FDA has stated that, "Insoluble glucose isomerase enzyme preparations are derived from recognized species of precisely classified, nonpathogenic, and nontoxicogenic microorganisms, including *Streptomyces rubiginosus*, *Actinoplane missouriensis*, *Streptomyces olivaceus*, *Streptomyces olivochromogenes* and *Bacillus coagulans* grown in a pure culture fermentation that produces no antibiotic."[5]

2.3. Bile Salt and Acid Tolerance

The tolerance capacity of *B. coagulans* SNZ1969 in varying concentrations of bile salts was investigated (Ashtekar, 2012). High bile salt percentage solutions were prepared by suspending bile salts [Hi-Media CR 008] at 1, 2 and 3% in a sterile sodium chloride solution (0.5%). In order to check the tolerance of *B. coagulans* strains, turbidity (optical density) of the cultures was hourly monitored at 600 nm. The results revealed that at low salt concentration there was a decrease in the growth of *B. coagulans* SNZ1969 with time. At higher concentration of bile salts static growth condition is seen for first 2 hours and then there is decrease by the 3rd hour. *B. coagulans* SNZ1969 is initially tolerant to 3% bile salts but with passage of time, it appears to be sensitive.

For acid tolerance capacity, a preculture of *B. coagulans* SNZ1969 was prepared by inoculating one loop onto PNY medium slants (Himedia M835) and incubated at 37°C for 24

[5] Available at: http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/MicroorganismsMicrobialDerivedIngredients/default.htm

hours. Overnight cultures were taken and washed off with 3 x 2 ml 0.5% saline. These cells were suspended in 0.1 N HCl and incubated at 37°C for 3 hours. In order to check the tolerance of *B. coagulans* strain, viable count of the cultures were monitored for every 30 minutes up to 3 hours. The results of this experiment revealed that *B. coagulans* SNZ 1969 was quite stable in acidic conditions with loss of approximately 20% viability by 2 hours.

2.4. Virulence

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

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

In a mouse model, Donskey et al. (2001) studied the effect of oral administration of *B. coagulans* on the density of vancomycin-resistant enterococci (VRE) in fecal matter. In this study, male CF-1 mice were first administered subcutaneously clindamycin (1.4 mg/day) once daily for two days before and three days after gastric inoculation of one of three different strains of VRE (VanB-1, Van A and VanB-2; 1 x 10^8 cfu/mouse). The findings from this study revealed that four days of oral *B. coagulans* therapy resulted in a statistically significant reduction in the density of stool VRE in mice colonized with one VanB strain, but not in a second VanB strain or in a VanA strain. *B. coagulans* remained detectable in the stool of mice four days after completion of therapy. These results lend support to the concept that *B. coagulans* treatment may reduce the density of colonization with some strains of VRE. The findings from this study suggest that *B. coagulans* treatment reduces the colonization of some strains of VRE infection.

2.5. Human Studies

As described below, orally administered spores of *B. coagulans* pass through the stomach and upon reaching the intestine germinate and multiply rapidly in about four hours following the administration. For approximately seven days after discontinuation of its ingestion, *B. coagulans* spores were detected in feces. In a long term clinical trial, administration of a *B. coagulans* preparation to children at a daily dose level of 1 x 10^8 spores for 12 months did not result in adverse effects. In several other clinical studies, no adverse effects of *B. coagulans* were noted. The available information supports the safety-in-use of *B. coagulans* for humans.

2.5.1. General Studies

The available information indicates that following oral administration, *B. coagulans* passes through the stomach in spore form to the duodenum, where it germinates and multiplies rapidly (Losada and Olleros, 2002). Following oral ingestion, it takes about 4 hours for the spores to travel to the duodenum and start germination. Once germinated in the intestine, *B. coagulans* becomes metabolically active as part of the facultative anaerobes and produces lactic acid as a fermentation product. In the human intestinal tract, *B. coagulans* has been reported to reside temporarily. Following discontinuation of the administration, spores of *B. coagulans* are excreted slowly via the feces for approximately seven days (Majeed and Prakash, 1998). The available evidence indicates that *B. coagulans* improves gastrointestinal ecology by replenishing the quantity of desirable obligatory bacteria and antagonizing pathogenic microorganism (Anonymous, 2002).

In a randomized, placebo controlled, double-blind clinical trail, Kajimoto et al. (2005) investigated the effects *B. coagulans* SANK70258 in subjects with seasonal allergic rhinitis. In this study, 55 subjects (20-65 years) healthy men and women with a history of Japanese cedar pollinosis were randomized to receive either test food containing 4 x 10^8 viable *B. coagulans* SANK70258 cells (n=29) or placebo (n=26) for eight weeks. The subjects were monitored for improvements from allergy and for safety related parameters including, hematology (9 commonly measured parameters) and clinical chemistry (25 commonly analyzed parameters). Additionally, adverse effects such as gastrointestinal symptoms and skin symptoms were recorded. The physical examination, hematology and clinical chemistry parameters did not reveal any abnormal changes between the groups. Similarly, no adverse reactions were noted in association with the intake of the test food. The results of this study suggest that ingestion of *B. coagulans* SANK70258 at a daily dose level of 4 x 10^8 cfu for eight weeks is safe for human consumption. The *B. coagulans* strain used in this clinical trial is the original or mother strain from which the subject strain (SNZ1969) of present GRAS determination has been obtained.

In an open label clinical trial, Mohan et al. (1990a; 1990b) investigated the effects of *B. coagulans* on serum lipid levels. In this trial, administration of *B. coagulans* spores at a dose level of 3.6 x 10^8 cfu/day for 12 weeks to 17 patients with type II hyperlipidemia resulted in a significant decrease in the total cholesterol *vs.* HDL cholesterol ratios by 24%, while their LDL *vs.* HDL ratios decreased by 33%. During the *B. coagulans* treatment, total cholesterol to HDL-cholesterol and LDL-cholesterol to HDL-cholesterol ratios was improved. A marginal increase in HDL-cholesterol from 43.6 to 46.8 mg/dl ($p<0.05$) was noted. No changes in the serum triglyceride levels of the patients were noted. No adverse effects of *B. coagulans* treatment were noted. Although the source of *B. coagulans* tablets was not mentioned in this study, it is likely to be *B. coagulans* SNZ1969 marketed in India.

In another study in 20 healthy adults, Ara et al. (2002) evaluated the effects of *B. coagulans* SANK 70258 on intestinal flora and decomposition products in the intestine, as well as on various dermal characteristics. The subjects were monitored for six weeks: two weeks before administration, two weeks during administration of *B. coagulans* (1 x 10^8 cfu/day) and two weeks after treatment. At initiation of the study, stool samples were collected before administration, 14 days after the start of administration and 14 days after the end of administration. The specimens were analyzed for decomposition products, with the volunteers recording their defecation frequency and assessing their fecal characteristics throughout the examination period. Ingestion of *B. coagulans* at 1 x 10^8 cfu/day revealed improvement in the

fecal shape, change of fecal color from dark brown to yellowish brown, decrease of fecal odor, the fecal pH, and an increase in defecation frequency of persons whose frequency was relatively low. The number of intestinal bifidobacteria was found to be significantly increased, whereas the number of intestinal *C. perfringens* significantly decreased after administration compared with the values before the intake. The concentrations of intestinal ammonia, indole and p-cresol content decreased. The results of this study indicate that the administration of *B. coagulans* improves the intestinal environment, defecation frequency, fecal characteristics and dermal characteristics. The *B. coagulans* strain SANK 70258 used in these investigations is the mother strain of the subject of present GRAS determination.

Ara et al. (2002) also investigated the effects of *B. coagulans* in 23 female volunteers (20 to 40 years old), with a tendency for constipation, on dermal characteristics as a result of the changes in the intestinal environment. The total study period was 12 weeks, with four weeks before administration, four weeks of administration of *B. coagulans* SANK 70258 (1 x 10^8 cfu/day), and four weeks of placebo administration. The volunteers were asked to record defecation frequency and fecal characteristics (fecal shape, color and odor) and skin characteristics (number of comedones) dairy. The skin was analyzed every two weeks by counting the number of skin eruptions (flares and papules). *B. coagulans* administration resulted in a significantly greater stool defecation frequency as compared to before the intake. Following ingestion of ingestion of 1x10^8 *B. coagulans* cfu/day, 72% of the subjects that complained of constipation or diarrhea before intake reported significant improvements after the treatment. No adverse effects of *B. coagulans* treatement were noted. The *B. coagulans* strain SANK 70258 used in these studies is the mother strain of the subject of this GRAS determination.

In two separate studies, Iino et al. (1997a, 1997b) studied the effects of *B. coagulans* on intestinal microflora. In the first study in 28 adult healthy Japanese women, Iino et al. (1997a) investigated the effects of *B. coagulans* on stool color, stool shape, stool frequency, defecation feeling and stool odor. For this study, lactose containing 1 x 10^8 *B. coagulans* cells/g was prepared and divided into sachets. The subjects ingested one sachet per day for two weeks. Improvements in stool properties (color, shape), along with increase in defecation frequency, was noted. No adverse effects of *B. coagulans* were reported. In the second study, Iino et al. (1997b) studied the effects of *B. coagulans* on intestinal flora, decayed products and stool property. In this study, 18 healthy adult women were divided in three groups to receive 0.2 x 10^8, 1.0 x 10^8 and 2.0 x 10^8 cells of *B. coagulans* SANK 70258 strain per day for two weeks. As regards adverse effects, no subject complained of gas generation, diarrhea or continuous abdominal pain problems due to intake of *B. coagulans*. Based on the results of this study, the investigators suggested that consumption of *B. coagulans* improves the bacterial flora and improves the health. The *B. coagulans* strain SANK 70258 used in these studies is the mother strain of the subject of this GRAS determination.

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

1.1 x 10^5 spores in the feces, while on day eight no *B. coagulans* spores were noted in the in the feces. The results of this study suggest that *B. coagulans* is transiently maintained in the intestinal tract.

In a randomized, double-blind, placebo-controlled clinical trial, 55 volunteers (including patients with diarrhea-predominant irritable bowel syndrome- IBS-D) received either *B. coagulans* (GBI-30, 6086) (n=26; 7 male, 19 female) or placebo (n=29; 6 male, 23 female) once a day for 8 weeks (Dolin, 2009). The active study capsule contained 2 x 10^9 cfu of *B. coagulans* and patients were instructed to take one active capsule or identically appearing placebo capsules containing microcrystalline cellulose, per day for 8 weeks. Adverse events reported during the course of study were, for the most part, mild to moderate and were generally self limiting. Five patients who received *B. coagulans* reported six adverse effects and six patients receiving placebo reported six adverse effects. One severe adverse effect (headache) was reported in the placebo group. In general, *B. coagulans* was well tolerated. Large variability in baseline scores prevented the assessment of severity scores and quality of life. The results of this study provides evidence that *B. coagulans* (GBI-30, 6086) is safe and effective for reducing daily bowel movements in patients with IBS-D (Dolin, 2009).

Astegiano et al. (2006) evaluated the effect of a dietary mixture (IBS Active) containing L-tryptophan, inulin, angelica, vegetal charcoal, vitamin PP, group B vitamins (B1, B2, B6) and probiotics (*Bacillus coagulans, Lactobacillus acidophilus, Streptococcus thermophilus*) in subjects with irritable bowl syndrome (IBS). In this study, the treatment group of 37 patients (11 men and 27 women; mean age, 44.3±5.1 years) received IBS Active (440 mg bid) over a period of 5 to 8 months (average 6 months). The control group consisted of 28 patients (6 men and 22 women; mean age, 48.6±3.7 years). Subjects were evaluated for abdominal pain and/or distension, constipation, diarrhea and alternating constipation and diarrhea. As compared with baseline values, the treatment group revealed reduction of 62% in abdominal pain, 55% in abdominal distension, 58% in constipation, 33% in diarrhea, and 62% in alternation between constipation and diarrhea. No statistically significant reduction in symptoms was found in the control group as compared to baseline value. The treatment with IBS Active reduced the symptoms and the difference was statistically significant for abdominal pain, abdominal distension and constipation between the treatment and control group. The results of this study indicate that use of *B. coagulans* as a dietary mixture with other substances is a positive adjunct in the treatment of IBS. No adverse effects were reported.

In a randomized double-blind, placebo-controlled clinical trial, Kalman et al. (2006) investigated the effects of *B. coagulans* on gastrointestinal symptoms in adults with post-prandial intestinal gas-related symptoms (abdominal pain, distention, flatulence). In this study, 61 adults volunteers (age 36.5±12.6 years; weight 75.4±17.3 kg) were randomized to receive *B. coagulans* GBI-30, 6086 preparation (n=30) or placebo (n=31) for four weeks. The subjects in the treatment group received one capsule containing 2.0 × 10^9 cfu *B. coagulans*/day for four weeks. Subjects were evaluated every two weeks and during each visit, the participants were asked to fill a series of questionnaires, in addition to hemodynamics (standard biochemical safety) and adverse event monitoring. The details of the hemodynamic parameters were not mentioned in the publication. The investigators concluded that the *B. coagulans*-based product was effective in improving the quality of life and reducing gastrointestinal symptoms in adults with post prandial intestinal gas-related symptoms and no GI diagnoses.

2.5.2. Infection-related Studies

The available infection-related published clinical studies with *B. coagulans* are summarized in Table 8. Cui et al. (2004) investigated the effects of *B. coagulans* in subjects with acute and chronic diarrhea. In this randomized, double-blind trial, 204 subject were divided in two groups, 101 in the control group (51 with acute diarrhea and 50 with chronic diarrhea) and 103 in the treatment group (51 with acute diarrhea and 52 with chronic diarrhea). The control group was treated with tablets containing Golden Bifid (*Bifidobacterium longum*) at a dose of 1 x 10^8 cfu three times daily for 3-7 days (acute diarrhea) and 14-21 days(chronic diarrhea). The treatment group received *B. coagulans* at a dose of 1 x 10^8 cfu, three times daily for 3-7 days (acute diarrhea) and 14-21 days (chronic diarrhea). In either group, no adverse effects of the treatment were noted. In both groups, significant increase in the number of *Bifidobacterium* and *Lactobacillus* species in the gut was noted. The investigators concluded that the *B. coagulans* species is an effective agent in the treatment of acute and chronic diarrhea and that its efficacy and safety are similar to that of Golden Bifid tablets.

Table 8. Summary of Published Clinical Studies of *B. coagulans*

Reference	No. of subjects	Treatment	Condition	Findings
Kalman et al. (2009)	61 (30 and 31/group)	2.0×10^9 cfu/capsule daily for 4 weeks	Gastrointestinal symptoms	Significant improvements in GSRS abdominal pain subscore was noted. No adverse effects were reported.
Hun (2009)	44 (22/group)	0.8×10^9 cfu daily for eight weeks	IBS-Abdominal pain and bloating patients	Improvements from baseline in abdominal pain and bloating scores in the treatment group. May be a safe and effective
Mandel et al. (2010)	45 (23 and 22/group)	2×10^9 cfu daily for sixty days	Rheumatoid arthritis	Appeared to be a safe. There were no serious adverse reactions reported throughout this study.
Dutta et al. (2010)	148 (78 and 70/group)	0.24×10^9 cfu daily for 5 days	Diarrhea in children	No therapeutic impact on management of acute dehydrating diarrhea

In a review article on the influence of fructo-oligosaccharide and lactobacilli on intestinal health, Losada and Olleros (2002) noted that *B. coagulans* has demonstrated its utility and advantages in various studies and also exhibits a high degree of safety. Doron et al. (2008) reviewed the articles describing meta-analyses of clinical trials in which probiotics were used in the prevention of antibiotic associated diarrhea. These investigators reported that *B. coagulans* is one of the most effective strains and are generally safe. In yet another review article, Johnston et al. (2007) assessed the efficacy and adverse effects of probiotics for the prevention of antibiotic-associated diarrhea in children. For this assessment, 10 clinical trials with Lactobacilli spp., Bifidobacterium spp., Streptococcus spp., or *Saccharomyces boulardii* alone or in combination, *Lactobacillus* GG, *B. coagulans*, *Saccharomyces boulardii* at 0.5 x 10^{10} to 4 x 10^{10} cfu/day were considered. Based on this assessment the most promising probiotics were considered as *Lactobacillus* GG, *B. coagulans*, *Saccharomyces boulardii* at 5 to 40 billion cfu/day).

B. coagulans product under the brand name Lacbon (marketed in India as Sporlac) has received approval by the Ministry of Health and Welfare, Japan, for its uses in the improvement

of symptoms caused by abnormalities in the intestinal flora or by dysbiosis. At 19 independent health care institutions in Japan, clinical trials with Lacbon tablets have been conducted. In these trials, 567 subjects received *B. coagulans* at doses ranging from 0.5 x 10^8 to 7.5 x 10^8 cfu/day for a period of 2 to 20 days. The findings from these studies show that *B. coagulans* is effective in treating diarrhea due to acute or chronic gastroenteritis, mal-digestion, infantile diarrhea and constipation. No adverse effects were reported. The details of these investigations were not available for independent review. The results from some of these clinical trials with Lacbon are summarized in Table 9 (Losada and Olleros, 2002).

Table 9. Summary of results of different trials with Lacbon (*B. coagulans*)* (Adapted from Losada and Olleros, 2002)**

Condition	No. of subjects	Treatment	Findings
Acute and chronic intestinal catarrh	38	1x10^8 – 6 x 0^8 spores/day for 2-12 days	~87 recovery from diarrhea to regular normal stools
Diarrhea	15	0.75 x 10^8 – 6 x 10^8 spores/day for 3-12 days	100% recovery from diarrhea to regular normal stools from third to fourth day
Constipation	10	3 x 10^8 - 7.5 x 10^8 spores/day for 2-10 days	70% recovery to normal stools and disappearance of abdominal distention
Abnormal intestinal fermentation	9	1 x 10^8 – 6 x 10^8 spores/day for 3-14 days	Disappearance of vomiting and nausea in all subject; appetite improved; stools became regular and normal; diarrhea and stomach pain relieved
Dyspepsia infantum	26	1 x 10^8 – 2 x 10^8 spores/day for 2-12 days	86% response; general condition and nature of stool improved; frequency of stool decreased to half or less than that before treatment
Allergic skin disease	5	2 x 10^8 - 4.5 x 10^8 spores/day for 4-12 days	80% response; obvious eruptions of strophulus and eczema decreased from the third day (topical therapy employed concomitantly
Miscellaneous symptoms	10	0.2 x 10^8 - 0.5 x 10^8 spores/day for 4-20 days	80% response seen in anorexia of nervous type and malnutrition in infants

*The *B. coagulans* strains used in these studies is the mother strain of the subject of this GRAS determination. **Additional details of these studies were not available for independent review.

2.5.3. Studies in Infants and Children

Chandra (2002) investigated the effects of *B. coagulans* (strain not specified; as the study is conducted in India- the strain is likely to be SNZ 1969; locally available) on the incidence and severity of acute rotavirus diarrhea. In this prospective, double-blind, randomized trial, 112 newborn healthy term infants in rural India were treated daily with an oral dose of 1 x 10^8 spores *B. coagulans* (n = 55) or a placebo (n = 57) for 12 months. The details of source material provider were not mentioned in the article. Morbidity was monitored each week for 12 months. During the course of study, the children were monitored for the number of episodes of rotavirus diarrhea, the duration of each episode and the general health of the infant (number of days ill *per* year). *B. coagulans* administration to the infants significantly decreased the number of episodes of rotavirus diarrhea and the duration of each episode, with a significant decrease in the number

of day ill/year (13 days ill in the *B. coagulans* group as compared to 35 in the control). The investigators did not report any adverse effects related to the treatment of *B. coagulans* administration.

In another study, Labalestra et al. (2008) evaluated the effect of a combination of symethicone and *B. Coagulans* (Colinox) on the gastric emptying time (GET) and relief of symptoms in infants with symptomatic gastroesophageal reflux (GER). In this randomized, double-blind, placebo-controlled, cross-over trial, 19 consecutive children, younger than one year of age, (11 female, 8 male; mean age: 5.5 months) with symptomatic GER were given the combination as an oral solution as well as a placebo for seven days administered four times daily. The wash-out period was seven days. The final GET (min) was significantly shorter in the group that received the combination of symethicone and *B. coagulans* as compared to placebo group. Additionally, a stronger improvement of the GER symptoms was noted in the group receiving the combination treatment as compared to the placebo group. No adverse effects were reported.

In an article published in Italian language, La Rosa et al. (2003) investigated the effects of *B. coagulans* and fructo-oligosaccharides (prebiotic/probiotic) preparation in the prevention of diarrhea due to antibiotics in childhood. In this randomized, double-blind, placebo-controlled trial, a total of 120 children, with active infections requiring antibiotics, were divided in two groups (60/group). The children were treated orally with probiotic/prebiotic preparation or a placebo (without prebiotic/probiotic). Children in the treatment group received daily mixture containing *B. coagulans* (5.5×10^8 cfu) and fructo-oligosaccharide (250 mg). The control group (n=60) received the placebo. The patients' diary and follow-up clinical examinations were used to monitor the changes. Of the 98 evaluable subjects, 71% in the group receiving the prebiotic/probiotic treatment had no diarrhea versus 38% in the placebo group. The duration of diarrhea in the treatment group was significantly lower (0.7 days) as compared to the placebo group (1.6 days). The study authors concluded that prophylaxis with the prebiotic/probiotic treatment significantly reduced the number of days and duration of events in children with antibiotic-induced diarrhea. No adverse effects of treatment were reported.

In an efficacy study, Dhongade and Anjaneyulu (1977) investigated the effects *B. coagulans* in the treatment of neonatal diarrhea. In this study, a clinical trial published as an abstract, sixty infants with confirmed cases of neonatal diarrhea were treated with 1.5×10^7 *B. coagulans* spores/day (Sporlac). Of the 60 subjects treated with Sporlac (*B. coagulans*), 49 responded within two days period. Based on the suggested dosage level of Sporlac at 5 million spores/kg body weight, each neonate received approximately 1.5×10^7 spores/day. No adverse effects of the treatment were noted. Additional details of the study were not available.

2.6. Animal Studies

As described in some publicly available reports, Sankyo Company Limited (Sankyo), Japan, investigated the acute and long-term effects of *B. coagulans* preparations (Sankyo, 1968; cited in Losada and Olleros, 2002; Anonymous, 2002). The *B. coagulans* strain used in these studies is the mother strain of the subject of this GRAS determination. The details of these studies were not published. In an acute study, *B. coagulans* powder containing 5×10^9 cfu (spores)/g was administered via gavage to male mice at dose levels of 1, 3 or 5 g/kg bw and the animals were observed for 7 days. No mortality was noted. No adverse effects, such as diarrhea was noted following the treatment. In the group receiving the highest dose (5 g/kg bw), slight

distension of the stomach was noted in "a few mice." However, these animals recovered to normal after a few hours. These observations suggest that the LD_{50} of *B. coagulans* powder preparation is greater than 5 g/kg bw. In another acute toxicity study, administration of *B. coagulans* GBI-30, 6086 cell mass at a dose level of 5 g/kg bw (5.2×10^{11} cfu/kg bw) to Wistar rats did not result in mortality or adverse effects. The results of this study suggest that the LD_{50} of the cell mass containing *B. coagulans* is greater than 5 g/kg bw (Endres et al., 2009).

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

In an acute and short-term oral toxicity study, Sudha et al. (2011) studied the potential toxicity of *B. coagulans* Unique IS-2 (MTCC- 5260) strain in rats. In this study, Sprague Dawley rats were orally fed with a single dose of 3250 and 6500 mg/kg bw/day (5×10^9 spores/g in water) dose of test organism for acute and sub-acute toxicity studies, respectively. While the experimental rats received doses of 130, 650, 1300 mg/kg bw/day (5×10^9 spores/g) for 14 consecutive days, other control animals received only water. The follow up study was carried out for 28 days. The results of this toxicity assessment did not reveal any treatment related changes in clinical signs, body weight, and feed intake. Additional parameters such as urine, hematological examinations, clinical chemistry, gross pathology and histopathology also did not show any treatment related significant changes at both time intervals. Based on the results of this study, the investigators determined 1300 mg/kg bw dose (5×10^9 cfu/g) of *B. coagulans* Unique IS-2 as the NOAEL, when administered for 14 days.

In a safety assessment, Endres et al. (2009) investigated subchronic toxicity potentials of a strain of *B. coagulans*. In this study, *B. coagulans* (GanedenBC^{30}™) cell mass (1.36×10^{11} cfu/g) was orally administered (gavage) to Wistar Crl:(WI) BR rats (10/sex/group) at dose levels of 0, 100, 300 and 1000 mg/kg bw/day for 90 consecutive days. The animals receiving the highest dose level received a dose of 1.36×10^{11} cfu *B. coagulans*/kg bw/day. The study was performed as per OECD guidelines. There were no deaths and no treatment-related signs were observed throughout the 13-week treatment period in any of the groups. Appearance and behavior of the animals were similar for all groups in the study. The findings from this study did not reveal any toxicologically significant differences between the treated and the control groups with respect to feed consumption, water consumption, sensory reactivity, general and behavioral conditions, hematological and clinical chemistry evaluations. Similarly, at the end of the study, no treatment-related macroscopic or microscopic changes in the organs were noted. The test item was well tolerated. The investigators determined that the no-observed-effect-levels (NOAEL) for both males and females to be >1000 mg (1.36×10^{11} cfu)/kg bw/day, the highest dose tested (Endres et al., 2009).

In another one-year study, Endres et al. (2011) investigated the safety of long-term consumption of *B. coagulans* in rats. The study was conducted as a combined study to investigate chronic oral toxicity (as per OECD and FDA Redbook guidelines) along with one-generation reproduction toxicity. *B. coagulans* preparation was fed to Wistar rats (20/sex/group) in their diet at levels of 0, 10,000, 20,000 and 33,300 mg/kg feed, corresponding to a dose level of 0, 600, 1200 and 2000 mg/kg bw/day, respectively, for 52 to 53 weeks. No test-article related mortality was noted. Similarly, clinical observations did not reveal any toxic signs related to the test article. No *B. coagulans* preparation treatment-related changes in body weight, body weight gain, or feed consumption were noted during the study. Blood samples collected at the end of the 3rd week and at 3, 6 or 12 months did not reveal any toxicological relevant changes in hematological, clinical chemistry or urine parameters. Statistically significant changes noted were either not dose-related, or were well within the historical background range or not correlated with other hematological or histopathological changes. Similarly, at termination, macroscopic and microscopic examinations did not reveal lesions attributable to treatment. The NOAEL in male and female rats was determined as 1948 and 2525 mg/kg bw/day, respectively, the highest dose tested (Endres et al., 2011). The intake at 2000 mg/kg bw/day will be equivalent to approximately 2.6 x 10^{13} cfu/person/day spores.

In parallel to the above described chronic oral toxicity study, a one-generation reproduction toxicity study was conducted as per OECD and FDA Redbook guidelines (Endres et al., 2011). For these investigations and similar to the chronic study, Wistar rats were divided in to four groups (10/sex/group) and were fed a diet containing *B. coagulans* preparation at a dose levels of 0, 600, 1200 and 2000 mg/kg bw/day. For these investigations, 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 findings from this study did not reveal any signs of treatment-related toxicity on the F0 (parental) generation (male or female). The NOEL for the parental group (reproductive performance) male and female rats was determined as 2372 and 3558 mg/kg bw/day, respectively. The NOEL for the F1 offspring was determined as 3558 mg/kg bw/day.

Rangarajan et al. (2005) investigated the effects of selected bacteria, including *B. coagulans,* in ameliorating biochemical imbalance, in 5/6th nephrectomized Sprague Dawley rats as a chronic renal failure model. In this study, cohorts of 6 nephrectomized rats, after 2-weeks of nephrectomy stabilization, were fed a casein-based diet plus *B. coagulans* for 16 weeks. The daily dose of *B. coagulans* was 1 x 10^8 cfu/day. During the course of the study, blood urea nitrogen, urine creatinine, body weight, and bacterial counts (feces) were measured at regular intervals. The results were compared with both the control and placebo groups. Feeding of the diet containing *B. coagulans* for 16 weeks significantly prolonged the life span of uremic rats, in addition to showing a reduction in blood urea nitrogen levels. The findings from this study suggest that *B. coagulans* supplementation to uremic rats slows the progression of azotemia, which may correlate with prolonged life span of uremic rats.

In a study in chicken, Cavazzoni et al. (1998) investigated the effects of *B. coagulans* during the first seven weeks of life. In this study, 75 male Ross strain chickens were randomly assigned to three treatment groups: Group C- received the standard diet without any additive; Group A- received the antibiotic virginiamycin (10 ppm) contained in the daily diet; and Group P - received *B. coagulans* daily at a dose level of 1.6 x 10^{10} cfu/kg/day (1000 ppm) for the first

seven days of life, then fed 4.0 x 10^9 cfu/kg/day (250 ppm) during days 8-49. The investigators noted that *B. coagulans* became integrated in the enteric microflora and did not interfere with other bacterial groups in this animal model. Additionally, *B. coagulans* was found to be transient, without any adhesion to the intestinal epithelium. One week post-administration, *B. coagulans* was detected in the feces (Cavazzoni et al., 1998).

In summary, the findings from animal studies suggest that oral administration of *B. coagulans* is unlikely to cause toxicity. No adverse effects of *B. coagulans* preparation containing 6.88 × 10^{10} cfu/g were noted in subchronic, chronic and reproductive toxicity studies. In these studies the lowest NOAEL of *B. coagulans* preparation was found to be 1948 mg/kg bw/day (13.40 x 10^{10} cfu/kg bw/day). In a long-term study, administration of *B. coagulans* to rats at a dose level of 5 g/kg/day (5x10^9 spores/g) for 15-months did not reveal toxic effects. Similarly, *B. coagulans* administration to chickens at a dose level of 4.0 x 10^9 cfu/kg/day for 7 weeks did not reveal any adverse effects.

3. SUMMARY AND DICUSSION

In recent years, spore forming bacteria such as *B. coagulans* are gaining importance for their potential health benefits because of certain advantages, such as resistance to heat and intestinal fluids, over other non-spore formers. Sanzyme Ltd. intends to use a well-characterized strain of *B. coagulans* SNZ1969 as a food ingredient in a number of foods for human consumption. *B. coagulans* is a gram-positive, catalase-positive, rod-shaped, slightly acidophilic, thermotolerant, aerobic to microaerophilic, highly resilient bacteria. *B. coagulans* SNZ1969 is a dark grayish white powder with a slightly sweet taste and characteristic odor. The intended use of *B. coagulans* SNZ1969 at a maximum level of 2 x 10^9 cfu/serving in a variety of foods such as 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 (excluding meat and poultry products).

B. coagulans was first isolated and described in 1932. As *B. coagulans* forms a spore, it possesses high heat and acid resistance. *B. coagulans* is used in the production of a protein-rich food known as *ugba* in African countries. This microorganism is also used as a probiotic to improve and maintain ecological balance of the intestinal microflora. The use of *B. coagulans* in the preparation of a traditional Nigerian food *(ugba)* demonstrates the use and dietary consumption of this microorganism. The approved use of *B. coagulans* in the production of glucose isomerase enzyme supports the conclusion that it is both nonpathogenic and nontoxicogenic in nature. In 2011, in response to a GRAS notice (GRN 399) for the use of *B. coagulans* strain in conventional foods resulting in a daily intake of 93.8 x 10^9 cfu/person/day, FDA issued a "no questions" letter.

The available animal and human scientific studies of *B. coagulans* further supports its safe use by humans. The available information also suggest that *B. coagulans* is well-tolerated, non-pathogenic and non-toxicogenic. Although details were not available for independent review, in a chronic animal study administration of *B. coagulans* to rats at a dose level of 5 g/kg/day (25 x 10^9 spores/kg bw/day) for 15-months did not reveal any adverse effects.

Similarly, *B. coagulans* administration to chickens at a dose level of 4.0 x 10^9 cfu/kg bw/day for up to 7 weeks did not reveal any adverse effects. *In vitro* studies show that *B. coagulans* spore preparation is not mutagenic or genotoxic. Additional studies in rats show that *B. coagulans* spore preparation does not induce acute, subchronic, chronic, or reproductive toxicity following consumption of up to 2000 mg/kg body weight per day (equivalent to approximately 2.6 x 10^{13} cfu per person per day) spores. All data from available animal studies support the conclusion that oral ingestion of *B. coagulans* is unlikely to cause adverse effects.

The available human studies suggest that following oral administration, *B. coagulans* passes through the stomach and germinates in the intestine within four hours. Upon discontinuation of oral *B. coagulans* administration, spores of this microorganism were noted in feces for up to seven days. The relatively rapid gastrointestinal-clearance time of *B. coagulans* and its inability to adhere to the gastrointestinal epithelium indicate that this microorganism is unlikely to lead to bacteremia, particularly in immunocompromised individuals. Oral administration of *B. coagulans* to infants at a dose of 1 x 10^8 cfu/day for 12 months did not reveal any adverse effects. Similarly, oral administration of *B. coagulans* at a dose of 1 x 10^8 cfu, three times daily to acute and chronic diarrhea subjects did not reveal any adverse effects. In hyperlipidemic subjects daily oral administration of 3.6 x 10^8 cfu of *B. coagulans* spores for 12 weeks was found to be safe. *B. coagulans* is considered to be one of the most effective and safe strains for the prevention of diarrhea. The traditional use as well as qualitative and quantitative scientific evidence supports the safety-in-use of a *B. coagulans* preparation as a food ingredient.

The safety determination of *B. coagulans* SNZ1969 is based on the totality of available evidence, including phenotypic and genotypic characterization, and animal and human studies, including those for other similar strains. The historical and dietary supplement uses of products containing *B. coagulans* strains further corroborate the safety evidence.

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

- Use in the production of a traditional protein-rich food known as *ugba*.
- No pathogenic and toxicogenic effects noted.
- Transient nature of *B. coagulans* in the GI tract and consumption either in foods or as a dietary supplement does not have any cumulative effect that would affect its safety.
- No toxicity reported in animal studies at doses up to 25 x 10^9 spores/kg bw/day.
- No adverse effects noted in several human studies, including studies of up to 1-year duration and in susceptible groups (children).
- *B. coagulans* SNZ1969 is marketed for over 40 years without any reports of significant adverse effects.

In summary, on the basis of scientific procedures[6] including knowledge from a history of exposure to *B. coagulans* SNZ1969, the consumption of *B. coagulans* SNZ1969 as an added food ingredient from its intended uses at levels up to 2 x 10^9 cfu/serving in a variety of specified foods and resulting in estimated daily intake of 36.4 x 10^9 cfu *B. coagulans* spores/day is considered safe. The intended uses are compatible with current regulations, *i.e., B. coagulans* SNZ1969 is used in specified foods (described in this document) and is produced according to current good manufacturing practices (cGMP).

[6] 21 CFR §170.3 Definitions. (h) Scientific procedures include those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance.

4. CONCLUSION

Based on a critical evaluation of the publicly available data summarized herein, the Expert Panel members whose signatures appear below, have individually and collectively concluded that a *Bacillus coagulans* SNZ1969 preparation, meeting the specifications cited above, and when used at maximum use levels of up to 2 x 10^9 cfu/serving (reference amounts customarily consumed, 21 CFR 101.12) in specific foods (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) when not otherwise precluded by a Standard of Identity as described in this dossier and resulting in estimated daily intake of 36.4 x 10^9 cfu *B. coagulans* spores/day is safe.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, we have also concluded that the use of this *B. coagulans* preparation in the foods at the levels specified above is GRAS.

Signatures

(b) (6)

Douglas Archer, Ph.D. — Feb. 5, 2014 — Date

(b) (6)

Robert L. Martin, Ph.D. — Feb. 4, 2014 — Date

(b) (6)

Madhusudan G. Soni, Ph.D., F.A.C.N., F.A.T.S. — Feb. 7. 2014 — Date

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6. APPENDIX I

16S rRNA profile of *Bacillus coagulans* SNZ1969

LOCUS	KC146407 1491 bp DNA linear BCT 31-OCT-2013
DEFINITION	Bacillus coagulans strain SNZ1969 16S ribosomal RNA gene, partial sequence.
ACCESSION	KC146407
VERSION	KC146407.1
KEYWORDS	
SOURCE	Bacillus coagulans
ORGANISM	Bacillus coagulans
	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE	1 (bases 1 to 1491)
AUTHORS	Swamy,M.V. and Soman,J.L.
TITLE	Screening and development of Bacillus coagulans for various probiotic applications
JOURNAL	Unpublished
REFERENCE	2 (bases 1 to 1491)
AUTHORS	Swamy,M.V. and Soman,J.L.
TITLE	Direct Submission
JOURNAL	Submitted (03-NOV-2012) Research and Development Division, Sanzyme Ltd, Gagan pahad, Shamshabad, Hyderabad, Andhrapradesh 501323, India
COMMENT	##Assembly-Data-START##
	Sequencing Technology :: Sanger dideoxy sequencing
	##Assembly-Data-END##
FEATURES	Location/Qualifiers
source	1..1491
	/organism="Bacillus coagulans"
	/mol_type="genomic DNA"
	/strain="SNZ1969"
	/isolation_source="green malt"
	/db_xref="taxon:1398"
	/country="India"
rRNA	<1..>1491
	/product="16S ribosomal RNA"

BASE COUNT 363 a 366 c 474 g 287 t

ORIGIN

```
   1 aggacgaacg ctggcggcgt gcctaataca tgcaagtcgt gcggaccttt taaaagcttg
  61 cttttaaaag gttagcggcg gacgggtgag taacacgtgg gcaacctgcc tgtaagatcg
 121 ggataacgcc gggaaaccgg ggctaatacc ggatagtttt ttcctccgca tggaggaaaa
 181 aggaaagacg gcttcggctg tcacttacag atgggcccgc ggcgcattag ctagttggtg
 241 gggtaacggc tcaccaaggc aacgatgcgt agccgacctg agagggtgat cggccacatt
 301 gggactgaga cacggcccaa actcctacgg gaggcagcag tagggaatct tccgcaatgg
 361 acgaaagtct gacggagcaa cgccgcgtga gtgaagaagg ccttcgggtc gtaaaactct
 421 gttgccgggg aagaacaagt gccgttcgaa cagggcggcg ccttgacggt acccggccag
 481 aaagccacgg ctaactacgt gccagcagcc gcggtaatac gtaggtggca agcgttgtcc
 541 ggaattatgg kgggcgtaaa gcgcgcgcag gcggcttctt aagtctgatg tgaaatcttg
 601 cggctcaacc gcaagcggtc attggaaact gggaggcttg agtgcagaag aggagagtgg
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1261 gaggttaagc caatcccaga aaaccattcc cagttcggat tgcaggctgc aacccgcctg
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1381 gccttgtaca caccgcccgt cacaccacga gagtttgtaa cacccgaagt cggtgaggta
1441 acctttacgg agccagccgc cgaaggtggg acagatgatt ggggtgatct a
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6.1. APPENDIX II

Analytical data from five manufacturing lots

Specifications of *B. coagulans* SNZ1969 from five non-consecutive manufacturing lots (Sanzyme, 2013)

Test	Standard specifications	Batch Numbers				
		BC-50-12-E01	**BC-50-12-L01**	**BC-50-13-D01**	**BC-50-13-E01**	**BC-50-13-H01**
Description	A dark grayish white powder slightly sweet in taste with characteristic odor.	Complies	Complies	Complies	Complies	Complies
LOD	NMT 5.0% w/w	3.53 % w/w	3.54 % w/w	3.69 % w/w	3.68 % w/w	3.65 % w/w
Lactic acid producing capacity	NLT 10 ml of 0.05 M NaOH is consumed.	13.05 ml	13.15 ml	13.35 ml	13.40 ml	13.55 ml
Assay (cfu/g)	NLT 50.0 x 10^9	59.98 x 10^9	62.98 x 10^9	65.06 x 10^9	60.85 x 10^9	66.70 x 10^9
Heat Resistant Ratio	NLT 70 %	80.83%	81.20%	80.89%	80.72%	81.52%
Heavy Metals						
Arsenic	NMT 3 ppm	0.42 ppm	1.9 ppm	0.83 ppm	0.83 ppm	0.14 ppm
Lead	NMT 3 ppm	1.08 ppm	2.1 ppm	1.05 ppm	1.02 ppm	0.78 ppm
Mercury	NMT 0.1 ppm	Not detected	0.07 ppm	Not detected	Not detected	0.06 ppm
Cadmium	NMT 1 ppm	0.51 ppm	0.71 ppm	0.15 ppm	0.16 ppm	0.12 ppm
Total Viable Aerobic Counts						
Other organisms	NMT 10,000 cfu/g	8,500 cfu/g	8,100 cfu/g	7,500 cfu/g	8,750 cfu/g	7,500 cfu/g
Fungus	NMT 10 cfu/g	5 cfu/g	5 cfu/g	5 cfu/g	5 cfu/g	5 cfu/g
Specified Pathogens						
Escherichia coli	Absent/10g	Absent	Absent	Absent	Absent	Absent
Salmonella	Absent/10g	Absent	Absent	Absent	Absent	Absent
Pseudomonas aeruginosa	Absent/1g	Absent	Absent	Absent	Absent	Absent
Staphylococcus aureus	Absent/1g	Absent	Absent	Absent	Absent	Absent
Bacillus cererus	Absent/1g	Absent	Absent	Absent	Absent	Absent
Lysteria monocytogenes	Absent/25g	Absent	Absent	Absent	Absent	Absent

SUBMISSION END