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ORIGINAL SUBMISSION

Soni & Associates Inc.

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September 15, 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* spore preparation (LactoSpore®)

Dear Sir/Madam:

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

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

Sincerely,

(b) (6)

(b) (6)

Madhu G. Soni, Ph.D., FACN, FATS

RECEIVED
SEP 21 2015
OFFICE OF
FOOD ADDITIVE SAFETY

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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)

Sabinsa Corporation (the notifier) has determined that *Bacillus coagulans* spore preparation (LactoSpore®) 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 *Bacillus coagulans* spore preparation (LactoSpore®) is exempt from the requirement of premarket approval.

Signed,

(b) (6)

Date Sept. 15, 2015

Madhu G. Soni, Ph.D., FACN, FATS

Agent for:

Sabinsa Corporation
20 Lake Drive
East Windsor, NJ 08520
USA

000003

B. Name and Address of Notifier:

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Marketing Director
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Payson, UT 84651, USA
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Email: Shaheen@sabinsa.com

C. Common or Usual Name of the Notified Substance:

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

D. Conditions of Intended Use in Food

Bacillus coagulans (MTCC 5856) spore preparation (LactoSpore®), 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 2×10^9 colony forming units (CFU)/serving (reference amounts customarily consumed, 21CFR 101.12). *B. coagulans* spore 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 LactoSpore® in the above mentioned food categories, is estimated to result in a maximum daily intake of 36.4×10^9 cfu/day.

E. Basis for GRAS Determination:

In accordance with 21 CFR 170.30, the intended use of *B. coagulans* spore preparation (LactoSpore®) 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* spore preparation (LactoSpore®). In recent years, *Bacillus coagulans* spore preparations has been the subject of two GRAS notifications (GRN 399; GRN 526). In response to all of these notices, FDA did not question the conclusions that the use of *Bacillus coagulans* spore preparation is GRAS under the conditions of use described in these notices. The safety determination of *Bacillus coagulans* spore preparation (LactoSpore®) for the present GRAS assessment is based on the totality of the available scientific evidence that includes human observations and a variety of preclinical

and clinical studies. An Expert Panel was assembled to evaluate the health aspects of *Bacillus coagulans* spore preparation (LactoSpore®). Based on the available safety-related information, the estimated daily intake, if ingested daily over a lifetime, the Expert Panel concluded that the intended uses of LactoSpore® as described herein are safe.

In the published literature, several safety-related animal and human studies of *B. coagulans* spore preparation have appeared. These studies include subchronic toxicity, chronic toxicity, reproduction toxicity, *in vitro* and *in vivo* genotoxicity and human clinical safety. All these studies support the safety in use of *B. coagulans* at the intended use levels. Sabinsa has conducted a series of phenotypic and genotypic studies, including whole genome sequencing of *B. coagulans* strain (MTCC 5856). These studies further support the non-toxigenic nature of this strain. On the basis of scientific procedures[1], Sabinsa Corporation (Sabinsa) considers the consumption of *B. coagulans* spore preparation (LactoSpore®), 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 at the offices of:

Madhu G. Soni, PhD, FATS
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Vero Beach, FL 32068

Telephone: +1- 772-299-0746
Email: msoni@soniassociates.net; sonim@bellsouth.net

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

Sabinsa's *Bacillus coagulans* spore preparation is a standardized (6 and 15 billion cfu/g product) white to off white powder with mild characteristic odor. It is a member of a subgroup of *Bacillus* spp.

A. Common Name:

Bacillus coagulans

B. Trade Name:

The subject of this notification will be marketed as LactoSpore®

C. Identity:

Bacillus coagulans is a Gram-positive, catalase-positive, spore forming, rod-shaped, slightly acidophilic, thermotolerant, aerobic to microaerophilic bacteria. LactoSpore® bearing Sabinsa's internal reference number SBC37-01 has been deposited with the Microbial Type Culture Collection (MTCC) and Gene Bank. The strain has been designated with accession

[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.

number of *Bacillus coagulans* MTCC 5856. This strain, the subject of present GRAS assessment, *B. coagulans* MTCC 5856 has been extensively studied for its physicochemical (phenotypic) and biological (including genotypic) characteristics. In order to confirm identity, the purity, isolation, morphological, physiological and biochemical studies have been carried out for *B. coagulans* MTCC 5856. In addition to phenotypic identification, genotyping studies, including whole genome sequencing, confirmed and identified the subject strain with a homology of 99.5% in consensus with *B. coagulans* ATCC 7050 (reference strain). Thus, the available information supports the characterization and identity of *B. coagulans* MTCC 5856, the subject of this GRAS assessment.

D. Physical Characteristics

White to off white powder with mild characteristic odor

E. Food Grade Specifications

Food grade specifications of *Bacillus coagulans* spore preparation (LactoSpore®) are presented in Tables II-E.1. Analytical data from five non-consecutive lots with strength 15×10^9 cfu/g are presented in Appendix I. These data suggest that *B. coagulans* spore preparation is consistently manufactured to meet the standard product specifications.

Table II-E.1. Specifications of *Bacillus coagulans* spore preparation (LactoSpore®)

Parameter	Characteristics
Appearance	Pale brown to brown powder (100 billion units/gm) White-to-off-white powder (15 billion units/gm)
Identity	The spores are ellipsoidal bodies, terminally placed at the end of every vegetative cell
Phenotyping	Characteristic for the strain
Genotyping	16S rRNA gene sequence study≠
Solubility	Slightly soluble in water. Insoluble in methanol and chloroform
Loss on drying	NMT 8%
Viable spore	NLT 15 billion (1.5×10^{10}) spores/g*
Other organisms	NMT 0.1 million CFU/g
Lactic acid producing capacity	NLT 10 ml of 0.05N NaOH consumed
Sieve test	Passes
-20 mesh	NLT 100%
-40 mesh	NLT 95%
-80 mesh	NLT 90%
Stability	Three years
Heavy metals	
Arsenic	NMT 1 ppm
Cadmium	NMT 1 ppm
Mercury	NMT 0.1 ppm
Lead	NMT 3 ppm
Microbiological assays	
Yeast and Mold	< 100 CFU/g
Escherichia coli	Negative /10g
Bile Tolerant Gram negative bacteria	NMT 100 cfu/g
Staphylococcus aureus	Negative /10g
Salmonella	Negative /10g
Pseudomonas aeruginosa	Negative /10g

*Based on information provided by Sabinsa Corporation; NMT = Not more than; NLT = Not less than; ≠: Performed once in a year; *Sabinsa Corporation produces LactoSpore® with different spore counts with identical specifications.

F. Manufacturing Process

Bacillus coagulans spore preparation (LactoSpore®) is manufactured according to current good manufacturing practices (GMP), as presented in Figure II.G. In brief, manufacturing of *B. coagulans* spores involves three steps: (1) Inoculum preparation, (2) Fermentation, and (3) Down stream processing.

In the first step, pure culture of *B coagulans* is inoculated to sterile seed medium that is incubated on shaker at 37-39°C for 22-24 hours. The seed culture is transferred to fermenter containing the fermentation medium for incubation at 37-39°C for 35-37 hours. The broth from incubation is harvested and further processed. In the downstream processing step, the broth harvest is centrifuged and bacterial cells are separated. The wet cake of sporulated bacterial cells is mixed with sterilized Demineralized (DM) water and filtered through mesh (100# size). The filtered slurry is spray dried and concentrated.

The spray-dried powder is mixed with maltodextrin to desired final concentration of *B. coagulans* spores. The manufacturing procedure assures a consistent and high-quality product that meets the specifications. The processing aids, processing aids, fermentation medium and diluents used in the manufacturing of LactoSpore® are either approved as food additives or as GRAS substances.

G. Manufacturing Flow Chart

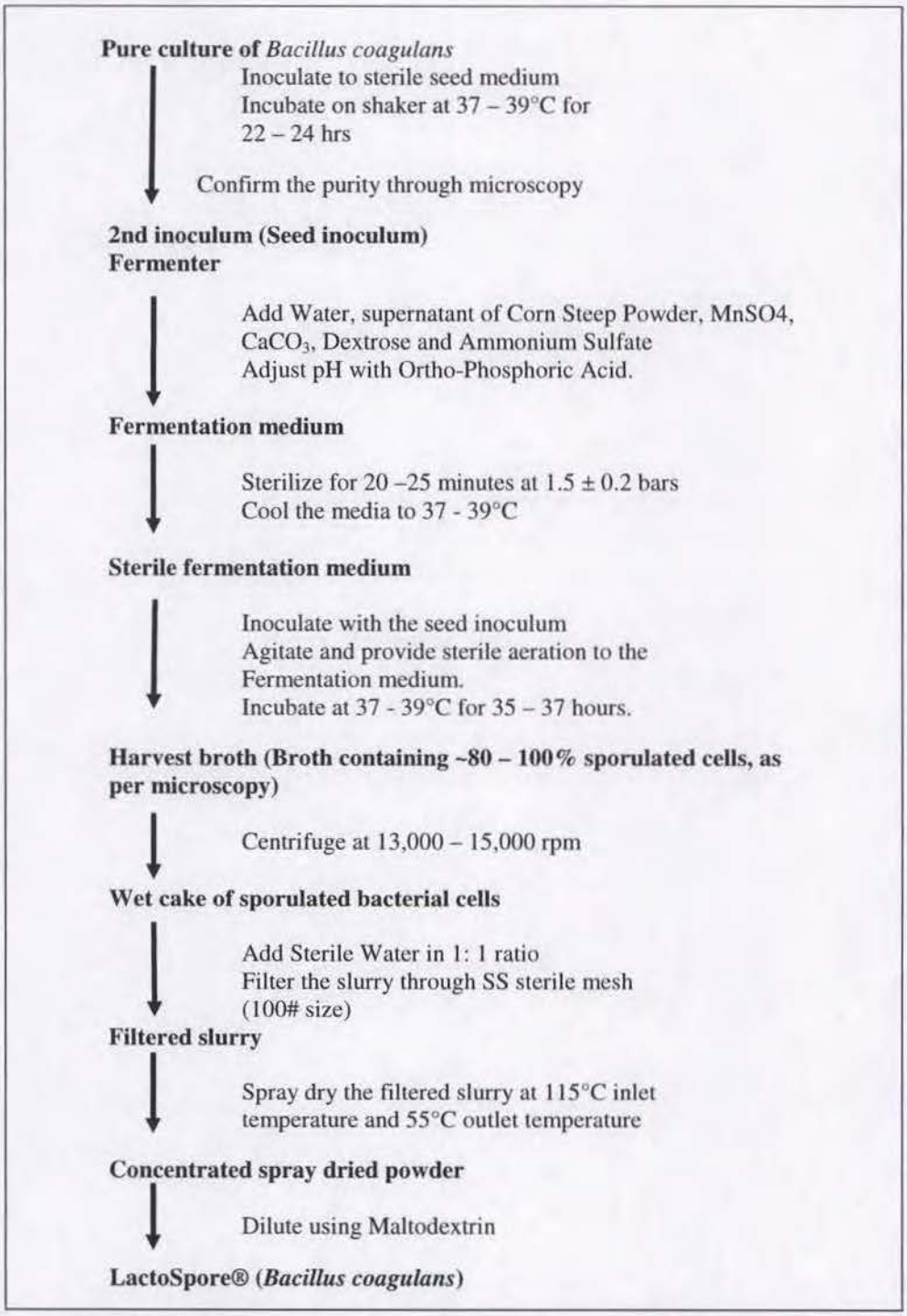

Figure II-G. Manufacturing process of *Bacillus coagulans* spore (LactoSpore®)

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

The determination that *Bacillus coagulans* spore preparation (LactoSpore®) is GRAS is based on scientific procedures. For this determination a comprehensive search of the scientific literature for safety and toxicity information on *Bacillus coagulans* was conducted through May 2015. In addition to several published studies of other similar strains of *B. coagulans,* the safety studies of *B. coagulans* MTCC 5856 includes survival in simulated gastric acid, bile tolerance, antibiotic sensitivity activity and human safety and tolerance study. In addition to chemical characteristics, *B. coagulans* has been characterized by genotypic analysis, including whole genome sequencing. 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. coagulans* spore preparation (LactoSpore®) to the selected foods described in this notice and at a maximum use level of approximately 2×10^9 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.

In coming to this decision that *B. coagulans* spore preparation (LactoSpore®) is GRAS, Sabinsa Corporation relied upon the conclusions that *B. coagulans* spore preparation (LactoSpore®) does not pose any toxicological hazards or safety concerns at the intended use levels, as well as on published toxicology studies 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* 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 to determine the safety of *B. coagulans* spore preparation (LactoSpore®) used as a food ingredient. Based on a critical evaluation of the pertinent data and information summarized herein, the Expert Panel members have individually and collectively determined by scientific procedures that the addition of *B. coagulans* spore preparation (LactoSpore®) 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 2×10^9 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 36.4×10^9 cfu *B. coagulans* spores/day is GRAS. It is also the opinion of the Expert Panelists that other qualified and

competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion.

Bacillus coagulans spore preparation was the subject of two GRAS notifications (GRN 526 and GRN 399) to the FDA for use as a food ingredient. 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 Sabinsa's proposed use of *Bacillus coagulans* spore preparation. A synopsis of the pertinent information in these documents is presented below (see attached Expert Panel Statement).

EXPERT PANEL STATEMENT

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF *Bacillus coagulans* MTCC 5856 (LACTOSPORE®)

Prepared for
Sabinsa Corporation
20 Lake Drive
East Windsor, NJ 08854

Prepared by
Soni & Associates Inc.
749 46th Square
Vero Beach, FL 32968

Panel Members

John A. Thomas, Ph.D., F.A.C.T., D.A.T.S.
Stanley M. Tarka, Jr., Ph.D.
Madhusudan G. Soni, Ph.D., F.A.C.N., F.A.T.S.

September 8, 2015

EXPERT PANEL STATEMENT

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF *Bacillus coagulans* MTCC 5856 (LACTOSPORE®)

TABLE OF CONTENTS

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF *Bacillus coagulans* MTCC 5856 (LACTOSPORE®) 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 Sabinsa Corporation, USA, to determine the Generally Recognized As Safe (GRAS) status of *Bacillus coagulans* spores preparation (LactoSpore®) 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 level of approximately 2×10^9 colony forming units (cfu)/serving. A comprehensive search of the scientific literature for safety and toxicity information on *Bacillus coagulans* was conducted through August 2015 and made available to the Expert Panel. The Expert Panel independently and critically evaluated materials submitted by Sabinsa Corporation and other information deemed appropriate or necessary. Following an independent, critical evaluation, the Expert Panel conferred and unanimously agreed to the decision described herein.

1.1. Background

Bacillus coagulans is a lactic acid producing bacteria with typical characteristics of both *Lactobacillus* and *Bacillus* genera. It was first isolated and described in 1932 (Horowitz-Wlassowa and Nowotelnow, 1932) and was considered to be a spore forming lactic acid bacteria in the Bergey's Manual (Bergey et al., 1939). As *B. coagulans* exhibits characteristics typical of both genera *Lactobacillus* and *Bacillus,* its taxonomic position between the families *Lactobacillaceae* and *Bacillaceae* was debated and later it was transferred to the genus *Bacillus*. It has been isolated from natural sources, such as heat-treated soil samples (Sneath et al., 1986). Similar to other species of the genus *Bacillus*, *B. coagulans* forms endospores, which are resistant to most chemical and physical conditions. *B. coagulans* has also been shown to maintain normal intestinal microflora and improve digestibility (Majeed and Prakash, 1998). Due to its spore forming ability, *B. coagulans* has high heat and acid resistance. This characteristic allows spores to survive industrial manufacturing and ensures a long-term viability, a property that more labile lactobacilli do not possess (Sanders et al., 2001). Given the stability limitations of conventional strains of probiotics, *B. coagulans* has been commonly marketed as a probiotic to maintain the ecological balance of the intestinal microflora and normal gut function.

In addition to its role as a probiotic, *B. coagulans* has been used as part of the fermenting process for the production of a protein-rich food known as *ugba* (Isu and Njoku, 1997)

[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.

commonly consumed in Nigeria. This food is produced by fermentation of the African oil bean seed (*Pentaclethra macrophylla* Bentham) over a 3-4 day period. The fermentation process enhances the meaty taste and other desirable organoleptic properties of *ugba*. It is considered a popular food delicacy in Nigeria, especially among the Ibo ethnic group. A significant number of bacteria and spores present in the product are consumed from this food. Up to five different types of bacteria (*Bacillus, Lactobacillus, Staphylococcus, Micrococcus* and members of the family *Enterobacteriaceae*) are used in the fermentation of *ugba*. However, the *Bacillus* spp. found in the samples tested (*i.e., B. coagulans, B. macerans, B. megaterium, B. pumilis* and *B. subtilis*), were the only species that could ferment the bean slices to produce the desired characteristic sensory qualities.

1.2. Description

B. coagulans is a Gram-positive, catalase-positive, spore forming, rod-shaped, slightly acidophilic, thermotolerant, aerobic to microaerophilic bacteria commonly found in the soil, air and dust. It is a highly resilient organism. The vegetative cells of *B. coagulans* can grow at temperatures as high as 65°C and the endospores can withstand temperatures in excess of 100°C. It can grow in a highly alkaline environment and the spores can also withstand the acidic environment of the stomach. *B. coagulans* produces the favored L (+) optical isomer of lactic acid and this effectively prevents the growth of numerous bacterial and fungal pathogens (Farmer, 2002). *B. coagulans* strain, the subject of this GRAS determination, has been deposited in the Microbial Type Culture Collection (MTCC) and Gene Bank and is designated with accession number of *Bacillus coagulans* MTCC5856 (in compliance with MTCC-IDA under the Budapest Treaty). This strain has been characterized in detail and its genome completely sequenced. Sabinsa Corporation intends to market this *B. coagulans* spore preparation under the name LactoSpore® as a standardized powder. General descriptive parameters and properties of *B. coagulans* preparations manufactured as LactoSpore® by Sabinsa are summarized in Table 1.

Table 1. General descriptive characteristics of *Bacillus coagulans* and LactoSpore®

Parameter	Description*
Source	*Bacillus coagulans*
Synonyms	*Lactobacillus sporogenes*
CAS No.	68038-65-3
Functional Use	Probiotic; enzyme production
Physical characteristics	Gram-positive, aerobic, endospore forming rod-like microorganism, slightly acidophilic and thermotolerant with a microaerophilic metabolism
Heat resistance: vegetative cells	**D95°C = 0.1 min
Heat resistance: spores at pH 7	**D111°C = 1.6 min
Odor	Characteristic

*Based on information provided by Sabinsa Corporation. **D = Decimal reduction time is the minutes of heating at temperature *t* for a log-cycle decrease in the number of survivors.

1.3. Specifications

Food grade specifications of LactoSpore® have been established by Sabinsa Corporation and are presented in Table 2. Analytical results from five non-consecutive lots (Appendix I) demonstrate that LactoSpore® is consistently manufactured to meet these specifications.

Table 2. Specifications of LactoSpore®

Parameter	Characteristics*
Appearance	Pale brown to brown powder (100 billion units/gm) White-to-off-white powder (15 billion units/gm)
Identity	The spores are ellipsoidal bodies, terminally placed at the end of every vegetative cell
Phenotyping	Characteristic for the strain
Genotyping	16S rRNA gene sequence study≠
Solubility	Slightly soluble in water. Insoluble in methanol and chloroform
Loss on drying	NMT 8%
Viable spore	NLT 15 billion (15×10^9) spores/g*
Other organisms	NMT 0.1 million cfu/g
Lactic acid producing capacity	NLT 10 ml of 0.05N NaOH consumed
Sieve test	Passes
-20 mesh	NLT 100%
-40 mesh	NLT 95%
-80 mesh	NLT 90%
Stability	Three years
Heavy metals	
Arsenic	NMT 1 ppm
Cadmium	NMT 1 ppm
Mercury	NMT 0.1 ppm
Lead	NMT 3 ppm
Microbiological assays	
Yeast and Mold	< 100 cfu/g
Escherichia coli	Negative /10g
Bile Tolerant Gram Negative bacteria	NMT100 cfu/g
Staphylococcus aureus	Negative /10g
Salmonella	Negative /10g
Pseudomonas aeruginosa	Negative /10g

*Based on information provided by Sabinsa Corporation; NMT = Not more than; NLT = Not less than; ≠: Performed once in a year; *Sabinsa Corporation produces LactoSpore® with different spore counts (ranging from 6 billion to 100 billion) with identical specifications.

1.3.1. Identification and Characterization

The purity, isolation, morphological, physiological and biochemical characteristics of *Bacillus coagulans* (MTCC 5856) was studied at an independent laboratory (Save, 2004). The spores were cultured on two separate media to establish genus Bacillus. The media employed were nutrient agar a general purpose medium and de Man, Rogosa and Sharpe agar a selective medium for Lactobacillus. The colonies grown on these media were subjected to Gram staining

and biochemical characterization. These investigations confirmed the identity and specifications of LactoSpore®. The biochemical characterization of Sabinsa's *Bacillus coagulans* strain showed 90% similarity between the variants and the reported characterization of *Bacillus coagulans* [Hammer 1915, Type Strain ATCC 7050]. The study report is included as Appendix II.

In an *in vitro* study, *Bacillus coagulans* SBC-37-01 strain (MTCC 5856) was tested for stability at varying pH and for bile salt tolerance as per FAO recommendations (Ali et al., 2013). The results of these experiments revealed that LactoSpore® was stable at different pH conditions and tolerated high bile salt concentrations. There was no significant difference in LactoSpore® growth and viability at maximum concentration tested (0.5% w/v). These findings suggest that the *Bacillus coagulans* SBC-37-01 (MTCC 5856) strain exhibits probiotic attributes and can survive at low pH and high bile concentration.

1.3.2. Genotypic Identification

In an attempt to further characterize *B. coagulans*, genotypic identification was carried out. For this identification Sabinsa's *Bacillus coagulans* (with internal reference number SBC37-01) was analyzed for the 16S rDNA gene sequence. *B. coagulans* strain (MTCC 5856), the subject of this GRAS assessment was found, through gene sequencing, to be identified as the historically identified and commonly recognized strain *B. coagulans* Hammer that has been deposited with American Type Culture Collection (ATCC) facility by Dr. N.R. Smith under the designation number 7050. The genotyping results confirmed and identified the strain as *Bacillus coagulans* with homology of 99.5% with *Bacillus coagulans* ATCC 7050 (Appendix III). These findings suggest that *B. coagulans* is homologous to *B. coagulans* Hammer.

1.3.3. Genome Sequencing

In an attempt to further characterize the subject of present GRAS, whole genome sequencing of *Bacillus coagulans* MTCC 5856 was undertaken using Illumina MiSeq sequencing platform. The whole genome sequencing work flow and analysis overview is presented in Figure 1. The genome of *B. coagulans* MTCC 5856 consists of a 3,018,045 bp long chromosome with a 46.74% GC content which is similar to what has been reported for *B. coagulans* (44 to 50%) (Sneath et al., 1986). The assembled sequences were uploaded to RAST server and annotated using *Bacillus coagulans* ATCC 7050 as reference. 3995 protein functions were predicted from the contigs generated. Entries involved in EPS Biosynthetic pathway were identified from the annotation column retrieved from the RAST server along with lipoteichoic acids. KAAS server was used for pathway identification with assembled sequences as input. Among the 98 contigs 3995 protein functions and 40 functional pathways have been predicted. 82 tRNA genes were identified using tRNAscan-SE-1.3.1 and 10 rRNA genes were identified using RNAmmer-1.2.

The Identification of Bacteriocin gene clusters in the genome of *B. coagulans* MTCC 5856 was performed using the assembled scaffolds as input to antiSMASH 2.0 [antiSMASH 2.0; Nucleic Acids Research (2013), doi: 10.1093/nar/gkt449]. AntiSMASH identified 4 bacteriocin gene clusters based on the location and order of genes present in the assembled contigs. The presence/production of bacteriocin is one of the desired properties of a probiotic strain.

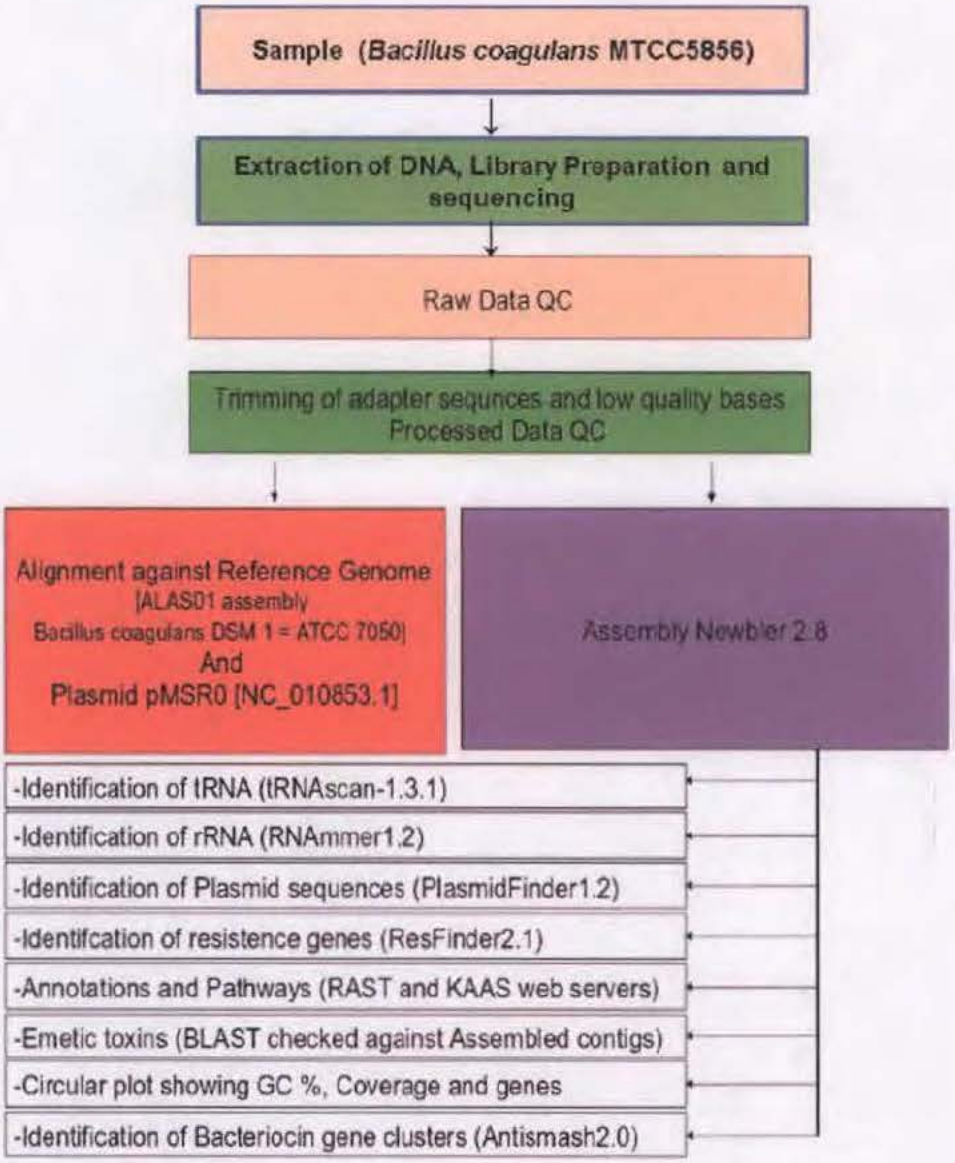

Figure 1. Overview of Whole Genome Sequencing Workflow and Analysis

The emetic toxin genes of *Bacillus spp.* were collected and their nucleotide sequences were downloaded from the NCBI database. Nucleotide blast was done using emetic nucleotide sequences as database and assembled contigs of *B. coagulans* MTCC 5856 as query. The results of blast did not yield any hits which suggested absence of any emetic toxin genes in the genome of *B. coagulans* MTCC 5856. Another concern is carrying any plasmid in the genome. The presence of other plasmids was checked by PlasmidFinder 1.2 (http://cge.cbs.dtu.dk/services/PlasmidFinder/) and both Enterobacteriaceae as well as Gram – positive plasmid database were selected. Assembled sequences were given as input. One hit was found to rep21 plasmid which contributes to 25% of the reference plasmid sequences. The genome study concluded the absence of any possible plasmid in the genome of *B. coagulans* MTCC 5856.

Further, ResFinder2.1 (http://cge.cbs.dtu.dk/services/ResFinder/) was used to identify drug resistance genes in the assembled sequences of *B. coagulans* MTCC 5856. ResFinder2.1 checks for resistance genes for Aminoglycoside, Beta-lactam, Fluoroquinolone, Fosfomycin, Fusidic Acid, MLS - Macrolide-Lincosamide-StreptograminB, Nitroimidazole, Phenicol, Rifampicin, Sulphonamide, Tetracycline, Trimethoprim and Glycopeptide. 98% sequence identity and 60% coverage was used as threshold for the identification. There were no resistance genes identified in the bacterial strain during analysis. Emerging resistance amongst bacteria to antimicrobials is a major concern world-wide. The probiotics are viable micro-organisms and consumed in various dietary and health products which may add to the pool of antimicrobial resistance genes already present in the gut bacterial population or otherwise increase the risk of transfer of drug resistance. Hence, it is strictly recommended by European Food Safety Authority to determine the minimum inhibitory concentration of the antimicrobials against such probiotic strains. The genome analysis confirmed the absence of antibiotic resistance genes in the genome of *B. coagulans* MTCC 5856.

1.4. Manufacturing Process

LactoSpore® is manufactured according to current good manufacturing practices (cGMP), as presented in Figure 2. Primarily manufacturing of *B. coagulans* involves three steps: (1) Inoculum preparation, (2) Fermentation, and (3) Down stream processing. In the first step, a pure culture of *B coagulans* is inoculated on sterile seed medium that is then incubated on shaker at 37-39°C for 22-24 hours. The seed culture is transferred to fermenter containing the fermentation medium for incubation at 37-39°C for 35-37 hours. The broth from incubation is harvested and further processed. In the downstream processing step, the broth harvest is centrifuged and bacterial cells are separated. The wet cake of sporulated bacterial cells is mixed with sterilized Demineralized (DM) water and filtered through mesh (100# size). The filtered slurry is spray-dried and concentrated. The spray-dried powder is mixed with maltodextrin to the desired final concentration of *B. coagulans* spores. The manufacturing procedure assures a consistent and high-quality product that meets the specifications (Table 2). The processing aids, fermentation medium and diluents used in the manufacturing of LactoSpore® are either approved as food additives or as GRAS substances.

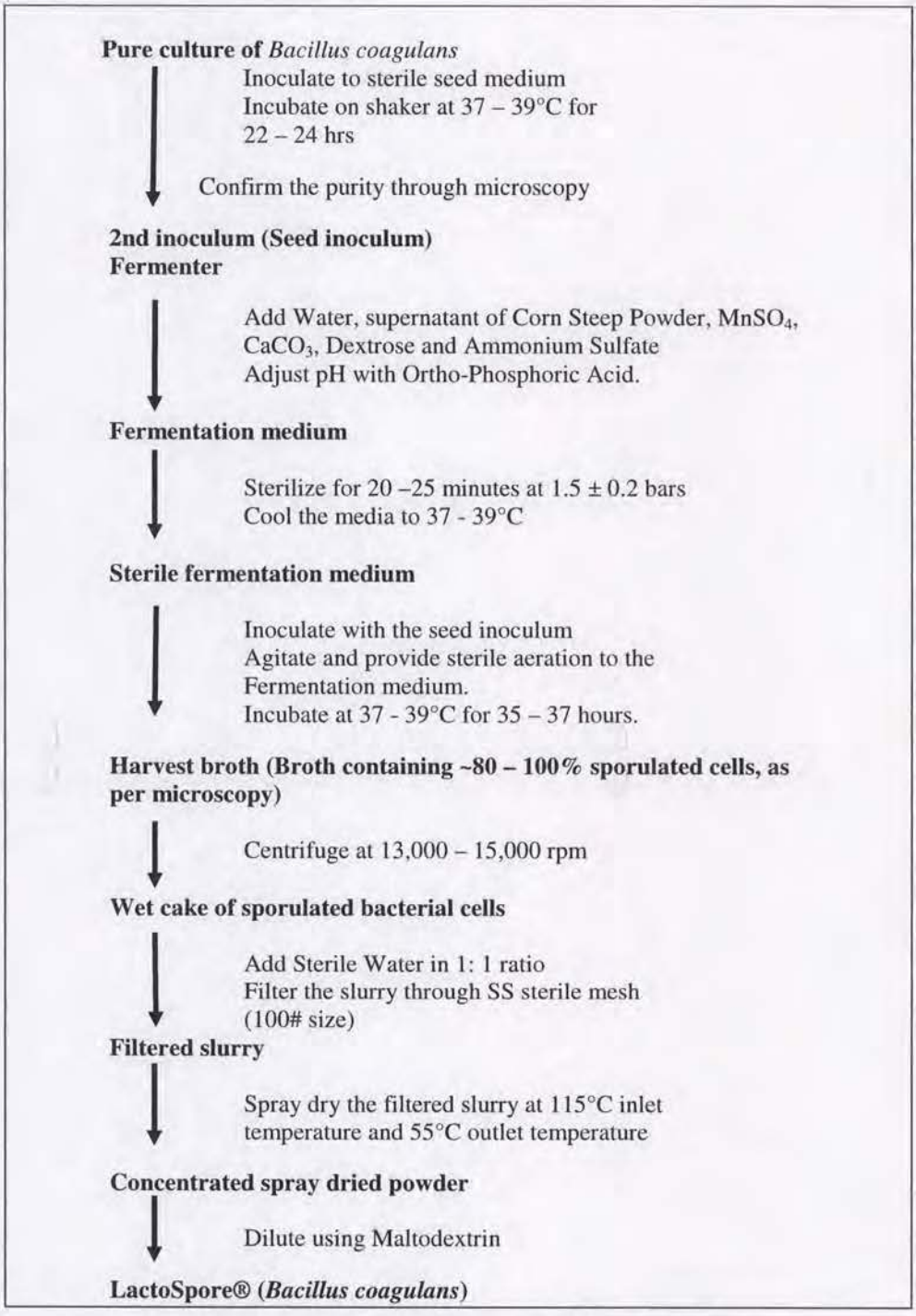

Figure 2. Manufacturing process of LactoSpore®

1.5. Current Uses and Regulatory Approvals

Currently, *B. coagulans* has been sold as a dietary supplement in the United States and across the world under different names such as Nature's Plus, Tancos, Pit-Stop, Fresh Start Bolus, Tarm-X Balans™, Sporolac®, Sanvita, Sanvitone, Bactlyte, Ampilac, etc. Several other products containing *B. coagulans* are available in the market. These formulations include *B.*

coagulans alone or combined with Lactobacilli or bifidobacteria, vitamins (particularly B complex), minerals, and prebiotics. The recommended dose of dry *B. coagulans* ranges from $3.6 \times 10^8 - 1.5 \times 10^9$ colony forming units (cfu)/capsule, two or three times *per* day with meals, for a healthy adult. At the highest recommended dose, the total daily intake of *B. coagulans* as a dietary supplement would be 1.1×10^9 cfu/day. Catanzaro and Green (1997) suggested a use of a standard dose of *B. coagulans* of 1.5×10^9 cfu once or twice per day. *B. coagulans* has been used as part of the fermenting process for the production of a protein-rich food known as *ugba* that is consumed in Nigeria (Isu and Njoku, 1997).

In Europe, *B. coagulans* has been granted Qualified Presumption of Safety (QPS) status since 2008 by the European Food Safety Authority (EFSA, 2012). The ATCC has classified *B. coagulans* as Biosafety Level 1, indicting that this bacteria is not known to cause disease in healthy humans. The Japanese Ministry of Health and Welfare has approved a *B. coagulans* product (Lacbon) for improvement in symptoms caused by abnormalities in the intestinal flora or in dysbiosis (Majeed and Prakash, 1998).

B. coagulans is approved for use in the preparation of enzymes used for food production (FDA, 2001). Under 21 CFR §184.1372, insoluble glucose isomerase enzyme produced from *B. coagulans* (a nonpathogenic and nontoxicogenic microorganism) is recognized as GRAS. FDA's Center for Veterinary Medicine has also approved the use of *B. coagulans* as GRAS for specified veterinary purposes. Similar to FDA, Health Canada has also permitted the use of *B. coagulans* in the production of glucose isomerase enzyme.

In addition to the above approved uses, FDA has received two GRAS notices on *B. coagulans* spore preparations. In August 2011, Ganeden Biotech Inc submitted a GRAS notification (GRN 399) to FDA for the use of *B. coagulans* strain in conventional foods (Ganeden, 2011). The notice indicated that *B. coagulans* preparation is to be used at a maximum level of approximately 2×10^9 cfu/serving in several food categories. FDA issued a "no questions" letter for GRN 399 on July 31, 2012 (FDA, 2012). In another recent GRAS notice, Unique Biotech Ltd. informed FDA that *B. coagulans* 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 2×10^9 cfu/serving (Unique, 2014). Following its review of the GRAS notice and other information available to FDA, the agency issued a "no questions" letter (FDA, 2015).

1.6. Intended Use Levels and Food Categories

Sabinsa Corporation intends to market LactoSpore®, *B. coagulans* preparation for use as a food ingredient in multiple food categories. Sabinsa's *B. coagulans* preparation is intended for use in the same foods and at the same use levels (2×10^9 cfu/serving) to those mentioned in the GRN 399 (Ganeden, 2011). There are no new or incremental food uses proposed for LactoSpore®. 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). The application of

LactoSpore® to the same foods and at the same levels (2×10^9 cfu/serving) as those in GRN 399 is not expected to affect the intake of *B. coagulans* 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 18.2×10^9 servings/day. Using this estimate of the number of servings/day at a level of 2×10^9 cfu/serving, Ganeden estimated the daily intake of *B. coagulans* spores at 36.4×10^9 cfu/day.

2. Common Knowledge of Safe Uses

2.1. Traditional Uses

Lactic acid bacteria have been used in foods for centuries and are generally considered as harmless (Lee and Salminen, 1995). These bacteria are widely used as starter cultures for fermentation in the dairy, meat and other food industries. To ensure the safety of these bacteria in food, many strains selected for such uses have been previously associated or endogenously found in humans. Their properties have been used to manufacture products like cheese, yoghurts, fermented milk products, beverages, sausages, and olives. These food-grade bacteria can also improve the safety, shelf life, nutritional value, flavor and quality of the product. Moreover, lactic acid bacteria can be used as cell factories for the production of food additives. When consumed, Lactic acid bacteria may also function as probiotics and contribute to the general health of the consumer.

The role of lactic acid bacteria in intestinal microecology has been extensively investigated. Lactic acid bacteria constitute an important element of the healthy digestive tract (Adams, 1999; Soomro et al., 2002; Ouwehand et al., 2004). Dietary ingestion of various lactic acid producing bacteria has a substantial history of use and their safety record is excellent. One lactic acid producing bacteria that has been used for its potential health benefits is *B. coagulans*. Of the 77 recognized *Bacillus* species, *B. coagulans* has been evaluated for probiotic functionality and sold worldwide for both human and animal uses (Sanders et al., 2003). *B. coagulans* is considered a beneficial bacteria for human health (Catanzaro and Green, 1997). *B. coagulans* Hammer (ATCC 31284) was first isolated at Yamanashi University in 1932 by Professor Nakayama.

In a series of studies from Portugal between 1958 and 1959, Guida and colleagues (Guida et al., 1958; Guida and Guida, 1959) investigated the potential gastrointestinal benefits of *B. coagulans* and other spore-forming bacteria. These articles were published in Portuguese and are cited in databases such as PubMed. The details of these articles were not available, however the available information indicate that *B. coagulans* was in use and consumed for many years.

2.2. Food Uses in Africa

Although fermented foods have a history of consumption in Africa, the absence of writing culture in most African countries makes their origin difficult to trace (Okonko et al., 2006). One such food, *ugba* is a popular protein-rich solid, flavorful alkaline food in the Ibo ethnic group of Nigeria. One of the species identified in the preparation of *ugba* by fermentation of the African oil bean is *B. coagulans*. Consumption of this food is known to result in the intake of *B. coagulans* (Isu and Njoku, 1997). The maximum density of bacillus cells found on day 3 of fermentation were $\log_{10} 9 - \log_{10} 11.9$ cfu/g. A large proportion (76%) of this population has been reported to use *ugba* as a snack (Onofiok et al., 1996). The presence of bacillus cells in

ugba at the levels noted above supports documentation of the intake of *B. coagulans*. Although the amount of consumption of *ugba* per occasion or daily was not available, the level of bacteria (*B. coagulans*) present in the *ugba* indicates that consumption of *B. coagulans* is greater than 1×10^9 cfu/day. This provides support for the historical use and consumption of this microorganism. These observations demonstrate that as part of *ugba, B. coagulans* is consumed as a food by humans.

3. TOXICOLOGY

In a number of preclinical investigations, the acute and long-term effects of *B. coagulans* have been investigated. The majority of the animal studies conducted with *B. coagulans* were undertaken to evaluate its efficacy for a probiotic effect. These studies are reviewed as part of the safety evaluation, as in addition to efficacy, relevant safety endpoints were also included. This monograph also reviews human studies performed to evaluate the safety and efficacy of *B. coagulans*. The assessment of efficacy studies is limited to a review of the results related to safety and tolerability. Although the form of *B. coagulans* used in the majority of the studies was not clear, it appears that exposure to *B. coagulans* in these investigations was in endospore form.

3.1. Animal Studies

In a series of preclinical studies, Sankyo Company Limited, Japan, investigated the acute and long-term effects of *B. coagulans* preparations. The details of these studies were not published but briefly cited in several reports (Sankyo, 1968; cited in Majeed and Prakash, 1998; Anonymous, 2002). In the acute toxicity study, a powder preparation of *B. coagulans* containing 5×10^9 cfu (spores)/g was administered by gavage to male mice at dose levels of 1, 3 or 5 g/kg. The mice were observed for 7 days. No deaths occurred, nor was there any abnormality such as diarrhea. In the group receiving 5 g/kg dose, "a few mice" showed slight distension of the stomach, which recovered to normal after a few hours. The results of this study suggest that the LD_{50} of a powder containing *B. coagulans* is greater than 5 g/kg. In an acute toxicity study (Endres et al., 2009), administration of *Bacillus coagulans* GBI-30, 6086 cell mass (1.04×10^{11} cfu/g) at a dose level of 5 g/kg (5.2×10^{11} cfu/kg bw) to Wistar rats did not result in mortality or adverse effects, suggesting the LD_{50} to be greater than 5 g/kg (5.2×10^{11} cfu/kg bw). In yet another acute toxicity study, Sudha et al. (2011a) reported that the LD_{50} of *B. coagulans* is greater than 6500 mg/kg bw (32.5×10^9 cfu/kg bw).

In additional studies, dogs (n=2), rabbits (n=3) and guinea pigs (n=15) were tested with orally administered maximum ingestible single daily doses (10 g/kg for dogs, 30 g/kg for rabbits and 50 g/kg for guinea pigs) of *B. coagulans* powder preparation for 7 days. No abnormalities were observed during the period of treatment as well as for 10 days subsequent to the withdrawal of treatment. 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. Body weight gains for treated groups were similar to those for the control group. Changes in organ weight showed no significant differences between treated and control groups. Additional details of the study were not available. Although details of these preclinical unpublished studies were not available for independent review, these studies indicate that *B. coagulans* preparation is non-toxic.

In a short-term, repeat-dose study, toxicity study, *B. coagulans* was administered (gavage) to four groups rats at dose levels of 0, 130, 650, 1300 mg *B. coagulans* preparation/kg bw/day for 14 consecutive days (Sudha et al., 2011a). The *B. coagulans* preparation contained 5×10^9

cfu/g. Half of the animals from each group were euthanized on day 15, while the remaining animals (recovery group) were euthanized on day 28 and examined for gross macroscopic lesions. No treatment-related changes were observed in clinical signs, bodyweights, feed intake, urine parameters, hematological examinations, clinical chemistry, gross pathology and histopathology. Based on the results of this study, the investigators concluded that *B. coagulans* was clinically well tolerated at doses up to 1300 mg (corresponding to 6.5×10^9 cfu/kg bw/day), when administered orally to Sprague Dawley rats for 14 days consecutively. The No Observed Adverse Effect Level (NOAEL) for *B. coagulans* was determined as 1300 mg (6.5×10^9 cfu)/kg bw/day, the highest dose tested (Sudha et al., 2011a).

In a subchronic toxicity study, *B. coagulans* (GanedenBC^{30}™) cell mass (1.36×10^{11} cfu/g) was administered via gavage to Wistar rats (10/sex/group) at doses of 0, 100, 300 and 1000 mg/kg bw/day for 90 consecutive days (Endres et al., 2009). As the concentration of the *B. coagulans* used in this study was 1.36×10^{11} cfu/g, the highest dose corresponds to 1.36×10^{11} cfu/kg bw/day. No mortality was noted during the course of the study. The results of this study did not reveal any toxicologically significant differences between the treated groups (100, 300 and 1000 mg/kg bw/day) and the controls with respect to feed consumption, water consumption, sensory reactivity, general and behavioral conditions, hematological and clinical chemistry evaluations. Additionally, exposure to *B. coagulans* did not reveal treatment-related macroscopic or microscopic signs or changes in the organ weights of the male and female rats at 100, 300 and 1000 mg/kg/day after the 13-week treatment period. 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.

In addition to the above described subchronic toxicity study, Endres et al. (2011) also conducted a one-year chronic oral toxicity study combined with a one-generation reproduction to further investigate the safety of long-term consumption of *B. coagulans*. In the chronic toxicity study conducted as per OECD and FDA Redbook guidelines, *B. coagulans* preparation was administered to Wistar rats (20/sex/group) in their diet at a dose level of 0, 600, 1200 and 2000 mg/kg bw/day for 52 to 53 weeks. The dose levels corresponded to a test article concentration in rat feed of 0, 10,000, 20,000 and 33,300 mg/kg. There was no test article-related mortality. General daily and detailed clinical observations did not reveal any toxic signs related to the test article. No test article-related changes in body weight, body weight gain, or feed consumption were observed during the study. Hematological, clinical chemistry and urinalysis parameters at the end of the 3rd week, as well as the 3rd, 6th or 12th months did not reveal toxicological relevant changes. Changes observable in all groups of treated male and female rats were either not related to administered dose, were well within the historical background range or were not correlated with other hematological or histopathological alterations. Macroscopic and microscopic examinations did not reveal lesions attributable to test article. The NOAEL in male and female rats was determined as 1948 and 2525 mg/kg bw/day, respectively, the highest dose tested.

The one-generation reproduction toxicity study was conducted according to the OECD and FDA Redbook guidance, and in parallel to the chronic oral toxicity study described above (Endres et al., 2011). For this study, Wistar rats were divided into four groups (10/sex/group) and were fed diet containing *B. coagulans* at a dose of 0, 600, 1200 and 2000 mg/kg bw/day. All animals were fed for ten weeks and during a three-week mating period. Male rats were fed 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 toxicity on the parental generation (male or female) of the same strain of rat during the course of the study with diet-mixed administration. The NOEL for the parental group male and female rats was determined as 2372 and 3558 mg/kg bw/day, respectively. The NOEL for the reproductive performance for the males and females was determined as 2372 and 3558 mg/kg bw/day. The NOEL for the F1 offspring was determined as 3558 mg/kg bw/day.

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

Cavazzoni et al. (1998) studied the use of *B. coagulans* as a probiotic for chickens during the first seven weeks of life. Seventy-five 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*, administered daily at 1.6×10^{10} cfu/kg/day (1000 ppm) for the first seven days of life, then fed 4.0×10^9 cfu/kg/day (250 ppm) during days 8-49. *B. coagulans* became integrated in the enteric microflora and did not interfere with other bacterial groups in this animal model. Furthermore, *B. coagulans* was transient, without any adhesion to the intestinal epithelium and was absent in the feces one week post-administration.

In summary, the data from the available animal studies indicate that administration of *B. coagulans* preparations is unlikely to cause adverse effects. The results of well designed subchronic, chronic and reproductive toxicity studies did not reveal adverse effects following administration of *B. coagulans* preparation containing 6.88×10^{10} cfu/g. The results of these studies revealed the lowest NOAEL of 1948 mg/kg bw/day (13.40×10^{10} cfu/kg bw/day). Although details of the study were not available for independent review, in a long-term study, administration of *B. coagulans* to rats at a dose level of 5 g/kg/day (5×10^9 spores/g) for 15-months did not reveal toxic effects. Similarly, *B. coagulans* administration to chickens at a dose level of 4.0×10^9 cfu/kg/day for 7 weeks did not reveal any adverse effects. Treatment of uremic rats (chronic renal failure) with *B. coagulans* (1×10^8 cfu/day) for 16 weeks prolonged the life span of these rats and also resulted in a reduction in blood urea nitrogen levels.

3.2. Human Studies

In a double-blind, randomized, placebo-controlled, parallel clinical trial (included by National Institute of Health in the list of clinical trials)[2], specifically designed to investigate the effects of *B. coagulans* MTCC 5856 spore preparation (LactoSpore®), 40 healthy adult volunteers (16 males and 24 females) were divided into two groups (Wilson, 2014). This study

[2] Available at: ClinicalTrials.gov- https://clinicaltrials.gov/ct2/show/NCT02176889

has been included by National Institute of Health in the list of clinical trails. Group one (n=20) received one LactoSpore® (containing 2 billion spores) tablet daily and the control group (n=20) received one placebo table daily, 30 minutes before meal for a period of 30 days. Safety and tolerability were investigated by examining hematology and clinical chemistry parameters, adverse events, tolerability questionnaire and bowel habits diary. At the end of 30 day, no changes in anthropometric parameters and laboratory parameters of safety were noted between the groups. The number of adverse effects in both groups were the same. There were two adverse events in the placebo group classified as abdominal pain (stomach pain), whereas only one such event in the treatment group was reported. No differences in daily number of bowel movements between the groups were noted. The results of this study suggest that administration of LactoSpore® at a dose level of 2 billion spores/day for 30 days is safe and well tolerated in healthy subjects.

In another study (pending publication) conducted with *B. coagulans* MTCC 5856 spore preparation (LactoSpore®) safety and efficacy of LactoSpore® - containing *B. coagulans* MTCC5856 (2 Billion spores) in comparison with placebo in patients receiving standard of care treatment for irritable bowl syndrome (IBS) were investigated (Srinivasa et al., 2014). In this randomized, double-blind, parallel group, placebo-controlled study, 36 subjects (18 to 55 years) were randomized into two groups that received either LactoSpore or placebo. Newly diagnosed or untreated patients who were not on any other treatment in the past 3 months with mild to moderate IBS in severity were enrolled into the study. Sompraz D (containing Domperidone 30mg and 40 mg of Esomeprizole) and Metrogyl 400 (Metronidazole 400 mg) once a day was considered as standard treatment of care for diarrhoea predominant IBS. In addition to this treatment, subjects were asked to self administer one Tablet per day (either *B. coagulans* MTCC 5856 or Placebo) at least 30 min before a meal, preferably in the morning as a dietary supplement for a period of 90 days. The following parameters were studied: Clinical laboratory parameters; Visual Analog Scale for abdominal pain (VAS); Gastrointestinal Discomfort Questionnaire; Bristol Stool Form Score; Physicians Global Assessment; Irritable Bowel Syndrome Quality of Life Questionnaire and; Pathogenic bacterial count in stools. No clinically significant abnormal lab values were identified and no statistically significant changes in the vitals were observed from the baseline to final visits. There were no serious adverse events or significant adverse events noted. Of the 36 participants, 31 completed the study. Five subjects dropped out of study, citing personal reasons. LactoSpore® treatment revealed a significant change/decrease in the clinical symptoms such as bloating, vomiting, diarrhea, abdominal pain and stool frequency towards end of the study. LactoSpore® treatment was considered as safe.

In a monocentric double-blind, placebo-controlled parallel group clinical trial, Urgesi et al. (2014) investigated the efficacy and the safety of a medical device containing a combination of Simethicone and *B. coagulans* in the treatment of IBS. In this study, 52 adult subjects (aged 18-75 years), suffering from IBS received the active treatment or placebo 3 times a day after each meal for 4 weeks of study period. The subjects were evaluated at 0, 14 and 28 days of treatment. Adverse effects were also recorded at 14 and 28 day evaluation. Although the investigators mentions safety was investigated, no safety related biochemical parameters were investigated. No serious adverse effects were recorded in both the groups.

Following oral administration, *B. coagulans* passes through the stomach in spore form and once inside the duodenum, germinates and multiplies rapidly (Majeed and Prakash, 1998; Losada and Olleros, 2002). The average duration between oral ingestion and germination has been estimated to be four hours. After germination, *B. coagulans* is metabolically active as part

of the facultative anaerobes in the intestine and produces lactic acid from fermentation. *B. coagulans* is considered a semi-resident, indicating it takes up only a temporary residence in the human intestinal tract. Spores of *B. coagulans* are excreted slowly via the feces for approximately seven days following discontinuation of its administration (Majeed and Prakash, 1998). *B. coagulans* has been suggested to improve gastrointestinal ecology by replenishing the quantity of desirable obligatory microorganisms and antagonizing pathogenic microbes (Anonymous, 2002).

Lactic acid producing bacteria can produce two isomeric forms of lactic acid-dextrorotatory [D (-)]-lactic acid and levorotatory [L(+)]-lactic acid. Of the two isomers, L(+)-lactic acid is completely metabolized in the body, while D(-)-lactic acid is partially metabolized, resulting in a degree of metabolic acidosis. As *B. coagulans* produces only L(+)-lactic acid, it is unlikely to cause acidosis or adverse effects (Majeed and Prakash, 1998).

Mohan et al. (1990a; 1990b) investigated the effects of *B. coagulans* on serum lipid levels in hypercholesterolemic patients. In this open-label clinical trial, daily administration of 3.6×10^8 cfu *B. coagulans* spores for 12 weeks to 17 patients with type II hyperlipidemia resulted in a decrease in the total *vs.* HDL cholesterol ratios by 24% ($p<0.001$), while their LDL *vs.* HDL ratios decreased by 33% ($p<0.001$). Total cholesterol to HDL-cholesterol and LDL-cholesterol to HDL-cholesterol ratios was improved during the three month treatment period with *B. coagulans*. HDL-cholesterol increased marginally from 43.6 to 46.8 mg/dl ($p<0.05$). There was no change in the serum triglyceride levels of the patients. No adverse effects of *B. coagulans* were noted.

Ara et al. (2002) investigated the effects of administration of *B. coagulans* SANK 70258 on the intestinal environment in 20 healthy adults, including an analysis of the bacterial flora and decomposition products in the intestine, with further evaluation of the dermal characteristics of the subjects. The test period lasted for six weeks: two weeks before administration, two weeks during administration of *B. coagulans* (1×10^8 cfu/day) and two weeks after administration. Stool samples were taken initially 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 1×10^8 cfu/day significantly increased the number of Bifidobacteria, while significantly decreasing the number of lecithinase-positive Clostridia ($P<0.05$). *B. coagulans* ingestion also significantly lowered the average fecal pH ($P<0.05$), while also inducing a decline in ammonium, *p*-cresol and indole concentrations in the fecal matter compared to the control period. No adverse effects of *B. coagulans* were noted.

In another experiment, Ara et al., (2002) investigated the effects of *B. coagulans* in 23 female subjects between the ages of 20 to 40, with a tendency for constipation, on their intestinal tract and skin blemishes. The test period was a total of 12 weeks: four weeks before administration, four weeks of administration of *B. coagulans* SANK 70258 (1×10^8 cfu/day), and four weeks of placebo administration. The subjects kept a daily record of their defecation frequency and their fecal characteristics (fecal shape, color and odor) and skin characteristics (number of comedones), with the skin analyzed every two weeks by counting the number of skin eruptions (flares and papules). Administration of *B. coagulans* resulted in greater stool defecation frequency than before intake ($P<0.05$), which the authors noted as benefiting the intestinal environment of the volunteers. Constipation is typically indicated by very hard or hard

fecal characteristics, with banana shaped or soft fecal characteristics indicating normal fecal consistency. Administration of *B. coagulans* to subjects with a propensity towards constipation resulted in an increased frequency of appearance of the banana-shaped and soft types of fecal material, while the hard and very hard types showed a decline, continuing even after *B. coagulans* administration was discontinued. Seventy-two *percent* of the subjects who had complained of constipation or diarrhea before intake recognized significant improvements ($P<0.05$) after ingestion of 1×10^8 *B. coagulans* cfu/day. Assessment of the dermal characteristics following *B. coagulans* administration revealed that the number of comedones significantly decreased ($P<0.05$) as constipation was alleviated, during and after the intake of *B. coagulans*.

The growth and proliferation of *B. coagulans* in the GI tract was investigated by the administration of 2.5 g/day of a preparation containing 1×10^8 cfu *B. coagulans* spores *per* gram (2.5×10^8 spores/day) for ten days to a subject (Ara et al., 2002). The number of *B. coagulans* found in the feces was used as an indicator of the total amount of *B. coagulans* remaining in the intestinal tract after discontinuation of *B. coagulans* ingestion. On the eighth day of administration, the total number of *B. coagulans* remaining in the intestine was 2.5×10^5 cfu. By the sixth day after discontinuation of treatment, less than ten *B. coagulans* spores were recovered in the feces. The same study was repeated, with administration of 8×10^8 spores/day of *B. coagulans* for four days. Prior to administration, no *B. coagulans* spores were found in the feces. By the second day of administration, 3.8×10^5 *B. coagulans* spores were noted in the feces. By the third day after discontinuation of *B. coagulans* ingestion, there were 1.1×10^5 spores in the feces, with an absence of *B. coagulans* spores in the feces by the eighth day post administration. This data indicates that *B. coagulans* is only transiently maintained in the intestinal tract.

In a case control study, Astegiano et al., (2006), investigated the effect of a dietary mixture (Active), composed of L-tryptophan, inulin, angelica, vegetal charcoal, vitamin PP, group B vitamins (B1, B2, B6) and probiotics (*Bacillus coagulans, Lactobacillus acidophilus, Streptococcus thermophilus*) in IBS patients. 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 mean period of 6 months (range, 5-8). The control group comprised 28 patients (6 men and 22 women; mean age, 48.6±3.7 years) served as controls. All subjects were assessed for the presence of abdominal pain and/or distension, constipation, diarrhea and alternating constipation and diarrhea. Compared with baseline values, the reduction in abdominal pain in the treatment group was 62%, 55% in abdominal distension, 58% in constipation, 33% in diarrhea, and 62% in alternation between constipation and diarrhea. Compared with baseline values, no statistically significant reduction in symptoms was found in the control group. Post-treatment comparison between the two groups showed that the treatment with ISB Active dietary integrator reduced symptoms and that the difference was statistically significant for abdominal pain, abdominal distension and constipation. 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.

In a randomized double-blind, placebo-controlled clinical trial, 61 adult volunteers (age 36.5 ± 12.6 years; height 165.1 ± 9.2 cm; 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 (Kalman et al., 2009). Subjects in the treatment group received daily one capsule containing 2.0×10^9 cfu *B. coagulans*. Subjects were evaluated every two weeks over a four-week period. During each visit, the participants were evaluated with a series of questionnaires in addition to hemodynamics (standard biochemical safety) and adverse event monitoring. The details of hemodynamic

parameters were not mentioned. The investigators concluded that the *B. coagulans*-based probiotic product was effective and safe for abating symptoms of gastrointestinal distress, particularly abdominal pain and distention in the post-prandial period.

In another 60-day clinical study, Sudha et al. (2011b) studied the effects of *B. coagulans*, on serum cholesterol. For these investigations, 30 hyperlipidemic subjects (15 male and 15 female; age 42-53 years) were divided into 3 groups (n=10). Subjects in two groups were given a daily dose of two capsules containing *B. coagulans* (10×10^9 cfu/capsule (Group A) and 20×10^9 cfu/capsule (Group B) and the subjects in the third group received standard medication. Serum lipid profiles were analyzed on day 0, 30 and 60 of the study period. At the end of study there were slight reductions in total cholesterol (11%) and LDL (0.8%), whereas an increase in HDL cholesterol levels (3.6%) was noted. No adverse effects were reported.

In yet another study, Sudha et al. (2012a) investigated the effects of *B. coagulans* on bacterial vaginosis in women (n=40) diagnosed with the condition by the presence of symptoms. The subjects were divided into two groups probiotic (n = 20) and control (n = 20) based on age (control group: 33 ± 3 years; probiotic group: 32.5 ± 3 years), history of previous vaginosis (control group: 75% or 15/20; probiotic group: 75% or 15/20) and severity of current vaginosis infection (burning micturation and itching, 35% in each group). The control group patients were given standard vaginosis treatment alone (Ofloxacin–Ornidazole). The probiotic group patients took standard vaginosis treatment plus two capsules of *B. coagulans* Unique IS2 (1×10^9 cfu/capsule) twice a day before meals for 90 days. At the end of the treatment, 80% of the probiotic group subjects showed a significant positive response as revealed by reduction of vaginosis symptoms as compared to the control group which exhibited reduction in 45% subjects only. No adverse effects of the treatment were reported.

3.2.1. Infection-related Studies

In an extensive review article describing meta-analyses of trials of probiotics for the prevention of antibiotic associated diarrhea, Doron et al. (2008) reported that *B. coagulans* is one of the most effective strains of probiotics for this use. Johnston et al. (2007) assessed the efficacy and adverse effects of probiotics, including *B. coagulans,* in the prevention of antibiotic-associated diarrhea in children. In this assessment of 10 clinical trials with Lactobacilli spp., Bifidobacterium spp., Streptococcus spp., or *Saccharomyces boulardii* alone or in combination, *Lactobacillus* GG, *B. coagulans*, *Saccharomyces boulardii* at 0.5×10^{10} to 4×10^{10} cfu/day were considered the most promising probiotics. In another 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.

In Japan, the Ministry of Health and Welfare has approved use of *B. coagulans* product (Lacbon) for improvement in symptoms caused by abnormalities in the intestinal flora or in dysbiosis (Majeed and Prakash, 1998). Clinical trials with Lacbon (*B. coagulans*) have been conducted at 19 independent health care institutions with participation from 567 subjects. The treatment period in these trials lasted from 2 to 20 days and the doses uses ranged from 0.5×10^8 to 7.5×10^8 cfu/day. These investigations revealed that Lacbon was effective in improving the symptoms due to abnormalities in intestinal flora such as 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 the

clinical trials with Lacbon are summarized in Table 3 (Majeed and Prakash, 1998, Losada and Olleros, 2002).

In a prospective, phase I clinical trial, Sudha et al. (2012b) evaluated the efficacy and safety of *B. coagulans* in the treatment of patients with acute diarrhea. In this study, a total of 28 patients (ages 18 to 55 years) with acute diarrhea received a capsule containing 2 billion or 2×10^9 cfu of *B. coagulans* two times a day for 10 days. Safety of *B. coagulans* was evaluated by assessment of incidence, type of adverse events, physical examination, and clinical laboratory test values (CBC, SGPT, serum creatinine, stool routine and microscopy). Treatment with *B. coagulans* decreased mean values for duration of diarrhea, frequency of defecation, and abdominal pain, while consistency of stool improved. Significant reductions in counts of RBC, WBC, and content of serum creatinine were observed, however the values were found to be in the normal range. No other significant changes in safety parameters were observed during the treatment. The investigators concluded that use of *B. coagulans* is effective and safe in the treatment of patients with acute diarrhea.

Table 3. Summary of results of different trials with Lacbon (*B. coagulans*) (Adapted from Majeed and Prakash, 1998; Losada and Olleros, 2002)*

Condition	No. of subjects	Treatment	Findings
Acute and chronic intestinal catarrh	38	1×10^8-6×10^8 spores/day for 2-12 days	~87 recovery from diarrhea to regular normal stools
Diarrhea	15	0.75×10^8-6×10^8 spores/day for 3-12 days	100% recovery from diarrhea to regular normal stools from third to fourth day
Constipation	10	3×10^8-7.5×10^8 spores/day for 2-10 days	70% recovery to normal stools and disappearance of abdominal distention
Abnormal intestinal fermentation	9	1×10^8-6×10^8 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	1×10^8-2×10^8 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	2×10^8- 4.5×10^8 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	0.2×10^8- 0.5×10^8 spores/day for 4-20 days	80% response seen in anorexia of nervous type and malnutrition in infants
Other studies from published literature			
Gastrointestinal symptoms (Kalman et al., 2009)	61 (30 and 31/group)	2.0×10^9 cfu/capsule daily for 4 weeks	Significant improvements in GSRS abdominal pain subscore was noted. No adverse effects were reported.
IBS- Abdominal pain and bloating patients (Hun, 2009)	44 (22/group)	0.8×10^9 cfu daily for eight weeks	Improvements from baseline in abdominal pain and bloating scores in the treatment group. May be a safe and effective
Rheumatoid arthritis (Mandel	45 (23 and 22/group)	2×10^9 cfu daily for sixty days	Appeared to be a safe and effective. There were no serious adverse reactions

et al., 2010)			reported throughout this study.
Diarrhea in children (Dutta et al., 2010)	148 (78 and 70/group)	0.24×10^9 cfu daily for 5 days	No therapeutic impact on management of acute dehydrating diarrhea

*Additional details of these studies were not available for independent review.

In a randomized, double-blind trial, Cui et al. (2004) investigated the safety and efficacy of *B. coagulans* in the treatment of acute and chronic diarrhea. In this trial 204 subject participated, 103 in the treatment group (51 with acute diarrhea and 52 with chronic diarrhea) and 101 in the control group (51 with acute diarrhea and 50 with chronic diarrhea). The treatment group received *B. coagulans* at a dose of 1×10^8 cfu, three times daily for 3-7 days (acute diarrhea) and 14-21 days (chronic diarrhea), while the control group received Golden Bifid (*Bifidobacterium longum*) tablets at a dose of 1×10^8 cfu three times daily for 3-7 days and 14-21 days. No adverse reactions were found in either group. The results did not reveal any statistically significant differences between the two groups. The number of *Bifidobacterium* and *Lactobacillus* species in the gut were both significantly increased. 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.

3.2.2. Studies in Infants and Children

In a prospective, double-blind, randomized trial, Chandra (2002) investigated the effects of *B. coagulans* on the incidence and severity of acute rotavirus diarrhea. In this study, 112 newborn healthy term infants in rural India received a daily oral dose of 1×10^8 spores *B. coagulans* (n = 55) or a placebo (n = 57) for 12 months. The infants 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). The administration of *B. coagulans* 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 days ill/year (13 days ill in the *B. coagulans* group *vs.* 35 in the control; $p<0.01$). No adverse effects related to the treatment of *B. coagulans* administration were reported.

In a randomized, double-blind, placebo-controlled, cross-over trial, Labalestra et al. (2008) evaluated the effect of a combination of symethicone and *B. coagulans* (Colinox) on the gastric emptying time (GET) and relief of symptoms in infants with symptomatic gastroesophageal reflux (GER). In this study, 19 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 ($p < 0.05$) shorter when the patient received the combination of symethicone and *B. coagulans* (125±20 min) as compared to placebo (165±30 min). Furthermore a stronger improvement of the GER symptoms was detected following the combination treatment than with placebo ($p < 0.01$). No adverse effects were reported.

In a randomized, double-blind, placebo-controlled trial, 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. A total of 120 children, with active infections requiring antibiotics, were enrolled in the study and received orally for 10 days either the probiotic/prebiotic preparation or a placebo (without prebiotic/probiotic). Children (n=60) in

the treatment group received a daily a mixture containing *B. coagulans* and fructo-oligosaccharide (Floxin) at a dose of 5.5×10^8 cfu and 250 mg, respectively. The other group (n=60) received the placebo. The results of the study were recorded from the patients' diary and from follow-up clinical examinations. 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 these groups was 0.7 vs 1.6 days (p=0.002), respectively. The investigators concluded that prophylaxis with the prebiotic/probiotic treatment significantly reduced the number of days and duration of events in children with antibiotic-induced diarrhea.

In another clinical trial published as an abstract, *B. coagulans* was investigated as a treatment for neonatal diarrhea (Dhongade and Anjaneyulu, 1977). In this study, sixty infants with confirmed cases of neonatal diarrhea were administered 1.5×10^7 *B. coagulans* spores *per* day (Sporlac). Of the 60 subjects, 49 responded within a 2-day period following the administration of Sporlac. Based on the suggested dosage level of Sporlac at 5 million spores/kg body weight, each neonate was given a spore level of about 1.5×10^7 spores/day. Although the objective of this study was to investigate the efficacy of Sporlac in the treatment of diarrhea, no adverse effects of the treatment were noted. Additional details of the study were not available.

3.3. Human Observation Summary

In summary, orally administered *B. coagulans* passes through the stomach in its spore form and germinates and multiplies rapidly in the intestine following administration. Spores of *B. coagulans* were noted in feces for approximately seven days after discontinuation of its ingestion. In a prospective, randomized, control trial, administration of a *B. coagulans* preparation to children at a daily dose level of 1×10^8 spores for 12 months did not result in adverse effects. In another randomized, double-blind trial, oral administration of *B. coagulans* at a dose of 1×10^8 cfu, three times daily to acute and chronic diarrhea subjects did not reveal any adverse effects. Daily administration of 3.6×10^8 cfu *B. coagulans* spores for 12 weeks to 17 patients with type II hyperlipidemia was found to be safe. *B. coagulans* is considered as one of the most effective strains of probiotics in the prevention of diarrhea. *B. coagulans* exhibits a high degree of safety.

3.4. Antibiotic Susceptibility of *Bacillus coagulans*

In order to assess the antibiotic susceptibility, a culture of *B. coagulans* strain (LactoSpore®) from Sabinsa was tested against a panel of antibiotics as described by Clincial Laboratory Standards Institute (CLSI, M07-A9, 2012). In this study, two LactoSpore® finished products batches (G130027, 15 billion units/g and G130042, 6 billion units/g) and LactoSpore® mother culture were studied. *B. coagulans* culture samples were tested by broth dilution method against nine antibiotics that included clindamycin, kanamycin, ampicillin, streptomycin, vancomycin, erythromycin, gentamycin, tetracycline and chloramphenicol. The results from these experiments are summarized in Table 4. The antibiotics tested showed a MIC range of 0.0078 to 1 mg/L against all the samples tested. The results of these investigations suggest that *B. coagulans* MTCC 5856 was found to be sensitive to all the antibiotics tested as per EFSA guidelines (EFSA, 2012b).

Table 4. Minimum Inhibitory Concentrations of Antibiotics Against LactoSpore® Cultures

Antibiotic tested	Mother culture	G130027	G139042
	MIC (mg/L)*		
Clindamycin hydrochloride	0.0078	0.0078	0.0078
Kanamycin sulphate	1.0	1.0	1.0
Ampicillin sodium	0.062	0.031	0.031
Streptomycin sulphate	1.0	1.0	1.0
Vancomycin hydrochloride	0.25	0.25	0.25
Erythromycin	0.125	0.125	0.125
Gentamycin sulphate	0.62	0.62	0.62
Tetracycline hydrochloride	0.62	0.62	0.62
Chloromphenicol	1.0	1.0	1.0

*Minimum Inhibitory Concentration

Moldenhauer et al. (1996) investigated the antimicrobial resistance of three species of Bacillus, including *B. coagulans* to thirty antimicrobial agents. In this study, Trypticase Soy Agar (TSA) plates were swabbed with 10^8 spores *per* ml suspension of *B. coagulans* ATCC 51232, 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 (Table 5). The results of this study suggest that *B. coagulans* is susceptible to commonly used antibiotics.

Table 5. Minimum inhibitory concentrations and minimum zones of inhibition by antibiotics against *B. coagulans* (adapted from Moldenhauer et al., 1996)

Antimicrobial Agent	Zone Diameter Requirements for Susceptibility (mm)*	MIC (µg /ml)
Amikacin (30µg)	≥17	≥17
Amoxicillin with Clavulanic Acid (20/10µg)	≥18	>40
Ampicillin (10µg / 25 µg)	≥30	>50
Aztreonam (30µg)	≥26	28
Bacitracin (10U)	≥22	37
Cefaclor (30µg)	≥18	43
Cefamandole (30µg)	≥18	47
Cefazolin (30µg)	≥18	>45
Cefonicid (30µg)	≥18	41
Cefoperazone (75µg)	≥21	46
Cefoxitin (30 mcg)	≥18	48
Ceftazidime (30µg)	≥18	35
Ceftizoxime (30µg)	≥20	>45
Cefuroxime (30µg)	≥18	>45
Cephalothin (30µg)	≥18	>45
Chloramphenicol (30µg / 50 µg)	≥18	33

Antimicrobial Agent	Zone Diameter Requirements for Susceptibility (mm)*	MIC (µg /ml)
Clindamycin (2µg)	≥21	38
Erythromycin (15µg)	≥21	34
Gentamicin (10µg)	≥15	38
Kanamycin (30µg)	≥18	30
Methicillin (5µg)	≥14	43
Nafcillin (1µg)	>13	35
Nitrofurantoin (100µg)	≥17	34
Norfloxacin (10µg)	≥17	32
Ofloxacin (5µg)	≥16	33
Penicillin (10 U / 1 U)	≥47	51
Streptomycin (10µg)	≥15	33
Sulfamethizole (0.25µg)	≥16	41
Tetracycline (5µg)	>19	36
Ticarcillin/Clavulanic Acid (75/10µg)	>20	51

*Data obtained from BBL Sensi-Disc package insert; #Zones of inhibition greater than 12 mm in this study were considered positive for susceptibility of *B. coagulans* to the corresponding antibiotic.

Farmer (2002) also reported the susceptibility of *B. coagulans* to commonly used antibiotic compound(s). In this investigation, antibiotic sensitivity of *B. coagulans* was studied using the Kirby-Bauer (counting colonies on plates) and Vitek (optical density of culture) susceptibility testing methodologies. In the Kirby-Bauer testing, *B. coagulans* was noted to be susceptible to: ampicillin; ciprofloxacin; erythromycin; gentamicin; oxacillin, rifampin; trimethoprim-sulfamethoxazole; vancomycin; and showed intermediate susceptibility to tetracycline. In the Vitek testing, *B. coagulans* was susceptible to: chloramphenicol; gentamicin (500 pg/ml); nitrofurantoin; norfloxacin; penicillin; streptomycin (2,000 µg/ml); vancomycin; and was resistant to tetracycline. The results of this investigation suggests that *B. coagulans* is susceptible to common antibiotics.

The available evidence indicates that *B. coagulans* does not produce antibiotic. 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" (FDA, 2001).

3.5. Cytotoxicity

In an attempt to determine the cytotoxic potential of *B. coagulans* samples from Sabinsa (LactoSpore®), the Vero Cell-based Cytotoxicity assay was performed to investigate detections of *Bacillus cereus*-like enterotoxin and to determine presence/absence of toxigenic activity along with positive control (*Bacillus cereus* ATCC 14579). The Vero cell cyotoxicity based assay

showed no swelling, rounding and disseminating of Vero cells under microscope when incubated with 100 µl of supernatant of the *B. coagulans* from Sabinsa samples for 5 hours when compared with *B. cereus* ATCC 14579, thus showing the absence of cytotoxicity.

In a Cell Viability MTT Assay, *B. coagulans* samples from Sabinsa were tested for viability along with positive control (*B. cereus* ATCC 14579). No significant viability reductions with respect to positive control were noted. The results of this study confirm the absence of cytotoxicity of the *B. coagulans*.

3.6. Virulence

In an attempt to determine the presence/absence of toxigenic activity, *B. coagulans* strain from Sabinsa, which is the subject of the present GRAS assessment, was tested using PCR-based detections of *Bacillus cereus*-like enterotoxin genes: *hblC, nheA,nheB, nheC* and *cytK*, along with positive control (*Bacillus cereus* ATCC 14579) (Hegde, 2013). The *B. coagulans* strain was analyzed for the enterotoxin genes according to the method of From et al. (2005). For this testing, primers were designed for all the 5 genes. Genomic DNA isolation was performed from the given bacterial cultures of LactoSpore® and *Bacillus cereus* ATCC 14579. The PCR conditions with the positive control were standardized and PCR amplification of the samples was undertaken with the standardized condition. The results indicated absence of the *Bacillus cereus*-like enterotoxin genes such as *hblC, nheA,nheB, nheC* and *cytK*.

There have been no reports in the literature of any strain of *B. coagulans* being involved in a pathogenic or opportunistic illness since its isolation. In general, lactic acid-producing bacteria are recognized as non-pathogenic bacteria to human health (Fooks and Gibson, 2002; Doron and Gorbach, 2006). Naturally-occurring and probiotic lactic acid bacteria have an excellent safety profile, and no major problems or health risks to humans have been noted during widespread use (Holzapfel et al., 1995; Salminen et al., 1996). *B. coagulans* has a longer history of use than most of the common *Lactobacillus* and *Bifidobacterium* species commonly sold at health food stores and/or used in the production of cultured dairy products. A critical review of pertinent studies and literature identified in searches conducted through online bibliographic retrieval systems, including PubMed, found no evidence of pathogenicity or toxicogenic effects of *B. coagulans*. In one patient bacteremia was suspected to be due to *B. coagulans*. The search criteria used to identify the studies that relate to this issue were: *B. coagulans* and pathogen, mycotoxin, toxigenic, infection or disease.

Baneriee et al. (1998) reviewed the University of Maryland Cancer Center records during the period of January 1978 to June 1986 and identified *B. coagulans* as a cause of one case out of 24 episodes of *Bacillus* bacteremias in 18 patients. Twelve of the 24 episodes of *Bacillus* bacteremia were considered possible infections; eleven of these patients had a diagnosis of acute leukemia, and one had metastatic breast cancer. Four of the twelve patients 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 immunocompromised population, and would not be defined as virulent. In the published literature, no information was found concerning *B. coagulans* causing an infection following oral ingestion.

Donskey et al. (2001) investigated the effect of oral administration of *B. coagulans* on the density of vancomycin-resistant enterococci (VRE) in fecal matter in a mouse model. 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; 1×10^8 cfu/mouse). Daily gavage of *B. coagulans* (10^7 cfu), bacitracin, or normal saline (control) was performed from day 4 (one day after clindamycin was discontinued) through day 7. In comparison with saline controls, *B. coagulans*-treated mice had significantly lower VRE (VanB- 1) densities on days 8 and 11 (P=0.03). Undetectable levels of VRE in 35% of the *B. coagulans* treated mice were noted on day 11, while none of the 11 saline controls had undetectable levels. 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. All of the mice inoculated with *B. coagulans* had detectable levels of *B. coagulans* in their stool on day 8 and day 11 of the study. These results suggest that *B. coagulans* treatment reduces the colonization of some strains of VRE infection.

3.7. Genomic Analysis

In addition to the above described specific pathogenic gene analysis, the genetic assessment of safety was performed on the genome sequence using a systematic approach in which all emetic toxin genes, enterotoxin genes, antibiotic resistance genes and presence of plasmid were screened. The results from this study revealed that no emetic toxin genes, enterotoxin genes and antibiotic resistance genes were found in the *B. coagulans* MTCC 5856 genome. The presence or production of enterotoxin was confirmed in in-vitro study using Vero cell line by MTT assay. Further, thephenotype of antibiotic resistance gene was also confirmed in in-vitro assay and *B. coagulans* MTCC 5856 was found to be susceptible to a panel of antibiotics as recommended by EFSA. In this analysis, sequences showing similarity with genes or gene products that could be of safety concern were shortlisted for further examination. The genomic analysis of *B. coagulans* did not reveal any significant safety risk. The findings from the genome analysis are summarized in Appendix IV. Additionally, the available evidence from published animal and human studies suggests that *B. coagulans* does not present significant risk following oral ingestion. In conclusion, the genomic analysis of *B. coagulans* provides no evidence that it could be harmful to human health following oral ingestion.

4. SUMMARY

Bacillus coagulans, first isolated and described in 1932, is a lactic acid producing bacteria with typical characteristics of both *Lactobacillus* and *Bacillus* genera. As *B. coagulans* forms a spore, it possesses high heat and acid resistance. In Nigeria, *B. coagulans* is used in a fermentation process for the production of a protein-rich food known as *ugba*. *B. coagulans* is used as a probiotic to maintain the ecological balance of the intestinal microflora and normal gut function. Sabinsa Corporation intends to use a standardized powder containing a *B. coagulans* preparation (LactoSpore®) at a maximum level of approximately 2×10^9 colony forming units (cfu)/serving in baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein

products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups.

The use of *B. coagulans* in the preparation of a traditional Nigerian food *(ugba)* demonstrates the use of this microorganism in a common food preparation. 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. The US FDA has approved the use of *B. coagulans* in the production of glucose isomerase enzyme. This approval supports the conclusion that this microorganism is nonpathogenic and nontoxicogenic (FDA, 2001). In 2011, FDA issued a "no questions" letter in response to a GRAS notice (GRN 399) for the use of *B. coagulans* strain in conventional foods resulting in a daily intake of 9.38×10^{10} cfu/person/day. The available published animal and human scientific studies of *B. coagulans* further supports its safety and use by humans. The available information suggest that *B. coagulans* is well-tolerated, non-pathogenic and non-toxicogenic.

The safety studies of *B. coagulans* spore preparation (LactoSpore®) includes survival in simulated gastric acid, bile tolerance, antibiotic sensitivity activity and human safety and tolerance study. In addition to its chemical characteristics, *B. coagulans* has been characterized by genotypic analysis, including whole genome sequencing. In addition to these specific studies of *B. coagulans* spore preparation (LactoSpore®), the available information from published studies with other strains of *B. coagulans* further supports the safety. Available 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 bw/day (equivalent to 2.6×10^{13} cfu/person/day) spores. Additionally, human studies suggest that following oral administration, *B. coagulans* passes through the stomach and germinates in the intestine within a few hours. Upon discontinuation of oral *B. coagulans* administration, spores of this microorganism were noted in feces for up to seven days. Oral administration of *B. coagulans* to infants at a daily dose level of 1×10^8 cfu for 12 months did not result in any adverse effects. Similarly, oral administration of *B. coagulans* at a dose of 1×10^8 cfu, three times daily to acute and chronic diarrhea subjects did not reveal any adverse effects. Daily oral administration of 3.6×10^8 cfu of *B. coagulans* spores for 12 weeks to hyperlipidemic subjects was found to be safe. *B. coagulans* is considered to be one of the most effective and safe strains of probiotics for use in the prevention of diarrhea.

The safety determination of *B. coagulans* spore preparation (LactoSpore®) 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 evidence of *B. coagulans* safety is supported by:

- Common use of products containing *B. coagulans* species. Use in the production of a traditional protein-rich food known as *ugba*.
- Complete characterization of the strain by phenotypic and genotypic means. No pathogenic and toxicogenic effects noted.
- Susceptibility to antibiotics.
- Available animal studies did not reveal any adverse effects of *B. coagulans*.
- Transient nature of *B. coagulans* in the gastrointestinal tract without any bioaccumulation.
- No adverse effects noted in several human studies, including studies of up to one year duration and in susceptible groups (children).

- Corroboration of safety from studies with substantially equivalent/similar strains

In summary, on the basis of scientific procedures[3] including knowledge of exposure from dietary supplement and food uses of *B. coagulans*, the consumption of *B. coagulans* spore preparation (LactoSpore®) from its use as an added food ingredient from its intended uses at levels up to 2×10^9 cfu/serving in a variety of specified foods and resulting in an estimated daily intake of 36.4×10^9 cfu *B. coagulans* spores/day is considered safe. The intended uses are compatible with current regulations, *i.e., B. coagulans* spore preparation (LactoSpore®) is used in specified foods (described in this document) and is produced according to current good manufacturing practices (cGMP).

[3] 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.

5. CONCLUSION

Based on a critical evaluation of the publicly available data summarized above, the Expert Panel members whose signatures appear below, have individually and collectively concluded that a *Bacillus coagulans* preparation (LactoSpore®), meeting the specifications cited above, and when used at maximum use levels of up to 2×10^9 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 36.4×10^9 cfu *B. coagulans* spores/day is safe and "Generally Recognized As Safe" (GRAS) based on scientific procedures.

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)

John A. Thomas, Ph.D., F.A.C.T., D.A.T.S. — 10 Sept 2015 Date

(b) (6)

Stanley M. Tarka, Jr., Ph.D. — 07 September 2015 Date

(b) (6)

Madhusudan G. Soni, Ph.D., F.A.C.N., F.A.T.S. — 12 Sept. 2015 Date

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7. APPENDIX I

Analytical data from five manufacturing lots

Specifications of LactoSpore® from five different manufacturing batches

Parameter	Standard*	Lot# G140202	Lot# G140307	Lot# G140373	Lot# G140482	Lot# G140483
Appearance	**White-to-off-white powder with mild characteristic odour**	Complies	Complies	Complies	Complies	Complies
Microscopy	**To be Comply**	Complies	Complies	Complies	Complies	Complies
Solubility	**Slightly soluble in water**	Complies	Complies	Complies	Complies	Complies
Loss on drying	**NMT 8% w/w**	0.30% w/w	0.60% w/w	2.34% w/w	0.38% w/w	1.21% w/w
Bacillus coagulans viable spore count	**NLT 15×10^9 spores/g**	17.31×10^9 spores/g	16.37×10^9 spores/g	17.90×10^9 spores/g	18.15×10^9 spores/g	17.80×10^9 spores/g
Other organisms	**NMT 0.1×10^6 cfu/g**	0.0031×10^6 cfu/g	0.00025×10^6 cfu/g	0.0012×10^6 cfu/g	0.0001×10^6 cfu/g	0.0001×10^6 cfu/g
Lactic acid producing capacity	**NLT 10 ml of 0.05N NaOH consumed**	13.4ml of 0.05N NaOH consumed	12.0ml of 0.05N NaOH consumed	12.0ml of 0.05N NaOH consumed	12.8ml of 0.05N NaOH consumed	12.6ml of 0.05N NaOH consumed
Sieve test	Passes					
-20 mesh	**NLT 100%**	100 %	100 %	100 %	100 %	100%
-40 mesh	**NLT 95%**	100 %	100 %	100 %	100 %	100%
-80 mesh	**NLT 90%**	93.10 %	90.95 %	90.07 %	93.51 %	96.38%
Heavy metals						
Arsenic	**NMT 1 ppm**	<0.2 ppm	0.38 ppm	<0.2 ppm	0.27 ppm	0.33 ppm
Lead	**NMT 3 ppm**	0.45 ppm	<0.2 ppm	0.25 ppm	0.44 ppm	0.44 ppm
Microbiological assays						
Yeast and Mold	**< 100 cfu/g**	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Escherichia coli	**Negative**	Negative/10g	Negative/10g	Negative/10g	Negative/10g	Negative/10g
Bile tolerant Gram negative bacteria	**≤ 10 CFU/g**	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Staphylococcus aureus	**Negative**	Negative/10g	Negative/10g	Negative/10g	Negative/10g	Negative/10g
Salmonella	**Negative**	Negative/10g	Negative/10g	Negative/10g	Negative/10g	Negative/10g
Pseudomonas aeruginosa	**Negative**	Negative/10g	Negative/10g	Negative/10g	Negative/10g	Negative/10g

***Standard specifications for marketed product**

8. APPENDIX II

Phenotyping Study Report

PHENOTYPING STUDY

Shri Vile Parle Kelavani Mandal's
SHRI CHHOTABHAI B. PATEL RESEARCH CENTRE
FOR CHEMISTRY & BIOLOGICAL SCIENCES
Regn. No. E 16340 (Mumbai)

Ref : Mic/04-05/007 Date : 30/7/2004

TEST REPORT

Name of the party	SAMI LABS LIMITED
Name of the samples	1) Lactospore powder 15BSG, 2) Bacillus coagulans slant.
Batch Nos.	1) G40486 2) 887/36
Ref. No. & date	17/6/2004.
Date of receipt	30/7/2004.
Test carried out	Species Identification.

Identification and specification of the isolates from Lactospore and Lactobacillus sorogenes slant.

The project was conceived with an objective to ascertain.

1) Purity of the spore culture
2) Isolation of species
3) Characterization using morphological and biochemical parameters.
4) Viable counts if more than one species is isolated

Procedure : The spores were cultured on two separate media to establish genus Lactobacillus / Bacillus. The media employed were nutrient agar a general purpose medium and Rogosa agar a selective medium for Lactobacilli.

The colonies grown on these media were subjected to gram staining and biochemical characterization. The results are tabulated.

Gram staining :- Gram positive.

3RD FLOOR, BHAIDAS SABHAGRIHA BUILDING, JUHU SCHEME, VILE PARLE (WEST), MUMBAI-400056
• TEL: 2617 5363 / 2615 4934 / 2611 9792 • FAX : (022) 2615 4851 • Email : cbprc@vsnl.net

000047

Shri Vile Parle Kelavani Mandal's
SHRI CHHOTABHAI B. PATEL RESEARCH CENTRE
FOR CHEMISTRY & BIOLOGICAL SCIENCES
Regn. No. E 16249 (Mumbai)

Observations & Conclusion

Two variants were isolated on the basis of quick growth on NA medium and slow development of colonies on the same medium.

The fast grower's as well as the 'slow growers' were Gram positive rods bearing terminal spores. On Rogosa agar specific for Lactobacillus, 'the fast grower' developed within 4 days whereas the slow grower took more than 6 days.

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Regn. No. E 16340 (Mumbai)

Biochemical Identification of organisms isolated from Lactospore powder and from the original slant provided by SAMI Labs Ltd.

Biochemical characteristics	Reported Results * For *B. coagulans*	Isolate from Spores		Isolate from Slant
		Fast grower	Slow grower	
Growth at different NaCl concentration.				
a) 2% NaCl	+ ve	+ ve	+ ve	+ ve
b) 5% NaCl	- ve	+ ve	- ve	- ve
c) 7% NaCl	- ve	- ve	- ve	- ve
d) 10% NaCl	- ve	- ve	- ve	- ve
Growth at different tempertures				
a) 5°C	- ve	- ve	- ve	- ve
b) 10°C	- ve	- ve	- ve	- ve
c) 37°C	+ ve	+ ve	+ ve	+ ve
d) 55°C	+ ve	+ ve	+ ve	+ ve
Glucose agar Gas from glucose	- ve	- ve	- ve	- ve
Voges proskar's test	+ ve	+ ve	+ ve	+ ve
Acid from Carbohydrates				
a) Glucose	+ ve	+ ve	+ ve	+ ve
b) Arabinose	d	+ ve	+ ve	+ ve
c) Xylose	d	+ ve	+ ve	+ ve
d) Mannitol	d	+ ve	+ ve	+ ve
Hyodrolysis of starch	+ ve	+ ve	+ ve	+ ve
Utilization of citrate	d	- ve	- ve	- ve
Nitrate reduction	d	- ve	- ve	- ve
Production of Indole	- ve	- ve	- ve	- ve
Production of dihydroxyacetone	d	+ ve	+ ve	+ ve
Decomposition of casein	d	+ ve	+ ve	+ ve
Liquifiation of gelatin	- ve	- ve	- ve	- ve
B-galactosidase	+ ve	+ ve	+ ve	+ ve
Lactic acid production	+ ve	+ ve	+ ve	+ ve
Catalase	+ ve	+ ve	+ ve	+ ve
Decomposition of urea	- ve	- ve	- ve	- ve
Degradation of Tyrosine	- ve	- ve	- ve	- ve

d = Variable + ve = positive reaction - ve = Negative reaction
* As compiled in Bergey's manual of systematic bacteriology, Volume 2, p.p.1122.

3RD FLOOR, BHAIDAS SABHAGRIHA BUILDING, JUHU SCHEME, VILE PARLE (WEST), MUMBAI-400056
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Regn. No.: E 16340 (Mumbai)

Biochemical characterization showed 90% similarity between the variants and the reported characteristics of *Bacillus coagulans*. [Hammer 1915. Type Strain ATCC 7050].

In the given spore sample, 'the fast grower' had a viable counts 1.6×10^7 CFU/gm on nutrient agar while 'the slow growers' had a count 5×10^9 CFU/gm on Rogosa agar.

On the basis of above, it may be concluded that both the variants are Lactic acid producing *Bacillus coagulans*.

The strains have been sent to MTCC, Chandigarh for DNA typing.

(b) (6)

(Dr. Supriya Save)
Scientific Officer.

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9. APPENDIX III

Genotyping Study Report

LactoSpore® Genotyping studies

In addition to the phenotype characterization performed on LactoSpore®, the 16SrDNA studies have been carried out.

The Sabinsa probiotic strain #01 sent for the study was labeled as SBC37-01. This strain earlier characterized as Bacillus coagulans by phenotyping studies grows optimally at 37^0C.

The genotyping result also confirmed and identified the strain as Bacillus coagulans with a homology of 99.5% in consensus with Bacillus coagulans ATCC 7050.

ATLANGENE
SILLIKER
Food Safety & Quality Solutions

A l'attention de :

SAMI LABS LIMITED

Numéro de dossier : A08.12.02.07
Date de réception : 02/12/2008
Analyse demandée : SEQUENÇAGE 16S – 1500pb
Date de l'analyse : 04/12/2008

N° Atlangene	*Description Echantillon*	*Résultat Identification*	*Homologie*	*pb Query / pb Database*
A08.0579	SBC37-01 PROBIOTIC BACTERIA SLANT	*Bacillus coagulans*	99.5%	1472/1482

Commentaire : Banque de données utilisée : Applied Biosystems , NCBI.

Date d'émission :
Responsable Technique
AL CARDOU

(b) (6)

SILLIKER SAS – Bio Ouest – Ile de Nantes – 21, rue La Noue Bras de Fer – 44200 NANTES
Tél. 02 40 92 14 14 – Fax. 02 40 92 05 06 – E-mail : info@silliker.fr – Web : www.atlangene.com
SAS au capital de 1 333 000 euros – 303 434 591 RCS Pontoise

000052

A08.0579a_consensusSequence.fsta

>Consensus for specimen A08.0579a

GGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGTGCGGACCTTTTAAAAGCTTGCTTTTAAAAGGTTAGCGGCGG
ACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGATCGGGATAACGCCGGGAAACCGGGGCtAAtACCGGATAGTTTTT
TCCTCCGCATGGAGGAAAAAGGAAAGACGGCTTYKGCTGTCACTTACAGATGGGCCCGCGGCGCATTAGCTaGTTGGTGG
GGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAA
CTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAAGAAGGC
CTTCGGGTCGTAAAACTCTGTTGCCGGGGAAGAACAAGTGCCGTTCGAACAGGGCGGCGCCTTGACGGTACCCGGCCAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCG
CGCGCAGGCGGCTTCTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAGCGGTCATTGGAAACTGGGAGGCTTGAGT
GCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTC
TCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGC
CGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
AACCTTACCAGGTCTTGACATCCTCTGACCTCCCTGGAGACAGGGCCTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGC
ATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGACCTTAGTTGCCAGCAT
TCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATG
ACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCGAGACCGCGAGGTTAAGCCAATCCCAGAAAACCATT
CCCAGTTCGGATTGCAGGCTGCAACCCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTACG
GAGCCAGCCGCCGAAGGTGGGACAGATGATTGGGGTGAAGTC

10. APPENDIX IV

Whole Genome Sequencing of *Bacillus coagulans* MTCC 5856 (LactoSpore®)

Report Attached separately (pages 1 to 11)

Whole Genome Sequencing of Bacillus coagulans MTCC 5856 (LactoSpore®)

Genotypic
genomics simplified

Genotypic Project ID:

SO_3543

For

SAMI LABS LIMITED, BANGALORE

Study Conducted By:

For Genotypic Technology

Name: Dr. Gopalakrishna Ramaswamy Date: 2/1/2015 (b) (6) Signature

Study Coordinated By:

Name: Dr. Dinesh. V Date: 2/1/2015 (b) (6) Signature

Data/Report Analyzed and Approved by:

Name Dr. Chellappa Gopalakrishnan Date: 2/1/2015 (b) (6) Signature

GENOTYPIC TECHNOLOGY PVT. LTD. BANGALORE

Genotypic Technology [P] Ltd., #2/13, Balaji Complex, 80 feet road, R.M.V. 2nd Stage, Bangalore-560094, INDIA

Phone: +91 80 40538202/ 8213; Fax: +91 80 40538222

Website: www.genotypic.co.in, **E-mail:** ngs.analysis@genotypic.co.in

000055

Data Analysis

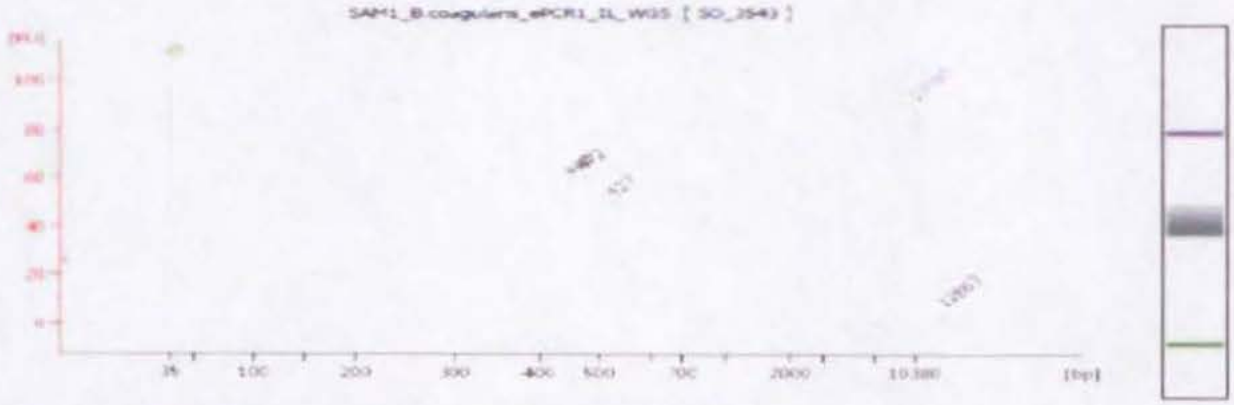
1.0: Objective of the project:

i. Alignment against existing reference sequence
ii. Presence of plasmid sequences
iii. Absence / presence of preselected set of genes /pathways defined by SAMI labs.

2.0: Sample details:
i. Samples received from SAMI Labs: 23 July 2014
ii. Sample shipment details: Sample received as a culture broth.

3.0: Sequencing platforms: Sequenced using Illumina MiSeq.

4.0: Data analysis tools: SeqQC_v2.2 [1], Bowtie2-2.1.0 [2], samtools-0.1.19 [3], Newbler v2.8 [4], PlasmidFinder1.2 [5], ResFinder2.1[6], RAST [7,8], KAAS web server [9], tRNAscan-SE-1.3.1[10,11], RNAmmer-1.2[12], ncbi-blast-2.2.26+[13], circos-0.64[14] and antiSMASH 2.0[15,16]



Overall Results for sample 1 : SAM1 B.coagulans ePCR1 IL WGS

Number of peaks found	4	Corr. Area :	706.8
Noise	0.2		

Peak table for sample 1 : SAM1 B.coagulans ePCR1 IL WGS

Peak		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1		35	125.00	5,411.3	Lower Marker
2		448	127.52	431.0	
3		472	154.96	503.5	
4		527	247.35	711.6	
5		10,380	75.00	10.9	Upper Marker
6		12,863	0.00	0.0	

Region table for sample 1 : SAM1 B.coagulans ePCR1 IL WGS

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
200	1,000	706.8	95	510	14.6	694.13	2,125.5	

Table **1.0: Bio-Analyzer profiles of libraries prepared for Illumina MiSeq sequencing:**

Comments: Illumina MiSeq library was optimal for sequencing.

(Refer "SO_3543_Library preparation report.pdf" for details)

000056

4.1: Paired End Sequencing Using Illumina Chemistry and analysis output:

Standard fragment libraries represent a collection of regions of 400-700 bp insert length obtained from randomly fragmented genomic DNA. Individual single-stranded inserts function as template molecules during sequencing. Two ends of such inserts are sequenced for a length of 300 nt each read in a 301 PE sequencing run. Illumina chemistry detects bases using a sequencing by synthesis approach where a DNA polymerase inserts fluorescent-tagged bases corresponding to the template molecules. This process is carried out in a massively parallel manner to enable the sequencing of millions of insert molecules simultaneously.

5.0: Whole Genome Sequencing workflow and analysis overview:

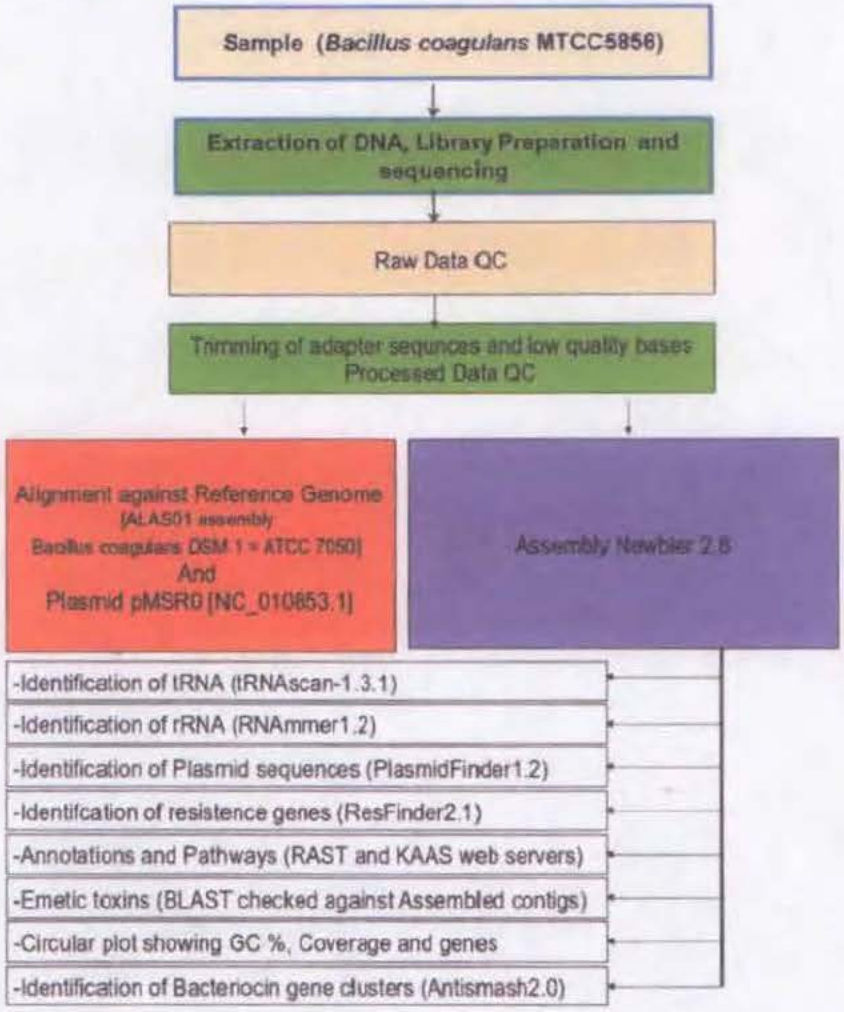


Figure.1.0: The workflow followed for sequencing and analysis of raw data.

5.1: Raw data processing and QC

A.The Illumina MiSeq paired end raw reads were quality checked using Genotypic Pvt. Ltd., proprietary tool SeqQC[1]. Illumina raw reads were processed by in-house script for Adapters and low quality bases trimming towards 3'-end.

Platform	Type of reads	Total number of raw reads	Total number of processed reads
Illumina MiSeq	Paired end (301bp max)	903999*2=1807998 **(542.39MB bases)**	893061*2=1786122 **(535.83 MB bases)**

Table2.0 : Statistics of Reads generated **Total Data Sequenced = 542.39 MB**

B.

GC content table:	GC %
Illumina MiSeq	46.74

Table.3.0: GC content table of sequenced processed data :

C.

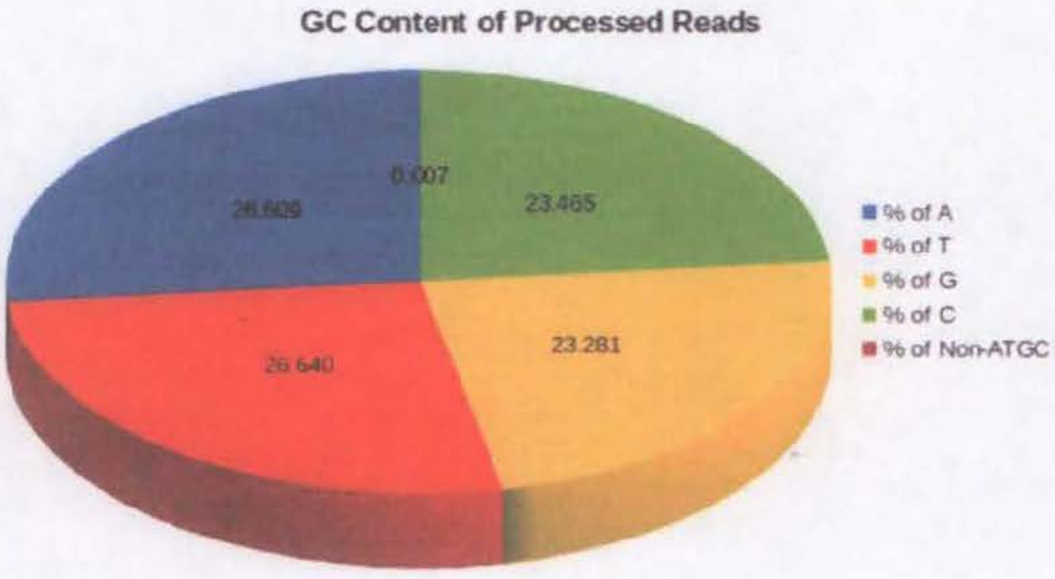


Figure.2.0: ATGC Composition of Illumina MiSeq processed data :

000058

5.2: Alignment against available reference genome:

The processed reads were aligned against the selected reference (**ALAS00000000/ALAS01000000**) genome (Bacillus coagulans DSM 1 = ATCC 7050) from NCBI. Bowtie2-2.1.0 program was used for the alignment and coverage statistics were generated using Genotypic Proprietary scripts. The reference contained 307 sequences with the total genome size of 3.018045 Mb. The genome was covered with an average read depth of ~112.

Alignment Statistics	SAM_1_B.Coagulans
Total Reads	**1786124**
Reads Aligned	**1194018**
% Reads Aligned	66.8497
Reference Sequence Length	**3018045**
Total Reference covered	**2599988**
%Total Reference covered	**86.1481**
%Total Reference covered with atleast 5X Read Depth	84.3293
%Total Reference covered with atleast 10X Read Depth	83.3594
%Total Reference covered with atleast 15X Read Depth	82.4982
%Total Reference covered with atleast 20X Read Depth	81.6431
Average Read Depth	**112.136**

Table.4.0: Alignment statistics with reference to *Bacillus coagulans* genome

(Refer "SO_3543_Alignment_Assembly_And_Annotation_Report.xls" for details.)

5.3: Assembly of sequenced genome data:

Assembly of Illumina MiSeq data was carried out with Newbler 2.8, generating 169 contigs with an N50 of 46.94Kb utilizing ~99% of reads. The scaffolds greater than 1kb were selected for further analysis.

Assembly tool	Newbler 2.8
Contigs Generated	169
Maximum Contig Length	1,99,094
Minimum Contig Length	1007
Average Contig Length	20,737.0 ± 26,907.7
Median Contig Length	8717
Total Contigs Length	35,04,549
Total Number of Non-ATGC Characters	0
Percentage of Non-ATGC Characters	0
Contigs >= 100 bp	169
Contigs >= 200 bp	169
Contigs >= 500 bp	169
Contigs >= 1 Kbp	169
Contigs >= 10 Kbp	81
Contigs >= 1 Mbp	0
N50 value	46941
GC Content	**Percentage**
Percent A	27.3022291884
Percent T	26.5370237369
Percent C	22.6668253176
Percent G	23.4939217571
Percent N	0

Table.5.0: Statistics of contigs generated

Total reads	1786122
Used reads	1765505
numAlignedReads	1771184
% of reads used	**99.16**

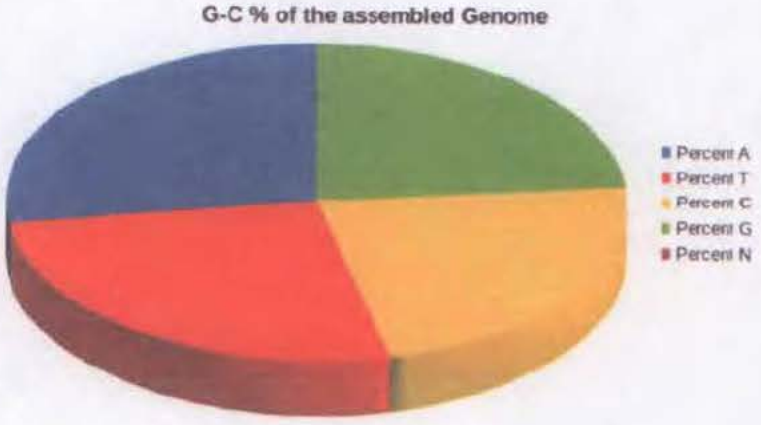


Figure.3.0: GC% of assembled Genome

5.4:Identification of Plasmid Sequences from the data:

The processed reads were aligned to the pMSR0 plasmid native to Bacillus coagulans DSM 1 = ATCC 7050. There were 0% reads aligned to the plasmid pMSR0 (NCBI accession ID: NC_010853.1). Presence of other plasmids was checked by PlasmidFinder 1.2 (http://cge.cbs.dtu.dk/services/PlasmidFinder/) and both enterobacteriaceae as well as gram – positive plasmid database were selected. Assembled sequences were given as input. "rep21" (Accession ID - AB254848.1) sequence was identified with 99% sequence identity, this contributes to 25% of the reference plasmid sequences. The sequence showing similarity was retrieved.

Plasmid_Check	
Plasmid	rep21
Identity	99.9
Query/HSP(Number of matches in query and subject sequence)	996/996
Contig ID of the assembled contig	contig00136
Position of the match in the assembled contig	391..1386
Information of the plasmid identified	rep(pSA1308)
Accession number of the subject sequence	AB254848.1

Table:6.0: Details of plasmid sequence identified

Refer "Contig00136_rep21_match_sequence.fa" for the fasta sequence.

(Refer "SO_3543_Plasmid_Check_alignment.txt" to view the alignment and "SO_3543_Plasmid_Check.xls")

5.5: Gene Annotation and Identification of Pathways:

The assembled sequences were uploaded to RAST server and annotated using Bacillus coagulans ATCC 7050 as reference. Entries involved in EPS Biosynthetic pathways was identified from the annotation column retrieved from the RAST server along with lipoteichoic acids. KAAS server was used for pathway identification with assembled sequences as input. Among the 98 contigs 3995 protein functions and 40 functional pathways have been predicted.

(Refer "SO_3543_Alignment_Assembly_And_Annotation_Report.xls" for details and refer "SO_3543_comparison_with_toxins_identified_from_Pathway_Report.xls" for details.)

5.6: Identification of resistance genes:

ResFinder2.1 (http://cge.cbs.dtu.dk/services/ResFinder/) was used to identify drug resistance genes in the assembled sequences. ResFinder2.1 checks for resistance genes for Aminoglycoside, Beta-lactam, Fluoroquinolone, Fosfomycin, Fusidic Acid, MLS - Macrolide-Lincosamide-StreptograminB, Nitroimidazole, Phenicol, Rifampicin, Sulphonamide, Tetracycline, Trimethoprim and Glycopeptide. 98% sequence identity and 60% coverage was used as threshold for the identification.
There were no resistance genes identified in the bacterial strain during analysis.

Resistance_against	Result
Aminoglycoside	No resistance genes found
Beta-lactam	No resistance genes found
Fluoroquinolone	No resistance genes found
Fosfomycin	No resistance genes found
Fusidic Acid	No resistance genes found
MLS - Macrolide-Lincosamide-StreptograminB	No resistance genes found
Nitroimidazole	No resistance genes found
Phenicol	No resistance genes found
Rifampicin	No resistance genes found
Sulphonamide	No resistance genes found
Tetracycline	No resistance genes found
Trimethoprim	No resistance genes found
Glycopeptide	No resistance genes found

Table: 8.0: Resistance genes found using ResFinder2.1

(Refer "SO_3543_Resistance_Check.xls" for details.)

5.7: Identification of tRNA and rRNA genes:

82 tRNA genes were identified using tRNAscan-SE-1.3.1 with "-s" option which uses default parameters and 10 rRNA genes were identified using RNAmmer-1.2 (http://www.cbs.dtu.dk/services/RNAmmer/).

number of sequences	169
number of bases tested (one strand)	3504549
number of bases tested (both strands)	7009098
number of predicted tRNA	82

Table:9.0: Summary of identified tRNA contigs

contig_id	attribute
contig00009	5s_rRNA
contig00074	5s_rRNA
contig00111	5s_rRNA
contig00017	5s_rRNA
contig00030	5s_rRNA
contig00036	5s_rRNA
contig00111	23s_rRNA
contig00120	23s_rRNA
contig00154	16s_rRNA
contig00156	16s_rRNA

Table.10.0: Attributes of rRNA contigs identified.

(Refer "SO_3543_tRNA_rRNA_Report.xls" for details.

5.8: Emetic toxin identification:

The genes were collected from literature shared. Nucleotide sequences were downloaded from NCBI. Nucleotide blast was done using emetic nucleotide sequences as database and assembled contigs as query. The blast did not yield any hit.

(Refer "SO_3543_Emetic_Toxin_Protein_Report.xls" for details.)

5.9: Circular plot of assembled contigs:

GC content and coverage for the contigs was calculated. Location of predicted and annotated genes were retrieved and plotted using the assembled contigs as the reference using Circos v0.64[14].

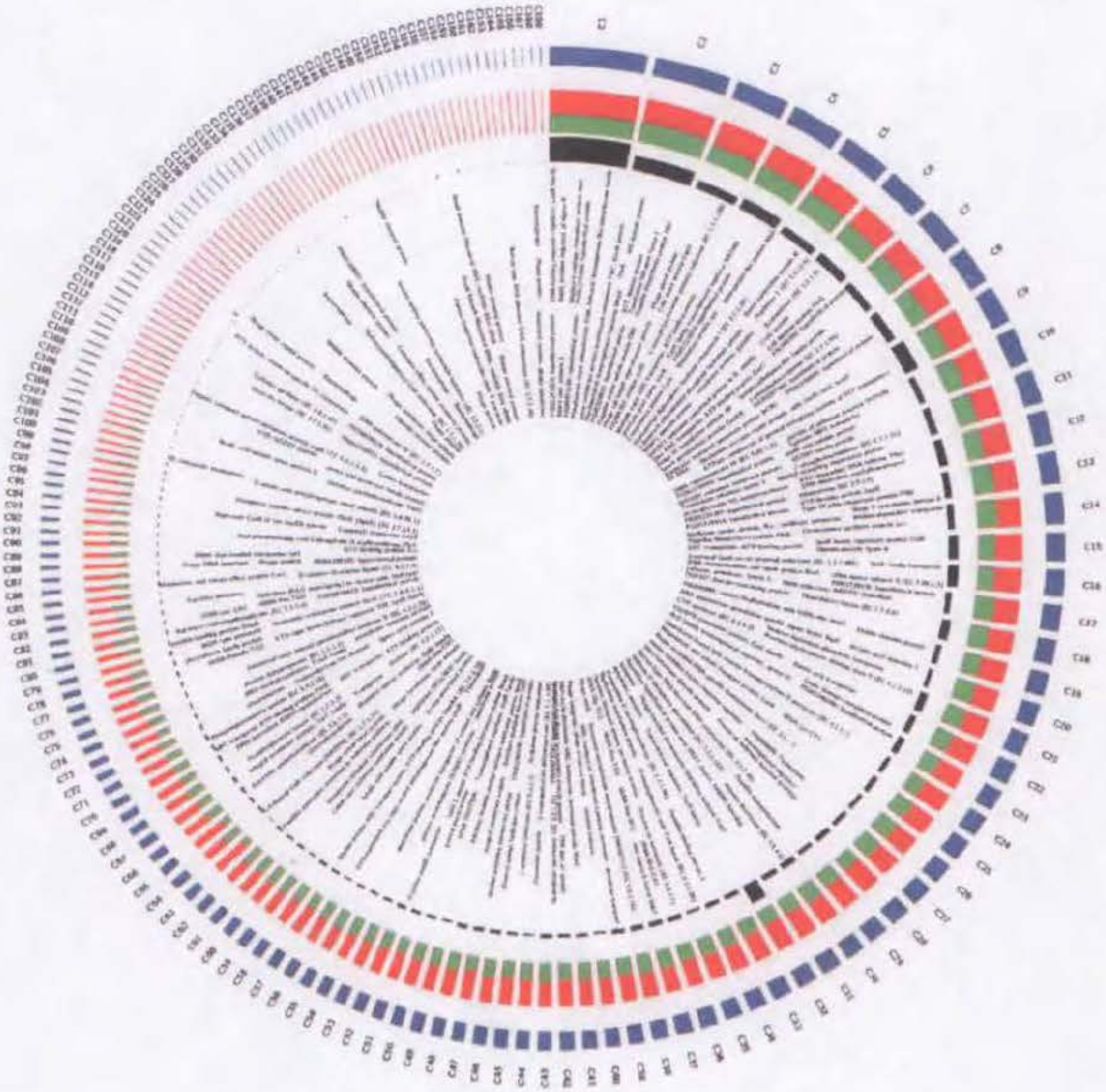


Figure.4.0: Circos plot

The outermost track contains the contig ID, followed by contigs in blue, AT% in red, GC% in green, coverage in black and the innermost track contains the genes identified based on their location on the assembled contigs.

Refer "SO_3543_Circos_Plot_Legend.pdf" and refer "SO_3543_Circos_Image.png" for raw image. "SO_3543_Circos_Plot.ppt" contains the ppt version of the pdf file.

5.10: Identification of Bacteriocin gene clusters:

The assembled scaffolds were given as input to antiSMASH 2.0[15,16]. antiSMASH identifies secondary metabolites from genome sequence. "Whole-genome PFAM analysis", "Subcluster Blast analysis", "Gene Cluster Blast analysis", "smCOG analysis for functional prediction and phylogenetic analysis of genes" options were selected. Further, the following 24 gene clusters were selected for analysis.

"polyketides (type I), polyketides (type II), polyketides (type III), heterocyst glycolipid-like polyketides, nonribosomal peptides, terpenes, lantibiotics, bacteriocins, beta-lactams, aminoglycosides / aminocyclitols, aminocoumarins, siderophores, ectoines, butyrolactones, indoles, nucleosides, phosphoglycolipids, melanins, oligosaccharides, furans, homoserine lactones, thiopeptides, phenazines, and others".

AntiSMASH identified 4 bacteriocin gene clusters based on the location and order of genes present in the assembled contigs.

Cluster	Type	From	To
The following clusters are from record contig00001:			
Cluster 1	Bacteriocin	1	5388
The following clusters are from record contig00015:			
Cluster 2	Bacteriocin	18583	28810
The following clusters are from record contig00154:			
Cluster 3	Bacteriocin	1	1157
The following clusters are from record contig00156:			
Cluster 4	Bacteriocin	1	1138

Table.12.0: Bacteriocin gene clusters identified

Refer "Bacteriocin_cluster_ReadMe.txt" for instructions on viewing the results and "contig00001.zip" for the results.

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SUBMISSION END