

ORIGINAL SUBMISSION

GRN 000660

**Notice to US Food and Drug Administration
that *Bacillus coagulans* GBI-30, 6086 is Generally
Recognized as Safe for use in Non-exempt Term
Infant Formula**

Submitted by the Notifier:

Ganeden Biotech, Inc.
5800 Landerbrook Drive, Suite 300
Mayfield Heights, Ohio 44124

Prepared by the Agent of the Notifier:

AIBMR Life Sciences, Inc.
2800 East Madison Street, Suite 202
Seattle, Washington 98112

July 11, 2016



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July 11, 2016

Antonia Mattia, PhD
Division Director
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
Department of Health and Human Services
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Mattia:

Following meetings with FDA on Tuesday, December 8, 2015 and Tuesday, June 28, 2016, pursuant to proposed regulation 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)), on behalf of Geneden Biotech, Inc. (the notifier), we are submitting, for FDA review, the enclosed notice that *Bacillus coagulans* GBI-30, 6086 is GRAS for use in non-exempt term infant formula.

As required, three copies of the notice are provided.

Should you have any questions or concerns regarding this notice, please contact me at 253-286-2888 or tim@aibmr.com.

Sincerely,

(b) (6)



Timothy S. Murbach, ND (agent of the notifier)
Senior Scientific & Regulatory Consultant
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1. GRAS Exemption Claim

Ganeden Biotech, Inc. (the notifier) has determined that *Bacillus coagulans* GBI-30, 6086 is Generally Recognized as Safe (GRAS) for its intended use in non-exempt term infant formula, consistent with section 201 (s) of the Federal Food, Drug and Cosmetic Act. The determination has been made based on scientific procedures. Therefore the use of *Bacillus coagulans* GBI-30, 6086 for its intended purpose is exempt from the requirement of pre-market approval.

(b) (6)

July, 2016

David Keller, DPM, MBA
Notifier

Date

1.1 Name and Address of the Notifier

Notifier

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1.2 Common or Usual Name

Bacillus coagulans GBI-30, 6086 (GanedenBC³⁰™)

1.3 Conditions of Use

Bacillus coagulans GBI-30, 6086 is intended to be added to non-exempt term infant formula at levels up to 2×10^8 CFUs per 100 mL infant formula as ready for consumption.

**B. coagulans* GBI-30, 6086 is not intended for use in any product that would require additional review by USDA.

1.4 Basis for GRAS determination

Scientific procedures are the basis for this GRAS determination. An independent and critical evaluation of the safety and GRAS status of the intended use of *B. coagulans* GBI-30, 6086 was conducted by an expert panel qualified by training and/or experience to evaluate the safety of food ingredients, including infant formula. The expert panel consisted of Judith Hauswirth, PhD (Hauswirth Consulting, Inc.; Former Branch Chief, Office of Pesticides Program, U.S. Environmental Protection Agency; Former Acting Director, Division of Drugs and Environmental Toxicology, U.S. Food and Drug Administration), John Thomas, PhD, DATS, FACT (Adjunct Professor, Department of Pharmacology & Toxicology, Indiana University School of Medicine), Ronald Kleinman, MD (Professor of Pediatrics, Massachusetts General Hospital for Children, Harvard Medical School), and John Endres, ND (Chief Scientific Officer, AIBMR Life Sciences, Inc.). The panel critically evaluated the scientific research available with regard to *B. coagulans* GBI-30, 6086 and collectively determined that the ingredient is GRAS for its intended use in infant formula. The expert panel believes that other qualified scientists reviewing the same publicly available data would come to the same conclusion.

1.5 Data/Information Availability Statement

The data and the information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of David Keller, DPM, MBA, Vice President of Scientific Operations, Ganeden Biotech, Inc., 5800 Landerbrook Drive, Suite 300, Mayfield Heights, Ohio 44124, Telephone: (440) 229-5204, email: keller@ganedenbiotech.com or will be sent to the FDA upon request.

2. Characterization

B. coagulans was first described in 1915 at the Iowa Agricultural Experiment Station, with regard to the coagulation of canned evaporated milk.¹ GanedenBC³⁰ is the trade name for a proprietary preparation of a *B. coagulans* strain designated as *B. coagulans* GBI-30, 6086. *B. coagulans* GBI-30, 6086 is a patented probiotic organism. It is L+ lactic acid producing, non-toxicogenic, and non-pathogenic. The organism is a gram-positive spore-forming rod that is aerobic to microaerophilic in nature. Its size is 0.9 µm x3.0 µm x5.0 µm. *B. coagulans* GBI-30, 6086 is manufactured as a pure cell mass consisting solely of *B. coagulans*. The pure cell mass is freeze-dried or spray-dried and blended with maltodextrin, microcrystalline cellulose, or sodium bicarbonate to achieve the desired concentration of the finished product.

B. coagulans GBI-30, 6086 was the subject of GRN 000399 submitted to FDA by Ganeden Biotech, Inc. and filed by the Agency on August 23, 2011. On July 31, 2012, GRN 000399 received the Agency's response letter with no questions pertaining to Ganeden's claim that *B. coagulans* GBI-30, 6086 is GRAS for its intended use as an ingredient in baked goods and baking mixes; beverages and beverage bases; breakfast cereals; chewing gum; coffee and tea; condiments and relishes; confections and frostings; dairy product analogs; fruit juices; frozen dairy desserts and mixes; fruit and water ices; gelatins, puddings, and fillings; grain products and pastas; hard candy; herbs, seeds, spices, seasonings, blends, extracts, and flavorings; jams and jellies; milk; milk products; nuts and nut products; plant protein products; processed fruits; processed vegetables and vegetable juices; snack foods; soft candy; soups and soup mixes; sugar; and sweet sauces, toppings, and syrups at a maximum level of approximately 2×10^9 colony forming units (CFU) per serving.

For the purpose of the toxicological studies described in GRN 000399 and discussed below, pure *B. coagulans* GBI-30, 6086 was used. The concentration varied slightly from batch to batch and, therefore, was reported for each study.

2.1 Identification

B. coagulans GBI-30, 6086 was identified to the genus level and then confirmed to be a pure strain of *B. coagulans* Hammer as reported in GRN 000399 under the subheading DNA Ribotyping Analysis on page 5, which is incorporated here by reference, where it is also noted that each lot of *B. coagulans* GBI-30, 6086 is subjected to 16S Ribosomal DNA base pair analysis as part of Ganeden's ongoing QC program.

To further characterize *B. coagulans* GBI-30, 6086, whole-genome sequencing was performed using the Illumina GAIIx platform at CRA-Genomics Research Centre (Piacenza, Italy) with a paired-end library.² This sequencing project has been deposited in DDBJ/EMBL/GenBank under the accession number

JPSK00000000. The genome consists of 3,458,655 base pairs with a GC% content of 46.38. From the total prediction of 3,373 genes, 3,197 coding sequences (CDS), 18 rRNAs, 82 tRNAs, 79 pseudogenes, 1 ncRNA, and 3 clustered regularly interspaced short palindromic region (CRISPR) arrays were identified. CDS genes were predicted to encode approximately 500 proteins related to energy metabolism and 80 related to dormancy and sporulation. Genes involved in adhesion (i.e., fibronectin- and mucus-binding proteins) and active metabolism (e.g., biotin biosynthesis) were also identified. The complete 16S rRNA gene sequence was searched against the ExTaxon database and was aligned with those of *B. coagulans* DSM 1^T, related taxa, and other representatives of the *Bacillus* genus using Clustal Omega^{3,4}. A phylogenetic tree was constructed using the number of differences algorithm as substitution model and neighbor-joining as tree inference method as implemented in MEGA v.6 software package.⁵ The 16S rRNA analysis demonstrated 99.92% identity compared to *B. coagulans* DSM 1^T, confirming that *B. coagulans* GBI-30, 6086 be allotted to species *B. coagulans*.

2.2 Other Ingredients

The other ingredients in the final product, *B. coagulans* GBI-30, 6086, consist of maltodextrin, microcrystalline cellulose, and/or sodium bicarbonate, which are used for bulking purposes, and milk powder and organic inulin, which can be used as diluents. Pursuant to 21 CFR 184.1444 (maltodextrin) and 184.1736 (sodium bicarbonate) these bulking agents are GRAS. The GRAS status of microcrystalline cellulose is included in the Select Committee on GRAS Substances (SCOGS) review of ethyl cellulose. Milk powder is GRAS due to common use pursuant to 21 CFR 182.1 and inulin is GRAS for use in infant term formulas pursuant to GRN Nos. 000477 and 000576. All corn-derived products used in the production of *Bacillus coagulans* GBI-30, 6086 are manufactured to meet European Union quality standards (EC 1829, EC 1830), which does not allow for GMO products.

3. Manufacturing and Production

The production process for *B. coagulans* GBI-30, 6086 consists of fermentation followed by recovery, followed by spray-drying or freeze-drying. The purpose of the recovery process (centrifugation) is to retrieve and concentrate *B. coagulans* post-fermentation.

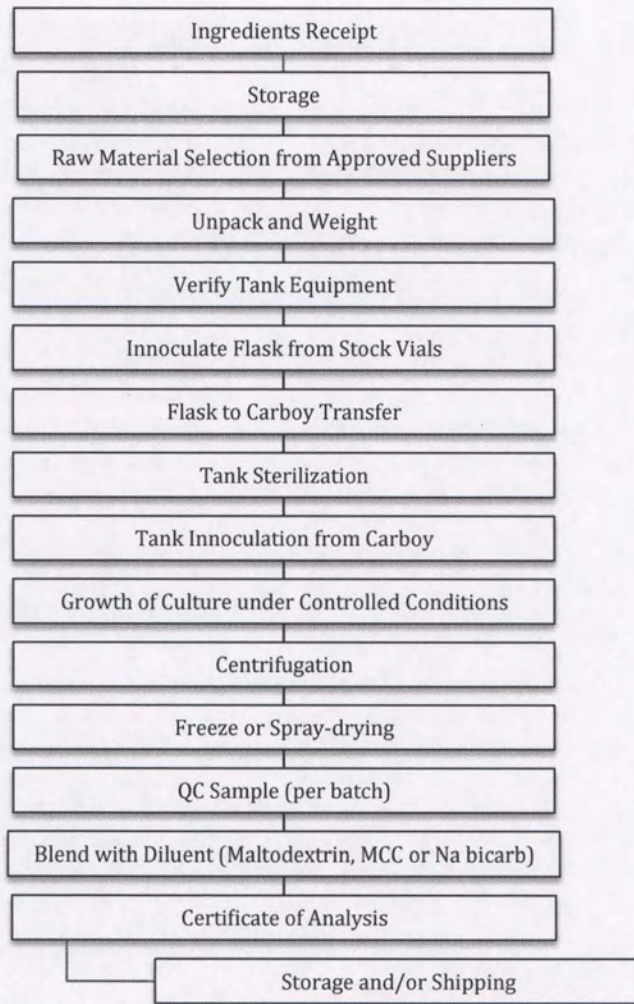


Figure 1. Manufacturing Flow Chart

3.1 Product Specifications

The product specifications for *B. coagulans* GBI-30, 6086, along with the specification methods, are listed in Table 1 below.

Table 1. Product specifications

Test Items	Specification	Method
Physical Characteristics		
Enumeration (CFU/g)	NLT 1.5×10^{10}	Ganeden TM-58
Appearance	Beige to light gray powder	Visual inspection
Moisture	NMT 6%	AOAC 926.08
Sieve Test	100% through 40 mesh	Internal Method
Sieve Test	98% through 80 mesh	Internal Method
Heavy Metals		
Arsenic	NMT 5 ppm	AOAC 984.27
Cadmium	NMT 5 ppm	AOAC 984.27
Lead	NMT 5 ppm	AOAC 984.27
Mercury	NMT 5 ppm	EPA 7471
Microbiological Tests		
Yeast and Mold	NMT 100 CFU/g	BAM CH. 18
Total Coliforms	NMT 10 CFU/g	BAM CH. 4
<i>E. coli</i>	None Detected	BAM CH. 4
<i>Staphylococci</i> (Coag. Pos.)	None Detected	AOAC 975.55
<i>Salmonella</i>	None Detected	AOAC 999.08
<i>P. aeruginosa</i>	None Detected	Internal Method

3.2 Non-sequential Lot Analysis

Production conformity and consistency of Ganeden's *B. coagulans* GBI-30, 6086 is tested in production lots. Five non-consecutive batch analyses, representing four years of production, examined by the Panel were reasonably consistent and met the product specifications for physical characteristics, heavy metals, and microbial analyses. Batch numbers reviewed and their manufacturing dates are as follows

1. Lot number (b) (6) July 25, 2012
2. Lot number [redacted] January 31, 2013
3. Lot number [redacted] January 16, 2014
4. Lot number [redacted] March 9, 2015
5. Lot number [redacted] March 10, 2016

3.3 Shelf-life Stability

A three-year shelf-life from the time of manufacture has been recommended as an appropriate expiration period for *B. coagulans* GBI-30, 6086. This recommendation is based upon accelerated and real-time shelf-life stability studies

performed to assess the stability of *B. coagulans* GBI-30, 6086. The accelerated test was conducted for three months under commercial packaging stored at 40°C and 75% relative humidity. The real-time shelf-life stability study was conducted for 36 months under commercial packaging stored at 25°C and 60% relative humidity. *B. coagulans* GBI-30, 6086 was stable and within specification throughout the tests with no significant changes occurring in the parameters assayed.

4. Self-limiting Levels of Use

There are no known inherent self-limiting levels of use.

5. Intended Use and Exposure

Bacillus coagulans GBI-30, 6086 is intended to be added to non-exempt term infant formula (including milk-based, soy-based, modified, hydrolyzed, and amino acid-based formula powders and liquids) at levels up to 2×10^8 CFUs per 100 mL infant formula as ready for consumption.

5.1 Estimated Daily Intake (EDI)—Exposure

Among healthy, full-term, formula-fed infants, highest energy consumption on a kcal/kg bw basis occurs in males 14–27 days old, who consume 121 and 143 kcal/kg bw/day at the 50th and 90th percentiles, respectively.⁶ In female infants, the highest energy consumption at the 50th percentile occurs in the same age group (14–27 days; 117 kcal/kg bw/day) while the highest consumption at the 90th percentile (143 kcal/kg bw/day) occurs in the 8–13 day old group (note, this is identical to 90th percentile consumption in 14–27 day old males and only slightly higher than 90th percentile consumption in 14–27 day old females at 136 kcal/kg bw/day). Although dated, Fomon's data is grossly consistent with more recent work^a based on the 2005–2012 National Health and Nutrition Examination Survey (NHANES) using the What We Eat In America (WWEIA) food category classification system⁷ and the Feeding Infants and Toddlers Study (FITS) 2008.⁸ According to the NHANES analysis by Grimes et al., mean calorie intake by infants 0–5.9 months is 612.5 ± 6.4 kcal/day while infants 6–11.9 months consume 847.3 ± 13.3 kcal/day.⁷ Based on the FITS 2008 analysis by Butte et al., mean and

^a As an illustration of the gross consistency of Fomon's data with that of the more recent estimates, considering the high 90th percentile intake of 143 kcal/kg bw/day by 14–27 day old males and 8–13 day old females and using U.S. Centers for Disease Control Infant Growth Chart Data, male body weight at the 90th percentile ranges from approximately 4.8 kg on day 15 to 5.2 kg on day 30 while female body weight at the 90th percentile is approximately 4.5 kg on day 15, total daily calorie intake would range from approximately 686.4–743.6 kcal in 14–27 day old males and approximately 643.5 kcal per day in 8–13 day old females. This appears grossly accurate compared to the Butte et al. estimate of 779 kcals/day in males and females 0–5.9 months old at the 90th percentile.

90th percentile calorie intake was 611 ± 6.9 and 779 kcal/day, respectively, in the 0–5 month age group and 854 ± 11.3 and 1183 kcal/day, respectively, in the 6–11 month age group.⁸ Fomon's data provides finer graduations, reporting break down of calorie intake by gender on a mg/kg basis at the 10th, 50th, and 90th percentiles from 8 days to 111 days old (approximately 3.5 months) divided into six age intervals (8–13, 14–27, 28–41, 42–55, 56–83, and 84–111 days).⁶ That Fomon's data spans only approximately 3.5 months versus data spanning 12 months provided by Grimes et al. and Butte et al. allows for a more conservative estimate as the percentage of calorie intake from infant formula declines with increasing age.⁷

Most cow milk-based formulas for term infants (i.e., “standard” infant formulas) provide 67 kcal/100 mL (20 kcal/fl oz.) when ready for consumption, but formula concentrates may be mixed to yield higher calorie densities up to 101 kcal/100 mL (30 kcal/fl oz.).⁹ Based on an infant formula comparison chart provided by Martinez and Ballew, 67 kcal/100 mL (20 kcal/fl oz.) is the recommended target for cow milk- soy protein-, and amino acid-based, as well as modified and extensively hydrolyzed, formulas for healthy term infants without special medical needs.⁹ Using the most conservative estimates of 143 kcals/kg bw/day (the 90th percentile in girls 8–13 days and boys 14–27 days) and 67 kcal/100 mL formula as ready to consume and assuming formula accounts for 100% of energy consumption, approximately 213.4 mL/kg bw/day of infant formula would be consumed. At the maximum addition level of 2×10^8 CFU *B. coagulans* GBI-30, 6086/100 mL of infant formula as ready for consumption a conservative high-end EDI is 4.27×10^8 CFU *B. coagulans* GBI-30, 6086/kg bw. Considering the lowest no-observed-effect-level (NOEL), which was 1.34×10^{11} CFU/kg bw/day (based on a concentration of 33,300 mg of the test article per kg of feed corresponding to 1948 mg/kg bw/day with a pure *B. coagulans* GBI-30, 6086 cell mass concentration of 6.88×10^{10} CFU/g), obtained from the one-year chronic repeated-dose oral toxicity study that was described in GRN 000399 on pages 13–16 and incorporated here by reference, this would result in a conservative margin of safety (calculated as the NOEL/EDI) of 314-fold and supports a conclusion that the expanded intended use of *B. coagulans* GBI-30, 6086 is reasonably certain to be safe. Because only very young, exclusively formula-fed infants would be expected to consume 100% of daily energy as infant formula and because the percent of daily energy intake for formula declines with increasing age, accounting for 65.4% and 47.1% of total daily energy intake in the 0–5.9 and 6–11.9 months groups, respectively,⁷ the true margin of safety is expected to be even greater.

Because *B. coagulans* GBI-30, 6086 is GRAS for use in a variety of foods as described in GRN 000399, it is possible that when solid food introduction to the infant is begun, exposure could occur from both infant formula as well as conventional food. Because energy intake from formula declines with increasing age and consumption of solid food and serving amounts of conventional foods are

expected to be much smaller than typical adult serving sizes and our EDI estimates are conservative, any potential exposure from conventional foods is expected to be largely substitutive to exposure from infant formula and remain well within an acceptable margin of safety.

6. Safety Assessment

6.1 Toxicological Studies on *Bacillus coagulans* GBI-30, 6086

The following toxicological studies were described in GRN 000399 under the subheading Toxicology Studies of the major heading Safety Assessment on pages 8–17, and are incorporated herein by reference:

- a bacterial reverse mutation assay conducted according to OECD 471¹⁰;
- an in vivo peripheral blood micronucleus assay in mice conducted according to OECD 474 and US FDA Redbook 2000 IV.C.1.d¹⁰;
- an in vitro chromosomal aberration assay conducted according to OECD 473¹⁰;
- an acute oral toxicity study in rats conducted according to OECD 423 and US FDA Redbook II—Draft, Acute oral Toxicity Tests (1993)¹⁰;
- a 90-day repeated-dose oral toxicity study in rats conducted according to OECD 408 and US FDA Redbook 2000 IV.C.4.a¹⁰;
- a one-year chronic repeated-dose oral toxicity study combined with a one-generation reproduction toxicity study in rats conducted according to OECD 452 and US FDA Redbook 2000 IV.C.5a and OECD 415 and US FDA Redbook 2000 IV.C.9a., respectively¹¹;
- an eye irritation study in rabbits conducted according to OECD 405¹⁰;
- a skin irritation study in rabbits conducted according to OECD 404.¹⁰

These studies showed no evidence of genotoxic potential, oral toxicity in acute, subchronic, and chronic studies in rats, reproductive toxicity, or eye and skin irritation in rabbits of *B. coagulans* GBI-30, 6086. Based on the results observed on the parameters evaluated in these studies, there was no indication of any toxicological effects on the organ systems evaluated, including those systems of particular susceptibility in infants (neurological, immune, skeletal, reproductive, and endocrine¹²).

Beyond the data provided by the genotoxicity, general oral toxicity, and reproductive performance evaluations, the postnatal observations in the F1 generation of the reproduction toxicity study is of some additional importance to the consideration of use of *B. coagulans* GBI-30, 6086 in infant formula. In this study, described in GRN 000399 on pages 13–17 and incorporated herein by reference, the F1 generation was observed, in accordance with the followed

guidelines, up to weaning (postnatal day 21).¹¹ Viability and lactation indices were similar among the control and treated groups. A statistically significant difference in mortality compared to controls appeared in the low-dose pups between postnatal days 7 and 14 and was not considered toxicologically relevant or related to administration of the test article due to the lack of a dose-response and the low magnitude of the difference. Clinically and biologically irrelevant but statistically significant minor increases in body weight and body weight gain in treated pup groups compared to controls were observed and were not related to dose (postnatal days 7-14-21). Therefore, these slight variations were not considered to be adverse or biologically significant outcomes. There were no statistically significant and/or clinically significant differences in clinical observations and postnatal development of the treated F1 pups compared to controls. All pups found dead were subject to necropsy. No test article-related alterations were observed on gross pathological examination of stillborns and pups found dead during the observation period. Necropsy observations were of normal physiological phenomena in fetuses/pups that occurred as spontaneous findings and with similar frequency among treated and control group pups and were recorded to determine whether dead newborns were live-born or stillborn. Pups that were cannibalized prior to discovery could not undergo necropsy; cannibalization occurred with similar frequency among treated and control groups and was not dose-related. Fetuses/pups were stillborn, found dead, and cannibalized, respectively, as follows: 2, 0, and 3 of 179 (controls); 4, 1, and 11 of 164 (low-dose); 2, 2, and 6 of 184 (mid-dose); and 0, 2, and 4 of 206 (high-dose). The NOEL for F1 offspring was considered as identical to that determined for the dams—3558 mg/kg bw/day (mean value) (equivalent to 2.45×10^{11} CFU/kg bw/day). However, due to the very low body weight of pups with respect to the dams, a very high, albeit transient, exposure to the test article can be presumed from the pups' sharing of the maternal feed when the pups started self-feeding (postnatal day 14).¹²

6.2 Neonatal Exposure to Bacteria

During the first months of life, barriers to bacterial colonization of the gut and translocation through the intestinal wall are immature compared to those found in childhood and older individuals. In addition the intestine is sterile at birth and is colonized rapidly as infants are exposed to microorganisms present in their environment (e.g., birth canal and maternal feces during non-cesarean birth, skin contact, breast milk, surrounding environment).^{13, 14} For example, human milk may contain up to 13 million CFU/mL of a variety of bacterial species.¹⁵ Thus, from an evolutionary perspective, infants are evolved to respond to oral exposure to a wide variety of pathogenic bacteria and other microorganisms without adverse outcomes, and in fact, this early exposure supports the development of a healthy gut barrier and host immune response.¹⁶ Furthermore, available data suggest that a stable intestinal microflora develops quickly in breastfed infants and that the introduction of solid food and/or infant formula (containing no added bacteria or

prebiotics) and weaning lead to a qualitative and quantitative change in this microflora (although the stability of the bifidobacterial component remains stable while populations of other species increase).¹⁷⁻¹⁹

Additionally, a number of other bacterial species have been determined GRAS for use in infant formula. In our searches of FDA's GRAS Notice Inventory Database, five GRAS notices pertaining to probiotic use in infant formula were located. These are summarized in the table below:

Table 2. FDA GRAS Notices for Probiotic use in Infant Formula

GRN No.	Organism	Intended Use	Addition Level*	EDI	Findings	Outcome / Date
49	<i>Bifidobacterium lactis</i> Bb12	Ingredient in milk-based infant formula by infants \geq 4 mos old	10^6 – 10^8 CFU/g	Not calculated	No adverse events were reported	FDA has no comments 3/19/2002
49	<i>Streptococcus thermophilus</i> Th4	Ingredient in milk-based infant formula by infants \geq 4 mos old	10^6 – 10^8 CFU/g	Not calculated	No adverse events were reported	FDA has no comments 3/19/2002
231	<i>Lactobacillus casei</i> subsp. <i>rhamnosus</i> GG	Ingredient in term infant formulas	10^8 CFU/g	10^8 – 10^{10} CFU/day	No adverse events were reported	FDA has no comments 5/29/2008
268	<i>Bifidobacterium longum</i> BB536	Ingredient in milk-based powdered follow-on infant formulas for term infants \geq 9 mos old	10^{10} CFU/g	12×10^{10} CFU/day for 9-12 month olds; 9.2×10^{10} CFU/day for 12-23 month olds	No evidence for a potential health hazard	FDA has no comments 7/8/2009
281	<i>Lactobacillus rhamnosus</i> HN001	Ingredient in milk-based powdered term infant formula and follow-on formula	10^8 CFU/g (1.35×10^9 CFU/100 mL ready to consume)	10^9 – 10^{10} CFU/day	No adverse events in studies relevant to human consumption were reported	FDA has no comments 8/31/2009
410	<i>Lactobacillus reuteri</i> DSM 17938	Ingredient in powdered whey-based term infant formula	10^6 – 10^8 CFU/g	a	No adverse events were reported	FDA has no comments 3/26/2012
454	<i>Bifidobacterium breve</i> M-16V	Ingredient in non-exempt powdered term infant formula containing partially hydrolyzed milk or soy proteins	10^8 CFU/g	9.9×10^9 CFU/day for one month old; 1.35×10^{10} CFU/day for 6 month old	No adverse events were reported	FDA has no comments 9/27/2013

GRN No.	Organism	Intended Use	Addition Level*	EDI	Findings	Outcome / Date
455	<i>Bifidobacterium breve</i> M-16V	Ingredient in exempt term powdered amino acid-based infant formulas	10 ⁸ CFU/g	9.9 x 10 ⁹ CFU/day for one month old; 1.35 x 10 ¹⁰ CFU/day for 6 month old	No adverse events were reported	FDA has no comments 9/30/2013
531	<i>Lactobacillus fermentum</i> CECT5716	Ingredient in powdered milk-based infant formulas	10 ⁷ CFU/g	2 x 10 ⁸ CFU/day	No adverse events were reported in animal and human studies; a single case report was noted for an adverse event in an immunocompromised individual	FDA has no comments 3/20/2013

*According to the reviewed GRNs, approximately 14 grams powdered formula is used to reconstitute 100 mL of ready to consume formula (FDA notes this amount varies slightly).

6.3 Studies on *Bacillus coagulans* in Infants

In addition to the lack of adverse effects of *B. coagulans* in human clinical trials in adults reported in GRN 000399 on page 19 under the subheading Additional Support of Safety and incorporated herein by reference, new literature searches were conducted in order to investigate results of studies conducted specifically in infants. A total of five randomized blinded placebo-controlled clinical trials in which infants were administered *B. coagulans* were located. None of the trials reported any treatment-related adverse effects. These trials are summarized with respect to safety aspects below.

The use of *B. coagulans* (miscalled by the authors of this study as *Lactobacillus sporogenes*^{b1, 20}) for prevention of necrotizing enterocolitis (NEC) in very low

^b As mentioned above, *Bacillus coagulans* was first described by Hammer in 1915. In 1933 Horowitz-Wlassowa and Nowotelnow isolated and described *Lactobacillus sporogenes*. Later, *L. sporogenes* was reclassified as *B. coagulans* based on shared characteristics, and this reclassification is well-known. In Bergey's Manual of Determinative Bacteriology, 8th edition, it is noted that spore-bearing rods producing lactic acid, facultative or aerobic and catalase positive are to be classified within the genus *Bacillus*. Unfortunately, the use of *L. sporogenes* still widely persists in the dietary supplement marketplace and also in the scientific literature.

birth weight infants (preterm infants <33 weeks gestational age or birth weight <1500 g who survived to feed enterally) was investigated.²¹ Infants of the treatment group were given a daily bolus of 3.5×10^8 CFU *B. coagulans* mixed in breast milk or mixed feeding (breast milk and formula) while the control group received breast milk or formula only (as the addition of treatment powder did not change the appearance of the test item, which was provided to subjects' caregivers under a blinded protocol). Treatment was initiated on the first feed and continued until discharge from the neonatal intensive care unit. Two hundred twenty-one infants completed the study and adverse outcomes (with the exception of feeding intolerance, which was significantly lower in the active treatment group) occurred with similar incidence in both groups. No adverse events attributable to the test item occurred during the study. While sepsis occurred with similar incidence in the active treatment and control groups (23.4 vs 26.4%, respectively, $p=0.613$), the pathogens were mostly catheter-related and *B. coagulans* was not found in any of the cultures.

One hundred twelve healthy term newborns were randomized to receive either 1×10^8 CFU *B. coagulans* (miscalled by the authors of this study as *L. sporogenes*—see footnote b) or placebo daily for 12 months in a clinical trial to investigate the potential preventive effect of the probiotic on acute diarrheal disease, a common cause of infant mortality in India.²² No adverse effects, withdrawals, or loss to follow up were reported, and while there was a trend for body weight at one year to be higher in the treated group, this wasn't statistically significant.

Another trial investigated the use of *B. coagulans* spores^c (miscalled by the authors of this study as *L. sporogenes* but also acknowledged in the paper's Introduction as *B. coagulans*—see also footnote b) versus placebo for acute watery diarrhea in 148 male subjects 6–24 months old.²³ Subjects received 1.2×10^8 CFU or placebo twice daily (2.4×10^8 daily) for up to five consecutive days or recovery, whichever occurred first. No adverse events or complications were observed during the treatment period or the 15-day no-treatment follow-up period.

In an Italian trial by La Rosa et. al. to investigate the effects of *B. coagulans* (misidentified by the authors of this study as *L. sporogenes*—see footnote b) with respect to antibiotic associated diarrhea, less than 10% of the 120 pediatric subjects were under the age of 2 years with the youngest subject being 4 months old; however, the number of subjects that were infants was not reported.²⁴ Subjects were administered a test item containing 5.5×10^8 CFU *B. coagulans* and 250 mg

^c Of the five clinical trials summarized here, this is the only one to specifically state spores were used; however, we are unaware of any application ever administering, or the availability of, *B. coagulans* in other but the spore form. And as it would be highly unusual for *B. coagulans* to be administered in the germinating form, we would expect that so doing would be cause for specific discussion in the study. Therefore, administration of spores is presumed in all studies reported.

of fructooligosaccharide (a prebiotic) or placebo for 10 days. The single adverse event reported occurred in the placebo group. Four subjects each of the placebo and treatment groups withdrew from the study for unidentified reasons.

No adverse events were reported in a published abstract summarizing a trial in which 19 infants (< 1 year of age; mean 5.5 months) with gastroesophageal reflux were given *B. coagulans* (dose not reported) for seven consecutive days.²⁵

6.4 Virulence and Toxicity

As shown in GRN 000399 under the subheading Additional Support of Safety on pages 18–19 and incorporated herein by reference, US FDA, Health Canada, and EFSA recognize *B. coagulans* as a non-pathogenic, non-toxicogenic organism. Furthermore, EFSA's granting of qualified presumption of safety (QPS) status to *B. coagulans* beginning in 2007 and maintained through the current 2015 publication, in addition to affirming non-pathogenicity, provides that the agency has no current safety concerns for the organism's intended uses in foods so long as the strain satisfies the qualifications of absence of toxigenic potential (e.g., toxins that can be produced in food and enterotoxins) and acquired antibiotic resistance genes.²⁶⁻³⁴

6.4.1 *Bacillus coagulans* GBI-30, 6086

An unpublished PCR assay, as reported in GRN 000399 on page 19 under the subheading Additional Support of Safety and incorporated herein by reference, showed no evidence that *B. coagulans* GBI-30, 6086 contains genes that encode for enterotoxins or hemolysins.

Since the submission of GRN 000399, additional work, based on the complete genomic sequence of *B. coagulans* GBI-30, 6086 has been carried out confirming and adding to this evidence.⁴ In this study, putative virulence factors (VF) were identified according the method of Chen et al.³⁵ using a local Protein-protein Basic Local Search Tool (BLASTP) with protein sequences derived from the *B. coagulans* GBI-30, 6086 annotated genes to query the Virulence Factor Database (VFDB). Basic Local Alignment Search Tool (BLAST) results were considered in the study if they demonstrated greater than 30% identity and 70% coverage.

Genes encoding for the hemolysin BL (HBL complex; hblC, hblD, hblA and hblB: AJ007794), the non-hemolytic enterotoxin NHE (NHE complex; nheA, nheB and nheC: Y19005), the enterotoxin T (bceT; D17312), the cytotoxin K (cytK; AJ277962), and the cereulide (cesA, cesH, cesP, cesT, cesB, cesC, cesD; DQ360825) were searched for on the genome of *B. coagulans* GBI-30, 6086 using BLASTX in order to further evaluate the potential for presence of enterotoxin genes. Additionally, searches were conducted to evaluate the presence of genes involved in the synthesis of lipopeptides as fengygin (fenA, AF023464; fenB, Appl Microbiol Biotechnol BACFENB; fenD, CAA09819; fenE, AF023465),

surfactins (srfAA, D13262; srfAB, AF233756; sfrAC, CAB12145), and lychenisin (lchAA, lchAB, lchAC; AJ005061).

B. coagulans GBI-30, 6086 was grown in the presence of arginine, histidine, lysine, ornithine, putrescine and tyrosine, and supernatants were obtained for HPLC determination and quantification of biogenic amine production according to the methods of Martuscelli et al., Tabanelli et al., and Tabanelli et al.,³⁶⁻³⁸ respectively. BLASTX was also searched against the genome of *B. coagulans* GBI-30, 6086 for the presence of genes related to biogenic amines.

The majority of the 200 putative VFs identified in the genome of *B. coagulans* GBI-30, 6086 using VFDB were defensive (multidrug transports and resistance proteins, a peroxidase, and an alkyl hydroperoxide reductase) or non-classical VFs related to normal cellular activities (Clusters of Orthologous Groups (COG) database (<http://www.ncbi.nlm.nih.gov/COG/>)). The majority of putative VFs were related to extracellular structures and may represent beneficial traits for adhesion to host cells or the sporulation mechanism (VFDB). Furthermore, possible VFs with relatively low similarity to known VFs can be detected by BLAST similarity. Therefore, the putative VFs identified in the genome of *B. coagulans* GBI-30, 6086 were not considered to be harmful.

B. coagulans GBI-30, 6086 does not carry any known enterotoxin or hemolysin genes as determined by the BLASTX analysis. Additionally, the genome of *B. coagulans* GBI-30, 6086 was determined not to contain genes encoding for the harmful cyclic lipopeptides—surfactins—or other lipopeptides with toxin activity, such as fengycin and lychenisin.

The HPLC analysis for biogenic amines demonstrated that *B. coagulans* GBI-30, 6086 did not produce tyramine, histamine, putrescine, cadaverine and phenylethylamine, and the polyamines, spermine and spermidine under the conditions of the assay. In the genomic analysis, with the exception of genes encoding for the metabolic pathways from arginine to putrescine and putrescine to spermidine, genes related to the production of biogenic amines were absent. Because the corresponding biogenic amines related to the identified pathways were not produced, it is presumed the genes were either not functional or not expressed at a sufficient level for production of detectable amounts.

6.4.2 *Bacillus coagulans* in general

The D(-)-lactate isomer of lactic acid is not produced in human metabolism, but human exposure can occur from bacterial production.¹⁴ Because human cells do not efficiently metabolize and excrete D(-)-lactate and infants have immature renal systems and decreased intestinal barrier function, newborns and neonates are at elevated risk of developing D-lactic acidosis if exposure occurs. *B. coagulans* does not produce D(-)-lactate.^{39, 40}

With the exception of the members of the *Bacillus cereus* group (e.g., *B. cereus*, *B. anthracis*, *B. thuringiensis*), the virulence of members of the *Bacillus* genus may be considered very low, and identified risk factors for *Bacillus* bacteremia include drug addiction, hemodialysis, and leukemia (all of which may contribute to immunosuppression).⁴¹ Based on two retrospective studies investigating *Bacillus* bacteremia, the presence of central venous catheters may increase risk of *Bacillus* bacteremia in immunocompromised patients.^{41, 42}

Of 1038 bacteremias occurring in patients of the University of Maryland Cancer Center, Baltimore between January 1978 and June 1986, 24 episodes of *Bacillus* bacteremia were documented, yet only a single case was documented as *B. coagulans* bacteremia.⁴² The classification of the *B. coagulans* bacteremia as a definite clinical infection or possible infection was not reported nor were the specific patient details (e.g., source of infection, signs, symptoms, outcomes). Based on the reported results and previous reports cited and discussed by the authors, no other cases of infection or possible infection from *B. coagulans* have been documented in the English literature dating to the 1950s, and the majority of cases of *Bacillus* bacteremias were due to *Bacillus cereus*. Thus, *B. coagulans* may be considered non-virulent.

6.5 Antibiotic Resistance

Phenotypic and genomic-sequencing analyses for antibiotic susceptibility/resistance of *B. coagulans* GBI-30, 6086 have been conducted with attention to transferable antibiotic resistance.⁴ Minimum inhibitory concentrations (MIC) of 15 antibiotics (ampicillin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, kanamycin, linezolid, neomycin, rifampicin, streptomycin, tetracycline, trimethoprim, vancomycin and virginiamycin) were determined according to standard protocol ISO 10932:2010 (IDF 223:2010). Putative antibiotic resistant genes were identified according to the method of McArthur et al.⁴³ using BLASTP with protein sequences derived from the *B. coagulans* GBI-30, 6086 annotated genes to query the Comprehensive Antibiotic Resistance Database (CARD) (<http://arpcard.mcmaster.ca>). ProphageFinder (<http://bioinformatics.uwp.edu/~phage/DOEResults.php>) was used to identify putative prophage sequences, and CRISPRFinder⁴⁴ was used to identify CRISPRs.

The results of the phenotypic analysis were compared to MIC cut-off values for *Bacillus* species as defined by EFSA,⁴⁵ and *B. coagulans* GBI-30, 6086 was found susceptible to 13 of the 15 antibiotics tested as follows: ampicillin (0.125 mg/L), chloramphenicol (0.25 mg/L), ciprofloxacin (0.03 mg/L), clindamycin (0.125 mg/L), erythromycin (0.125 mg/L), gentamicin (0.031 mg/L), linezolid (0.06 mg/L), neomycin (2 mg/L), rifampicin (0.016 mg/L), tetracycline (0.25 mg/L), trimethoprim (0.063 mg/L), vancomycin (0.063 mg/L) and virginiamycin (0.016 mg/L). *B. coagulans* GBI-30, 6086 was resistant to the two of the 15 antibiotics, kanamycin and streptomycin, with MICs greater than 1500 mg/L.

The majority, 57, of the 109 putative antibiotic resistance genes identified in the genomic analysis were transporters. The remaining are broken down as follows: genes modulating the antibiotic efflux (9); genes associated with resistance to daptomycin (6), polymyxin (1), streptothricin (1), penicillin (5), vancomycin (13), elfamycin (1), rifampin (2), sulphonamide (1), macrolides (as erythromycin, streptogramin and chloramphenicol) (2), fluoroquinolone (2), aminocoumarin (2), and trimethoprim (1); other genes related to a non-specified antibiotic resistance (4); and aminoglycosides (2).

Because the resistance exhibited in the phenotypic analysis was confined to aminoglycoside antibiotics, these two genes were further evaluated while it was assumed that the additional genes retrieved *in silico* were not functional, not expressed at sufficient levels, or only partially similar to known antibiotic resistance genes, and these genes were, therefore, not considered to represent harmful traits of *B. coagulans* GBI-30, 6086. The two identified aminoglycoside resistance genes were IE89_07115 and IE89_03650.

IE89_07115 encodes for the ribosomal protein S12 of subunit 30S, and the authors reported that the mechanism of aminoglycoside resistance “can be mediated by 16S rRNA methylases and methyltransferases or intrinsic mechanisms as chromosomal mutations.” Because no active rRNA methylases and only a single active 16S rRNA methyltransferase, lacking similarity to genes involved in aminoglycoside resistance, was retrieved by CARD in the *B. coagulans* GBI-30, 6086 genome, intrinsic resistance due to mutation was assumed. The genomic regions surrounding IE89_07115 lack mobile elements and the gene is co-localized with other genes that encode for essential chromosomal genetic information. Based on the above factors, the region was considered highly stable and the risk of gene transfer was considered low.

IE89_03650 encodes for an aminoglycoside 3-N-acetyltransferase as determined by the CARD query. Flanking regions lack mobile elements and show that IE89_03650 is co-localized with a gene encoding for a multidrug transporter MatE (an organization detectable in all available *B. coagulans* genomes cataloged in NCBI). Again, these factors indicate a very low risk of transferability of this gene.

Nine complete transposase-encoding genes were identified in the genome of *B. coagulans* GBI-30, 6086 using the NCBI Prokaryotic Genomes Annotation Pipeline; however, their flanking regions were not associated with antibiotic resistance or other putatively adverse genes. Two prophage-like elements were identified using ProphageFinder; however, these were considered defective and non-functional phages as the essential tail tape measure protein gene was not present, and *attL* and *attR* sites in both prophage regions were not identified. Three CRISPR arrays were identified. Two were located in adjacent contigs, suggesting the possibility that they are part of the same array, and five CRISPR-associated (cas) proteins near the CRISPR *locus* on the first contig were also identified. The

third CRISPR array was located in a contig with no proteins annotated. The presence of a CRISPR system represents a barrier to entry of foreign DNA, and may promote genome stability, including limiting of the spread of antibiotic resistance by counteracting multiple routes of horizontal gene transfer.

Based on the above analysis, it is concluded that *B. coagulans* GBI-30, 6086 is susceptible to the majority of major classes of antibiotics representing a wide range of determinants of resistance and currently does not harbor transferable antibiotic resistance genes.

6.6 Allergenicity

In addition to culture media containing soy and milk derived proteins, a version of *B. coagulans* GBI-30, 6086 is also manufactured on allergen-free culture media. Each version of *B. coagulans* GBI-30, 6086 is manufactured according to the processes, standard operating procedures, and specifications described in GRN 000399 and this current submission and labeled as appropriate and required according to allergens.

No reports of allergic reactions to *B. coagulans* were found in our searches of the scientific literature and government databases, including FDA's Safety Information and Adverse Event Reporting Program, MedWatch, and FDA's Recalls, Market Withdrawals, & Safety Alerts search engine (accessed June 6, 2016).

7. Justification for Use in Non-exempt Infant Formula

No studies reviewed for this submission, including those described in GRN 000399 and incorporated herein by reference and studies on infant populations, have revealed a toxicological adverse effect of *B. coagulans* GBI-30, 6086 on growth, which is a primary determinant of safety for infant formulas¹⁴ or any other toxicologically relevant outcome measures. Toxicological studies have also failed to identify any target organs, including those systems of particular susceptibility in infants. Furthermore, studies have established that *B. coagulans* GBI-30, 6086 is non-pathogenic, non-toxicogenic, and does not harbor transferable antibiotic resistance genes.

The scientific procedures forming the pivotal and corroborative data of the safety assessment (i.e., the technical element) for this intended use of *B. coagulans* GBI-30, 6086 in non-exempt term infant formula are published and available in the public domain. This published data and the consensus of the Expert Panel satisfy the common knowledge element of the GRAS standard and provide ample evidence that there is reasonable certainty that consumption of *B. coagulans* GBI-30, 6086 for its intended use in non-exempt term infant formula up to a maximum addition level of 2×10^8 CFU/100 mL is not harmful.

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