

Comprehensive GRAS Assessment

Of

Bifidobacterium longum subsp. *infantis*
Bi-26TM

For Usage Conditions for
General Recognition of Safety
for

DuPont Nutrition & Biosciences

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Part 1 – Signed statements and certification

December 15, 2020
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
HFS-200,
5001 Campus Drive
College Park, MD 20740-3835

Re: GRAS Notice – Exemption claim for the use of *Bifidobacterium longum* subsp. *infantis* Bi-26™

Dear Office of Food Additive Safety:

In accordance with the U.S. Food and Drug Administration's (FDA) Substances Generally Recognized as Safe; Final Rule, (81 FR 54959) relating to the filing of notices for substances that are considered to be generally recognized as safe (GRAS), please accept this claim and the attached information, submitted in triplicate, for that purpose as it relates to the use of *Bifidobacterium longum* subsp. *infantis* Bi-26™ (hereafter *B. infantis* Bi-26™). Specifically, we claim that the use of *B. infantis* Bi-26™ in non-exempt infant and toddler powdered formulas is exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act based on its determination that such uses are GRAS. This conclusion was made in concert with a panel of experts qualified by scientific training and experience.

No information used in this part of this notification is trade secret or confidential commercial information. In accordance with the requirements outlined in 21 CFR 170, Subpart E of the final rule, the following information is included with this exemption claim:

- (i) Name and address of the Notifier:
Jayne Chalfin Davies
Regulatory Affairs
DuPont Nutrition & Biosciences
200 Powder Mill Road
Wilmington, DE 19803
- (ii) Common or Usual Name of the Notified Substance:
Bifidobacterium longum subsp. *infantis* Bi-26™
- (iii) Intended Conditions of Use:
B. infantis Bi-26™ is manufactured in compliance with current Good Manufacturing Practice as specified in 21 CFR Parts 111 and 117. *B. infantis* Bi-26™ is intended to be added to non-exempt infant and toddler formulas at a level of 1×10^8 CFU/g to ensure at least 1×10^6 CFU/g serving throughout the 12 – 18 month life of the product. *B. infantis* Bi-26™ is intended to be added as a live microbial ingredient.
- (iv) Basis for the GRAS Determination:
This GRAS conclusion is based on scientific procedures (21 CFR 170.30 (a) and (b)) as discussed in the detailed description provided below.
- (v) Availability to FDA of Data and Information that are the Basis of Determination:

The data and information forming the basis for this GRAS determination and the exemption claim asserted herein are available for FDA review and copying during customary business hours at the following address, or will be sent to FDA either in an electronic format that is accessible for FDA evaluation or on paper, upon request:

Jayne Chalfin Davies
Regulatory Affairs
DuPont Nutrition & Biosciences
200 Powder Mill Road
Wilmington, DE 19803

610-864-7219
jayne.c.davies@dupont.com

- (vi) No data or information contained in parts 2 through 7 of this GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.
- (vii) If applicable and necessary, as required by §170.270 I authorize FDA to send any trade secrets to the Food Safety Inspection Service (FSIS) of the U. S. Department of Agriculture.
- (viii) I certify that, to the best of my knowledge, this GRAS notice for *B. infantis* Bi-26™ is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to me and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

Should you have any questions regarding the submission of this notice, please contact Jayne Davies of DuPont Nutrition & Health. Thank you for your prompt consideration of, and response to, this notice.

Sincerely,

Jayne Chalfin Davies
Regulatory Affairs
DuPont Nutrition & Biosciences

Part 2 – Identity, method of manufacture, specifications, and physical or technical effect

A. Identity:

1. **Name of the GRAS organisms:** *Bifidobacterium longum* subsp. *infantis* Bi-26™. The strain is also referred to in the DuPont Global Culture Collection (DGCC) as 11473 and has been deposited in the ATCC Culture Collection as SD 6720.
2. **Source of the GRAS organisms:** *B. infantis* Bi-26™ originated as a human isolate and was identified according to standard taxonomic guidelines.

a) **The taxonomic lineage is:**

Kingdom: Bacteria

Phylum: Actinobacteria

Class: Actinobacteridae

Order: Bifidobacteriales

Family: Bifidobacteriaceae

Genus: *Bifidobacterium*

Species: *longum*; Entrez Genome ID: 183. Number of genomes of this species sequenced: 80 (GOLD), 31 (NCBI).

Subspecies: *infantis*

Strain: Bi-26™

b) **Description of the GRAS organisms:**

Bifidobacteria are non-pathogenic, non-toxicogenic bacteria species that are normal inhabitants of the human gastro-intestinal tract. The genus *Bifidobacterium*, though phylogenetically unrelated to the other lactic acid bacteria (LAB), is often also included in the LAB category on the grounds of similarities in its biochemistry, physiology and ecology (Adams, 1999). *Bifidobacterium* spp. are anaerobic, non-spore forming Gram-positive, non-motile and catalase-negative with a range of cell morphologies including short curved rods, club-shaped rods and Y-shaped branches (Gomes and Malcata, 1999).

Bifidobacterium infantis is a Gram-positive, non-spore forming, anaerobic, irregularly shaped rod microorganism. It is typically found in the human infant gastrointestinal tract. Due to its homology with *Bifidobacterium longum*, it has been joined to this species and renamed *Bifidobacterium longum* subsp. *infantis* (Mattarelli P, 2008).

B. infantis Bi-26™ is a lyophilized bacteria fermentation product that is produced in accordance with cGMPs as provided for in 21 CFR Parts 111 and 117. *B. infantis* Bi-26™ was sourced from a human isolate and has been in commercial use in foods since 2014.

c) Genomic Analysis:

RiboPrinter® analysis

RiboPrinter® analysis targets the 5S, 16S, and 23S regions plus intragenic spacers regions within the genome. This automated Southern blot technology provides a genetic fingerprint that allows identification to the Genus and species level, but may also discriminate within a species. The Bi-26™ RiboPrinter® pattern matches those for *B. infantis* within the RiboPrinter® database. See attached example RiboPrinter® report in Appendix A.

16S rRNA Alignment

Additionally, the identity of Bi-26™ can be confirmed through 16S rRNA full sequence and alignment testing. Results for an example lot are included in Appendix B. The 1486 base pair sequence alignment of Bi-26™ matched the 16S rRNA region of *Bifidobacterium longum* subsp. *infantis* with 99.76% homology.

Genome summary

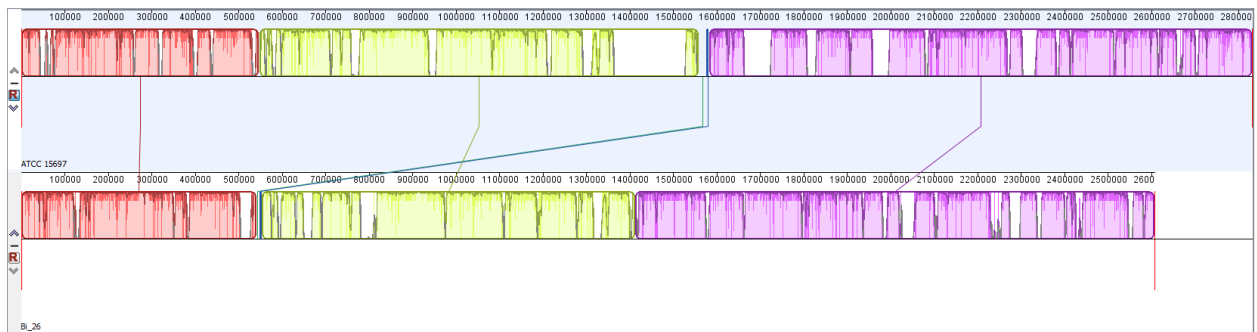
The type strain DNA sequences for *Bifidobacterium longum* subsp. *infantis* were obtained as references for the strain's taxonomic designations. High-quality full length 16S sequence of the DuPont manufactured strain was determined via PCR and Sanger sequencing. Phylogenetically based clustering was performed using the most closely related species of these strains. The high-quality, full length 16S rRNA sequence of *Bifidobacterium longum* subsp. *infantis* Bi-26™ was compared to the type strain and other neighboring sequences. In each case, the designation of species provided is validated by greater than 99% identity to the type strain sequence.

In addition to the full-length sequencing of the 16S rRNA gene of *B. infantis* Bi-26™ whole genome sequencing has been completed. Comparative genomics has been performed with the resulting genomic information. Comparison to other privately held and publicly available genomic references confirm the overall genetic synteny of *B. infantis* Bi-26™ to others of the same species.

Genetic comparison to public strains

A whole genome comparison of *B. infantis* Bi-26™ to the public type strain *B. infantis* ATCC 15697 appear in Figure 1. A taxonomic assessment of Bi-26™ using the 16S rRNA gene and several public databases was performed, as well as a metabolic comparison of Bi-26™ to type strain.

Figure 1. Mauve alignment of the *B. infantis* Bi-26™ strain to the *B. infantis* type strain ATCC 15697 genome.



Conclusions:

- Whole genome alignments show Bi-26™ to be most similar to strain ATCC 15697 (Figure 1).
- The 16S rRNA gene of Bi-26™ is most similar to *B. infantis* ATCC 15697 using BLAST in NCBI.

- There were no virulence factors that are suspect for actively producing hazardous compounds.
- A few genes related to prophage, plasmids, and transposases were located in the genome, but do not show evidence for transferring antibiotic resistance or virulence genes.
- No safety concerns were identified in Bi-26™ when compared to strain ATCC 15697.

d) Nutrient Metabolism:

Bi-26 Metabolism of Human Milk Oligosaccharide 2'-fucosyllactose

Zabel et al., (2019) investigated the ability of Bi-26 to utilize a human milk oligosaccharide (HMO) 2'-fucosyllactose (2'-FL) and the impact of resulting metabolite production. An analysis of the metabolism of HMO 2'-FL in *B. infantis* Bi-26 revealed a number of relevant upregulated gene clusters, including three novel ABC-type sugar transport clusters coinciding with metabolism of 2'-FL and its monomers glucose, fucose, and galactose. The formation of acetate, formate, and lactate was confirmed, indicating that the cell uses metabolites to produce higher levels of ATP. The authors concluded that metabolism of 2'-FL involves a more complex and diverse metabolite production compared to lactose.

B. Method of manufacture:

Danisco operates multiple DuPont Nutrition and Health culture production and blending facilities in the United States, Europe, and Asia. The Danisco USA, Inc. manufacturing site in Madison, Wisconsin is an entity of DuPont Nutrition and Health (hereafter DuPont). The site consists of two adjacent buildings (Culture Plant and Freeze Dry/Natural Extracts Plant) at 3322 and 3326 Agriculture Drive, Madison, Wisconsin, USA 53716. Other DuPont culture production/blending sites are located in Rochester, New York, France (Dange, Epernon, Sassenage, and Vinay), Germany (Niebüll), and China (Beijing). For a number of DuPont bacterial culture products, production may involve more than one manufacturing facility.

B. infantis Bi-26™ strain is produced in the Madison, WI facility, where fermentation occurs starting from the culture working seed through large scale fermentation. The bacteria are harvested and concentrated into pellet form and then freeze dried in a qualified facility. The milling and bulk packaging for *B. infantis* Bi-26™ take place in the Madison, WI facility.

The DuPont Madison plant manufacturing process, for production of cultures, is a batch type fermentation process where a blend of proteins, carbohydrate, and other vitamins and minerals are blended with water, sterilized, and then inoculated with the selected bacteria. Each fermentation product has a defined growth medium and fermentation growth conditions (pH, temperature).

B. infantis Bi-26™ is manufactured in compliance with the U.S. Food and Drug Administration's current Good Manufacturing Practice guidelines (21 CFR 111 and 21 CFR 117) in FDA regulated and inspected facilities. All ingredients utilized are food grade or approved for use by the FDA (Appendix C). The manufacturing process is summarized below.

Master Seed

The source organism used is *B. infantis* Bi-26™. The cultures are maintained in the culture bank of DuPont as frozen 1mL vials at -180°C. DuPont independently verifies the identity of each culture. Each seed lot in the culture bank is fully characterized to insure the identity of the seed strains. From the seed vials, DuPont produces concentrated starter for the industrial fermentation.

As the bacteria fermentation products produced by DuPont are destined to be either directly consumed or used as starter cultures for food fermentations such as yogurt manufacture, DuPont takes great care to ensure the quality of the product. These quality control processes begin with the identification, storage, and handling of the bacteria seed stocks.

Genus and species designation for each bacterial species have been determined by 16S rRNA testing. For identification on strain level, a specific DNA-fingerprinting technique is applied that ensures identity of the seed stocks. The fingerprinting technique is applied prior to preservation of every strain.

A Master Seed repository is maintained for each of the bacterial strains at the DuPont Global Culture Collection (DGCC) in Niebüll, Germany. The repository is a collection of purified, tested, and qualified Master Seed stocks derived from single strain isolates stored at -180°C in liquid nitrogen to maintain long term cell viability.

The microbiological quality of the Master Seeds is determined by testing for microbiological contamination at the DGCC. These identity and purity specifications are absolute acceptance criteria for the Master Seeds. If a Master Seed vial lot fails any of the required tests, the lot is placed on Quality Control (QC) hold to prohibit use and the lot is subsequently destroyed.

Working seed

Working seeds are prepared under controlled conditions from master seed stock maintaining effective acceptance criteria at DGCC. All Working Seeds are prepared under controlled conditions from Master Seed stock meeting established acceptance criteria and each new lot of Working Seeds is held in “quarantine” pending QC testing (strain identity and purity as described for the Master Seeds) and release. If the Working Seed vial lot fails any of the required tests, the lot is placed on QC hold and destroyed. Qualified, tested Working Seed stocks are stored at -80°C until used in production fermentation.

The use of tandem Master and Working seed inventories reduces the risk of genetic drift over time due to excessive sub-culturing of strains and ensures the integrity of the strain collection. All steps in the preparation of Master and Working seed are documented in a specified database, allowing traceability of every seed preparation down to each single batch of raw material used.

Fermentation process

The fermentation begins by withdrawing one of the working seed vials and scaling-up via a series of fermentations until a commercial size batch is complete. The fermentation starts off in a 100mL vessel, then transferred sequentially to a 6 L vessel, then to 300L vessel and finally to the largest (30,000 L) vessel where fermentation is completed.

As each organism produces organic acids during metabolism, an ammonium hydroxide base must be injected into the medium to maintain pH at the proper set point to for optimization of pH during growth.

The fermentation production process of each scale-up is a closed system with no product exposure from seed inoculation to cell harvest. Prior to each fermentation batch, all mixing tanks, heat exchangers, lines, fermenters and centrifuges are cleaned via automated clean-in-place systems. Systems are then either steamed or chemically sanitized prior to product contact.

At the DuPont Madison plant, there are two methods to measure growth in the fermenter. First, flow meters on the ammonium hydroxide feed lines to the fermenters measure the volume of base used to

maintain optimum growth pH of the culture. The base addition rate is proportional to the acid developed in the fermentation, which is proportional to cell growth rates. Second, the pH in the fermenter is monitored on digital display and on recording charts. By consulting these charts, the growth characteristic of a given fermentation can be determined.

Fermenters are normally cooled to stop the fermentation when the pH and base addition data indicate that the fermentation has entered stationary phase. Cooled fermentate is pumped through continuous flow centrifuges and the bacteria are concentrated. Cryoprotectant is added to cooled concentrate and the mixture is then pelletized by immersion of concentrate droplets in liquid nitrogen. These concentrate pellets are then freeze-dried in a qualified facility.

Milling process

The milling process takes place entirely in the DuPont Madison facility. The freeze-dried pellets are milled according to standard procedures utilizing a Fitzpatrick mill fitted with a mesh screen operating at 2000 rpm. Production batch records contain mill charge and appropriate operator sign-off.

Blending process

The blending process is performed in the Madison, WI facility under 21 CFR 111 and 21 CFR 117 cGMPs. Blending can occur by either blending in Marion and/or V-blender mixers, or by utilizing Intermediate Bulk Containers. The processes are slightly different, but are used interchangeably depending on available resources.

Milled pellets, along with approved excipients, are added to the blender. All ingredients added to the blender, both milled pellets and excipients, are documented on production batch record containing traceability information and appropriate operator sign off. Milling and ingredient addition is performed in a controlled environment.

The blender is allowed to mix for an established amount of time prior to packaging to ensure homogeneity. Product is dispensed out of blender and through metal detector prior to packaging.

Packaging

Bulk packaging of the product is carried out in a controlled environment within the DuPont Madison facility. The HVAC system consists of an air-handling unit with air-cooled direct expansion type condenser including ducted heater for reheating. Pressure relief dampers operate in conjunction with the fresh air intake system maintaining the whole area at a positive pressure to prevent contaminant infiltration to the packaging room. The area design conditions are as follows:

Dry Bulb Temperature	72° F
Relative Humidity	≤ 35% RH

A HEPA filter is used in the packaging room for high performance in these demanding operating conditions as the final filter for particulate removal.

Quality Systems

The DuPont Madison plant has fully implemented HACCP plans, Standard Operating Procedures and Quality Control programs to ensure the quality of each product. DuPont Madison has numerous certifications, including ISO FSSC 22000 food safety certification, ISO 9001 Quality Management System certification, and NSF Dietary Supplements cGMP certification.

A quality control laboratory is maintained on site. Quality control personnel are qualified by training and experience to test products and to release product based on specifications. In addition, a third-party laboratory with ISO 17025 certification, located in Madison WI, performs QC testing for DuPont under contract.

The Quality Control unit utilizes a SAP computer quality control system for the specification, quality control data entry and product release. No product can be released for use without acceptance by the Quality Control unit according to specified acceptance criteria.

Each bacteria fermentation product must meet specifications and must have a confirmation of identity (compared to the Master Seed) by 16S rDNA sequence analysis or RiboPrinter® analysis for release of the product. Microbiological testing is performed by trained QC microbiologists in the Madison plant laboratory and certified external laboratory using standard methods.

Cleaning and quality testing of the process rooms and equipment are under the control of Manufacturing and Quality Assurance, following the established SOPs. Fermentation rooms are isolated from the freeze-drying processes and access is controlled. Materials cannot enter the milling and blending process areas prior to cleaning, sanitation and subsequent surface testing for cleanliness via ATP testing. Room access is controlled by appropriate signage, and additional protective gowning must be worn in processing rooms where product is potentially exposed. Operator sign-off for clean, sanitation and testing is required on the lot batch ticket. Quality Assurance is responsible for review of completed batch tickets.

Process rooms are segregated from other manufacturing areas with appropriate closures. Room air quality is controlled via HEPA air filtration of incoming air and maintenance of positive pressure in the process rooms relative to adjacent processing areas. HEPA filtration operation is monitored for performance; air quality is monitored monthly by Quality Assurance. Operators may not bring materials into process areas where HEPA filtration is not functioning to specification. Operators sign-off on the lot batch ticket for temperature and humidity and record the temperature and humidity on the batch ticket. Quality Assurance is responsible for review of completed batch tickets.

Rooms and equipment used in manufacturing are approved for production only after cleaning, sanitization, and quality inspection. Prior to qualification of the process room for production, as specified in the appropriate SOP, the blending room is sprayed from ceiling to floor with 145-160°F water. All large equipment having any product contact surfaces is thoroughly scrubbed / foamed with a neutral detergent cleaner, rinsed with cold water, sanitized with an acid/iodine based sanitizer at 50 ppm and re-rinsed with cold water. The floor is sanitized with acid/iodine sanitizer at not less than 50 ppm.

Process rooms and equipment are tested by Quality Assurance following cleaning and sanitation. Cleaning is verified both visually and through sampling. Sampling is done through ATP and Microbiological swabs. The microbial swabs are tested for sanitation indicators (coliform, *E. coli*, and TPC). Room and equipment surfaces must be negative by test to qualify for use in production. All results are documented and signed off by Quality Assurance.

Batch records are maintained as per Standard Operating Procedures and are provided to Quality Assurance for each lot produced. Quality Assurance is responsible for batch ticket review.

A schematic overview of the fermentation and freeze dry processes are presented in Figures 2 and 3 below. The Hazard Analysis and Critical Control Point (HACCP) Flow Diagram is presented in Figure 4. Specifications are listed in Table 1.

Figure 2: Fermentation Process Diagram

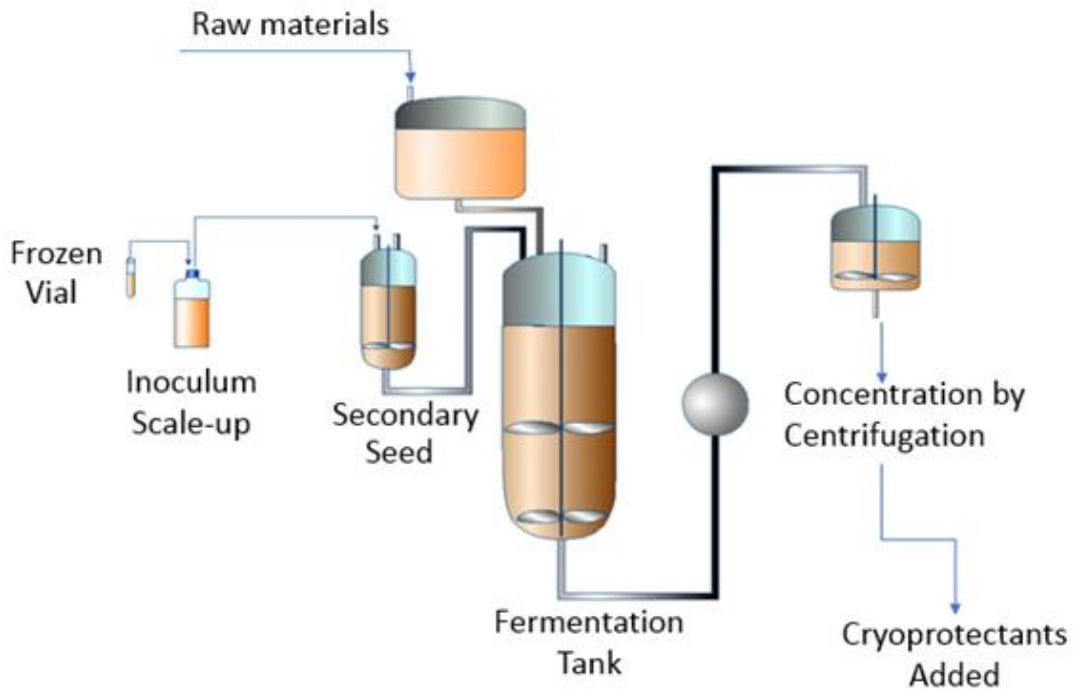


Figure 3: Freeze Drying and Milling Process Diagram

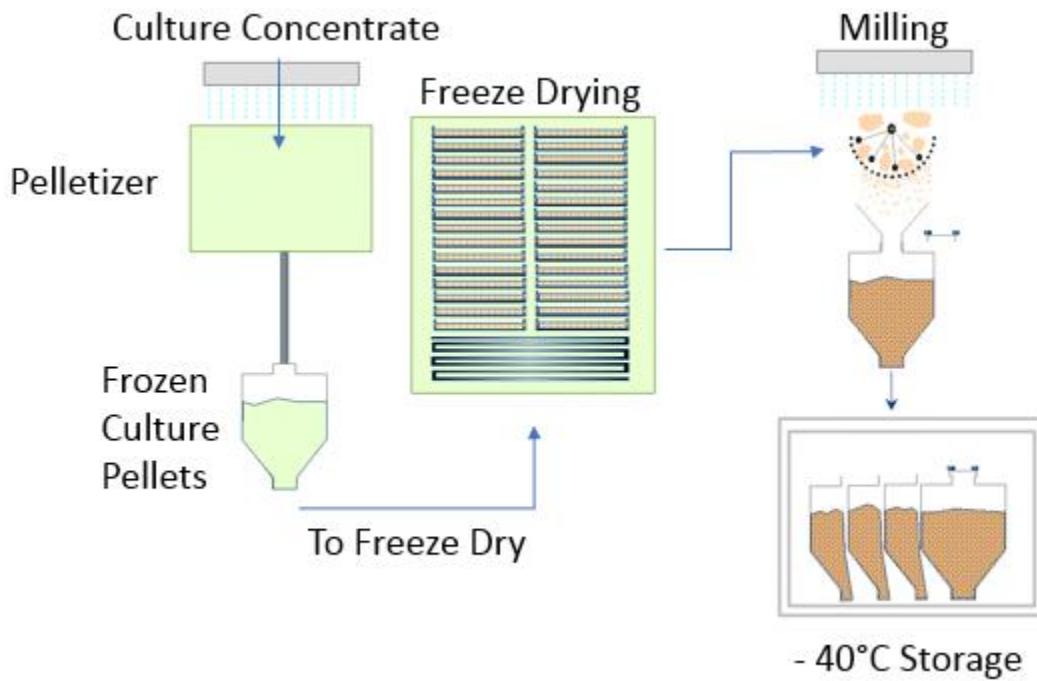
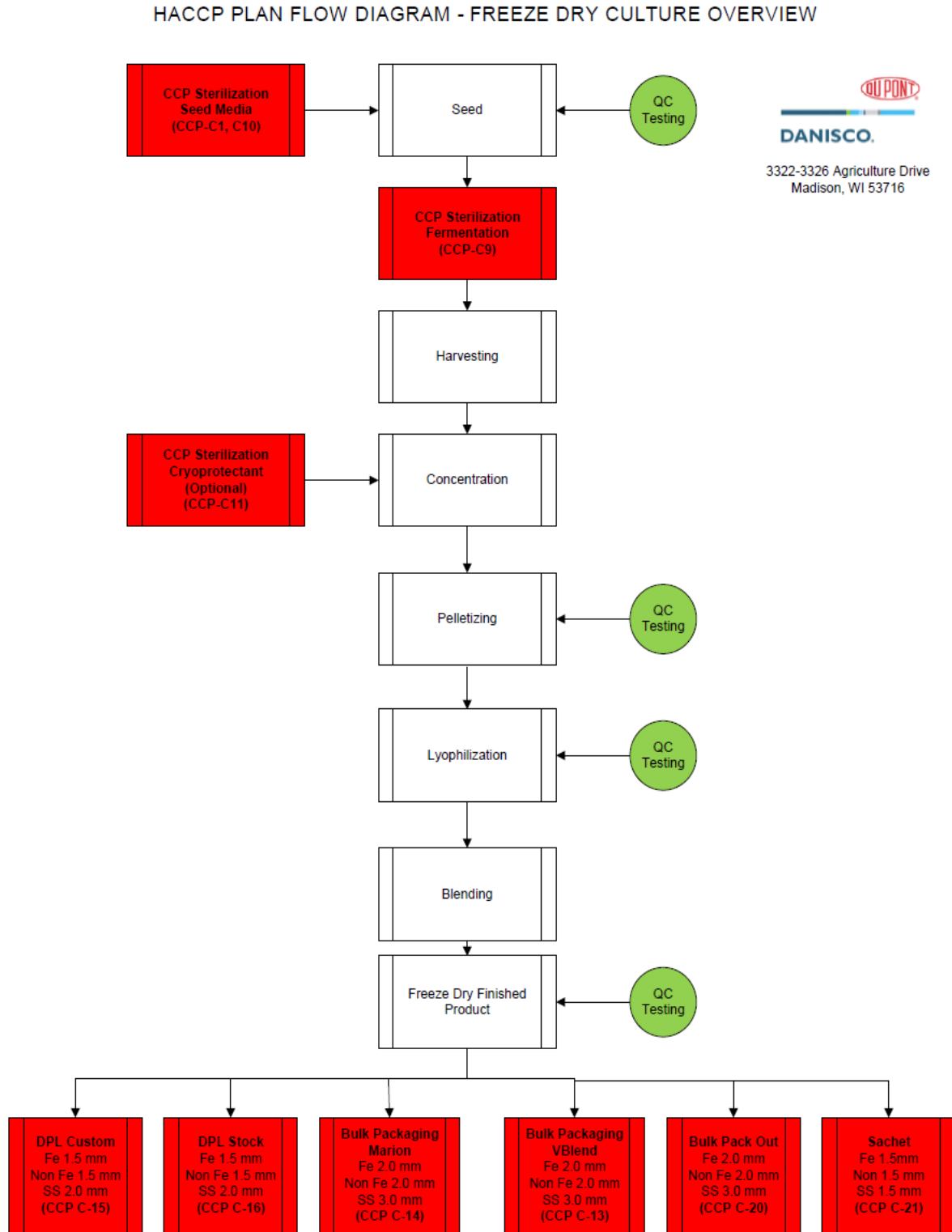


Figure 4: Manufacturing Flow Diagram



C. Product specifications

Table 1: Product Specifications

Parameter	Specification	Method
Form ¹	Freeze-dried powder	Visual inspection
Color ¹	White to cream	Visual inspection
Particle size ²	40 mesh	Fitzmill Screen
Viable cell Count ³	≥ 5.00 x10 ¹⁰ CFU/g	ISO 7889/IDF 117
Proximates ⁴		
Carbohydrate	42.6 g/100 g	Calculation
Protein	47.7 g/100 g	AOAC 992.23
Moisture	4.1 g/100 g	AOAC 926.08
Fats	1.4 g/100 g	AOAC 996.06
Fiber	0.0 g/100 g	AOAC 991.43
Sodium	426 mg/100 g	AOAC 984.27
Heavy metals ⁵		
Arsenic	< 1.0 ppm	AOAC 984.27
Lead	<0.5 ppm	AOAC 984.27
Cadmium	<0.2 ppm	AOAC 984.27
Mercury	<0.05 ppm	EPA 7471
Microbiological purity		
Non-Lactic Cell Count ³	< 5,000/g	ISO 13559
Enterococci (CFU/g) ³	< 100/g	SMEDP, 17th Ed.
Coliform (MPN) ³	Negative by test (< 10/g)	AOAC 966.24
<i>Escherichia coli</i> (MPN) ³	Negative by test (< 0.3/g)	AOAC 966.24
<i>Staphylococcus</i> (coagulase +) ³	Negative by test (< 10/g)	AOAC 975.55
<i>Salmonella</i> ³	Negative in 40 g	AOAC 2004.03
<i>Listeria</i> ³	Negative in 25 g	AOAC 999.06
Molds and Yeast ⁶	< 100 CFU/g	USP

1 Specification provided on Product Description sheet; 2 Internal Specification recorded in Batch Record; 3 Specification reported on Certificate of Analysis; 4 Specification provided on product Nutritional statement; 5 Specification provided on product Heavy Metal statement; 6 Internal Specification tested on bulk intermediate powder, not reported on COA

Batch analysis

Certificates of analysis of 4 non-consecutive batches of finished product are included in Appendix D. These indicate that the manufacturing process consistently meets product specifications and is not contaminated. A heavy metal statement is provided in Appendix E and a nutritional statement is provided in Appendix F. Please note, as stated in footnotes of Table 1, some analytes are evaluated separately as part of a routine surveillance testing program and are not reported on the certificates of analysis.

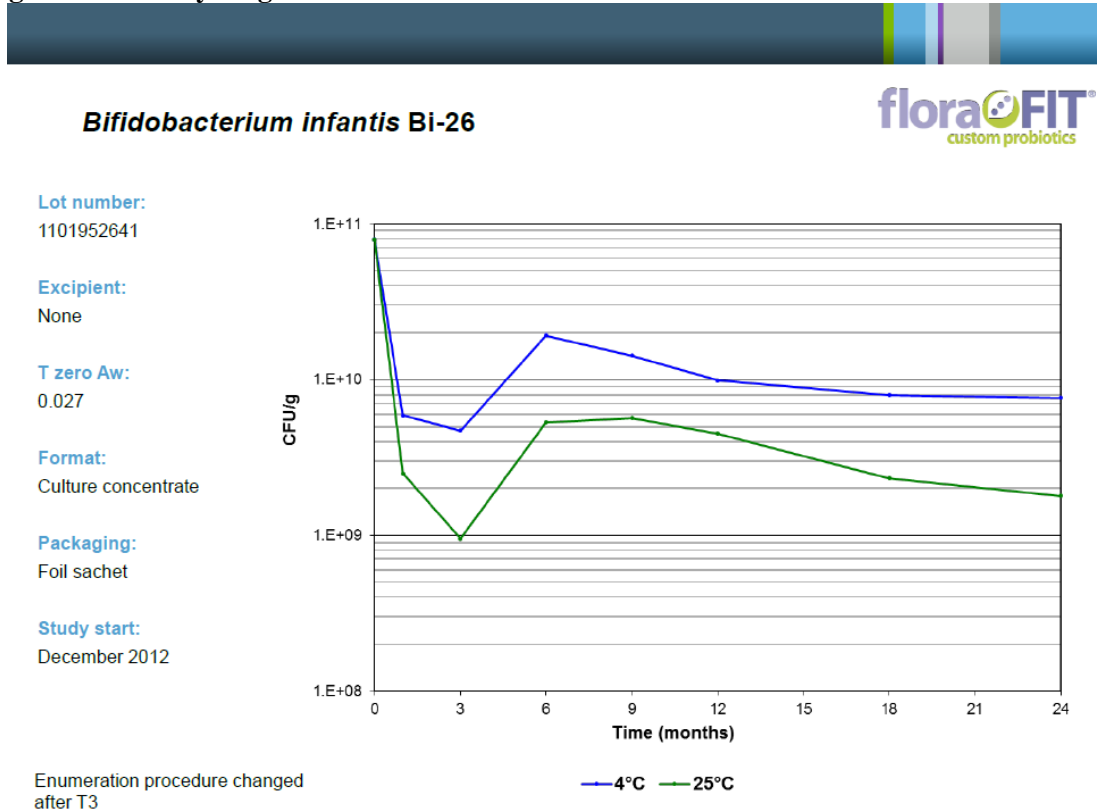
Enumeration

Enumeration is performed to obtain the total bacterial cell count per gram in a sample. The results of this test are used to determine if a sample has the required number of bacteria to qualify for an intermediate or final product.

Stability

The stability of *B. infantis* Bi-26™ was analyzed at refrigerated (4°C) and at room temperature (25°C) over a 24-month period by monitoring viable cell counts at regular intervals, See Figure 5. This is excellent stability for a live microorganism powder. This type of stability allows the deliverability of a target amount of live culture throughout shelf life of the final product.

Figure 5: Stability diagram



GMO Status

Since FDA does not have specific regulations nor a definition addressing genetically modified organisms, DuPont Nutrition & Health adheres to EC Directives on such and certifies that *B. infantis* Bi-26™ is conventional (non-GMO). The culture strain Bi-26™ has itself not been genetically

modified in accordance with to Directive 2001/18/EC and is neither subject to the labeling requirement of (EC) 1830/2003 nor to the authorization procedure of Regulation (EC) 1829/2003 (Appendix G).

Allergens

B. infantis Bi-26™, produced by DuPont as a single strain with no added excipients, does not contain allergens as determined by The Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA), including protein derived from milk, egg, fish, crustacean shellfish, tree nuts, wheat, peanuts, soybeans (Appendix G). Neither *Bifidobacteriae* or *B. infantis* are known to be food allergens {Castellazzi, 2013 #228} and there have been no reported allergenic responses in the *B. infantis* clinical studies.

Part 3 – Dietary exposure

A. Current dietary exposure of *B. infantis* Bi-26™

DuPont notes the current use of *B. infantis* Bi-26™ is in yogurt and other dairy products, soy products, beverages, chewing gum, confectionary, snacks and other foods. It is added to conventional foods at levels sufficient to ensure at least 1×10^{10} CFU/serving throughout the shelf life of the product.

B. Intended human food uses (estimated daily intake)

DuPont proposes the use of the *B. infantis* Bi-26™ in non-exempt infant formulas and toddler formulas (toddler formulas refer to products intended for infants and young children for 12 months of age and older) at a level of 1×10^8 CFU per g of powdered formula that is intended for consumption by term infants and toddlers from the time of birth through 2 years of age. This level of *B. infantis* Bi-26™ is intended to ensure a minimum concentration of 10^6 CFU/g throughout the 12-18 month shelf life of the infant formula powder. With normal dilution of the infant formula powder in water according to the label directions (i.e., 13.5 g/ 100 mL) and assuming an average daily formula intake of 800 milliliters, DuPont estimates that the daily intake of *B. infantis* Bi26™ microorganism would be approximately 10^9 - 10^{10} CFU per day. *B. infantis* Bi-26™ will serve as a live microbial ingredient. It is not intended for use by immune-compromised infants or toddlers.

Part 4 – Self-limiting levels of use

There is no self-limiting level of use for *B. infantis* Bi-26™, and use will be restricted to those food types that can support viability of *B. infantis* Bi-26™ throughout the shelf-life of the product.

Part 5 - Experience based on common use in food before 1958

The statutory basis for the GRAS conclusion for *B. infantis* Bi-26™ is not based on common use in food, but on scientific procedures (21 CFR 170.30 (a) and (b)).

Part 6 - Narrative

A. Review of safety information

1. History of consumption of *B. infantis* Bi-26™

B. infantis strain Bi-26™ is a human isolate, identified according to standard taxonomic guidelines. *B. infantis* Bi-26™ has been in commercial use since 2014 and is a lyophilized bacteria fermentation product that is produced in accordance with cGMP as provided for in 21 CFR 111 and 21 CFR 117. DuPont sells *B. infantis* Bi-26™ for inclusion in food and supplement products globally. *B. infantis* Bi-26™ has been sold worldwide, including in North America, China, South Africa, Middle East, Europe and Asia/Pacific countries. Over 23000 Kg of *B. infantis* Bi-26™ has been sold since 2012; DuPont affirms that no safety-related complaints related to *B. infantis* Bi-26™ have been received.

2. Regulatory History of *B. infantis*, related Bifidobacterium and other lactic acid producing bacteria

Bifidobacterium longum has been included as one of the many microorganisms intentionally added to food that should be regarded as safe based on the European Food Safety Authority's (EFSA) comprehensive assessment of safety. A list of qualifying microorganisms was compiled to represent those that meet the criteria of Qualified Presumption of Safety (QPS) and do not raise safety concerns (EFSA, 2007). This QPS list has been updated frequently and the *B. longum* listing is included. The most recent update indicates no safety concerns, and so the listing of *B. longum* remains in the 2018 QPS update (EFSA, 2018).

The Natural and Non-Prescription Health Product Directorate (NNHPD) of Canada has developed a Natural Health Product Ingredient Database to include approved substances for use in Natural Health Products. This database includes a list of approved bacteria for use as medicinal ingredients in Natural Health Products. Several Bifidobacteria species are approved for use as medicinal ingredients, including *B. longum* subsp. *infantis*. (Health Canada, 2015).

In the United States, several GRAS notifications for Bifidobacteria, including *B. longum* and *B. infantis*, for use in infant formula and conventional foods have been reviewed by FDA and filed with no questions. Below please find a summary of relevant notifications. Table 2 highlights GRAS notifications for live microorganism use in infant formula.

GRAS notification was submitted to FDA on *Bifidobacterium lactis* Bb12, and *Streptococcus thermophilus* Th4 (GRN 49) as ingredients in milk-based infant formula intended for consumption by infants four months and older, at GMP levels ($10^7 - 10^8$ CFU/g). FDA responded to GRN 49 that it had no questions (CFSAN, 2000).

GRAS notification (GRN 268) was submitted to FDA for the use of *Bifidobacterium longum* BB536 to be added to a variety of foods. The list of this variety of foods consisted of 11 subcategories of breads/baked goods, two subcategories of cereals, 17 subcategories of dairy products/dairy-based foods and dairy substitutes, three subcategories of fruit products, and 12 subcategories under miscellaneous. The maximum level of use proposed for this Bifidobacterium species for each

proposed usage was 1×10^{10} CFU per serving. The GRAS notification GRN 268 was reviewed by FDA and the Agency responded that it had no questions (CFSAN, 2009).

B. animalis subsp. *lactis* was submitted as a GRAS notification (GRN 377) to FDA. In this notification, it was proposed that the strain, *B. lactis* Bf-6 was to be added as an ingredient to foods. The list was devised on the principle of foods that can sustain viable *B. lactis* for the shelf life of the food, which included such dairy foods as fluid milk, yogurt, milk-based desserts and gravies, and cheese; dry seeds, nuts, and nut butters; grain products such as flour, yeast breads, quick breads, cakes, cookies, pies, pastries, crackers, pancakes, waffles, French toast, and crepes; pasta; cooked and ready-to-eat cereals, grain mixtures and meat substitutes; fruit and fruit beverages; dark-green vegetables, olives, pickles, relishes, and vegetable soups; salad dressings; sugars and sugar substitutes, syrups, honey, molasses, jellies, jams, preserves, gelatin desserts, ices and popsicles, candies, and chewing gum, and carbonated soft drinks, sport drinks, and thirst quenchers, energy drinks and water. The proposed maximum level of use is 10^{11} CFU/serving for each of these uses. FDA reviewed the GRAS notification GRN 377 and responded that it had no questions (CFSAN, 2011).

Four *B. animalis* subsp. *lactis* strains, HN019, Bi-07, B1-04, and B420 were submitted in a GRAS notification (GRN 445) by Danisco USA, Inc. to FDA. These four isolates are to be added as ingredients to foods such as ready-to-eat breakfast cereals, bars, cheese, milk drinks and milk products, bottled water and teas, fruit juices, fruit nectars, fruit-ades, and fruit drinks, chewing gum and confections. The proposed level of these isolates in each of these foods was 5×10^9 CFU/serving at consumption. FDA reviewed the GRAS notification GRN 445 and responded that it had no questions (CFSAN, 2012).

GRAS notification (GRN 453) was submitted to FDA for *Bifidobacterium breve* M-16V to be used as an ingredient in baked goods, breakfast cereals, fruit juices and nectars, fruit ices, vegetable juices, milk-based drinks and powders, dairy product analogues, frozen dairy desserts, processed cheese, imitation cheese, cheese spreads, butter-type products, snack foods, gelatin, puddings, fillings, meal replacement snack bars, nut and peanut spreads, hard and soft candies, cocoa-type powder, and condiment sauces. *B. breve* M-16V was proposed to be added to the selected food product for the general population and medical foods, with the levels of use not to exceed 5×10^9 CFU/serving in selected foods, and 10^8 CFU/g in medical foods. FDA reviewed the GRAS notification GRN 453 and responded that it had no questions (CFSAN, 2013a).

GRAS notification (GRN 454) was submitted to FDA for *Bifidobacterium breve* M-16V, for use in term infant formulas for healthy infants and exempt term infant formulas containing hydrolyzed proteins and/or amino acid mixtures. The level of use was up to 10^8 CFU/g of infant formula powder. FDA reviewed the GRAS notification and responded that it had no questions (CFSAN, 2013b).

GRAS notification (GRN 455) was submitted to FDA, for *Bifidobacterium breve* M-16V to be added to powdered amino acid-based exempt term infant formulas including powdered amino acid-based exempt term infant formulas for the management of allergies in infants at levels providing 10^8 CFU/g of infant formula powder. FDA reviewed the GRAS notification GRN 455 and responded that it had no questions (CFSAN, 2013c).

GRAS notification (GRN 572) was submitted to the U.S. Food and Drug Administration (FDA) for the use of lactase enzyme preparation produced by the submerged fermentation of a genetically modified strain of *B. bifidum* produced in *Bacillus licheniformis*. The lactase is used to catalyze the hydrolysis of lactase in the dairy industry for making lactose reduced/free products, e.g. milk, yogurt, cream and ice cream. The level of use is described as “levels not to exceed the minimum amount

necessary to achieve the intended technical effect". FDA reviewed the GRAS notification and responded that it had no questions (CFSAN, 2015a).

Danisco USA, Inc. submitted a similar GRAS notification (GRN 579) to FDA for the use of lactase enzyme preparation produced by the submerged fermentation of *Bacillus subtilis* carrying the lactase gene from *B. bifidum* encoding the wild-type truncated lactase enzyme for use in the production of galacto-oligosaccharide for infant formula and in the production of fresh dairy products. The FDA reviewed the GRAS notification and responded that it had no questions (CFSAN, 2015b).

GRAS notification (GRN) 758 was filed to demonstrate the safety of *Lactobacillus helveticus* R0052, *Bifidobacterium longum* subsp. *infantis* R0033, and *Bifidobacterium bifidum* R0071, both individually and in combination. On the basis of scientific procedures, these strains were determined to be safe as ingredients in powdered infant formula at 5×10^7 CFU/g. The notice was reviewed by the FDA and the Agency responded that it had no questions. (CFSAN, 2018).

GRAS notification (GRN) 813 was submitted to the FDA for use of *Bifidobacterium longum* BORI for use in powdered non-exempt infant formula up to 10^8 cfu/gram of powdered formula as well as wide range of conventional foods. On the basis of scientific procedures, these strains were determined to be safe as ingredients in powdered infant formula and included conventional foods. The notice was reviewed by the FDA and the Agency responded that it had no questions. (CFSAN, 2019a)

GRAS notification (GRN) 814 was submitted to the FDA for use of *Bifidobacterium bifidum* BGN4 for use in powdered non-exempt infant formula up to 10^8 cfu/gram of powdered formula as well as wide range of conventional foods. On the basis of scientific procedures, these strains were determined to be safe as ingredients in powdered infant formula and included conventional foods. The notice was reviewed by the FDA and the Agency responded that it had no questions. (CFSAN, 2019b)

Table 2: GRAS Notifications for Live Microorganism Use in Infant Formula

GRN Number	Species	Date
49	<i>Bifidobacterium lactis</i> Bb12	2002
231	<i>Lactobacillus rhamnosus</i> GG	2008
268	<i>Bifidobacterium longum</i> BB536	2009
281	<i>Lactobacillus rhamnosus</i> HN001	2009
410	<i>Lactobacillus reuteri</i> DSM 17938	2012
454	<i>Bifidobacterium breve</i> M-16V	2013
455	<i>Bifidobacterium breve</i> M-16V	2013
531	<i>Lactobacillus fermentum</i> CECT 5716	2015
758	<i>Lactobacillus helveticus</i> R0052 <i>Bifidobacterium longum</i> subsp <i>infantis</i> R0033 <i>Bifidobacterium bifidum</i> R0071	2018

3. Safety of *B. infantis*, Bifidobacterium genus, and lactic acid bacteria

a) *Bifidobacterium longum* subsp. *infantis* strain Bi-26™

Acute toxicity

Bifidobacterium longum subsp. *infantis* Bi-26™ was administered by gavage to five fasted female Crl:CD(SD) rats at a dose of 5000 mg/kg, which corresponded to an overall dose of 1.94×10^{12} CFU/kg body weight consistent with OECD 425. The rats were then observed for mortality, body weight effects, and clinical signs for 15 days after dosing. The rats were necropsied to detect grossly observable evidence of organ or tissue damage. There was no incidence of mortality, clinical abnormalities, or overall body weight losses. No gross lesions were reported at necropsy. It was concluded that under the conditions of this study, *B. infantis* Bi-26™ was not considered acutely toxic via the oral route of exposure in female rats. (Appendix H)

Antibiotic resistance

Antimicrobial resistance in bacteria can be mediated by many different mechanisms that range from unknown and non-specific to fully understood and well-studied. To address the question of transferability of antibiotic resistance, it is best to define the two types of resistance, intrinsic and acquired. Intrinsic resistance reflects an organism's ability to thrive in the presence of an antimicrobial agent, is not horizontally transferable, and is typical of the strains of a given species (Mathur and Singh, 2005). Acquired resistance occurs when a strain is resistant to a drug to which the species is typically sensitive and may be mediated by mutation of indigenous genes or by added genes (EFSA, 2012).

The primary concern of acquired resistance is not the acquisition of a gene or mutation that provides resistance, but the ability of that resistance to be horizontally transferred. Therefore, the focus has been on acquired resistance genes with the belief that they present a greater risk of transfer of resistance via horizontal gene transfer within and between species (Mathur and Singh, 2005). Bacteria have been reported to have both intrinsic and acquired resistances to many classes of antibiotics, only some of which are known to be transferable (Nawaz et al., 2011; Zhang et al., 2011).

There are three identified mechanisms of horizontal gene transfer (HGT) in bacteria; natural transformation, conjugation and transduction. Because not all species have these abilities, strain level differences need to be evaluated to determine if HGT is possible (Marshall et al., 2009; Ouoba et al., 2008). Three types of HGT were evaluated in this investigation, conjugative plasmids, transposases, and prophage/bacteriophage elements. Antibiotic resistance has been previously documented to be transferable on plasmids, transposases and phage (Aires et al., 2007; Colomer-Lluch et al., 2011; Marshall et al., 2009; Wang et al., 2006). Therefore, the highest risk of an antibiotic gene being mobilized to another strain/species involves these mechanisms of HGT, all of which have previously been reported in LAB in both *in vitro* and *in vivo* studies (Mathur and Singh, 2005).

Type of analysis conducted:

In each case, a whole genome sequence of the manufactured strain was obtained and analysed for the mechanisms of HGT by comparison to known drug resistance markers. When the mechanism of resistance was well documented and genomically located in the sequence, an evaluation of the flanking regions and the sequence identity was conducted. When a mechanism of resistance was not well understood, examination of all the known HGT mechanisms in that strain was completed to rule out a possibility of a resistance gene located in the vicinity. Note that not all drug resistances were evaluated. Only the genes responsible for the drug resistance over the EFSA breakpoint for clinically relevant antibiotics were investigated.

Analysis of *B. infantis* Bi-26™ (DGCC 11473):

An antibiogram of *B. infantis* Bi-26™ (DGCC 11473) was established using the ISO 10932 IDF223 method and VetMIC Lact-1 and 2 micro-dilution plates that included all antibiotics recommended by the FEEDAP. Recorded Minimum Inhibitory Concentrations (MICs) are displayed in Table 2. MIC values are below or equal to the Microbial Break Points (MBPs) defined for *Bifidobacterium* (EFSA, 2012). According to these results, Bi-26™ (DGCC 11473) does not bear acquired antibiotic resistance.

Table 3: Antibiogram of *Bifidobacterium infantis* Bi-26™

APPENDIX : Antibiotic Susceptibility Profile										
Method used : ISO 10932 IDF 223 with VetMIC Lact 1 and 2 microdilution plates										
	Gentamycin	Kanamycin	Streptomycin	Tetracycline	Erythromycin	Clindamycin	Chloramphenicol	Ampicillin	Vancomycin	Virginamycin*
	Gm	Km	Sm	Tc	Em	Cl	Ch	Amp	Va	Vi*
	MIC µg/ml									
DGCC 11473	Max.	Max.	Max.	Max.	Max.	Max.	Max.	Max.	Max.	Max.
<i>Bifidobacterium longum</i> subsp. <i>infantis</i>	8	128	4	2	0,25	0,25	2	0,25	0,5	0,25
MBP for <i>Bifidobacterium</i> **	64	NR***	128	8	1	1	4	2	2	1

* Virginamycin is no more included in the FEEDAP recommended list of antibiotics (june 2012) **EFSA Journal 2012;10(6):2740
NR***: not required

Virulence factors

The genome of *Bifidobacterium longum* subsp. *infantis* Bi-26™ was analyzed for bacteriocins, toxin genes, and genes associated with hemolysin production. First, the “Virulence, Disease and Defense” subsystem feature in RAST was mined. The annotations of the genome were mined for key words using the Geneious 6.1.8 viewer. Suspect genes were confirmed using BLAST protein (blastp) in NCBI. Finally, local searches were performed using Geneious 11.0.4 with the custom Basic Local Alignment Search Tool (BLAST) function. The following different databases were used:

- **BAGELdb.** The database for bacteriocins (<http://bagel.molgenrug.nl/>).
- **NCBI_bacteriocindb.** 138,176 proteins that are a result from a “bacteriocin” search *Gene* in National Center for Biotechnology Information (NCBI).
- **T3db.** A collection of toxin genes from the Toxin and Toxin-Target Database (<http://www.t3db.ca/>).
- **DBETHdb.** A collection of 229 bacterial endotoxins from 26 pathogenic bacteria (<http://www.hpppi.iicb.res.in/btox/>).
- **Pioneer_toxin_2016db.** A collection of 7,639 toxin protein sequences from an internal database at DuPont Pioneer.

The protein sequences of Bi-26™ annotations were compared to all these databases. As noted in the guidelines from European Food Safety Authority in regards to allergen presence, results that match at least 35% of sequence identities in a sliding 80 amino acid window were considered suspect and analysed further. Searches from the various collections were refined based on target, as the searches can broadly incorporate elements that are not related to the query (for example, if “bacteriocin” is in the title of the reference organism). Suspect proteins were assessed using *blastp* and UniProt (www.uniprot.org).

Summary:

- No bacteriocins were identified by database searches or in the literature.
- *mazF* and *mazG* genes and antitoxin gene were located, but both are part of the type II *mazEF* toxin-antitoxin system, which is not harmful to hosts.
- No additional toxins were located by database search.
- Five genes that are associated with hemolysin were identified, but were shown to have transport functions only and not to produce the hemolysin protein.

Results:

Bacteriocins

No bacteriocins were identified in RAST, by either database or during literature searches.

Toxin Production

The BLAST search identified *mazF* and *mazG* genes, which are toxin components in the *mazEF* toxin-antitoxin system. Toxin-antitoxin systems are intracellular regulatory mechanisms that are thought to enable different functions like gene regulation, growth control, and programmed cell death (Magnuson, 2007). As such, they pose no danger to hosts. This system is an mRNA-degrading endonuclease that mediates programmed cell death during stress (Kolodkin-Gal et al., 2007). Local BLAST matches using the criteria above did not detect any known toxin producing genes, only genes involved in transport and normal cellular functions.

Hemolysin

Five genes were located in Bi-26TM with protein sequences related to hemolysins that also match known genes in other Bifidobacterium species. Studies show that four separate genes are necessary for hemolysin production and excretion in *E. coli* (Wagner et al., 1983). Two genes, *hylA* and *hylC* synthesize active hemolysin proteins. The genes *hylBa* and *hylBb* work to transport the protein through the periplasm and through the outer membrane, respectively. Partial decomposition of hemoglobin, α -hemolysis, can be caused by hydrogen peroxide (Barnard et al., 1996), while β -hemolysis is the complete lysis of blood cells and γ -hemolysis means bacteria have no effect on blood cells. Bi-26TM grew on blood agar plates and was shown to be α -hemolytic. Further, *hylA* and *hylC* protein sequences from *Aquifex aeolicus* and *E. coli*, respectively, had no matches in Bi-26TM. It is thus concluded that the genes noted above are involved in cellular transportation, and not in the production of virulent hemolysin.

Conclusions:

- No bacteriocins were identified by database searches or in the literature.
- Only a toxin-antitoxin system that targets Bi-26TM was located. There are no toxin genes that are virulent to hosts.
- Bi-26TM is α -hemolytic, meaning that the bacterium produces hydrogen peroxide which partially degrades blood cells.

Lactic Acid Production

Lactic acid is the most important metabolic end-product of fermentation processes by lactic acid bacteria and other microorganisms (Pfeiler and Klaenhammer, 2007). For thousands of years, lactic acid fermentation has been used in the production of fermented foods (Bourdichon et al., 2012). Due to its molecular structure, lactic acid has two optical isomers L (+)-lactic acid and its mirror image, D(-)-lactic acid (Flint et al., 2015). *B. infantis* Bi-26TM only produces L (+) lactic acid. In humans, animals, plants, and microorganisms, L (+)-lactic acid is a normal intermediate or end-product of the

carbohydrate and amino acid metabolisms. It is important for the generation of energy under anaerobic conditions (Ewaschuk et al., 2005).

Production of biogenic amines

Histamines: In lactic acid bacteria, production of histamine results from the catabolism of histidine by a histidine decarboxylase. A specific detection method for histidine decarboxylase genes has been developed internally by DuPont based on the scientific literature and on the most updated genomic databases. Applied to Bi-26™, the method failed to detect a histidine decarboxylase gene. Consequently, Bi-26™ is unlikely to produce histamine.

Tyramine: In lactic acid bacteria, production of tyramine results from the catabolism of tyrosine by a tyrosine decarboxylase. A specific detection method for tyrosine decarboxylase genes has been developed internally by DuPont based on the scientific literature and on the most updated genomic databases. Applied to Bi-26™, the method failed to detect a tyrosine decarboxylase gene. Consequently, Bi-26™ is unlikely to produce tyramine.

Summary

In addition to the previously established strain identity of Bi-26™, the acute toxicity analysis, absence of transferable antibiotic resistance elements, and absence of virulence factors are all consistent with benign microorganisms.

b) *Bifidobacterium longum* subsp. *infantis*

The safety of microorganisms at the species level has globally been recognized; we therefore include a comprehensive review of the safety information for the species *B. infantis* and compare the whole genome sequence of the DuPont strain Bi-26™ to those within the same species that are publicly available and well characterized. This comparative genomics has shown the overall genetic synteny of *B. infantis* Bi-26™ to others of the same species.

Bifidobacterium longum subsp. *infantis* has been documented as having a technical role in fermented food products. *Bifidobacterium* species have a long history of safe use when consumed as part of dairy food and supplement products, with eight *Bifidobacterium* species listed in IDF Bulletin No. 377: *Inventories of Microorganisms with a Documented History of Use in Food* (Mogensen et al., 2002). A more recent IDF Bulletin No. 455, *Safety Demonstration of Microbial Food Cultures in Fermented Food Products*, provides an update to the aforementioned inventory of microbial species, taking a global perspective versus the original focus on European dairy products. The updated inventory lists a reorganization of the *Bifidobacterium* species included, with eight species of *Bifidobacterium* listed, and references *B. longum* use in dairy as early as 1963 (Mathieu-Chanderlier et al., 1998). This reorganization included a taxonomic shift wherein *B. infantis* was transferred to *B. longum* as *B. longum* subsp. *infantis*. (Bourdichon et al., 2012)

A literature search was performed to evaluate clinical safety of *Bifidobacterium longum* subsp. *infantis*. Forty-seven relevant studies were identified and reviewed. Studies were published between 1999 and 2018. Sixteen studies were randomized, blinded, placebo-control trials, the remaining studies were either partially randomized/blinded trials, observational cohorts, uncontrolled, or open-label.

A total of 13,707 subjects were included in the 47 studies and the total number of treatment days was 2.74×10^7 . The duration of treatment ranged from 2 days to 12 months. Doses ranged from 1×10^7 – 1.8×10^{11} CFU/day, but the dose in most studies clustered around 10^9 – 10^{10} CFU/day. The median

dose was 3×10^9 CFU/day. Stratified by health status, 12 studies were conducted on healthy subjects and 36 studies conducted on subjects compromised by such factors as low birth weight, premature birth, necrotizing enterocolitis, diarrhea, irritable bowel syndrome, or other disorders. Stratified by age, studies on infants, children, and adults were the subject of 20, 8, and 10 studies, respectively. Four of the studies were case reports on 9 subjects.

In the 20 studies on infants, the number of treated subjects was 12,410, the number of treatment days was 1.65×10^7 , and the median dose was 3×10^9 CFU/day.

Other than the case studies, the studies either reported no treatment-related adverse events, described the *B. infantis* treatment as well tolerated, or did not report any safety-related endpoints. In twelve of the infant studies, the subjects were either extremely low birthweight, very low birthweight, or premature infants. When adverse events were reported they were generally confined to gastrointestinal issues, were equally distributed between treatment and control groups, were typically considered mild and reversible, and were not considered related to *B. infantis* treatment. Bacteremia was reported in some preterm infants with extremely low birth weight or major gastrointestinal or immunocompromising disorders (i.e. bowel perforations, necrotizing enterocolitis, short bowel syndrome). These were all case reports, and in each case the bacteremia was resolved on discontinuation of treatment, Bertelli et al. (2014), Zbinden et al. (2015), Esaiassen et al. (2016).

These studies using *B. infantis* are summarized below and tabulated in Appendix I.

In a recent randomized, double-blind, placebo-controlled (RDBPC) trial, Enani et al. (2018) young (age 18-35) and older (age 60-85) subjects consumed a mixture of *B. infantis* CCUG 52486 (10^9 CFU/day) and gluco-oligosaccharide (GOS) (n=60) or placebo (n=64) for eight weeks. Safety parameters were not the primary outcome of the study, however, adverse events were monitored and reported to include only mild gastrointestinal symptoms which did not differ between study and control groups.

Escribano et al. (2018) reported on a RDBPC study in healthy infants. Formula-fed infants (age <3 months) received a supplemented formula containing 10^7 CFU/g of *B. infantis* (n=93) or standard formula (n=97) for 12 weeks. ”Supplemented formula was concluded to be “safe, well tolerated and associated with lower constipation prevalence”.

Kumar et al. (2018) reported on healthy adults (age 22-64) who received *B. infantis* 35624 supplementation (dose not reported) for two weeks.. Safety parameters were not the primary outcome of the study; however, subjects completed a patient assessment of upper gastrointestinal symptoms (PAGI-SYM), which identified no significant differences between pre- and post-supplementation scoring.

Del Giudice et al. (2017) reported on a RDBPC parallel arm clinical trial wherein subjects (age 4-17) consumed a live microorganism blend of *B. longum* BB536 (3×10^9 CFU), *B. infantis* M-63 (1×10^9 CFU), and *B. breve* M-16 V (1×10^9 CFU) (n=20) or placebo (n=20) daily for four weeks.. Adverse events were monitored and authors concluded that “both treatments were well tolerated and there were no clinically relevant side effects in children of both groups”.

In an observational, population-based cohort study, Härtel et al. (2017) reported on the effect of supplementation with a combination of live microorganisms on growth of very low birth weight (VLBW) infants. Hospitalized VLBW infants (< 33 weeks’ gestation) consumed 10^9 CFU/day each of *B. infantis* and *L. acidophilus* (n=6229) or no supplementation (n=2305) for the duration of their stay (greater than 28 days). Adverse events were not reported.

Manzano et al. (2017) reported on the safety and tolerance of three live microorganism strains, including *B. infantis* R0033 in a RDBPC, parallel arm trial on healthy infants. Subjects (age 3-12 months) in four groups received *B. infantis* R0033 (n=53), *B. bifidum* R0071 (n=51), or *L. helveticus* R0052 (n=52) at a dose of 3×10^9 CFU/day, or placebo (n=52) for eight weeks. The authors reported various safety parameters, including adverse events, D-lactic acid concentration in urine, and characteristics of stools. Results showed equivalent recorded adverse events across groups. No serious adverse events were reported. The authors thus concluded that *B. infantis* and the other microorganism are “safe, and well tolerated”.

Ringel-Kulka et al. (2017) reported on an RDBPC parallel arm trial to assess the impact of *B. infantis* 35624. Subjects (n=275), mean age 42 years, received either placebo or *B. infantis* 35624 (10^9 CFU/day) for four weeks. The authors reported the regime as “well tolerated.”

A phase I clinical trial was undertaken by Smilowitz et al. (2017), to evaluate the safety and tolerance of *B. infantis* EVC001 administration to breastfed infants. Subjects were given $1.8-2.8 \times 10^{10}$ CFU/day of *B. infantis* in breast milk (n=34) for 21 days, or received no supplement (n=34). No differences in symptomatic outcomes was reported between study and control groups, and the authors concluded that *B. infantis* was safely consumed and well-tolerated.

Esaiassen et al. (2016) conducted a review of the consumption of the live microorganism combination, Infloran® (*L. acidophilus* 10^9 CFU and *B. infantis* 10^9 CFU) in capsule form in Norway in extremely preterm infants between April 2014 and August 2015 and reported three cases of bacteremia. *B. longum* was identified in blood cultures and matched by comparative analysis to the *B. longum* strain cultured from the capsule. All three infants were extremely preterm (≤ 24 weeks' gestation) and had respiratory distress syndrome. Oral microorganism combination ($\frac{1}{2}$ capsule/day) were given during the first week of life and increased to 1 capsule/day after days 4–7 (CFU not reported). Patient 1 presented with sepsis and severe hypotension at day 8. Surgery revealed ileal perforations, bowel necrosis, and histologic features of necrotizing enterocolitis (NEC). Patient 2 presented with apnea, bradycardia and temperature instability at day 12. Patient 3 had sepsis and NEC at day 9, and surgery revealed bowel perforations. Initial culture did not show *B. longum* growth, and consumption were continued. On day 46, Patient 3 presented with hypotension and metabolic acidosis, at which time *B. longum* was detected. All three patients recovered from infection upon discontinuation of the microorganism combination and treatment with antibiotics.

Hoy-Shultz et al. (2016) reported on the safety and tolerability of *L. reuteri* DSM 17938 and *B. infantis* 35624 in healthy infants (age 4-12 weeks) in a Phase I randomized, parallel arm study. Three groups received the live microorganism supplement (10^8 *L. reuteri* and 10^9 *B. infantis*) (n=89): daily dosing (29 doses overall), weekly dosing (five doses), or every two week dosing (three doses); and a fourth control group received no supplement (n=24). “No differences in rates of any reported symptoms were observed among arms; additionally, no sudden adverse or allergic reactions were found after microorganism administration, and no hospitalizations were deemed related to microorganism administration.” The authors concluded that the “treatment was safe”.

Powell et al. (2016) reported on a RDBPC pilot study, in which subjects (>34 weeks' gestation) received *B. infantis* ATCC 15697 (10^9 CFU) (n=10) or placebo (n=11) twice daily for six weeks or until discharge. No serious adverse events were reported.

Stojković et al. (2016) reported on the optimal duration of intervention with a combination of live microorganisms and fructo-oligosaccharides in a randomized, observational cohort study. Children under 5 (n=78) who had been hospitalized for a respiratory infection in the preceding year were given

a combination blend of *L. acidophilus* Rosell-52, *B. infantis* Rosell-33, *B. bifidum* Rosell-71 (5×10^9 CFU total) and fructo-oligosaccharides. The authors concluded that blend “is well tolerated in young children”.

Tehrani et al. (2016) reported on a RDBPC parallel arm clinical trial in which healthy children, aged 3-6 years, received a combination drop containing *L. rhamnosus* ATCC 15820 (1×10^{10} CFU/mL), *L. reuteri* ATCC 55730 (2×10^9 CFU/mL), and *B. infantis* ATCC 15697 (1.5×10^9 CFU/mL). Combination drops (n=30) or placebo drops (n=23) were administered daily for two weeks. No safety related endpoints were reported.

Langkamp-Henken et al. (2015) reported on a RDBPC trial that assessed the effect of live microorganism consumption in an academically stressed undergraduate population. Full time students (age ≥ 18) consumed 3×10^9 CFU of either *L. helveticus* R0052 (n=146), *B. infantis* R0033 (n=142), or *B. bifidum* R0071 (n=142) or placebo (n=147) daily for six weeks. Safety parameters were not discussed, however withdrawals related to adverse events were similar between groups and mostly associated with mild gastrointestinal symptoms. One participant in the *B. infantis* group withdrew after 1 day because of abdominal pain, 1 participant in the placebo group withdrew after 25 days because of abdominal pain, and 1 participant in the placebo group withdrew due to diarrhea.

Van Niekerk et al. (2015) reported on a RDBPC study aiming to assess the impact of a microorganism blend in HIV-exposed and HIV-unexposed populations of VLBW infants. Subjects (<34 weeks' gestation, <1250 g) received a live microorganism blend of *L. rhamnosus* and *B. infantis* (3.5×10^8 CFU/day of each strain) (n= 91) or placebo (n= 93) for 28 days or until discharge. The authors concluded that the intervention “proved to be safe” but recommended caution in use due to lack of clinical studies in similar populations.

A retrospective review of three cases of bacteremia in preterm infants at the University Hospital of Zurich was reported by Zbinden et al. (2015). Three VLBW infants (gestational age ≤ 30 weeks) undergoing therapy with a microorganism combination, Infloran[®] (*L. acidophilus* 10^9 CFU and *B. infantis* 10^9 CFU) presented varied respiratory and other complications. Patient 1 presented with nosocomial infection, and *B. longum* was detected in cultures from day 20. Patient 1 “displayed a complicated clinical course with sequential antibiotic therapy, so the Infloran was continuously administered from birth to day 28”. The infant improved and cultures taken on day 27 were negative. Patient 2 had multiple episodes of mechanical ventilation and developed bronchopulmonary dysplasia and nosocomial infection. The patient improved after cessation of microorganism combination and antibiotic treatment. Cultures obtained on day 20 displayed *B. longum* growth. Patient 3 was diagnosed with NEC stage III, with *B. longum* detected in blood cultures from day 11. Analysis of all *B. longum* isolates identified them as homologous to the Infloran[®] combination. The authors reported that in a 5-year review period, *B. longum* bacteremia was identified in these three cases (0.4%), and emphasize caution with the use of microorganism combinations in preterm neonates.

Bertelli et al. (2014) reported two cases of *B. longum* bacteremia in premature newborns receiving live microbial organisms. In Case 1, a patient born at 26 weeks' gestation was administered Infloran[®] (*L. acidophilus* (5×10^8 CFU) and *B. infantis* (5×10^8 CFU) twice daily for 10 days. Symptoms of sepsis developed and patient was treated with antibiotics. The patient was later diagnosed with ileoileal intussusception. Case 2 involved an infant born at 28 weeks gestation, receiving Infloran for 5 days. The patient was diagnosed with NEC stage III, and the authors concluded that the presence of the *Bifidobacterium* in the blood was “likely to be the consequence of intestinal necrosis rather than the cause”. Full genome comparisons were not performed to verify the identity of the infecting strains as Infloran[®]. Analysis of single-nucleotide polymorphisms (SNPs) was carried out and “the small number of SNPs identified between both strains isolated from blood culture and all strains recovered

from [live microbial ingredients] suggests that the strains involved in bacteremia were originating from [live microbial ingredients]”. The authors recommend that future clinical trials include monitoring of blood cultures.

A retrospective cohort study was conducted by Li et al. (2014) to review the use of live microorganism supplement in VLBW infants. Infants (< 1500 g) were separated into a study group of those admitted Aug. 2007- Jul. 2011 receiving *B. infantis* and *L. rhamnosus* ($0.5-1.05 \times 10^9$ CFU/day) (n=291) and control group of those admitted Aug. 2003-Jul. 2007 receiving no supplementation (n=289). The authors reported that no cases of infection were related to the strains and that the supplement was “well tolerated with no major adverse events”.

Tobin et al. (2014) reported on a small open label study to assess the value of rapid qPCR assays for confirmation of microorganism colonization after supplementation. Healthy adults (n=7) and preterm infants (n=6) consumed the live microorganism blend ABC Dophilus for Infants® containing 2×10^8 *B. infantis*, 2.3×10^8 *B. lactis*, and 2.3×10^8 *S. thermophilus* per g (15 g for adults and 1.5 g for infants) daily for seven days.. No safety related endpoints were reported.

Charbonneau et al. (2013) reported on the levels of *B. infantis* in the fecal excretion of healthy patients and those with irritable bowel syndrome (IBS) after live microorganism administration. Subjects (age 18-65) received 10^9 CFU/day of *B. infantis* 35624 (healthy n=41, IBS n=39), or placebo (IBS n=37) for eight weeks. A review of adverse events showed no significant differences between groups, and no adverse events (AEs) were attributed to microorganism administration.

A RDBPC pilot study was reported by Ellis et al. (2013) with the aim of assessing the impact of *B. infantis* supplementation on infants with congenital heart disease. Subjects were given 4.2×10^9 CFU twice daily of *B. infantis* (n=8) or placebo (n=8) for eight weeks.. No safety related endpoints were discussed..

Three RDBPC trials were reported by Groeger et al. (2013) to assess the effect of *B. infantis* on in patients with ulcerative colitis (n = 22), chronic fatigue syndrome (n = 48) and psoriasis (n = 26). Adult subjects (age 18-75) in these subgroups and a healthy control group (n=22) received 10^{10} CFU/day of *B. infantis* 35624 for 6-8 weeks. No safety related endpoints were reported.

Jacobs et al. (2013) reported on a RDBPC trial aimed at determining the effect of administration of a combination of live microorganisms in ‘very preterm’ infants. Subjects (<32 weeks’ gestation, weighing <1500 g) received the intervention of *B. infantis*, *S. thermophilus*, and *B. lactis* (containing 1×10^9 total organisms, 3×10^8 of *B. infantis*) (n=548) or placebo (n=551) until discharge or 12 months’ corrected age. No serious adverse events were recorded and authors reported that the microorganism combination “appears to be safe”.

A RDBPC trial investigating the effect of *B. infantis* consumption in adults (age 18-75) with celiac disease was reported by Smecuol et al. (2013). Subjects received 4×10^9 CFU/day of *B. infantis* (n=12) or placebo (n=10) for three weeks. The authors reported “No serious adverse effects or significant biochemical changes were reported by patients in either treatment arm.”

Underwood et al. (2013) reported on a comparison of preterm infants after increasing doses of Bifidobacteria consumption by formula-fed subjects and consumption of live microorganisms by human milk-fed subjects. Formula-fed, preterm infants (<33 weeks’ gestation, <1500 g) received increasing doses of *B. infantis* (n=6) or *B. lactis* (n=6) over five weeks, up to 8.4×10^9 CFU/day. Additionally, human milk-fed subjects consumed both strains alternately (8×10^9 CFU/day) with washout period (n=9). The authors noted that both microorganisms were well tolerated.

Pantovic et al. (2013) reported on the optimal time of supplementation with Biostime (Probiokid®), a 1:1:1 mixture of *L. helveticus* R0052, *B. infantis* R0033, and *B. bifidum* R0071 plus fructo-oligosaccharide (FOS) in an uncontrolled, open-label trial in children age 0 – 42 months (n=31), hospitalized with respiratory and/or ear infections. Subjects received 3×10^9 CFU/day for 6 months. No adverse events were reported in this poorly described study that lacked elements of good clinical practices (GCP).

A single center, RDB active control trial to compare Smecta (a hydroscopic montmorillonite suspension) plus Biostime to Smecta alone in infants with non-infectious diarrhea was reported by Wu et al. (2013). Infants less than 12 months of age (n=32) received 5×10^9 CFU Biostime, a 1:1:1 mixture of *L. helveticus* R0052, *B. infantis* R0033, and *B. bifidum* R0071 plus FOS. Infants age 13-24 months (n=35) received 5×10^9 CFU Biostime, and infants 25 – 36 months (n=17) received 15×10^9 CFU Biostime for 3 days. All dose groups, including the control group (n=64) received oral Smecta. No adverse reactions were reported in any of the experimental groups.

In an RDB active control trial, Xi et al. (2013) reported on the effect on children with thrush (age 1-36 months) of Biostime in combination with sodium carbonate and nystatin compared to nystatin alone (control, n=35). The subjects (n=35) received 1×10^{11} CFU Biostim/day for 17 days. No adverse reactions were reported.

A multicenter, randomized, controlled, double-blinded clinical study in extremely low birth weight (ELBW) infants was reported by Al-Hosni et al. (2012). Subjects weighing less than 1000 g were randomized into consumption of a combination of live microorganisms (n=50) and control (n=51) groups. The live microorganism combination group consumed *B. infantis* and *L. rhamnosus* (5×10^9 CFU/day each) until 34 weeks' gestational age or discharge. Adverse events were monitored and none were attributed to consumption of the live microorganism combination.

A single center, RDB active control trial to compare Smecta (a hydroscopic montmorillonite suspension) plus Biostime to Smecta alone in infants with non-infectious diarrhea was reported by Gao et al. (2012). Infants less than 12 months of age (n=14) received 5×10^9 CFU Biostime, a 1:1:1 mixture of *L. helveticus* R0052, *B. infantis* R0033, and *B. bifidum* R0071 plus FOS. Infants age 13-24 months (n=14) received 5×10^9 CFU Biostime, and infants 25 – 36 months (n=15) received 15×10^9 CFU Biostime for 3 days. All dose groups, including the control group (n=43) received oral Smecta. No adverse reactions were reported in any group.

A single center, RDB active control trial to compare Smecta (a hydroscopic montmorillonite suspension) plus Biostime to Smecta alone was reported by Wang et al, (2012). Infants less than 12 months of age (n=33) received 5×10^9 CFU Biostime a 1:1:1 mixture of *L. helveticus* R0052, *B. infantis* R0033, and *B. bifidum* R0071 plus FOS. Infants age 13- 24 months (n=43) received 5×10^9 CFU Biostime, and infants 25 – 36 months (n=28) received 15×10^9 CFU Biostime for 3 days. All dose groups, including the control group (n=43) received oral Smecta. No adverse reactions were reported in any group.

Frech et al. (2011) reported on the effect of microorganism supplementation in adults with systemic sclerosis in an open label study. Ten subjects consumed either *B. infantis* or *L. rhamnosus* (10^9 CFU/day) for two months. The authors reported no complications with microorganism use.

Jenke et al. (2011) reported on a case of Bifidobacterium septicaemia in an extremely low birthweight infant. Patient was born at 27 weeks, weighing 600 g, and received Infloran® enterally beginning on day 9. Symptoms of septicaemia presented on day 18. Blood cultures grew Bifidobacterium, and PCR

analysis reveal two strains, one of *Bifidobacterium longum* and one of *B. infantis*. The authors recommended a cautious risk-benefit consideration in the use of live microorganisms in extremely low birthweight infants.

Cazzola et al. (2010) reported on a RDBPC multicenter study that investigated a live microorganism and oligosaccharide blend from Biostime, a 1:1:1 mixture of *L. helveticus* R0052, *B. infantis* R0033, and *B. bifidum* R0071 plus FOS) in children. Healthy children reporting at least three respiratory or gastrointestinal episodes during the previous winter received the blend (n=62) or placebo (n=73) daily for three months. The authors reported that no adverse events were associated with the blend, and considered it well tolerated.

In a RDBPC parallel arm trial on hospitalized children (age 6-30 mo) with rotaviral infection and diarrhea, Yang et al. (2010) reported on the effect of feeding with lactose-free-milk powder plus 5×10^9 CFU Biostime, a 1:1:1 mixture of *L. helveticus* R0052, *B. infantis* R0033, and *B. bifidum* R0071 plus FOS (n=58) or breastfed or formula fed control children (n=40). The children received treatment until discharge (mean 8.5 ± 2.3 days). No adverse events were reported.

In a RDBPC, parallel arm, active control trial on hospitalized or outpatient children (age 3-24 mo, n=32) with persistent diarrhea, Jiang et al. (2008) reported on the effect of feeding with Biostime, a 1:1:1 mixture of *L. helveticus* R0052, *B. infantis* R0033, and *B. bifidum* R0071 plus FOS. Infants under the age of 6 months received 5×10^9 CFU/day (n=32), children 6-12 months received 10×10^9 CFU/day, children age 12-24 months received $10 - 20 \times 10^9$ CFU/day, and the control group received Golden Bifido (n=20). The subjects received treatment until discharge (mean 7.1 days). No adverse events were reported.

In a RDBPC, parallel arm, active control trial on children (age 0 – 5 years) with persistent diarrhea, Mei & Chang (2008) reported on consumption of 1×10^{11} CFU/day Biostime, a 1:1:1 mixture of *L. helveticus* R0052, *B. infantis* R0033, and *B. bifidum* R0071 plus FOS. The treatment (n=39) and control (n=39) groups received Ribaviren throughout the 7-day study. There was no report of adverse events.

A RDBPC, parallel arm, active control trial on children (age 0 – 4 years, n=28) with persistent diarrhea, conducted by Chen (2007) reported on the consumption of 1×10^{11} CFU/day Biostime, a 1:1:1 mixture of *L. helveticus* R0052, *B. infantis* R0033, and *B. bifidum* R0071 plus FOS for 13 days. The treatment stratified into 4 age groups. Four children in each age group received the Biostime treatment and two age matched children were controls. No adverse events were reported.

In an open, randomized, control trial on hospitalized infants (age 1-24 months) with acute watery diarrhea, Vivatvakin and Kowitdamrong (2006) reported on patients receiving a live microorganism blend of 3×10^9 CFU/day plus an oral rehydration solution (n=35) or rehydration alone (n=36) for two days. The authors reported “no difference of the patients' characteristics between the study group and the control group.”

Whorwell et al. (2006) reported on *B. infantis* 35624 at various dosages in women with IBS. Subjects consumed placebo or live microorganism doses of 1×10^6 , 1×10^8 , or 1×10^{10} CFU/mL for four weeks (n=90 per group). No significant adverse events were recorded in any group.

Lin et al. (2005) reported on a masked, randomized control study to evaluate live microorganism consumption in VLBW infants. Infants beyond day 7 of life received the live microorganism combination, Infloran® (*B. infantis* and *L. acidophilus*, dose not reported) twice daily with milk (n=180) or control (n=187) until discharge. While safety determination was not a primary outcome of

the study, positive blood cultures were tested and none grew *Lactobacillus* or *Bifidobacterium* species.

O'Mahony et al. (2005) reported on a RDBPC study in which seventy-seven adult subjects (age 18-75) were randomized into three groups, either consuming *B. infantis* 10^{10} CFU/day, *L. salivarius*, or placebo for eight weeks. The authors reported that the live microorganism supplementation was well tolerated and free of significant adverse events.

In a RDBPC, parallel arm, active control trial on children (age 6 – 24 months) with pediatric rotavirus gastroenteritis, Cui & Wure (2003) reported on the effect of dietary treatment with Biostime, a 1:1:1 mixture of *L. helveticus* R0052, *B. infantis* R0033, and *B. bifidum* R0071 plus FOS. Children under the age of 12 months (n=62) received 5×10^9 CFU/day Biostime, children age 12 – 24 months (n=60) received 10×10^9 CFU Biostime plus Lacidophilin. Both groups received Ribavirin. Treatment continued until diarrhea was resolved for at least 72 hours. There was no report of adverse events.

Lee et al. (2001) reported on a prospective clinical study to determine the impact of live microorganism consumption in hospitalized children. Patients (age 6-60 months) received rehydration and 10^9 CFU/day each of *L. acidophilus* and *B. infantis* (n=50), or rehydration alone (n=50) for 4 days. The authors considered the live microorganism blend capable of being “safely administered during an episode of acute diarrhea.”

Hoyos (1999) reported on an open label study of the effect of *B. infantis* and *L. acidophilus* in newborns from October 1994 - October 1995. During the study period, all newborns (average gestational age 35 weeks) received 2.5×10^8 CFU/day each of *B. infantis* and *L. acidophilus* for the duration of their hospital stay (average 5.5-8.5 days). Data were compared to a historic control group of newborns admitted during the previous year. Results included no complications attributed to the daily administration of *L. acidophilus* and *B. infantis*.”

c) **Bifidobacterium and lactic acid bacteria**

Bifidobacterium species have historically been considered safe and suitable for human consumption with several published studies addressing its safety (Aguirre, M., 1993) (Gasser, 1994) (Salminen, S. 1998). *Bifidobacterium* have long been considered to be non-pathogenic and have been isolated from the gastrointestinal tracts of healthy animals and humans.

The Food and Agriculture Organization and World Health Organization expert consultation reported that “no pathogenic or virulence properties have been found for lactobacilli, bifidobacteria, or lactococci” (FAO/WHO, 2002). The safety of live microorganisms was recently reviewed (Sanders et al., 2010; Sanders et al., 2007). The available evidence demonstrates that any strain, including members of the genera *Lactococcus*, *Lactobacillus*, and *Bifidobacterium* is considered safe, as long as the strain is devoid of any transferable antibiotic resistance genes.

Infections in humans by these genera are extremely rare (Sanders et al., 2007). Cases of infection due to bifidobacteria are extremely rare (Borriello et al., 2003). Immunocompromised patients generally are more vulnerable to infection with pathogens and have a higher incidence of opportunistic infections. However, there is no published evidence that consumption of live microorganisms that contain lactobacilli or bifidobacteria increases the risk of opportunistic infection among such individuals (Borriello et al., 2003). Weber et al. (2015) identified only 21 cases of bacteremia due to *Bifidobacterium* species and only 7 were identified as possibly related to consumption of live

microbial ingredients by preterm infants. Thus, the risk of infection by these genera is in the "negligible" range, considering that exposure to them is universal and persistent, not only through live microbial containing foods and products but also as common colonizers of the human body (the digestive tract and oral and vaginal cavities). This lack of pathogenicity extends across all age groups (including preterm infants and pregnant women) (Lin et al., 2005, Saavedra et al., 2004). Further investigation is warranted for live microorganism use in at-risk human populations such as severely immunocompromised subjects, neonates, or hospitalized patients (Snydman, 2008).

In a comprehensive, evidence-based review and meta-analysis of the literature regarding the safety of live microorganisms, 622 peer-reviewed research articles were evaluated (Hempel et al., 2011). Of these, 235 studies reported only nonspecific safety statements such as "well tolerated" but did not indicate specific adverse events or the kinds of events that were monitored. The remaining 387 studies predominantly investigated *Lactobacillus*, alone or in combination with other genera, most often *Bifidobacterium*. These studies were pooled to evaluate the relative risks (RR) of use of microorganisms, active or lyophilized, single ingredients or in combination, in all delivery vehicles when used to improve health. The following key relative risk results germane to the current report are listed along with 95% confidence intervals (CI), p value, and the number of randomized clinical trials (RCT) included in the pool.

- There was **no evidence of increased risk from interventions with live microorganisms** compared to control groups
 - a. based on the number of participants with adverse events (RR 0.98, CI: 0.93 – 1.04, p=0.537, 121 RCT)
 - b. based on the number of adverse-event incidences (RR 1.00, CI: 0.93 – 1.07, p=0.999, 208 RCT)
 - c. **"None of the case series, controlled clinical trials, or parallel and crossover RCT reported an infection caused by the administered live microbial ingredients"** though few reported that they monitored for this
- There was no indication participants using live organisms experienced more:
 - a. Gastrointestinal events (RR 1.03, CI: 0.89 – 1.18, p=0.693, 126 RCT)
 - b. Infections (RR 1.00, CI: 0.87 – 1.16, p=0.967, 65 RCT)
 - c. Or other adverse events (RR 1.01, CI: 0.91 – 1.12, p=0.923, 131 RCT)
- Stratified by genus there was **no indication that participants using *Lactobacillus* experienced an increased risk.** (RR 0.98, CI: 0.87 – 1.11, p=0.785)
- **Stratified by age there was no indication of increased risk of adverse events for children, adults, or elderly.**
- Although case studies have reported serious adverse events in health compromised, not generally healthy participants, **subgroup analyses of RCT did not show an increased risk of adverse events** in either:
 - a. **Medium health-compromised participants** (RR 1.03, CI: 0.94 – 1.13, p=0.491)
 - b. **Critically ill patients** (RR 0.79, CI: 0.51 – 1.22, p=0.286)
- There was **no indication that consumption of live microorganisms led to hospital admission or lengthened hospitalization.** Most of these studies were based on *Lactobacillus* interventions.

- (RR 1.06, CI: 0.97 – 1.16, p=0.201, 66 RCT)
- There was no indication that consumption of live microorganisms increased the risk of adverse events in individuals concomitantly taking:
 - a. Antibiotics
(RR 1.07, CI: 0.94 – 1.23, p=0.271)
 - b. Corticosteroids
(RR 1.04, CI: 0.88 – 1.22, p=0.650)

The strength of these conclusions is somewhat mitigated by the inconsistency between the results of RCT and case studies, the lack of systematic reporting of adverse events, and poor documentation in the studies evaluated. The authors conclude the RCT-based evidence does not indicate an increased risk of adverse events. “The available evidence in RCTs does not indicate an increased risk; however, rare adverse events are difficult to assess and despite the substantial number of publications, the current literature is not well equipped to answer questions on the safety of [live microbial ingredient]interventions with confidence.”

Bifidobacterium have long been considered non-pathogenic, safe and suitable for human consumption. Very few instances of infection have been associated with these bacteria and several published studies have addressed their safety (Aguirre and Collins, 1993; Gasser, 1994; Gueimonde et al., 2004; Salminen et al., 1998; Sanders et al., 2010).

4. Adverse events in clinical trials

No known adverse events have been associated with the administration of *B. infantis* Bi-26™ and no complaints related to the safety of this ingredient have been reported to DuPont.

B. Inconsistent information

DuPont Nutrition & Health (formerly Danisco) and an external expert have reviewed the available data and information and are not aware of any data and information that are, or may appear to be, inconsistent with this conclusion of GRAS status.

C. Expert Panel Evaluation

The GRAS Panel individually and collectively critically evaluated the materials summarized above. The GRAS Panel also evaluated the safety of *B. infantis* Bi-26™ using a decision tree analysis developed by (Pariza et al., 2015). Based on their critical evaluation of the information on the safety of *B. infantis* Bi-26™ summarized above, they unanimously concluded that DuPont’s *B. infantis* Bi-26™, manufactured consistent with cGMP and meeting food grade specifications, is Generally Recognized As Safe (GRAS) based on scientific procedures for use in infant formulas and toddler formulas, at a level of 1×10^8 CFU per g of infant formula powder. This level of *B. infantis* Bi-26™ is intended to ensure a minimum concentration of 10^6 CFU/g throughout the 12-18 month shelf life of the infant formula powder. It is the GRAS Panel’s opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusions. See Appendix J GRAS Panel Statement.

D. Common knowledge elements of GRAS conclusions

The first common knowledge element for a conclusion of GRAS status is that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing published, peer-reviewed scientific journals for the safety assessment. The studies on which this GRAS conclusion has been based have been published in the scientific literature.

The second common knowledge element required for a GRAS determination is that consensus among qualified scientists about the safety of the substance with its intended use must be demonstrated. It is agreed that there is adequate data in the scientific literature to conclude that *B. infantis* Bi-26™ is a common component of food sources for man and animals, and is nutritionally efficacious without any evidence of adverse effects.

Finally, *B. longum* is QPS in Europe, and *B. longum* is in common use as a food preparation in fermented food.

E. Final conclusion

Based on scientific procedures, the above data and the information presented herein, DuPont Nutrition & Biosciences has concluded that the proposed use of *B. infantis* Bi-16™ at a level of 1×10^8 CFU/g of infant formula that is intended for consumption by term infants from the time of birth through 2 years of age is GRAS. This level of *B. infantis* Bi-26™ is intended to ensure a minimum concentration of 10^6 CFU/g throughout the 12-18 month shelf life of the infant formula powder formula. DuPont bases this opinion in part on the published clinical studies on various strains of the species *Bifidobacterium longum* subsp. *infantis*, safety reviews by various national and international regulatory bodies on the safety of *Bifidobacterium* in general and *B. longum* subsp. *infantis* in particular, and genotypic analysis and phenotypic properties of the *B. infantis* Bi-26™ strain. DuPont believes *B. infantis* Bi-26™ does not present a significant or unreasonable risk of illness or injury at this level for these uses. General recognition of DuPont's GRAS determination is supported by the consensus rendered by an independent expert panel, qualified by experience and scientific training to evaluate the proposed uses for *B. infantis* Bi-26™. The GRAS Panel convened by DuPont Nutrition & Biosciences independently and critically evaluated all data and information presented herein, and concluded that DuPont Nutrition & Biosciences' *B. infantis* Bi-26™ is GRAS based on scientific procedures for use in infant formulas and toddler formulas.

Part 7 - List of supporting data and information in GRAS notice

A. List of Abbreviations

AE	adverse event
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
CD	celiac disease
CFSAN	Center for Food Safety and Nutrition
CFU	colony forming unit
CGMPs	Current Good Manufacturing Practices
CI	confidence interval
COA	certificate of analysis
DGCC	DuPont Global Cultures Collection
EFSA	European Food Safety Authority
ELBW	extremely low birth weight
FCC	Food Chemicals Codex
FDA	Food and Drug Administration
FOS	fructo-oligosaccharide
FSIS	Food Safety Inspection Service
GMO	genetically modified organism
GOS	gluco-oligosaccharide
GRAS	Generally Recognized As Safe
GRN	GRAS notification
HAACP	Hazard Analysis and Critical Control Points
HEPA	high efficiency particulate air
HGT	horizontal gene transfer
HIV	human immunodeficiency virus
HMO	human milk oligosaccharide
HVAC	heating, ventilation, and air conditioning
IBS	irritable bowel syndrome
IDF	International Dairy Federation
ISO	International Standardization Organization
LAB	lactic acid bacteria
LBT	lactulose breath test
MBP	microbial break point
MIC	minimum inhibitory concentration
NEC	necrotizing enterocolitis
NSF	National Science Foundation
OECD	Organization for Economic Cooperation and Development
ORS	oral rehydration solution
PAGI-SYM	patient assessment of upper gastrointestinal symptom
PCR	polymerase chain reaction
ppm	parts per million
QC	quality control
QPS	qualified presumption of safety

RCT	randomized clinical trial
RDBPC	randomized, double-blind, placebo-controlled
RR	relative risk
rRNA	ribosomal ribonucleic acid
SNP	single-nucleotide polymorphisms
SOP	standard operating procedure
VLBW	very low birth weight

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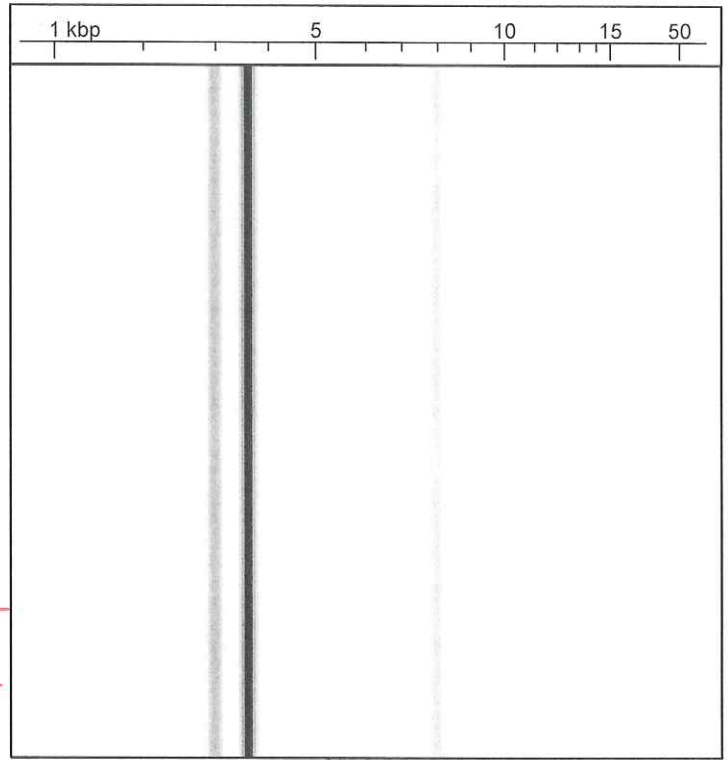
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C. Appendices

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Created	3/15/2016 7:54:45 PM
Batch Extraction Status	Passed
User - Submitted	RP\jawe
Process	KHA
Enzyme	EcoRI
Isolated From	STDUT
Label	BI26
Sample Comment	1102701119
Batch	475-750
Operator	JAWE



Identification

PASS / FAIL

Signature: _____
Date: 3/17/16

	Type	Number	Similarity	Label	1 kbp RiboPrint™ Pattern 15 50
1	Custom ID	Madison_EcoRI-4	0.90	Bifidobacterium longum infantis	
2	RiboGroup	ECORI 475-56-S-8	0.95		



Alignment Report - 16S Full Gene Identification

Customer: Freeburg , Barbara

Company: Danisco

Address: 3322 Agriculture Dr, Madison, WI 53716 USA

125 SANDY DRIVE•NEWARK, DE 19713•PH 302-737-4297•FX 302-737-7781•WWW.MIDILABS.COM

MD16M2 Library Revision: 2.22

Created: 3/7/2018 11:02:56 AM

Sample ID: C1802241922-Bi-26 1103190483

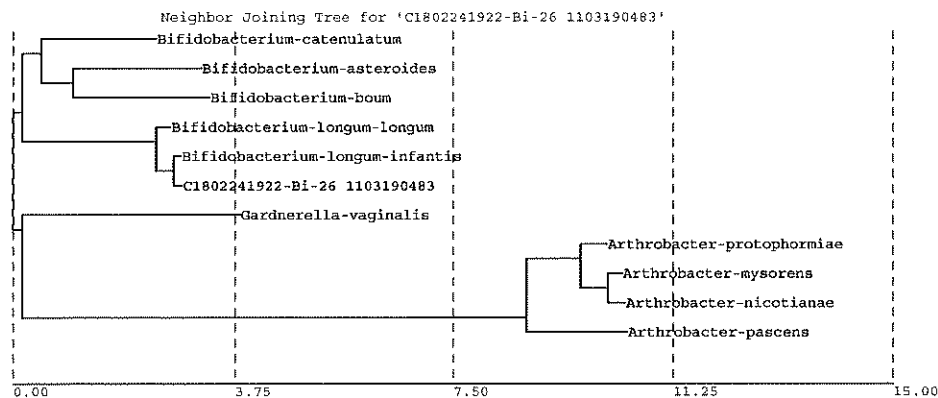
16S DNA: 1486 base pairs

MD16M2 DNA Match Report

Match	%Diff	Length	Library Entry Name
1	0.24	1521	Bifidobacterium-longum-infantis
2	0.54	1520	Bifidobacterium-longum-longum
3	4.69	1523	Bifidobacterium-catenulatum
4	5.97	1524	Bifidobacterium-boum
5	6.38	1520	Bifidobacterium-asteroides
6	6.56	1523	Gardnerella-vaginalis
7	12.84	1521	Arthrobacter-protophormiae
8	13.24	1521	Arthrobacter-mysorens
9	13.38	1521	Arthrobacter-nicotianae
10	13.46	1520	Arthrobacter-pascens

Concise Alignment (maximum difference 3.02):

	74	274	450	984	995	1113
Sample:	AAGC-	C	T	ATCCC	GGGGT	G
Lib Match 1:	GGGCT	M	T	ATCCC	GGGGT	G
Lib Match 2:	AAGC-	C	A	GTCGT	ACGGC	A
	** *	*	*	* **	** *	*



Closest Match: Bifidobacterium-longum

Confidence Level: Species

Reviewer's Signature: 03-07-18

LeAnne Gandolfo, Lab Manager



Danisco USA, Inc.
3329 Agriculture Drive
Madison, WI 53716
800-255-6837 Tel
608-395-2603 Fax

FOOD GRADE STATEMENT

Date: April 12, 2018

Product: 1283879 Bi-26™ 50B - 1KG

Dear Valued Customer,

The above listed product is produced in accordance with the U.S. FDA's current Good Manufacturing Practices guidelines (21 CFR 117), and is considered Food Grade and safe for human consumption.

This information is given in respect of DuPont's policy of openness and transparency with its customers.

Sincerely,



Sarah Pace
Quality & Food Safety Coordinator
DuPont - Nutrition & Health

Certificate of Analysis

Date: 13 Mar 2018
 Our ref. no.: 0
 Your ref.:

Material:	1283879	Bi-26 50B - 1KG	
Batch No.:	1102993037	Best before date:	17 Oct 2018
Quantity:	0.000	Production date:	17 Apr 2017

Test	Result	Specification	Unit	Reference
Viable Cell Count	2.42E+11	> 5.00E+10	/g	ISO 7889/ IDF 117
Enterococcus	< 100	< 100	/g	SMEDP
Non Lactics	< 5000	< 5000	/g	ISO 13559
Coliforms	< 10.0	< 10.0	/g	AOAC
E. coli, neg. by test (<0.3/g)	Negative	Negative		AOAC
Staph. aureus, neg. by test (<10/g)	Negative	Negative		AOAC
Salmonella, negative in 40 g	Negative	Negative		AOAC
Listeria, negative in 25 g	Negative	Negative		AOAC

Comments

Exceeds 50 billion CFU/gm of freeze-dried Bifidobacterium infantis.

The above product has been analyzed by Danisco and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

Best if used before the date listed above when stored at or below 4°C.

AOAC references above reflect the current edition of AOAC.

Culture identity is confirmed to Genus/species level (or sub-species level where applicable) based on DNA Fingerprinting Analysis generated by Automated Ribotyping.

Certificate of Analysis

Date: 13 Mar 2018
Our ref. no.: 0
Your ref.

Material:	1283879	Bi-26 50B - 1KG	
Batch No.:	1102993037	Best before date:	17 Oct 2018
Quantity:	0.000	Production date:	17 Apr 2017

This certificate is generated automatically



Phil Ihrke

Quality Control Department

Certificate of Analysis

Date: 13 Mar 2018

Our ref. no.: 0

Your ref.

Material:	1283879	Bi-26 50B - 1KG	
Batch No.:	1103021382	Best before date:	01 Dec 2018
Quantity:	0.000	Production date:	01 Jun 2017

Test	Result	Specification	Unit	Reference
Viable Cell Count	3.90E+11	> 5.00E+10	/g	ISO 7889/ IDF 117
Enterococcus	< 100	< 100	/g	SMEDP
Non Lactics	< 5000	< 5000	/g	ISO 13559
Coliforms	< 10.0	< 10.0	/g	AOAC
E. coli, neg. by test (<0.3/g)	Negative	Negative		AOAC
Staph. aureus, neg. by test (<10/g)	Negative	Negative		AOAC
Salmonella, negative in 40 g	Negative	Negative		AOAC
Listeria, negative in 25 g	Negative	Negative		AOAC

Comments

Exceeds 50 billion CFU/gm of freeze-dried Bifidobacterium infantis.

The above product has been analyzed by Danisco and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

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Certificate of Analysis

Date: 13 Mar 2018

Our ref. no.: 0

Your ref.

Material:	1283879	Bi-26 50B - 1KG	
Batch No.:	1103021382	Best before date:	01 Dec 2018
Quantity:	0.000	Production date:	01 Jun 2017

This certificate is generated automatically



Phil Ihrke

Quality Control Department

Certificate of Analysis

Date: 13 Mar 2018

Our ref. no.: 0

Your ref.

Material:	1283879	Bi-26 50B - 1KG	
Batch No.:	1103080295	Best before date:	28 Feb 2019
Quantity:	0.000	Production date:	29 Aug 2017

Test	Result	Specification	Unit	Reference
Viable Cell Count	2.95E+11	> 5.00E+10	/g	ISO 7889/ IDF 117
Enterococcus	< 100	< 100	/g	SMEDP
Non Lactics	< 5000	< 5000	/g	ISO 13559
Coliforms	< 10.0	< 10.0	/g	AOAC
E. coli, neg. by test (<0.3/g)	Negative	Negative		AOAC
Staph. aureus, neg. by test (<10/g)	Negative	Negative		AOAC
Salmonella, negative in 40 g	Negative	Negative		AOAC
Listeria, negative in 25 g	Negative	Negative		AOAC

Comments

Exceeds 50 billion CFU/gm of freeze-dried Bifidobacterium infantis.

The above product has been analyzed by Danisco and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

Best if used before the date listed above when stored at or below 4°C.

AOAC references above reflect the current edition of AOAC.

Culture identity is confirmed to Genus/species level (or sub-species level where applicable) based on DNA Fingerprinting Analysis generated by Automated Ribotyping.

Certificate of Analysis

Date: 13 Mar 2018

Our ref. no.: 0

Your ref.

Material:	1283879	Bi-26 50B - 1KG	
Batch No.:	1103080295	Best before date:	28 Feb 2019
Quantity:	0.000	Production date:	29 Aug 2017

This certificate is generated automatically



Phil Ihrke

Quality Control Department

Certificate of Analysis

Date: 13 Mar 2018

Our ref. no.: 0

Your ref.

Material:	1283879	Bi-26 50B - 1KG	
Batch No.:	1103116756	Best before date:	28 Apr 2019
Quantity:	0.000	Production date:	27 Oct 2017

Test	Result	Specification	Unit	Reference
Viable Cell Count	1.65E+11	> 5.00E+10	/g	ISO 7889/ IDF 117
Enterococcus	< 100	< 100	/g	SMEDP
Non Lactics	< 5000	< 5000	/g	ISO 13559
Coliforms	< 10.0	< 10.0	/g	AOAC
E. coli, neg. by test (<0.3/g)	Negative	Negative		AOAC
Staph. aureus, neg. by test (<10/g)	Negative	Negative		AOAC
Salmonella, negative in 40 g	Negative	Negative		AOAC
Listeria, negative in 25 g	Negative	Negative		AOAC

Comments

Exceeds 50 billion CFU/gm of freeze-dried Bifidobacterium infantis.

The above product has been analyzed by Danisco and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

Best if used before the date listed above when stored at or below 4°C.

AOAC references above reflect the current edition of AOAC.

Culture identity is confirmed to Genus/species level (or sub-species level where applicable) based on DNA Fingerprinting Analysis generated by Automated Ribotyping.

Certificate of Analysis

Date: 13 Mar 2018
Our ref. no.: 0
Your ref.

Material:	1283879	Bi-26 50B - 1KG	
Batch No.:	1103116756	Best before date:	28 Apr 2019
Quantity:	0.000	Production date:	27 Oct 2017

This certificate is generated automatically



Phil Ihrke

Quality Control Department



Danisco USA, Inc.
3329 Agriculture Drive
Madison, WI 53716
800-255-6837 Tel
608-395-2603 Fax

HEAVY METALS / PESTICIDES STATEMENT

Date: March 8, 2018

Product Code: 1283879

Product Name: Bi-26™ 50B - 1 KG

Dear Valued Customer,

Please be informed that DuPont Nutrition and Health performs annual surveillance testing for heavy metals and pesticides on samples of finished products.

Based on historical data we can state that the product above complies with the FCC specification:

- Arsenic <1 ppm
- Lead <0.5 ppm
- Mercury <0.05 ppm
- Cadmium <0.2 ppm

It is also in compliance with the Environmental Protection Act 40 CFR Part 180 Tolerances and Exemptions for Pesticide chemical residues in Food. We do not test for toxic residues such as Aflatoxins, PCB-Dioxin, Mycotoxins, PAH, 3 MCPD, etc.

This product is produced in accordance with the U.S. Food and Drug Administration's Current Good Manufacturing Practices guidelines, in an FDA regulated and inspected facility.

This information is given in respect of DuPont's policy of openness and transparency with its customers.

Sincerely,



Sarah Pace
Quality & Food Safety Coordinator
DuPont® - Nutrition & Health



NUTRITIONAL INFORMATION

Product Name: Bi-26 50B - 1KG

Product Code: 1283879

Parameter		Amount per 100 grams of product
Total Calories	(kcal)	373.3
- Calories from fat	(kcal)	12.3
Total fat	(g)	1.4
- Saturated Fat	(g)	0.5
- Trans fatty acids	(g)	0.0 **
Cholesterol	(mg)	0.0 **
Sodium	(mg)	426.0
Carbohydrates	(g)	42.6
- Dietary fiber	(g)	0.0 **
- Total sugars* (added)	(g)	35.8
Protein	(g)	47.7
Vitamin A	(IU)	<5
Vitamin C	(IU)	302.4
Vitamin D	(IU)	0.0 **
Calcium	(mg)	49.4
Iron	(mg)	1.3
Potassium	(mg)	568.0
Moisture	(g)	4.1
Ash	(g)	4.2

* Total sugars is the same as added sugar

** Not a significant source

The above nutritional information is analyzed, and to the best of our knowledge, is reliable. Users should conduct their own tests to determine the suitability of this data and this product for their own specific purposes. Statements contained herein should not be considered as a warranty of any kind, expressed or implied.

DuPont Nutrition & Health, 3329 Agriculture Drive, Madison, WI 53716 USA

PRODUCT DESCRIPTION - PD 245316-4.2EN**Material no. 1283879****Bi-26 50B - 1KG**

FloraFIT® Probiotics

Description

Freeze-dried probiotic powder. White to cream-color in appearance.

Country of origin

Made in the USA using foreign and domestic ingredients

Directions for use

See Danisco Cultures Usage & Handling Guide

Composition

Bifidobacterium infantis Bi-26™

Microbiological specifications

Cell count	> 5.00E+10 CFU / g
Non-Lactic Count	< 5000 / g
Enterococci	< 100 / g
Coliforms	< 10 / g
E. coli	neg. by test (< 0.3 / g)
Staphylococcus (coag. pos.)	neg. by test (< 10 / g)
Salmonella	neg. (40 g enrichment)
Listeria	neg. (25 g enrichment)

Storage

Shelf life is 18 months when stored in the original, sealed package at or below 4°C. Frozen storage will extend shelf life.

Packaging

High barrier foil laminated pouch

Quantity

1 kg

Purity and legal status

Local regulations should always be consulted concerning the status of this product, as legislation regarding its intended use may vary from country to country.

Safety and handling

MSDS is available on request.

Kosher status

Circle K certification

Halal status

IFANCA certification

PRODUCT DESCRIPTION - PD 245316-4.2EN

Material no. 1283879

Bi-26 50B - 1KG

FloraFIT® Probiotics

Allergens

Below table indicates the presence (as added component) of the following allergens and products thereof:

Yes	No	Allergens	Description of components
	X	wheat	
	X	other cereals containing gluten	
	X	crustacean shellfish	
	X	eggs	
	X	fish	
	X	peanuts	
	X	soybeans	
	X	milk (including lactose)	
	X	nuts	
	X	celery	
	X	mustard	
	X	sesame seeds	
	X	sulphur dioxide and sulphites (> 10 mg/kg)	
	X	lupin	
	X	molluscs	

Local regulation has always to be consulted as allergen labelling requirements may vary from country to country.

GMO status

Bi-26 50B - 1KG does not consist of, nor contains, nor is produced from genetically modified organisms according to the definitions of Regulation (EC) 1829/2003 and Regulation (EC) 1830/2003 of the European Parliament and of the Council of 22 September 2003.

TRADE SECRET

Unpublished Work
Copyright ©2016 E.I. du Pont de Nemours and Company

STUDY TITLE: Bifidobacterium infantis Bi-26: Acute Oral Toxicity Study in Rats - Up-and-Down Procedure

TEST GUIDELINES: U.S. EPA Health Effects Test Guidelines
OPPTS 870.1100 (2002)

OECD Guideline for the Testing of Chemicals
Section 4 (Part 425) (2008)

U.S. FDA Redbook 2000: IV.C.2: Acute Oral Toxicity Tests
(1993)

AUTHOR: Pushkor Mukerji, B.A.

STUDY COMPLETED ON: September 15, 2016

PERFORMING LABORATORY: E.I. du Pont de Nemours and Company
DuPont Haskell Global Centers
for Health & Environmental Sciences
P.O. Box 30
Newark, Delaware 19714
U.S.A.

LABORATORY PROJECT ID: DuPont-21549-834

WORK REQUEST NUMBER: 21549

SERVICE CODE NUMBER: 834

SPONSOR: E.I. du Pont de Nemours and Company
Wilmington, Delaware 19805
U.S.A.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in compliance with U.S. FDA (21 CFR part 58) Good Laboratory Practice Standards, which are compatible with current OECD Good Laboratory Practices, except for the items documented below. None of the items listed impact the validity of the study.

1. The characterization of the test substance was not performed under full compliance with GLPs. The procedures were conducted by trained personnel using established methods; therefore the accuracy of the data was considered sufficient for the purposes of this study.
2. The dosing formulation used in the study was analyzed for concentration, but not stability or homogeneity. Analyses of stability and homogeneity were not considered necessary to meet study objectives, because the formulation was prepared on the day of dosing and stirred prior to and throughout the dosing procedure.

Sponsor: E.I. du Pont de Nemours and Company
Wilmington, Delaware 19805
U.S.A.

Study Director: _____

Pushkor Mukerji, B.A.
Staff Toxicologist
E.I. du Pont de Nemours and Company

15-Sep 2016
Date

Sponsor: _____

Sponsor Representative

_____ Date

QUALITY ASSURANCE STATEMENT

Work Request Number: 21549
Service Code Number: 834

Key inspections for the above referenced study were completed by the Quality Assurance Unit of DuPont Haskell and the findings were submitted on the following dates:

<i>Audit Dates</i>	<i>Date Reported to Study Director</i>	<i>Date Reported to Management</i>
<u>Protocol:</u> 16 May 2016	16 May 2016	16 May 2016
<u>Conduct:</u> 26 May 2016	26 May 2016	26 May 2016
<u>Report/Records:</u> 10 August 2016	10 August 2016	10 August 2016

Reported by:




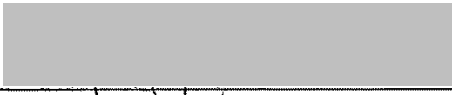
Jessica Garcia-Arbuet
Quality Assurance Auditor


25 August 2016
Date

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Analytical Evaluation by:  26-Aug-2016
Date
Abby Myhre, M.S.
Staff Scientist

Anatomic Pathology
Evaluation Reported by:  29-Aug-2016
Date
Nancy P. Betts, B.S.
Associate Scientist

Anatomic Pathology
Evaluation Reviewed by:  14 Sept 2016
Date
Steven R. Frame, D.V.M., Ph.D., Diplomate ACVP
Senior Research Fellow

Reviewed by:  31 August 2016
Date
Melissa N. Fallers, B.A.
Associate Scientist


Issued by Study Director:  15 Sep 2016
Date
Pushkor Mukerji, B.A.
Staff Toxicologist

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STUDY INFORMATION

Substance Tested: Bifidobacterium infantis Bi-26

Haskell Number: 31906

Composition: Bifidobacterium infantis

Purity: Not applicable

Physical Characteristics: Off white solid

Study Initiated/Completed: May 13, 2016 / (see report cover page)

Experimental Start/Termination: May 24, 2016 / September 14, 2016

In-Life Initiated/Completed: May 24, 2016 / June 7, 2016

SUMMARY

A single dose of Bifidobacterium infantis Bi-26 was administered by oral gavage to fasted female rats at a dose of 5000 mg/kg. The five rats were dosed on the same day. All rats were observed for mortality, body weight effects, and clinical signs for 14 days after dosing. The rats were necropsied to detect grossly observable evidence of organ or tissue damage. The concentration of colony forming units in the dosing formulation was analyzed by enumeration of the actual dosed formulation.

No deaths occurred. There were no clinical abnormalities or overall (test day 1-15) body weight losses. No gross lesions were present in the rats at necropsy.

Under the conditions of this study, Bifidobacterium infantis Bi-26 was not considered acutely toxic *via* the oral route of exposure in female rats at a dose level of 5000 mg/kg (equivalent to 1.94×10^{12} cfu/kg by analysis, and corresponding to a range of 4.36×10^{11} to 4.78×10^{11} cfu/animal). In the absence of test substance related mortality, an LD₅₀ was not calculated.

According to the guidance provided by the U.S. EPA for classification and label statements regarding hazards due to pesticides (Label Review Manual, Chapter 7: Precautionary Statements, revised July 2014), Bifidobacterium infantis Bi-26 is classified in Toxicity Category IV.

In accordance with the provisions of Regulation (EC) Number 1272/2008 amended by Commission Regulation (EU) Number 286/2011 of 10 March 2011 (ATP002) on the Classification, Labeling, and Packaging of Substances and Mixtures, classification is not required.

According to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Sixth revised edition 2015, classification is not required.

INTRODUCTION

The purpose of this study was to assess the acute oral toxicity of Bifidobacterium infantis Bi-26 when administered by oral gavage to female rats. Per the test guidelines, the dose level of 5000 mg/kg was chosen based on the lack of toxicity historically observed for probiotic test substances.

ANIMAL WELFARE ACT COMPLIANCE

This study complied with all applicable sections of the Guidelines from the Guide for the Care and Use of Laboratory Animals (NRC 2011). All studies conducted by or for DuPont Haskell Global Centers for Health & Environmental Sciences (DuPont Haskell) adhere to the following principles:

- The sponsor and/or the study director ensure that the study described in this report does not unnecessarily duplicate previous experiments, and is in compliance with the DuPont Policy on Animal Testing.
- Whenever possible, procedures used in this study have been designed to implement a reduction, replacement, and/or refinement in the use of animals in an effort to avoid or minimize discomfort, distress or pain to animals. All methods are described in this study report or in written laboratory standard operating procedures.
- DuPont Haskell policy is that animals experiencing severe pain or distress that cannot be relieved are painlessly euthanized, as deemed appropriate by the veterinary staff and study director or appropriate designee.
- Methods of euthanasia used during this study were in conformance with the above referenced regulation and the recommendations of the American Veterinary Medical Association (AVMA 2013) Guidelines on Euthanasia.
- Animals were provided with species-appropriate environmental enrichment.
- The procedures in the protocol have been reviewed by the Haskell Animal Welfare Committee and comply with acceptable standards for animal welfare and humane care.

DuPont Haskell is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

MATERIALS AND METHODS

A. Test Guidelines

The study design complied with the following test guidelines:

- U.S. EPA, OPPTS 870.1100: Acute Oral Toxicity, *Health Effects Test Guidelines* (2002)

- OECD, Section 4 (Part 425): Acute Oral Toxicity: Up-and-Down Procedure, *Guideline for the Testing of Chemicals* (2008)
- U.S. FDA, Redbook 2000: IV.C.2: Acute Oral Toxicity Tests (1993)

B. Test Substance

(Appendix A)

The test substance, Bifidobacterium infantis Bi-26, was supplied by the sponsor as an off white solid (powder), and was assigned Haskell number 31906. The test substance appeared to be stable under the conditions of the study. No evidence of instability, such as a change in color or physical state, was observed.

C. Test System

Female (nulliparous and non-pregnant) CrI:CD(SD) rats were received from Charles River Laboratories International, Inc., Raleigh, North Carolina, U.S.A.

The CrI:CD(SD) rat was selected based on consistently acceptable health status and on extensive experience with the strain at DuPont Haskell.

D. Animal Husbandry

1. Housing

Animals were housed individually in solid-bottom caging with bedding and appropriate species-specific enrichment.

2. Environmental Conditions

Animal rooms were maintained at a temperature of 20-26°C (68-79°F) and a relative humidity of 30-70%. Animal rooms were artificially illuminated (fluorescent light) on an approximate 12-hour light/dark cycle. Excursions outside of these ranges were of insufficient magnitude and/or duration to have adversely affected the validity of the study.

3. Feed and Water

PMI[®] Nutrition International, LLC Certified Rodent LabDiet[®] 5002 and water were available *ad libitum* except as noted in section F. Dosing.

4. Identification

Each rat was assigned an identification number, which was written on each rat's tail with a water-insoluble marker.

5. Acclimation

The rats were weighed and observed for general health during the 6-day quarantine period.

6. Animal Health and Environmental Monitoring Program

As specified in the DuPont Haskell animal health and environmental monitoring program, the following procedures are performed periodically to ensure that contaminant levels are below those that would be expected to impact the scientific integrity of the study:

- Water samples are analyzed for total bacterial counts, and the presence of coliforms, lead, and other contaminants.
- Samples from freshly washed cages and cage racks are analyzed to ensure adequate sanitation by the cagewashers.

Certified animal feed is used, guaranteed by the manufacturer to meet specified nutritional requirements and not to exceed stated maximum concentrations of key contaminants, including specified heavy metals, aflatoxin, chlorinated hydrocarbons, and organophosphates. The presence of these contaminants below the maximum concentration stated by the manufacturer would not be expected to impact the integrity of the study.

The animal health and environmental monitoring program is administered by the attending laboratory animal veterinarian. Evaluation of these data did not indicate any conditions that affected the validity of the study.

E. Formulation Samples

Duplicate samples were collected on the day of study start. Analysis was conducted on the same day as the dose was prepared. Analysis was conducted by enumerating colony forming units. Detailed methods are in Appendix A.

The duplicate, back-up, sample was saved at $<-60^{\circ}\text{C}$ and then discarded because no additional analysis was necessary.

F. Dosing

A single oral dose of Bifidobacterium infantis Bi-26, suspended in phosphate buffered saline, was administered oral gavage to fasted female rats at a dose level of 5000 mg/kg. The five rats were dosed on the same day.

The rats were approximately 10 weeks old on the day of dosing. The rats were fasted approximately 18.25 hours prior to dosing, with food being returned to the rats approximately 3 hours after dosing. Individual dose volumes were calculated using the fasted body weights obtained prior to dosing. The rats were dosed at a volume of 20 mL per kg of body weight. The weight of each animal was within the $\pm 20\%$ of the mean weight for the group of animals. The dosing formulations were stirred prior to and throughout the dosing procedure.

G. Observations, Body Weights, and Anatomic Pathology

Daily animal health observations were conducted throughout the study for mortality and signs of illness, injury, or abnormal behavior. Animals were weighed on test days -1, 1, 8, and 15, and

were observed for clinical signs at the beginning of fasting, just before dosing (test day 1), once during the first 30 minutes after dosing and 2 more times on the day of dosing, and once each day thereafter. On test day 15, the rats were euthanized and necropsied to detect grossly observable evidence of organ or tissue damage. The rats were euthanized by exsanguination while under isoflurane anesthesia.

The complete GI tract (esophagus to rectum) from each animal was excised and preserved in formalin. Because no further examination was required, the organs were discarded at the conclusion of the study.

RESULTS AND DISCUSSION

Analytical Evaluation

A. Dose Formulation Analysis

(Appendix A)

The concentration of Bifidobacterium infantis Bi-26 in the sample was measured at 9.68×10^{10} cfu/mL (colony forming units per mL), corresponding to an administered dose of 1.94×10^{12} cfu/kg bodyweight. The administered dose is calculated for each animal below.

Animal	Body Weight on Test Day 1 (g)	cfu/Animal ($\times 10^{11}$)
7983	229.1	4.44
7984	246.4	4.78
7985	227.0	4.40
7986	224.7	4.36
7987	235.6	4.57

In-life Toxicology

A. Dose Progression and Mortality

No deaths occurred.

B. Clinical Signs and Body Weights

(Appendix B through Appendix D)

There were no clinical abnormalities or overall (test day 1-15) body weight losses.

Anatomic Pathology Evaluation

A. Gross Observations

(Appendix E)

No gross lesions were present in the rats at necropsy.

CONCLUSIONS

Under the conditions of this study, Bifidobacterium infantis Bi-26 was not considered acutely toxic *via* the oral route of exposure in female rats at a dose level of 5000 mg/kg (equivalent to

1.94×10^{12} cfu/kg by analysis, and corresponding to a range of 4.36×10^{11} to 4.78×10^{11} cfu/animal). In the absence of test substance related mortality, an LD₅₀ was not calculated.

According to the guidance provided by the U.S. EPA for classification and label statements regarding hazards due to pesticides (Label Review Manual, Chapter 7: Precautionary Statements, revised July 2014), Bifidobacterium infantis Bi-26 is classified in Toxicity Category IV.

In accordance with the provisions of Regulation (EC) Number 1272/2008 amended by Commission Regulation (EU) Number 286/2011 of 10 March 2011 (ATP002) on the Classification, Labeling, and Packaging of Substances and Mixtures, classification is not required.

According to the United Nations Globally Harmonized System of Classification and Labelling of Chemicals (GHS), Sixth revised edition 2015, classification is not required.

RECORDS AND SAMPLE STORAGE

Raw data, the protocol, amendments (if any), and the final report will be retained at DuPont Haskell, Newark, Delaware, U.S.A. or Iron Mountain Records Management, Wilmington, Delaware, U.S.A.

APPENDICES

Appendix A
Certificate of Analysis, Analytical Results, and Analytical Methods



Certificate of Analysis

Date: 29 Apr 2016
Our ref. no.: 0
Your ref.

Material:	MSAMPBI-26	BI-26 50B 100GM STD SAMPLE
Batch No.:	1102552819	Best before date: 08 Dec 2016
		Production date: 09 Jun 2015

Test	Result	Specification	Unit	Reference
Viable Cell Count	1.82E+11	> 5.00E+10	/g	ISO 7889/ IDF 117
Enterococcus	< 100	< 100	/g	CMMEF
Non Lactics	< 5000	< 5000	/g	ISO 13559
Coliforms	< 10.0	< 10.0	/g	AOAC
E. coli, neg. by test (<0.3/g)	Negative	Negative		AOAC
Staph. aureus, neg. by test (<10/g)	Negative	Negative		AOAC
Salmonella, negative in 40 g	Negative	Negative		AOAC
Listeria, negative in 25 g	Negative	Negative		AOAC

Comments

Exceeds 50 billion CFU/gm of freeze-dried Bif. infantis.

The above product has been analyzed by Danisco and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

Best if used before the date listed above when stored at or below 4°C.

AOAC references above reflect the current edition of AOAC.

Culture identity is confirmed to Genus/species level (or sub-species level where applicable) based on DNA



Certificate of Analysis

Date: 29 Apr 2016
Our ref. no.: 0
Your ref.

Material:	MSAMPBI-26	BI-26 50B 100GM STD SAMPLE
Batch No.:	1102552819	Best before date: 08 Dec 2016
		Production date: 09 Jun 2015

Fingerprinting Analysis generated by Automated Ribotyping.

This certificate is generated automatically



Phil Ihrke

Quality Control Department

Danisco US - Madison Plant
CULTURE PLANT
3322 Agriculture Dr.
MADISON WI 53716

Page:2/ 2

Analytical Method and Results

An enumeration was conducted on the formulated test substance in order to determine the bacterial load received by the animals. One mL of the fresh dose preparation was added to 99 mL phosphate buffer and mixed thoroughly resulting in a 10^{-2} stock dilution. The 10^{-2} dilution was further serially diluted in phosphate buffered saline until a 10^{-8} and 10^{-9} dilution was achieved. The serial dilution of the 10^{-2} stock was conducted in duplicate. Two dilution levels were selected for plating to ensure at least one set of plates resulted in a countable number of colonies. The dilution scheme was selected based on the theoretical colony forming units (cfu)/mL calculated from the COA value. Both replicates of the 10^{-8} and 10^{-9} dilutions were plated in triplicate using the pour plate method. One mL of the 10^{-9} dilution or 0.5 mL of the 10^{-8} dilution was added to the bottom of each sterile petri dish. Fifteen mL of MRS/0.05% Cysteine-HCl agar was added to each dish and swirled gently. Once solidified the plates were inverted and incubated in an anaerobic environment at 38°C for approximately 72 hours. Following the incubation period all plates were counted and number of cfu recorded.

The counts from the 10^{-9} dilution were selected for calculating the bacterial load.

Replicate Plate Counts						Dilution	CFU/mL	Analysis date
1	2	3	4	5	6			
91	104	86	88	96	116	1.00E-09	9.68E+10	5/24/2016

Appendix B
Individual Body Weights

INDIVIDUAL BODY WEIGHTS

EXPLANATORY NOTES

ABBREVIATIONS:

g - grams

Individual Body Weights

Sex: Female Bodyweight (g)

5000 mg/kg	Day(s) Relative to Start Date			
	-1	1	8	15
7983	241.1	229.1	258.5	273.7
7984	255.1	246.4	264.2	285.2
7985	245.4	227.0	264.7	284.6
7986	234.4	224.7	251.0	258.4
7987	242.7	235.6	262.5	272.9
Mean	243.7	232.6	260.2	275.0
SD	7.5	8.7	5.7	10.9
N	5	5	5	5

Appendix C
Individual Body Weight Gains

INDIVIDUAL BODY WEIGHT GAINS

EXPLANATORY NOTES

ABBREVIATIONS:

g - grams

Individual Body Weight Gains

Sex: Female Body Weight Gain (g)

5000 mg/kg	Day(s) Relative to Start Date		
	1 → 8	8 → 15	1 → 15
7983	29.4	15.2	44.6
7984	17.8	21.0	38.8
7985	37.7	19.9	57.6
7986	26.3	7.4	33.7
7987	26.9	10.4	37.3
Mean	27.6	14.8	42.4
SD	7.1	5.9	9.4
N	5	5	5

Appendix D
Individual Clinical Observations and Mortality Records

INDIVIDUAL CLINICAL OBSERVATIONS AND MORTALITY RECORDS

EXPLANATORY NOTES

ABBREVIATIONS:

- A, B - time slot for observations
- Ts1 - post-dose observation 1 (within 30 minutes of dosing)
- Ts2 - post-dose observation 2
- Ts3 - post-dose observation 3
- X - present

Individual Clinical Observations and Mortality Records

Group	Sex	Animal	Clinical Sign	Site	Day numbers relative to Start Date														
					-1 A	1 A	1 Ts1	1 Ts2	1 Ts3	2 A	3 A	4 A	5 A	6 A	7 A	8 A	9 A	10 A	
1	f	7983	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	
			Scheduled sacrifice	
		7984	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Scheduled sacrifice	
		7985	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Scheduled sacrifice	
		7986	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Scheduled sacrifice	
		7987	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Scheduled sacrifice	

 Severity Codes: X = Present

Group 1 - 5000 mg/kg

Individual Clinical Observations and Mortality Records

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	11 A	12 A	13 A	14 A	15 A	
1	f	7983	No Abnormalities Detected		X	X	X	X	X	
			Scheduled sacrifice		X	
		7984	No Abnormalities Detected		X	X	X	X	X	X
			Scheduled sacrifice		X
		7985	No Abnormalities Detected		X	X	X	X	X	X
			Scheduled sacrifice		X
		7986	No Abnormalities Detected		X	X	X	X	X	X
			Scheduled sacrifice		X
		7987	No Abnormalities Detected		X	X	X	X	X	X
			Scheduled sacrifice		X

 Severity Codes: X = Present

Group 1 - 5000 mg/kg

Appendix E
Individual Animal Gross Observations

Individual Animal Gross Observations

Group: 1 Dose: 5000 mg/kg Sex: Female

Animal Ref.	Mode Of Death	Death Day (Week)	Observation(s)
7983	SCHEDULED SACRIFICE	15 (3)	No Visible Lesions
7984	SCHEDULED SACRIFICE	15 (3)	No Visible Lesions
7985	SCHEDULED SACRIFICE	15 (3)	No Visible Lesions
7986	SCHEDULED SACRIFICE	15 (3)	No Visible Lesions
7987	SCHEDULED SACRIFICE	15 (3)	No Visible Lesions

Appendix I

Reference	Design	Subjects	Strain/Dose	Duration	Safety-Related Results
Ma et al., 2019	Open-label, no control, before-and-after	Flood victims with IBS (age \geq 18 yr) received strain (n=20) or controls (n=30)	<i>B. infantis</i> M-63 (10^9 CFU/day)	12 weeks	"No additional symptoms or adverse events were reported from participants from either group during the entire period of intervention."
Enani et al., 2018	RDBPC parallel arm trial	Young subjects (age 18-35) and older subjects (age 60-85) received strain and gluco-oligosaccharide(n=60) or placebo (n=64).	<i>B. infantis</i> CCUG 52486 (10^9 CFU/day) + gluco-oligosaccharide	8 weeks	Two mild AEs of gastrointestinal bloating reported, one in study group and one in placebo.
Escribano et al., 2018	Multicentre RDBPC trial	Infants (age <3 months) received strain (n=93) or unsupplemented formula (n=97).	<i>B. infantis</i> IM1® (10^7 CFU/g)	12 weeks	Supplemented formula reported as safe and well tolerated.
Kumar et al., 2018	Open-label, no control	Healthy adults (age 22-64) received <i>B. infantis</i> (n=19)	<i>B. infantis</i> 35624, dose not reported	2 week treatment with LBT before and after	No significant difference was found when comparing pre- and post-supplementation gastrointestinal symptoms
Del Giudice et al., 2017	RDBPC parallel arm trial	40 Children (age 4-17) with asthma received strain combination or placebo (1:1)	<i>B. longum</i> BB536 (3×10^9 CFU), <i>B. infantis</i> M-63 (1×10^9 CFU), and <i>B. breve</i> M-16 V (1×10^9 CFU) daily	4 weeks	Both strain combination and placebo were well tolerated. No clinically significant side effects in either group.
Härtel et al., 2017	Observational population-based cohort study	VLBW infants (<33 weeks' gestation, subgroup 28-32 weeks) received strains (n=6229) or no strains (n=2305)	Infloran® <i>L. acidophilus</i> (10^9) and <i>B. infantis</i> (10^9) daily	Strains consumed for the duration of primary stay in hospital (\geq 28 d), with 24 mo and 5 year follow up analysis in a smaller subset	No safety related endpoints or adverse events were discussed.

Reference	Design	Subjects	Strain/Dose	Duration	Safety-Related Results
Manzano et al., 2017	RDBPC parallel arm trial	Healthy infants (age 3-12 months) received one of three strains (n=53 for B. infantis) or placebo (n=52)	B.infantis R0033, B. bifidum R0071, or L. helveticus R0052 (3x10 ⁹ CFU/day)	8 weeks	No serious adverse events. Total number of AEs recorded was equivalent in all groups.
Ringel-Kulka et al., 2017	RDBPC parallel arm trial	Adults experiencing abdominal discomfort and bloating ≥2 times per week for at least 3 months (n=275 total) not under physician supervision or treatment	B.infantis 35624 (10 ⁹ CFU/day)	2 week placebo run-in followed by 4 week intervention	Placebo and strain were reported as well tolerated.
Smilowitz et al., 2017	Parallel, partially-randomized Phase I clinical trial	Breastfeeding infants from day 7-28 of life given strain (n=34) or not (n=34).	B. infantis EVC001 (1.8x10 ¹⁰ CFU/day)	3 weeks	No observed different in infant illness or adverse events between study and control groups.
Esaiassen et al., 2016	Review/Case Studies	3 patients diagnosed with B. longum bacteremia. All patients extremely premature with impaired immune systems. Patients 1 & 3 additionally had severe gastrointestinal complications.	Infloran® L. acidophilus (10 ⁹) and B. infantis (10 ⁹); 1/2 capsule daily for first week, then 1 capsule daily	8-12 days	Infants developed bacteremia which was resolved with cessation of strain treatment and antibiotics.
Guthman et al., 2016	Retrospective cohort study	Review of VLBW infants receiving strain supplementation (n=591) or not (n=633) in German and Swiss hospitals	L. acidophilus and B. infantis (10 ⁹ CFU/day each)	10 or 14 days	Strain was effective in reducing rate of NEC. No safety related endpoints or adverse events were discussed.

Reference	Design	Subjects	Strain/Dose	Duration	Safety-Related Results
Hoy-Schulz et al., 2016	Randomized parallel arm trial	Healthy infants (age 4-12 weeks) received strain blend (n=89) or no supplementation (n=24).	L. reuteri DSM 17938 (10^8 CFU) and B. infantis 35624 (10^9 CFU)	Daily dosing (29 doses overall), weekly dosing (five doses), or every two week dosing (three doses)	No differences in rates of any reported symptoms were observed among arms; additionally, no sudden adverse or allergic reactions were found after strain administration, and no hospitalizations were deemed related to strain administration.
Powell et al., 2016	RDBPC pilot trial	Infants with gastroschisis received strain (n=10) or placebo (n=11).	B. infantis ATCC 15697 (10^9 CFU twice daily)	6 weeks or until discharge	Administration of the strain or placebo was well tolerated, even during the period of gastric suctioning.
Stojković et al., 2016	Observational cohort study	Children (age < 5) hospitalized in previous year due to respiratory infection consumed strains and oligosaccharide(n=78).	L. acidophilus Rosell-52, B. infantis Rosell-33, B. bifidum Rosell-71 (5×10^9 CFU total) + FOS	3-9 months	No side effects of strains and oligosaccharide were identified in the examined children and it was well tolerated.
Tehrani et al., 2016	RDBPC parallel arm trial	Healthy children (age 3-6 y) in placebo (n=23) or strain combination (n=30) groups	5 drops containing L. rhamnosus ATCC 15820 (1×10^{10} CFU/mL), L. reuteri ATCC 55730 (2×10^9 CFU/mL), and B. infantis ATCC 15697 (1.5×10^9 CFU/mL) daily	14 days	No safety related endpoints or adverse events were discussed.

Reference	Design	Subjects	Strain/Dose	Duration	Safety-Related Results
Langkamp-Henken et al., 2015	RDBPC parallel arm trial	Healthy undergraduate students (age ≥ 18) received one of three strains (n=142 for B. infantis) or placebo (n=147)	L. helveticus R0052, B. infantis R0033, or B. bifidum R0071 (3×10^9 CFU/day)	6 weeks	No safety related endpoints were discussed. Withdrawals related to mild symptoms did not differ significantly between study and control groups. One participant in the B infantis group withdrew after 1 day because of abdominal pain, 1 participant in the placebo group withdrew after 25 days because of abdominal pain and 1 participant in the placebo group withdrew due to diarrhea.
Van Niekerk et al., 2015	RDBPC parallel arm trial	Premature and VLBW infants (<34 weeks' gestation; <1250 g) separated into HIV-exposed and HIV-unexposed groups received strain combination (n=37+54) or placebo (n=37+56).	L. rhamnosus GG and B. infantis (3.5×10^8 CFU each/day)	28 days or upon discharge	None of the positive blood cultures taken from patients presenting septicaemia grew Lactobacillus or Bifidobacterium species.
Zbinden et al., 2015	Review/Case Studies	3 VLBW preterm infants (<30 week gestational age) diagnosed with B. longum bacteremia.	Infloran® L. acidophilus and B. infantis; Dose not reported	11-28 days	Infants developed bacteremia which was resolved with cessation of strain consumption and antibiotics.
Bertelli et al., 2014	Case Report	2 VLBW preterm infants (≤ 28 weeks gestation). Patient 1 diagnosed with ileoileal intussusception. Patient 2 with NEC.	Infloran® L. acidophilus (5×10^8 CFU) and B. infantis (5×10^8 CFU) twice daily	Patient 1: 10 days; Patient 2: 5 days	Infants developed bacteremia which was resolved with cessation of strain consumption and antibiotics.
Härtel et al., 2014	Observational population-based cohort study	VLBW infants (<1500 g and gestational age >22 and <32 weeks) received strain combination (n=2310) or no strains (n=518)	Infloran® L. acidophilus (10^9) and B. infantis (10^9) daily	From day 2 or 3 of life for 14 days or until full enteral feeds.	No safety related endpoints or adverse events were discussed.

Reference	Design	Subjects	Strain/Dose	Duration	Safety-Related Results
Li et al., 2014	Retrospective cohort study	VLBW infants (< 1500 g). Study group of patients admitted Aug. 2007- Jul. 2011 receiving strain combination (n=291) and control group of patients admitted Aug. 2003-Jul. 2007 receiving no strains (n=289)	S. thermophilus, B. infantis and B. bifidum (0.5-1.05x10 ⁹ CFU total/day)	Until corrected gestational age of 36 weeks or discharge.	Strain consumption was well tolerated with no major adverse events.
Tobin et al., 2014	Open label	Preterm infants (<32 weeks gestation, weighing <1500 g) received strain combination (n=6) or breastmilk (n=6). Healthy adults received strain combination (n=7)	ABC Dophilus Probiotic Powder for Infants®: B. infantis, S.thermophilus and B. lactis (1-3x10 ⁹ CFU/day of B. infantis)	7 days	No safety related endpoints or adverse events were discussed.
Charbonneau et al., 2013	Single center, double blind, randomized trial.	Patients (age 18-65) with IBS received strain (n=39) or placebo (n=37), and healthy subjects received strain (n=41)	B. infantis 35624, 10 ⁹ CFU/day	8 weeks	AEs were mild and did not differ significantly between groups. No AEs were attributed to the treatment.
Ellis et al., 2013	RDBPC Pilot study	Infants with congenital heart disease received B. infantis (n=8) or placebo (n=8).	B. infantis (8.4x10 ⁹ CFU/day)	8 weeks	No safety related endpoints or adverse events were discussed.
Groeger et al., 2013	Three separate RDBPC trials	Patients (age 18-75); healthy (n=22) and with ulcerative colitis (n = 22), chronic fatigue syndrome (n = 48) and psoriasis (n = 26).	B. infantis 35624, 10 ¹⁰ CFU/day	6-8 weeks	No safety related endpoints or adverse events were discussed.
Jacobs et al., 2013	Multicentre RDBPC trial	Preterm infants (<32 weeks gestation, weighing <1500 g) received strain combination (n=548) or placebo (n=551)	ABC Dophilus Probiotic Powder for Infants®: B. infantis, S.thermophilus and B. lactis (300x10 ⁶ CFU/day of B. infantis)	Strains consumed until discharge or 12 month corrected age	No serious adverse events reported. Strain consumption appears to be safe.

Reference	Design	Subjects	Strain/Dose	Duration	Safety-Related Results
Smecuol et al., 2013	Exploratory RDBPC study	Subjects (age 18-75) received strain (n=12 or placebo (n=10)	B. infantis (Lifestart 2), 4×10^9 CFU/day	3 weeks	No serious adverse effects or significant biochemical changes were reported by patients in either group.
Pantovic, 2013	Uncontrolled, open-label trial	Hospitalized children (0-42 mo, n=31)	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS) 3×10^9 CFU	6 months	No adverse events were reported
Underwood et al., 2013	Randomized Phase I trial	Preterm infants (<33 weeks gestation, <1500 g) received increasing doses of B. infantis (n=6) or B. lactis (n=6) with formula. Second trial of subjects consumed both strains alternately with washout period (n=9).	B. infantis or B. lactis (up to 8.4×10^9 CFU/day)	2-5 weeks	Strains were well tolerated.
Wu, 2013	Single center RDB active control trial	Hospitalized children (0-36 mo, n=84)	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS) < 12 mo (n=32) 5×10^9 CFU 13-24 mo (n=35) 5×10^9 CFU 25-36 mo (n=17) 15×10^9 CFU All dose groups received oral Smecta Active control (n=64) groups matched for age all received only oral Smecta	3 days	No adverse reactions, No adverse reactions were observed in any group

Reference	Design	Subjects	Strain/Dose	Duration	Safety-Related Results
Xi et al, 2013	Randomized, DB, active control trial	Children (1-36 mo) diagnosed with thrush	Probiokid a 1:1:1 mixture of <i>L. helveticus</i> R0052, <i>B. infantis</i> R0033, <i>B. bifidum</i> R0071 + FOS) (n=35) 2% Na ₂ CO ₃ + nystatin + Biostime 1 x 10 ¹⁰ CFU	17 days	No adverse reaction was reported
Al-Hosni et al, 2012	Multicenter randomized controlled double-blinded clinical study	ELBW infants (<1000 g) separated into strain (n=50) and control (n=51) groups	<i>L. rhamnosus</i> GG and <i>B. infantis</i> (5x10 ⁹ CFU of each/day)	Until 34 weeks postmenstrual age or discharge	No sepsis was detected related to strain consumption and no reports of adverse events were attributed to train.
Gao, 2012	Single center RDB active control trial	Hospitalized children (0-36 mo, n=86)	Probiokid a 1:1:1 mixture of <i>L. helveticus</i> R0052, <i>B. infantis</i> R0033, <i>B. bifidum</i> R0071 + FOS) < 12 mo (n=14) 5 x 10 ⁹ CFU 13-24 mo (n=14) 5 x 10 ⁹ CFU 25-36 mo (n=15) 15 x 10 ⁹ CFU All dose groups received oral Smecta Active control (n=43) groups matched for age all received only oral Smecta	3 days	No adverse reactions, No adverse reactions were observed in any group

Reference	Design	Subjects	Strain/Dose	Duration	Safety-Related Results
Wang, 2012	Single center RDB active control trial	Children (3-36 mo, n=194) with non-infectious diarrhea	Probiokid a 1:1:1 mixture of <i>L. helveticus</i> R0052, <i>B. infantis</i> R0033, <i>B. bifidum</i> R0071, plus FOS) < 12 mo (n=33) 5 x 10 ⁹ CFU 13-24 mo (n=43) 5 x 10 ⁹ CFU 25-36 mo (n=28) 15 x 10 ⁹ CFU All dose groups received oral Smecta Active control groups matched for age all received only oral Smecta	3 days	No adverse reactions. There was no report of adverse events.
Frech et al., 2011	Open label	10 Adult patients with systemic sclerosis and symptoms of bloating consumed <i>B. infantis</i> or <i>L. rhamnosus</i>	<i>L. rhamnosus</i> or <i>B. infantis</i> (10 ⁹ CFU/day)	2 months	No complication with strain use were reported.
Jenke et al., 2011	Case Study	VLBW Infant (27 wks gestation, 600 g)	Infloran® <i>L. acidophilus</i> and <i>B. infantis</i> ; Dose not reported	10 days	Infant developed symptoms of septicaemia which were resolved with cessation of strain consumption ,and antibiotics.
Cazzola et al., 2010	Multicentre RDBPC trial	Healthy children (age 3-7 y) who reported at least 3 episodes of illness in previous winter received combination of strains and oligosaccharide (n=62) or placebo (n=73)	Probiokid a 1:1:1 mixture of <i>L. helveticus</i> R0052, <i>B. infantis</i> R0033, <i>B. bifidum</i> R0071, and FOS (3x10 ⁹ CFU/day total)	3 months	Reported adverse events were not serious and did not differ between study groups. None were attributed to strain treatment.

Reference	Design	Subjects	Strain/Dose	Duration	Safety-Related Results
Yang et al, 2010	Randomized, DB, PC trial	Hospitalized children (6-30 mo) with rotaviral infection and diarrhea	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS) (n=58) 5 x 10 ⁹ CFU plus lactose-free milk powder formula or control (n=40 breast-fed or formula fed)	Until discharge (mean 8.5 ± 2.3 days)	No adverse events were reported
Jiang, 2008	RDB active control trial	Hospitalized or outpatient children (3-24 mo) with persistent diarrhea	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS)(n=32) < 6 mo 5 x 10 ⁹ CFU 12-24 mo 10 - 20 x 10 ⁹ CFU Golden Bifido control (n=20)	Until diarrhea resolved (mean 7.1 days)	No adverse events were reported
Mei & Chen, 2008	RDB active control trial	Children (0-7 yr, n=78) diagnosed with rotavirus infection	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS)(n=32) 1 x 10 ¹⁰ CFU + Ribaviren vs Ribavirin only control (n=39)	7 days	There was no report of adverse events

Reference	Design	Subjects	Strain/Dose	Duration	Safety-Related Results
Chen et al, 2007	Control, parallel arm trial	Children (<1 - 4 years, n=28)	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS) (n=28, 1 x 10 ¹⁰ CFU) Age-matched healthy controls (n=8)	13 days	No adverse events were reported
Cui & Wure, 2007	RDB active control trial	Hospitalized children (6 - 24 mo, n=62) diagnosed with rotavirus infection who had diarrhea for less than 3 days	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS)(n=62) < 12 mo 5 x 10 ⁹ CFU 12 -24 mo 1 x 10 ¹⁰ CFU Lacidophilin control (n=60)	Until diarrhea resolved (at least 72 hrs)	There was no report of adverse events
Vivatvakin et al., 2006	Open, randomized control trial	Infants admitted to hospital with acute watery diarrhea (age 1-24 months) received strain + ORS (n=35) or ORS only (n=36)	L. acidophilus and B. infantis (3x10 ⁹ CFU/day total)	2 days	No adverse events were reported
Whorwell et al., 2006	RDBPC parallel arm trial	IBS patients allocated to placebo, or strain at doses of 10 ⁶ , 10 ⁸ , or 10 ¹⁰ CFU (n=90 per group)	B. infantis 35624 (10 ⁶ , 10 ⁸ , or 10 ¹⁰ CFU/day)	4 weeks	No significant adverse events were recorded.
Lin, et al., 2005	Masked, randomized control trial	VLBW infants received strain combination(n=180) or control (n=187)	Infloran® L. acidophilus and B. infantis with breastmilk twice daily, dose not reported	Strains consumed for the duration of hospital stay, beginning at day 7 of life	Primary outcome was not safety, but no complications were observed related to treatment (such as Lactobacillus or Bifidobacterium sepsis)

Reference	Design	Subjects	Strain/Dose	Duration	Safety-Related Results
O'Mahony et al., 2005	RDBPC parallel arm trial	Adults with IBS (age 18-75) (n=77) randomized to receive B. infantis, L. salivarius, or placebo.	L. salivarius UCC4331 or B. infantis 35624 (10^{10} CFU/day)	8 weeks	Therapy was well tolerated and free of significant adverse events
Lee et al., 2001	Prospective clinical study	Children (6-60 months) allocated to receive strain combination (n=50) or rehydration alone (n=50)	L. acidophilus and B. infantis (10^9 CFU/day each)	4 days	Other than primary outcome, no safety endpoints were discussed. Authors considered therapy to be safe and effective.
Hoyos, 1999	Single center, open-label comparison study	Newborns admitted to hospital (average gestational age 35 weeks) during October 1994-October 1995 received strains (n=1237). Data was compared to previous year admissions receiving no strain (n=1282).	L. acidophilus and B. infantis (2.5×10^8 CFU each) daily	Length of hospital stay (averages ranging from 5.5-8.5 days)	No complications attributed to the use of the strain preparation were observed.

Appendix J

Table 1: Decision Tree Analysis for Determining the Safety of Microbial Cultures for Consumption

1. Has the strain ⁱ been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? ⁱⁱ (If YES, go to 2. If NO, the strain must be characterized and unambiguously identified before proceeding).	YES
2. Has the strain genome been sequenced? (If YES, go to 3. If NO, the genome must be sequenced before proceeding to 3.) ⁱⁱⁱ	YES
3. Is the strain genome free of genetic elements ^{iv} encoding virulence factors ^v and/or toxins ^v associated with pathogenicity? ^{vi} (If YES, go to 4. If NO, go to 15.)	YES
4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? ^{vii} (If YES, go to 5. If NO, go to 15.)	YES
5. Does the strain produce antimicrobial substances? ^{viii} (If NO, go to 6. If YES, go to 15.)	NO
6. Has the strain been genetically modified using rDNA techniques? (If YES, go to 7. If NO, go to 8.)	NO
7. Do the expressed product(s) that are encoded by the introduced DNA have a history of safe use in food ^{ix} ? (If YES, go to 8. If NO, the expressed product(s) must be shown to be safe before proceeding to 8.) ^x	YES
8. Was the strain isolated from a food that has a history of safe consumption for which the species , to which the strain belongs, is a substantial ^{xi} and characterizing ^{xii} component (not simply an 'incidental isolate')? (If YES, go to 9. If NO, go to 13.) ^{xiii}	YES
9. Has the species , to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been affirmed to be safe for food use by an authoritative group of qualified scientific experts? ^{xiv} (If YES, go to 10. If NO, go to 13.)	YES
10. Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9a continue to support the conclusion that the species , to which the strain belongs, is safe for use in food? (If YES, go to 11. If NO, go to 13.)	YES
11. Will the intended use of the strain expand exposure to the species beyond the group(s) that typically consume the species in "traditional" food(s) in which it is typically found (for example, will a strain that was isolated from a fermented food typically consumed by healthy adults be used in food intended for an 'at risk' group)? (If NO, go to 12. If YES, go to 13.)	NO

<p>12. Will the intended use of the strain expand intake of the species (for example, increasing the number of foods beyond the traditional foods in which the species typically found, or using the strain as a probiotic rather than as a fermented food starter culture, which may significantly increase the single dose and/or chronic exposure)? (If NO, go to 14. If YES, go to 13.)</p>	<p>YES</p>
<p>13. Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? ^{xv} If yes, go to 15. If no, go to 14.)</p>	<p>NO</p>
<p>14. The strain is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.</p>	<p>YES</p>
<p>15. The strain is NOT APPROPRIATE for human or animal consumption.^{xvi}</p>	

ⁱ A strain is a “population of organisms that descends from a single organism or pure culture isolate.” P. 392, Prescott, Harley and Klein, 1996, Microbiology, Wiley. We recognize that the genotype and/or phenotype of a strain may change slightly when carried in culture, but such changes are irrelevant to safety considerations because there is no known mechanism or precedent for isolated strains in culture to begin spontaneously expressing pathogenic traits, unless that potential was already present in the genome at the time of isolation.

ⁱⁱ Whole Genome Sequencing provides distinct advantages for identification and characterization of microorganisms. In-depth analysis, including functional and comparative genomic studies, is afforded by sequencing the whole genome. This technology can provide a wealth of information that can be used for identification and characterization, including evidence of genetic evolution for adaptation of a species to a nutrient-rich environment, such as dairy products or the gastrointestinal tract (Pfeiler, EA, Klaenhammer, TR. 2007. The genomics of lactic acid bacteria. TRENDS in Microbiol, 15(12); 546-553). Less comprehensive molecular analysis, such as RAPD, FISH, and MLST, may also provide adequate information for identification, but the characterization ability is often times limited within a bacterial species (Gosiewski, T, Chmielarczyk, A, Strus M, Brzychczy-Wloch M, Heczko PB. 2012. The application of genetics methods to differentiation of three *Lactobacillus* species of human origin. Ann Microbiol 62:1437-1445).

ⁱⁱⁱ The genomic sequence provides the tools to mine the genome for a number of functions, uncovering information spanning from safety to host-cell interactions (Callanan, M. 2005. Mining the Probiotic Genome: Advanced Strategies, Enhanced Benefits, Perceived Obstacles. Current Pharmaceutical Design, 11: 25-36). From a regulatory perspective, the ability to show percentage/regions of similarity and differentiation between a new strain of interest in comparison with a *type strain*, or an accepted strain with history of safe use, is beneficial (U.S. FDA; July 2011. Draft Guidance for Industry: Dietary Supplements: New Dietary Ingredient Notifications and Related Issues). The genome sequence is analogous to a chemical specification for a food ingredient, that is, it defines precisely what is being evaluated and permits a genetic assessment of pathogenic and toxigenic potential. Isolates from a type-strain culture collection, or a strain collection held by a commercial culture manufacturer, may be considered to have the same safety characteristics as, and to be substantially equivalent to, the original source pure culture, so in

these cases the requirement for genome sequencing may be satisfied by sequencing the genome of the original source pure culture.

^{iv} The term "genetic elements" refers to gene sequences encoded in the chromosome or extra-chromosomal DNA.

^v Known genetic element sequences for virulence factors and protein toxins are searchable, e.g. the MvirDb database of microbial virulence factors (<http://mvirdb.lnl.gov>) [ref Nucl. Acids Res.(2007) 35 (suppl 1): D391-D394.doi: 10.1093/nar/gkl791].

^{vi} In considering the issue of "pathogenicity" and the potential to produce an infection, it is important to distinguish between *true pathogens* (i.e., microbes that possess virulence factors and are therefore capable of crossing or evading non-compromised host barriers) versus *opportunistic pathogens* (i.e., microbes that do not possess the required virulence factors to produce an infection in a non-compromised host). Typically this can be accomplished via genome analysis for known virulence factors coupled with a comprehensive search of the peer-reviewed scientific literature for infectious potential.

^{vii} A functional antibiotic resistance gene results in an antibiotic resistance phenotype.

^{viii} In this context, the term 'antimicrobial substances' refers to antibiotics that are used in medical or veterinary applications, for example substances that are positive in the JECFA test (FAO. 1981. *FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives*, Appendix A, pp. 317–318, FAO/WHO, Geneva, Switzerland.)

^{ix} The use of the terms "food" and "feed" includes supplements, which are in most jurisdictions considered to be a subset of the general categories.

^x Demonstration of the safety of the expressed product may be accomplished by testing, e.g. toxicological testing as required by various regulatory bodies such as the US FDA Redbook 2000 (<http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditive/sGRASPackaging/ucm2006826.htm>) or by establishing a substantial equivalence of the test article to a substance with a safe history of use in food, or, in the case of animal feed additives, establishing a substantial equivalence of the test article to a substance with a history of safe use in target animal feeds.

^{xi} Food fermentations, e.g. Cheddar cheese or yogurt, commonly result in "substantial" microbial food culture populations of 10^6 - 10^8 colony forming units per gram of the food. Significance should be judged relative to the fermented food, i.e. numbers of different organisms in a microbial population may change during the course of the life of the fermented food, e.g. Lactobacilli counts in Cheddar cheese are routinely low in the initial stages of cheese maturation, but begin to increase in numbers while the Lactococci, responsible for initial acid production, count decreases as the cheese ripens and pH decrease. [Spatial and temporal distribution of non-starter lactic acid bacteria in Cheddar cheese. N.A. Fitzsimons, T.M. Cogan, S. Condon, T. Beresford. *Journal of Applied Microbiology* 90(4): 600–608, 2001; Kosikowski, F. V., and V. V. Mistry. *Cheese and Fermented Milk Foods*. 1997. 3rd Ed. F. V. Kosikowski, L. L. C. Westport, CT.]

^{xii} A species is a "characterizing" component of a food if it has a measurable impact on flavor, texture, stability or preservation properties that are characteristic of the food, e.g. typical color and flavor of "blue" cheeses derived

from *Penicillium roqueforti*; or surface texture, flavor and odor of Limburger cheese resulting from *Brevibacterium linens* growth on the surface. The color and flavor of “blue” cheese and the aroma, flavor and texture of Limburger cheese are characteristic of the food and the microbial cultures that are responsible for these traits are characterizing components.

^{xiii} A strain that was isolated from a type-strain or a commercial culture, with a history of safe use in food fermentations, is deemed to have satisfied this requirement and may proceed to 9a.

^{xiv} For example, the Qualified Presumption of Safety list (<http://www.efsa.europa.eu/en/topics/topic/qps.htm>) prepared and periodically updated by the European Food Safety Authority is the output from a systematic safety review of the included microorganisms by qualified experts.

^{xv} Experimental evidence of safety is required. Such evidence may include, but is not necessarily limited to, studies in appropriate animal models, and clinical trials in humans.

^{xvi} In some cases, the strain may be shown to be appropriate by test and re-application of the decision tree, e.g., where an undesirable genetic element has been removed from a strain's genome.

Appendix K

Michael W. Pariza Consulting LLC
7102 Valhalla Trail
Madison, WI 53719
(608) 271-5169
mwpariza@gmail.com

Michael W. Pariza, Ph.D., Member

January 2, 2020

Elizabeth McCartney
Regulatory Affairs Specialist
Nutrition & Biosciences
Dow DuPont Specialty Products (DuPont) Division
3329 Agriculture Drive
Madison, WI 53716

RE: GRAS opinion on the intended uses of DuPont's *Bifidobacterium longum* subsp. *infantis* Bi-26™

Dear Ms. McCartney,

I am writing regarding your request for an evaluation of safety of DuPont's *Bifidobacterium longum* subsp. *infantis* Bi-26™ (Bi-26™) for use in yogurt and other dairy products, soy products, beverages, chewing gum, confectionary, snacks and other foods, and also dietary supplement formats including powders, sticks, sachets, tablets, and capsules, at levels that will provide a total intake not to exceed 5×10^{12} CFU/day. In conducting this evaluation, I considered the biology of *Bifidobacterium* and *B. longum* subsp. *infantis*, relevant information available in the peer-reviewed scientific literature, and information that you provided in the document entitled, "Comprehensive GRAS Assessment Of *Bifidobacterium longum* subsp. *infantis* Bi-26™".

Bifidobacterium spp. are Gram-positive, non-spore forming, anaerobic, pleomorphic bacilli that constitute one of the major microbial genera of the human colonic microbiota. The *Bifidobacterium* genus does not contain pathogenic or toxigenic species. *Bifidobacteria* were discovered in the feces of breast-fed infants and are regarded as a primary reason for the greater resistance of breast-fed infants to disease. *Bifidobacteria* also appear to play key positive roles in the intestinal health of humans throughout life.

Bifidobacterium longum subsp. *infantis* has a history of safe use as a probiotic. For example, the organism has been approved for use as a medicinal ingredient in Natural Health Products by the Natural and Non-Prescription Health Product Directorate of Canada. The genomes of

several strains of *Bifidobacterium longum* subsp. *infantis*, including Bi-26™, have been sequenced and found to contain no evidence of pathogenic or toxigenic traits. To the contrary, analysis of the *B. Longum* subsp. *infantis* genome revealed genes for several physiological traits that appear to explain in part the successful and beneficial adaptation of this bacterium to the human colon (Sela *et al.*, The genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome. *Proc Natl Acad Sci US* 105: 18964–18969, 2008).

Bi-26™ was isolated from a healthy human. The safety of the strain has been evaluated using the decision tree of Pariza *et al.* (Determining the safety of microbial cultures for consumption by humans and animals. *Regul Toxicol Pharmacol* 73:164-171, 2015). Since 2014, Bi-26™ has been sold in North America, Europe, the Middle East, China and other Asia/Pacific countries, and South Africa. Bi-26™ is produced under cGMP using only good grade ingredients. The specifications for DuPont's Bi-26™ product are appropriate for a microorganism that is used in human food, and the proposed use levels are appropriate for a species that normally resides in the human colon and is associated with beneficial health effects.


From these considerations, I agree that DuPont's *Bifidobacterium longum* subsp. *infantis* Bi-26™ product, manufactured consistent with cGMP and meeting food grade specifications, is *Generally Recognized As Safe* (GRAS) for direct addition to foods including, but not limited to, yogurt and other dairy products, soy products, beverages, chewing gum, confectionary, and snacks at levels that will provide a total intake not to exceed 5×10^{12} CFU/day.

I also agree that DuPont's *Bifidobacterium longum* subsp. *infantis* Bi-26™ product, manufactured consistent with cGMP and meeting food grade specifications, is safe and does not present a significant or unreasonable risk of illness or injury under the conditions of use in dietary supplement formats including powders, sticks, sachets, tablets, and capsules at levels that will provide a total intake not to exceed 5×10^{12} CFU/day.

It is my professional opinion that other qualified experts would concur in these conclusions.

Please note that this is a professional opinion directed at safety considerations only and not an endorsement, warranty or recommendation regarding the possible use of the subject product by you or others.

Sincerely,



Michael W. Pariza, Ph. D.
Member, Michael W. Pariza Consulting, LLC
Professor Emeritus, Food Science
Director Emeritus, Food Research Institute
University of Wisconsin-Madison

FDA USE ONLY

GRN NUMBER 000985	DATE OF RECEIPT Dec 15, 2020
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration
**GENERALLY RECOGNIZED AS SAFE
(GRAS) NOTICE** (Subpart E of Part 170)

Transmit completed form and attachments electronically via the Electronic Submission Gateway (*see Instructions*); OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety (*HFS-200*), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission (*Check one*)
 New Amendment to GRN No. _____ Supplement to GRN No. _____

2. All electronic files included in this submission have been checked and found to be virus free. (*Check box to verify*)

3. Most recent presubmission meeting (*if any*) with FDA on the subject substance (*yyyy/mm/dd*): _____

4. For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? (*Check one*)
 Yes If yes, enter the date of communication (*yyyy/mm/dd*): _____
 No

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier	Name of Contact Person Jayne Chalfin Davies		Position or Title Regulatory Affairs	
	Organization (<i>if applicable</i>) Danisco USA, Inc.			
	Mailing Address (<i>number and street</i>) 200 Powder Mill Road E320			
City Wilmington		State or Province Delaware	Zip Code/Postal Code 19803	Country United States
Telephone Number 610 864 7219		Fax Number	E-Mail Address jayne.c.davies@dupont.com	
1b. Agent or Attorney (if applicable)	Name of Contact Person		Position or Title	
	Organization (<i>if applicable</i>)			
	Mailing Address (<i>number and street</i>)			
City		State or Province	Zip Code/Postal Code	Country
Telephone Number		Fax Number	E-Mail Address	

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Bifidobacterium longum subsp. infantis Bi-26™

2. Submission Format: (Check appropriate box(es))

- Electronic Submission Gateway Electronic files on physical media
 Paper
If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? (Check one)

- Yes (Proceed to Item 5) No (Proceed to Item 6)

5. The submission incorporates information from a previous submission to FDA as indicated below (Check all that apply)

- a) GRAS Notice No. GRN _____
 b) GRAS Affirmation Petition No. GRP _____
 c) Food Additive Petition No. FAP _____
 d) Food Master File No. FMF _____
 e) Other or Additional (describe or enter information as above) _____

6. Statutory basis for conclusions of GRAS status (Check one)

- Scientific procedures (21 CFR 170.30(a) and (b)) Experience based on common use in food (21 CFR 170.30(a) and (c))

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

- Yes (Proceed to Item 8)
 No (Proceed to Section D)

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information (Check all that apply)

- Yes, information is designated at the place where it occurs in the submission
 No

9. Have you attached a redacted copy of some or all of the submission? (Check one)

- Yes, a redacted copy of the complete submission
 Yes, a redacted copy of part(s) of the submission
 No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

B infantis Bi-26™ is manufactured in compliance with current Good Manufacturing Practice as specified in 21 CFR Part 111. B infantis Bi-26™ is intended to be added as a live microbial ingredient to non-exempt infant and toddler formula at a level of 1×10^8 CFU/g to ensure at least 1×10^6 CFU/g serving throughout the 12-18 month life of the product.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

- Yes No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

- Yes No, you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

- PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).
- PART 3 of a GRAS notice: Dietary exposure (170.235).
- PART 4 of a GRAS notice: Self-limiting levels of use (170.240).
- PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).
- PART 6 of a GRAS notice: Narrative (170.250).
- PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

Yes No

Did you include this other information in the list of attachments?

Yes No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Jayne Chalfin Davies

(name of notifier)

has concluded that the intended use(s) of Bifidobacterium longum subsp. infantis Bi-26™

(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Jayne Chalfin Davies *(name of notifier)* agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

DuPont Nutrition&Biosciences 200 Powder Mill Road, Building 320 Wilmington DE 19803

(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance. The notifying party certifies that the information provided herein is accurate and complete to the best of his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official,
Agent, or Attorney

Jayne Chalfin Davies Digitally signed by Jayne Chalfin Davies
Date: 2020.12.15 14:59:38 -05'00'

Printed Name and Title

Jayne Chalfin Davies

Date (mm/dd/yyyy)

12/15/2020

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)

OMB Statement: Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services, Food and Drug Administration, Office of Chief Information Officer, PRASStaff@fda.hhs.gov. (Please do NOT return the form to this address). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

Part 1 – Signed statements and certification

April 1, 2021
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
HFS-200,
5001 Campus Drive
College Park, MD 20740-3835

Re: GRAS Notice – Exemption claim for the use of *Bifidobacterium longum* subsp. *infantis* Bi-26™

Dear Office of Food Additive Safety:

In accordance with the U.S. Food and Drug Administration's (FDA) Substances Generally Recognized as Safe; Final Rule, (81 FR 54959) relating to the filing of notices for substances that are considered to be generally recognized as safe (GRAS), please accept this claim and the attached information, submitted in triplicate, for that purpose as it relates to the use of *Bifidobacterium longum* subsp. *infantis* Bi-26™ (hereafter *B. infantis* Bi-26™). Specifically, we claim that the use of *B. infantis* Bi-26™ in non-exempt infant and toddler powdered formulas is exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act based on its determination that such uses are GRAS. This conclusion was made in concert with a panel of experts qualified by scientific training and experience.

No information used in this part of this notification is trade secret or confidential commercial information. In accordance with the requirements outlined in 21 CFR 170, Subpart E of the final rule, the following information is included with this exemption claim:

- (i) Name and address of the Notifier:
Danisco USA Inc., a wholly owned subsidiary of International Flavors and Fragrances, Inc.
DuPont Experimental Station – E320
200 Powder Mill Road
Wilmington DE 19803
- (ii) Common or Usual Name of the Notified Substance:
Bifidobacterium longum subsp. *infantis* Bi-26™
- (iii) Intended Conditions of Use:
B. infantis Bi-26™ is manufactured in compliance with current Good Manufacturing Practice as specified in 21 CFR Parts 111 and 117. *B. infantis* Bi-26™ is intended to be added to non-exempt infant and toddler formulas at a level of 1×10^8 CFU/g to ensure at least 1×10^6 CFU/g serving throughout the 12 – 18 month life of the product. *B. infantis* Bi-26™ is intended to be added as a live microbial ingredient.
- (iv) Basis for the GRAS Determination:
This GRAS conclusion is based on scientific procedures (21 CFR 170.30 (a) and (b)) as discussed in the detailed description provided below.

- (v) Availability to FDA of Data and Information that are the Basis of Determination: The data and information forming the basis for this GRAS determination and the exemption claim asserted herein are available for FDA review and copying during customary business hours at the following address, or will be sent to FDA either in an electronic format that is accessible for FDA evaluation or on paper, upon request:

Jayne Chalfin Davies
Global Regulatory Affairs
Danisco, USA Inc.
DuPont Experimental Station – E320
200 Powder Mill Road
Wilmington DE 19803

Tel: 610-864-7219
Jayne.Davies@IFF.com

- (vi) No data or information contained in parts 2 through 7 of this GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.
- (vii) If applicable and necessary, as required by §170.270 I authorize FDA to send any trade secrets to the Food Safety Inspection Service (FSIS) of the U. S. Department of Agriculture.
- (viii) I certify that, to the best of my knowledge, this GRAS notice for *B. infantis* Bi-26™ is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to me and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

Should you have any questions regarding the submission of this notice, please contact Jayne Davies. Thank you for your prompt consideration of, and response to, this notice.

Sincerely,



Jayne Chalfin Davies
Global Regulatory Affairs
Danisco, USA Inc.

June 23, 2021

Ellen Anderson
Division of Food Ingredients
Center of Food Safety and Applied Nutrition
US Food and Drug Administration



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Re: GRAS Notice No. GRN 000985

Dear Ms. Anderson:

Below please find Danisco's responses to FDA's questions of June 3, 2021 regarding our GRAS Notice GRN 000985 for the intended use of *Bifidobacterium longum* subsp. *infantis* ATCC SD 6720 (*B. longum* subsp. *infantis* ATCC SD6720). The FDA's questions are italicized followed by our response in plain text.

1. On page 5, the notifier states, "Bifidobacteria are non-pathogenic, non-toxicogenic bacteria species". For the administrative record, please confirm that Bifidobacterium longum subsp. infantis strain ATCC SD 6720 is non-pathogenic and non-toxicogenic.

Response:

For the administrative record, Danisco confirms that *B. longum* subsp. *infantis* ATCC SD6720 is non-pathogenic and non-toxicogenic.

2. Please describe whether B. longum subsp. infantis strain ATCC SD 6720 produces antibiotics.

Response:

This strain doesn't produce antibiotics, these are typically produced by fungi.

3. The notifier references their "... manufactured strain" on pages 6 and 19. For the administrative record, please clarify if B. longum subsp. infantis strain ATCC SD 6720 is genetically engineered.

Response:

For the administrative record, Danisco would like to clarify that *B. longum* subsp. *infantis* ATCC SD6720 is not genetically engineered.

4. On page 6, the notifier states, "In addition to the full-length sequencing of the 16S rRNA gene of B. infantis Bi-26™ whole genome sequencing has been completed." Please discuss whether the full genomic sequences are publicly available and provide the corresponding NCBI accession number.

Response: The whole genome is available. Please see NCBI accession number NZ_CP054425 (link: https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP054425.1?report=genbank)

200 Powder Mill Road, E320
Experimental Station
Wilmington, DE.
T 610.864.7219
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5. In Table 1 on page 13, the notifier lists the following specifications:
a. Non-lactic cell count as “< 5000/g”. For the administrative record, please provide the unit of measurement for this specification.

Response:

The unit of measure for this specification is cfu/g (colony forming units per gram).

b. Coliforms as “(MPN), negative by test (< 10/g)”. Appendix D and Appendix G lists the specification as “< 10/g” only. For the administrative record, please clarify this discrepancy and confirm the unit of measurement for this specification.

Response:

Danisco would like to clarify that for coliforms, the specification listed in Appendix D and Appendix G should align with that in Table 1, “negative by test (<10/g). The units are most probable number per gram (MPN/g) since coliform is tested via MPN technique. The limit is 10 where values between 0-10 are acceptable.

c. *Escherichia coli* as “(MPN), negative by test (< 0.3/g)”. For the administrative record, please confirm the unit of measurement for this specification.

Response:

Danisco wishes to confirm for the administrative record, that the unit of measurement for this specification is MPN/g as *Escherichia coli* is tested via MPN technique.

d. *Staphylococcus (coagulase+)* as “negative by test (< 10/g)”. For the administrative record, please provide the unit of measurement for this specification.

Response:

Danisco wishes to confirm for the administrative record, that the unit of measurement for this specification is cfu/g.

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6. For the administrative record, please specify whether “*Listeria*” that appears in Table 1 on page 13 refers to *Listeria monocytogenes*.

Response:

For the administrative record, Danisco clarifies that Table 1 on page 13 should refer to *Listeria* species.

7. For the administrative record, please provide the full citation for “SMEDP, 17th ed” that appears in Table 1 on page 13.



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Response:

For the administrative record, the complete citation for “SMEDP, 17th ed.” is Wehr, M and Frank, JF. 2004. Standard Methods for the Examination of Dairy Products, 17th Edition.

8. *In Table 1 on page 13, the notifier states that the method used to detect yeast and mold is “USP”. For the administrative record, please provide the complete citation for the referenced method.*

Response:

For the administrative record, the citation should be United States Pharmacopeia 31, Chapter 61 MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: MICROBIAL ENUMERATION TESTS, Plate count method for (TYMC) total combined yeasts and molds count, May 2009.

https://www.uspnf.com/sites/default/files/usp_pdf/EN/USPNF/generalChapter61.pdf

9. *In Table 1 on page 13, the notifier lists the following methods:*

a. *AOAC 992.23 used to analyze protein, which, per AOAC is “Applicable to cereal grains and oil seeds containing 0.2–20% N”. Please clarify if this method is appropriate and fit for purpose.*

b. *AOAC 926.08 used to analyze moisture, which, per AOAC corresponds to “Loss of drying (moisture) in cheese”. Please clarify if this method is appropriate and fit for purpose.*

c. *EPA 7471 used to detect mercury, which corresponds to detection of mercury in solid or semisolid wastes. Please clarify if this method is appropriate and fit for purpose.*

d. *AOAC 999.06 used to detect Listeria, which, per AOAC corresponds to detection of Listeria spp. in dairy products, vegetables, seafood, raw meat and poultry, processed meat and poultry. Please clarify if this method is appropriate and fit for purpose.*

e. *AOAC 966.24 used to detect coliforms and Escherichia coli, which per AOAC corresponds to detection of coliforms and E. coli in nuts and nut products/tree nut meats. Please clarify if this method is appropriate and fit for purpose.*

Response:

Danisco confirms that all the above cited methods are applicable, appropriate and fit for the intended purpose as presented in Table 1 on page 13.

10. *The notifier provides a specification for Salmonella serovars, listed as negative by test in 40 grams. The method referenced is AOAC 2004.03. We note that this method requires pre-enrichment to initiate the growth of salmonellae. The method states, “... procedure must be performed as described in [AOAC] 967.26 (see 17.9.02) or as in Bacteriological Analytical Manual”; both AOAC 967.26 and the Bacteriological Analytical Manual Chapter 5: Salmonella are based on the analysis of a 25-gram test portion. Please clarify the analytical method used to detect Salmonella serovars has been validated for that purpose.*



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Response:

The above referenced method AOAC 2004.03 has been validated for the intended purpose to detect Salmonella serovars in 40 grams.

11. *Please state whether all analytical methods used to analyze the batches for conformance with the stated specifications have been validated for that particular purpose.*

Response:

Dansico utilizes official methods (AOAC, ISO, USP, etc.) for analysis of *B. longum subsp. infantis strain ATCC SD 6720* to demonstrate conformance with the stated specifications. The use of official methods prevents the need for method validation. Any deviations (i.e. sample size) from the official methods would be validated.

12. *As the intended uses of B. longum subsp. infantis strain ATCC SD 6720 includes use in infant formula, please provide the following:*
a. *A specification for Cronobacter sakazakii, along with the respective analytical methods and results from three non-consecutive batch analyses to demonstrate that B. longum subsp. infantis ATCC SD 6720 meets the established specifications.*

Response will be provided July 26, 2021.

b. *Specify the intended protein source(s) of the non-exempt infant formulas.*

Response:

The intended protein sources of the non-exempt infant formulas are cow's milk and soybean.

13. *On page 15, the notifier states, "B. infantis Bi-26™, produced by DuPont as a single strain with no added excipients, does not contain allergens as determined by The Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA), including protein derived from milk, egg, fish, crustacean shellfish, tree nuts, wheat, peanuts, soybeans." For the administrative record, please state whether any of the raw materials used in the fermentation media are allergens or are derived from allergens.*

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Response:

Dansico confirms for the administrative record that none of the raw materials used in the fermentation media are allergens or derived from allergens. See Attachment 1 which supplements information provided in Appendix G.

14. *On page 15, the notifier estimates the dietary exposure to B. longum subsp. infantis strain ATCC SD 6720 to be 10^9 to 10^{10} CFU per day. However, we note that this was based on an average daily formula intake. Please provide:*

a. *A citation for the reference value of average infant formula intake, including whether it was the average for 0-6 months or 0-12 months.*

Response:



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The average formula intake was derived from human milk consumption cited in the 10th edition of the Recommended Dietary Allowances (National Research Council 1989). This reference states "Average milk consumption for infants born at term is now accepted to be 750 ml for the first 6 months (with a coefficient of variation of approximately 12.5%) and 600 ml during the next 6 months when complementary foods are given."

The intake of 800 ml is an average value for 0-6 months and was determined by rounding up the average intake of 750 ml for 0-6 months. We assumed that infant formula consumption would match average milk consumption.

This value of 800 ml per day can also be supported by recent analysis of 2005-2012 NHANES data. The mean intake of infant formula by 0-5.9-month-old was 834 ml and by 6-11.9-month-old was 783 ml (Grimes et al 2017).

Cited references:

Grimes, Carley A.; Szymlek-Gay, Ewa A.; Nicklas, Theresa A. 2017. "Beverage Consumption among U.S. Children Aged 0–24 Months: National Health and Nutrition Examination Survey (NHANES)" *Nutrients* 9, no. 3: 264. <https://doi.org/10.3390/nu9030264>.

National Research Council (1989). Subcommittee on the Tenth Edition of the RDAs, Food and Nutrition Board (1989) Recommended Dietary Allowances. 10th Ed. National Academy Press, Washington D.C.

b. *A dietary exposure estimate for B. longum subsp. infantis strain ATCC SD 6720 at the 90th percentile for infants 0 to 6 months of age and 6 to 12 months of age.*

Response:

Using the food consumption data reported in a recent National Health and Nutrition Examination Survey (NHANES; 2015-2016) dataset compiled by the U.S. Department of Health and Human Services, National Center for Health Statistics, and the Nutrition Coordinating Center (as cited in GRN 952), the estimated dietary intake of *B. longum subsp. infantis* strain ATCC SD 6720 from infant formula (as consumed, ready-to-drink or reconstituted formula prepared from powder) at the 90th percentile for 0-6 mo. and 6-12 mo. can be calculated.

Using 90th percentile intake for 3-5.9 mo. as representative of infants 0-6 mo., formula volume is 1239 ml/d and provides 1.67×10^{10} cfu/d (assuming addition level of 10^8 cfu/g).

Using 90th percentile intake for 9-11.9 mo. as representative for infants 6-12 mo., formula volume is 1097 ml/d and provides 1.48×10^{10} cfu/d (assuming addition level of 10^8 cfu/g).



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15. On page 15, the notifier discusses the current dietary exposure to *B. longum* subsp. *infantis* strain ATCC SD 6720 in conventional foods. However, a cumulative dietary exposure was not provided. Please discuss the cumulative dietary exposure for the populations discussed in the notice (i.e., children up to 2 years of age) that may also consume conventional foods containing added *B. longum* subsp. *infantis* strain ATCC SD 6720. If the notified use could be considered as partially or completely substitution for existing uses of *B. longum* subsp. *infantis* strain ATCC SD 6720, please provide a narrative indicating that, if there is an increase in the cumulative exposure to *B. longum* subsp. *infantis* strain ATCC SD 6720, you have concluded that the cumulative exposure is still consistent with safe use of this ingredient.

Response:

Within the context of this notice, children up to 2 years of age (also referred to as toddlers) is the population with the highest potential dietary exposure to *B. longum* subsp. *infantis* strain ATCC SD 6720 because they consume both toddler formula and conventional foods. A conservative estimate of cumulative dietary exposure to *B. longum* subsp. *infantis* strain ATCC SD 6720 by children up to 2 years of age including contribution from conventional foods can be determined from daily food intake data from 2007- 2016 NHANES. USDA and HHS report that daily, toddlers at two years of age consume approximately six servings (250 g) of food and beverages (as two 1 cup servings of milk) a day (USDA and HHS 2020). Assuming half of these foods and beverages contain added *B. longum* subsp. *infantis* strain ATCC SD 6720 at an addition level of 1×10^{11} CFU per serving to provide 1×10^{10} per serving at end of the shelf, total dietary intake from conventional foods and beverages would be 3×10^{11} cfu per day.

If total beverage intake (i.e. 2 -1 cup servings) was substituted in total with a toddler formula containing added *B. longum* subsp. *infantis* strain ATCC SD 6720 (addition level of 10^8 cfu/g) and half of the foods contained the added strain, total dietary intake would be 2.06×10^{11} CFU per day. A 'worse-case' assessment using the 90tile formula volume intake of 1097 ml/d containing 1.48×10^{10} cfu/d would result in total dietary intake of 2.15×10^{11} CFU/d.

The above demonstrates that with the addition of *B. longum* subsp. *infantis* strain ATCC SD 6720 to infant and toddler formula, cumulative intake does not appear to increase.

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These examples most likely overestimate actual dietary exposure since it is not expected that a consumer would consume 3 servings of foods containing the strain and the number of CFU decline over the shelf-life of the food or beverage. (Kailasapathy and Chin, 2000). The incorporation of microbial cultures into processed food products and subsequent storage can be stressful for the bacterial cells, and their viability may decrease due to the food matrix chosen, water activity, and pH of the final product (Min et al., 2017).

In summary, it is likely the maximum ingestion (from conventional foods alone or with toddler formula) would be less than 3×10^{11} cfu per day and well within levels that have been shown to be safe.



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Cited references:

Kailasapathy, K. and Chin, J., (2000). Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. *Immunology and Cell Biology* 78, 80-88.

Min, M., Bunt, C. R., Mason, S. L., Bennett, G. N., & Hussain, M. A. (2017). Effect of non-dairy food matrices on the survival of probiotic bacteria during storage. *Microorganisms*, 5(3), 43.

USDA, (2020). U.S. Department of Agriculture and U.S. Department of Health and Human Services. *Dietary Guidelines for Americans, 2020-2025*. 9th Edition. Figure 2-2, p. 65.

16. *On pages 16-18, the notifier summarizes several previously submitted GRAS notices for Bifidobacteria used in infant formula and various conventional foods. We note that some details in the notifier's summaries do not accurately reflect the information contained in the response letters to the GRAS notices listed below. For the administrative record, please make a statement that corrects these references:*

a. *On page 17, the notifier states that an intended use of Bifidobacterium breve M-16V in GRN 000453 is in medical foods. We note that the intended use in medical foods is not included in our response letter for GRN 000453.*

Response:

For the administrative record, Danisco corrects the reference to the intended use of *Bifidobacterium breve* M-16V which does not include medical foods.

b. *On page 17, the notifier states that an intended use of Bifidobacterium breve M-16V in GRN 000454 is in "exempt term infant formulas containing hydrolyzed proteins and/or amino acid mixtures". We note that this intended use is described as "exempt powdered term infant formula containing partially-hydrolyzed milk or soy proteins" in our response letter for GRN 000454.*

Response:

For the administrative record, Danisco corrects the reference to the intended use of *Bifidobacterium breve* M-16V. It should include exempt powdered term infant formula containing partially-hydrolyzed milk or soy proteins.

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c. *On pages 17-18, the notifier reiterates the intended uses displayed in the [online GRAS inventory](#) for GRNs 000455, 000572, 000579, 000758, 000813, and 000814. We note that these descriptions are not entirely accurate. For future submissions, please refer to the response letters when summarizing previously submitted GRAS notices.*

Response:

In future submissions, Danisco will refer to the Agency response letters when summarizing previously submitted GRAS notices.

17. *On page 19, the notifier states, "... a whole genome sequence of the manufactured strain was obtained and analysed for the mechanisms of HGT by comparison to known drug resistance markers. When the mechanism of resistance was well documented and genomically located in the sequence, an*

evaluation of the flanking regions and the sequence identity was conducted. When a mechanism of resistance was not well understood, examination of all the known HGT mechanisms in that strain was completed to rule out a possibility of a resistance gene located in the vicinity.” Please briefly summarize the results from the whole genome sequence analysis of mechanisms of horizontal gene transfer (HGT).



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Response:

Dansico notes that the above text was followed by the statement: “Only the genes responsible for the drug resistance over the EFSA breakpoint for clinically relevant antibiotics were investigated.” Results were not provided since as demonstrated on page 20 of the notice *B. longum* subsp. *infantis* strain ATCC SD 6720 was below EFSA breakpoints for all antibiotics.

18. Please provide an updated literature search that discusses the safety of B. longum subsp. infantis, including the date (month and year) the literature search was performed and discuss whether there are any study results that may be contradictory to a GRAS conclusion.

Response:

See Attachment 2 for an updated literature search (performed in June 2021) that includes clinical studies conducted in *B. longum* subsp. *infantis*, published since January 2019. The search identified 7 additional clinical studies. (Yousef et al 2020, Alcon-Giner et al 2020, van Best et al 2020, Mariben et al 2019, Sanctuary et al 2019, Smeucol et al 2019, Henrick et al 2019). In these trials, a total of 229 additional subjects received *B. longum* subsp. *infantis* with 12 adults with celiac disease, 8 children ages 2-11y with Autism Spectrum Disorder, 20 term infants and 189 preterm infants. One publication presents a protocol of a clinical trial with a target sample size of 654 preterm infants but results were not included (Mariben et al 2019). None of these studies provided results that are contradictory to a conclusion that *B. longum* subsp. *infantis* strain ATCC SD 6720 is GRAS for the intended use.

Cited references:

Alcon- Giner, C et al (2020). Microbiota Supplementation with Bifidobacterium and Lactobacillus Modifies the Preterm Infant Gut Microbiota and Metabolome: An Observational Study Cell Rep Med. Aug 25; 1(5): 100077

Henrick, B.M., Chew, S., Casaburi, G. et al. Colonization by *B. infantis* EVC001 modulates enteric inflammation in exclusively breastfed infants (2019). *Pediatr Res* 86, 749–757.

Marißen J, Haiß A, Meyer C, et al. (2019). Efficacy of Bifidobacterium animalis subsp. lactis (BB-12), *B. infantis* and Lactobacillus acidophilus (La-5) probiotics to prevent gut dysbiosis in preterm infants of 28+0–32+6 weeks of gestation: a randomised, placebo- controlled, double-blind, multicentre trial: the PRIMAL Clinical Study Protocol. *BMJ Open*;9: e032617.

Sanctuary MR, Kain JN, Chen SY, Kalanetra K, Lemay DG, Rose DR, et al. (2019). Pilot study of probiotic/colostrum supplementation on gut function in children with autism and gastrointestinal symptoms. *PLoS ONE* 14(1): e0210064. <https://doi.org/10.1371/journal.pone.0210064>.
Smeucol E, et al. (2020) Effect of Bifidobacterium infantis NLS super strain in symptomatic coeliac disease patients on long-term gluten-free diet - an exploratory study. *Benef Microbes*. Oct 12;11(6):527-534.

van Best, N et al, (2020). Influence of probiotic supplementation on the developing microbiota in human preterm neonates, Gut Microbes, 12:1, DOI: 10.1080/19490976.2020.1826747

Yousuf EI, et al (2020). Persistence of Suspected Probiotic Organisms in Preterm Infant Gut Microbiota Weeks After Probiotic Supplementation in the NICU. Front Microbiol. Sep 25; 11:574137.



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19. *On page 31, the notifier states, “The GRAS Panel individually and collectively critically evaluated the materials summarized above,” and refers to the GRAS panel statement in Appendix J. Appendix J (electronic page 99) contains a table titled “Decision Tree Analysis for Determining the Safety of Microbial Cultures for Consumption.” Appendix K (electronic page 103) contains a letter written by Dr. Michael Pariza dated January 2, 2020, with the subject “Re: GRAS opinion on the intended uses of DuPont’s Bifidobacterium longum subsp. infantis Bi-26™”. This letter discusses intended uses and use levels that are not included in GRN 000985. Please explain the relevance of Appendix K to GRN 000985 and revise any statements regarding a GRAS expert panel evaluation, if necessary.*

Response:

In error, Danisco included the incorrect GRAS panel statement in Appendix J. We have provided the correct GRAS panel statement as Attachment 3, entitled *GRAS Panel Report on the Generally Recognized as Safe (GRAS) Status of the Proposed Uses of Bifidobacterium longum subsp. infantis Bi-26™ in non-exempt infant and toddler formula*. This statement confirms the unanimous conclusion that this strain manufactured consistent with cGMP and meeting food grade specifications, is Generally Recognized as Safe (GRAS) based on scientific procedures for use in infant formulas and toddler formulas.

20. *Appendix H (electronic page 57) is marked “Trade Secret”. Please clarify if the information contained in Appendix H is considered confidential and protected from public release. If Appendix H is confirmed to contain trade secret information, please clarify if this designation has any impact on the conclusion that the intended use of B. longum subsp. infantis strain ATCC SD 6720 is GRAS.*

Response:

Danisco clarifies that Appendix H is not confidential nor protected from public release and therefore can be included in the GRAS determination.

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Sincerely,

Jayne Chalfin Davies
Global Regulatory Affairs



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June 22, 2021



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Product: Bifidobacterium infantis Bi-26™

To Whom It May Concern,

IFF Health & Biosciences - Madison certifies that Bifidobacterium infantis Bi-26™ does not contain allergens as determined by The Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) (Public Law 108-282) (U.S. Food and Drug Administration, 2006), including protein derived from milk, eggs, fish, crustacean shellfish, mollusks, tree nuts, wheat, peanuts, and soybeans. This includes raw materials used in fermentation.

Sincerely,



Sarah Pace
Quality & Food Safety Coordinator
IFF Health & Biosciences - Madison

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Attachment 2- Literature Search on Safety of *B. longum* subsp. *infantis*- Updated June 2021

Reference	Objective	Design	Subjects	Strain/Dose	Duration	Safety-Related Results
Yousuf El, et al . 2020	Compare prevalence and abundance of Bifidobacterium and Lactobacillus in the gut of preterm infants who were exposed to a probiotic supplement in-hospital to those who did not receive such supplementation. Infants were followed to 5 months corrected age.	Prospective cohort pilot study	22 early preterm infants, 8 received probiotics, 14 no probiotic.	Blend of 2 billion CFU bacteria per single dose sachet, including: Bifidobacterium longum subsp. infantis (HA-116), and Bifidobacterium longum subsp. longum (HA-135)	from birth to hospital discharge for a duration of between 3.29 and 13.57 weeks;	No safety related outcomes or adverse events reported.
Alcon-Giner et al . 2020	To explore the gut microbiota composition and fecal me-tabolome in preterm infants receiving routine probiotic supplementation compared to preterm infants from NICUs not using probiotic supplementation.	observational longitudinal study comprising two preterm groups.	Preterm infants: 101 infants orally supplemented with Bifidobacterium and Lactobacillus and 133 infants non-supplemented (control) matched by age, sex and delivery method.	Twice daily dose of Infloran [®] - 1 billion CFU Bifidobacterium bifidum NCDO 2203 and 1 billion CFU Lactobacillus acidophilus NCDO 1748	Samples were collected corresponding to four time points at 0–9, 10–29, 30–49, and 50–99 days of age from birth	Authors report "no adverse effects were observed to result from over 5 years of routine clinical use of probiotics used in this study."
Best et al 2020	To characterize the persistence of live microbial organism after oral administration of two different strains and their influence on the microbial ecosystem during and after the intervention and their association with the development of NEC.	observational study, 2 different supplementation vs no supplementation	80 preterm neonates born at a gestational age <32-weeks	2 different products: "probiotic Total dose 10 ⁹ 1"-Lactobacillus acidophilus (ATCC 4356) and Bifidobacterium longum subspecies infantis (ATCC 15697) "probiotic 2"-of Lactobacillus acidophilus La-14 (ATCC SD5212), Bifidobacterium longum subsp. longum BI-05 (ATCC SD5588) subsequently referred to as B. longum, Lactobacillus casei Lc-11 (ATCC SD5213), and Bifidobacterium animalis subsp. lactis (ATCC SD5215)	Infants were fed from <32 weeks (26-30 weeks median) gestational age to 36 weeks gestational age. Samples collected before, during, after supplementation	Authors report "no adverse effects or infections by the administered probiotic were observed during the study period."

Mariben et al 2019	Publication of protocol for a study to evaluate effect of 2 live microbial strains in the prevention of gut dysbiosis and safety in preterm infants The efficacy endpoint was the prevention of gut dysbiosis at day 30 of life.	Randomized, placebo-controlled, double-blind, multicentre study	654 preterm infants of 28+0–32+6 weeks of gestation	Bifidobacterium animalis subsp. lactis, B. infantis and Lactobacillus acidophilus. Dose is 1.5×10 ⁹ (CFUs) of each of the strains:	first 48 hr of life to 28 days	Study results not presented
Smecuol E et al, Oct 2020	To evaluate effect of live microbial ingredient on persistent gastrointestinal symptoms in adults with celiac disease	Randomised, cross-over, double-blind, placebo-controlled trial	12 Adults with celiac disease on gluten free diet for at least 2 yrs.	Bifidobacterium infantis NLS super strain (B. infantis NLS-SS), dose not specified	After one-week run-in, patients were randomised to B. infantis NLS-SS or placebo for 3 weeks with cross-over after a 2-week wash-out period.	No differences in adverse events between the 2 treatments
Hendrick et al 2019	To investigate the impact of B. infantis EVC001 colonization on enteric inflammation in a subset of exclusively breastfed term infants from a larger clinical study. This was a subset of the data presented in Smilowitz et al 2017	Parallel, partially-randomized, controlled 2-month trial	exclusively breastfed term infants who were fed EVC001 (n = 20) and control infants (n = 20)	1.8 × 10 ¹⁰ colony-forming units (CFU) B. infantis EVC001	53 days, 21 d of supplementation beginning at day 7 postnatal with follow up until postnatal day 60	No safety outcomes reported in this paper. Safety outcomes in primary trial reported in Smilowitz et al 2017 below
Sanctuary et al 2019	Pilot study evaluating tolerability of a probiotic (Bifidobacterium infantis) in combination with a bovine colostrum product (BCP) as a source of prebiotic oligosaccharides and to evaluate GI, microbiome and immune factors in children with ASD and GI co-morbidities.	Randomized, double blind, controlled trial	Children 2-11 y with Autism Spectrum Disorder and GI co-morbidities (n=8)	Bifidobacterium longum subsp. infantis (UCD272) Dose of 2x10 ¹⁰ CFU per day	12-week study - 5 weeks of probiotic-supplementation, followed by a two-week washout and 5 weeks of prebiotic only supplementation.	No adverse events reported. Probiotic combination was well tolerated.
Ma et al., 2019	To determine if B. infantis M-63 improved symptoms of Irritable Bowel Syndrome (IBS)	Open-label, no control, before-and-after	Flood victims with IBS (age ≥ 18 yr) received probiotic (n=20) or controls (n=30)	day,	12 weeks	"No additional symptoms or adverse events were reported from participants from either group during the entire period of intervention."

Enani et al., 2018	To evaluate the effect of synbiotic on the B and T cell response to seasonal influenza vaccination in young and older subjects.	RDBPC parallel arm trial	Young subjects (age 18-35) and older subjects (age 60-85) received synbiotic (n=60) or placebo (n=64).	B. infantis CUG 52486 (10^9 CFU/day) + gluco-oligosaccharide	8 weeks	Two mild AEs of gastrointestinal bloating reported, one in study group and one in placebo.
Escribano et al., 2018	To determine effectiveness of B. infantis CECT7210 in reducing incidence of diarrhea in healthy infants	Multicentre RDBPC trial	Infants (age <3 months) received probiotic (n=93) or unsupplemented formula (n=97).	B. infantis IM1® (10^7 CFU/g)	12 weeks	Supplemented formula reported as safe and well tolerated.
Kumar et al., 2018	To determine the effect of B. infantis 35624 on hydrogen and methane excretion during LBT.	Open-label, no control	Healthy adults (age 22-64) received B. infantis (n=19)	B. infantis 35624, dose not reported	2 week treatment with LBT before and after	No significant difference was found when comparing pre- and post-probiotic PGI-SYM scoring.
Del Giudice et al., 2017	Investigation of whether a Bifidobacteria mixture could relieve nasal symptoms, and affect quality of life in children with asthma due to Parietaria allergy.	RDBPC parallel arm trial	40 Children (age 4-17) with asthma received probiotic or placebo (1:1)	B. longum BB536 (3×10^9 CFU), B. infantis M-63 (1×10^9 CFU), and B. breve M-16 V (1×10^9 CFU) daily	4 weeks	Both probiotic and placebo were well tolerated. No clinically significant side effects in either group.
Härtel et al., 2017	To assess the effect of L. acidophilus/B. infantis probiotics on growth in VLBW infants during primary stay in hospital and to determine whether this effect is modified by antibiotic exposure.	Observational population-based cohort study	VLBW infants (<33 weeks' gestation, subgroup 28-32 weeks) received probiotics (n=6229) or no probiotics (n=2305)	Infloran® L. acidophilus (10^9) and B. infantis (10^9) daily	Probiotics consumed for the duration of primary stay in hospital (≥ 28 d), with 24 mo and 5 year follow up analysis in a smaller subset	No safety related endpoints or adverse events were discussed. Probiotics use was associated with improved weight gain and higher growth rates for body length and head circumference.
Manzano et al., 2017	To evaluate the safety and tolerance of three probiotic strains in healthy infants aged 3 to 12 months.	RDBPC parallel arm trial	Healthy infants (age 3-12 months) received one of three probiotics (n=53 for B. infantis) or placebo (n=52)	B. infantis R0033, B. bifidum R0071, or L. helveticus R0052 (3×10^9 CFU/day)	8 weeks	No serious adverse events. Total number of AEs recorded was equivalent in all groups.
Ringel-Kulka et al., 2017	To assess the efficacy of B. infantis 35624 for the relief of abdominal discomfort and bloating in a non-patient population.	RDBPC parallel arm trial	Adults experiencing abdominal discomfort and bloating ≥ 2 times per week for at least 3 months (n=275 total) not under physician supervision or treatment	B. infantis 35624 (10^9 CFU/day)	2 week placebo run-in followed by 4 week intervention	Placebo and probiotic were reported as well tolerated.

Smilowitz et al., 2017	To determine the safety and tolerability of supplementing breastfed infants with <i>B. infantis</i> (EVC001)	Parallel, partially-randomized Phase I clinical trial	Breastfeeding infants from day 7-28 of life given probiotic (n=34) or not (n=34).	<i>B. infantis</i> EVC001 (1.8×10^{10} CFU/day)	3 weeks	No observed difference in infant illness or adverse events between study and control groups.
Esaiassen et al., 2016	Investigation of consumption of Infloran by extremely preterm infants in Norway April 2014-August 2015.	Review/Case Studies	3 patients diagnosed with <i>B. longum</i> bacteremia. All patients extremely premature with impaired immune systems. Patients 1 & 3 additionally had severe gastrointestinal complications.	Infloran® <i>L. acidophilus</i> (10^9) and <i>B. infantis</i> (10^9); 1/2 capsule daily for first week, then 1 capsule daily	8-12 days	Infants developed bacteremia which was resolved with cessation of probiotic treatment and antibiotics.
Guthman et al., 2016	To investigate the effect of a short course of probiotics in the reduction of NEC.	Retrospective cohort study	Review of VLBW infants receiving probiotic supplementation (n=591) or not (n=633) in German and Swiss hospitals	<i>L. acidophilus</i> and <i>B. infantis</i> (10^9 CFU/day each)	10 or 14 days	Treatment was effective in reducing rate of NEC. No safety related endpoints or adverse events were discussed.
Hoy-Schulz et al., 2016	To investigate the safety and suitability of <i>L. reuteri</i> DSM 17938 and <i>B. infantis</i> 35624 in healthy infants.	Randomized parallel arm trial	Healthy infants (age 4-12 weeks) received probiotic blend (n=89) or nothing (n=24).	<i>L. reuteri</i> DSM 17938 (10^8 CFU) and <i>B. infantis</i> 35624 (10^9 CFU)	Daily dosing (29 doses overall), weekly dosing (five doses), or every two week dosing (three doses)	No differences in rates of any reported symptoms were observed among arms; additionally, no sudden adverse or allergic reactions were found after probiotic administration, and no hospitalizations were deemed related to probiotics administration.
Powell et al., 2016	To assess the impact of probiotic administration on microbiota and length of hospitalization of infants with gastroschisis.	RDBPC pilot trial	Infants with gastroschisis received probiotic (n=10) or placebo (n=11).	<i>B. infantis</i> ATCC 15697 (10^9 CFU twice daily)	6 weeks or until discharge	Administration of the probiotic or placebo was well tolerated, even during the period of gastric suctioning.
Stojković et al., 2016	To determine optimal duration of administration of synbiotic blend for control of respiratory infections.	Observational cohort study	Children (age < 5) hospitalized in previous year due to respiratory infection consumed synbiotic (n=78).	<i>L. acidophilus</i> Rosell-52, <i>B. infantis</i> Rosell-33, <i>B. bifidum</i> Rosell-71 (5×10^9 CFU total) + FOS	3-9 months	No side effects of synbiotic were identified in the examined children and it was well tolerated.
Tehrani et al., 2016	To evaluate the effect of a probiotic drop on salivary counts of <i>Streptococcus mutans</i> and <i>Lactobacillus</i> in children.	RDBPC parallel arm trial	Healthy children (age 3-6 y) in placebo (n=23) or probiotic (n=30) groups	5 drops containing <i>L. rhamnosus</i> ATCC 15820 (1×10^{10} CFU/mL), <i>L. reuteri</i> ATCC 55730 (2×10^9 CFU/mL), and <i>B. infantis</i> ATCC 15697 (1.5×10^9 CFU/mL) daily	14 days	No safety related endpoints or adverse events were discussed.

Langkamp-Henken et al., 2015	To examine the effect of probiotic consumption on the number of healthy days in an academically stressed undergraduate population.	RDBPC parallel arm trial	Healthy undergraduate students (age ≥ 18) received one of three probiotics (n=142 for B. infantis) or placebo (n=147)	L. helveticus R0052, B. infantis R0033, or B. bifidum R0071 (3×10^9 CFU/day)	6 weeks	No safety related endpoints were discussed. Withdrawals related to mild symptoms did not differ significantly between study and control groups. One participant in the B infantis group withdrew after 1 day because of abdominal pain, 1 participant in the placebo group withdrew after 25 days because of abdominal pain and 1 participant in the placebo group withdrew due to diarrhea.
Van Niekerk et al., 2015	To assess the effect of probiotics on the incidence of NEC in premature infants born to HIV-positive and HIV-negative women.	RDBPC parallel arm trial	Premature and VLBW infants (<34 weeks' gestation; <1250 g) separated into HIV-exposed and HIV-unexposed groups received probiotic (n=37+54) or placebo (n=37+56).	L. rhamnosus GG and B. infantis (3.5×10^8 CFU each/day)	28 days or upon discharge	None of the positive blood cultures taken from patients presenting septicaemia grew Lactobacillus or Bifidobacterium species.
Zbinden et al., 2015	Retrospective review of three cases of bacteremia in preterm infants at University Hospital of Zurich July-Dec. 2012	Review/Case Studies	3 VLBW preterm infants (<30 week gestational age) diagnosed with B. longum bacteremia.	Infloran® L. acidophilus and B. infantis; Dose not reported	11-28 days	Infants developed bacteremia which was resolved with cessation of probiotic treatment and antibiotics.
Bertelli et al., 2014	Cases of bacteriemia in preterm infants	Case Report	2 VLBW preterm infants (≤ 28 weeks gestation). Patient 1 diagnosed with ileoiliac intussusception. Patient 2 with NEC.	Infloran® L. acidophilus (5×10^8 CFU) and B. infantis (5×10^8 CFU) twice daily	Patient 1: 10 days; Patient 2: 5 days	Infants developed bacteremia which was resolved with cessation of probiotic treatment and antibiotics.
Härtel et al., 2014	To evaluate prophylactic use of L. acidophilus/B. infantis probiotics in VLBW infants.	Observational population-based cohort study	VLBW infants (<1500 g and gestational age >22 and <32 weeks) received probiotics (n=2310) or no probiotics (n=518)	Infloran® L. acidophilus (10^9) and B. infantis (10^9) daily	From day 2 or 3 of life for 14 days or until full enteral feeds.	No safety related endpoints or adverse events were discussed.

Li et al., 2014	Review of use of probiotic therapy for the prevention of NEC in VLBM infants.	Retrospective cohort study	VLBW infants (< 1500 g). Study group of patients admitted Aug. 2007- Jul. 2011 receiving probiotics (n=291) and control group of patients admitted Aug. 2003-Jul. 2007 receiving no probiotic (n=289)	S. thermophilus, B. infantis and B. bifidum (0.5-1.05x10 ⁹ CFU total/day)	Until corrected gestational age of 36 weeks or discharge.	Prophylactic therapy was well tolerated with no major adverse events.
Tobin et al., 2014	To assess the value of rapid qPCR assays for detection of probiotic species in preterm infant and adult participants.	Open label	Preterm infants (<32 weeks gestation, weighing <1500 g) received probiotic (n=6) or breastmilk (n=6). Healthy adults received probiotic (n=7)	ABC Dophilus Probiotic Powder for Infants®: B. infantis, S.thermophilus and B. lactis (1-3x10 ⁹ CFU/day of B. infantis)	7 days	No safety related endpoints or adverse events were discussed.
Charbonneau et al., 2013	To compare fecal excretion of B. infantis before, during, and after probiotic use in healthy patients and those with IBS.	Single center, double blind, randomized trial.	Patients (age 18-65) with IBS received probiotic (n=39) or placebo (n=37), and healthy subjects received probiotic (n=41)	B. infantis 35624, 10 ⁹ CFU/day	8 weeks	AEs were mild and did not differ significantly between groups. No AEs were attributed to the treatment.
Ellis et al., 2013	Investigate the impact of probiotic Bifidobacterium longum ssp infantis on the fecal microbiota and plasma cytokines in neonates with congenital heart disease.	RDBPC Pilot study	Infants with congenital heart disease received B. infantis (n=8) or placebo (n=8).	B. infantis (8.4x10 ⁹ CFU/day)	8 weeks	No safety related endpoints or adverse events were discussed.
Groeger et al., 2013	To assess the impact of B. infantis 35624 administration on inflammatory biomarker and plasma cytokine levels.	Three separate RDBPC trials	Patients (age 18-75); healthy (n=22) and with ulcerative colitis (n = 22), chronic fatigue syndrome (n = 48) and psoriasis (n = 26).	B. infantis 35624, 10 ¹⁰ CFU/day	6-8 weeks	No safety related endpoints or adverse events were discussed.
Jacobs et al., 2013	To determine the effect of administering a specific combination of probiotics to very preterm infants on culture-proven late-onset sepsis.	Multicentre RDBPC trial	Preterm infants (<32 weeks gestation, weighing <1500 g) received probiotic (n=548) or placebo (n=551)	ABC Dophilus Probiotic Powder for Infants®: B. infantis, S.thermophilus and B. lactis (300x10 ⁶ CFU/day of B. infantis)	Probiotics consumed until discharge or 12 month corrected age	No serious adverse events reported. Treatment appears to be safe.
Smecul et al., 2013	To assess the effect of B. infantis natrene life start strain on patients with untreated celiac disease (CD).	Exploratory RDBPC study	Subjects (age 18-75) with CD received probiotic (n=12 or placebo (n=10)	B. infantis (Lifestart 2), 4x10 ⁹ CFU/day	3 weeks	No serious adverse effects or significant biochemical changes were reported by patients in either treatment arm.
Pantovic, 2013	To investigate the optimal time of supplementation of Probiokid in children with common respiratory and/or ear infections	Uncontrolled, open-label trial	Hospitalized children (0-42 mo, n=31)	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS) 3 x 10 ⁹ CFU	6 months	No adverse events were reported

Underwood et al., 2013	To determine the impact of two probiotic bifidobacteria on the fecal microbiota of premature infants fed either human milk or formula.	Randomized Phase I trial	Preterm infants (<33 weeks gestation, <1500 g) received increasing doses of B. infantis (n=6) or B. lactis (n=6) with formula. Second trial of subjects consumed both strains alternately with washout period (n=9).	B. infantis or B. lactis (up to 8.4×10^9 CFU/day)	2-5 weeks	Probiotics were well tolerated.
Wu, 2013	To test the effectiveness of Smecta (a hydroscopic dioctahedral montmorillonite suspension) and Biostime (Probiokid) in infants with non-infectious diarrhea	Single center RDB active control trial	Hospitalized children (0-36 mo, n=84)	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS < 12 mo (n=32) 5×10^9 CFU 13-24 mo (n=35) 5×10^9 CFU 25-36 mo (n=17) 15×10^9 CFU All dose groups received oral Smecta Active control (n=64) groups matched for age all received only oral Smecta	3 days	No adverse reactions, No adverse reactions were observed in any group
Xi et al, 2013	To investigate the effect of Biostime (Probiokid) on oral thrush	Randomized, DB, active control trial	Children (1-36 mo) diagnosed with thrush	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS) (n=35) 2% Na_2CO_3 + nystatin + Biostime 1×10^{10} CFU	17 days	No adverse reaction was reported
Al-Hosni et al, 2012	To determine whether probiotic supplementation improves growth in extremely low birth weight infants.	Multicenter randomized controlled double-blinded clinical study	ELBW infants (<1000 g) separated into probiotic (n=50) and control (n=51) groups	L. rhamnosus GG and B. infantis (5×10^9 CFU of each/day)	Until 34 weeks postmenstrual age or discharge	No sepsis was detected related to probiotics, and no reports of adverse events were attributed to probiotic.
Gao, 2012	To test the effectiveness of Smecta (a hydroscopic dioctahedral montmorillonite suspension) and Biostime (Probiokid) in infants with non-infectious diarrhea	Single center RDB active control trial	Hospitalized children (0-36 mo, n=86)	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS) < 12 mo (n=14) 5×10^9 CFU 13-24 mo (n=14) 5×10^9 CFU 25-36 mo (n=15) 15×10^9 CFU All dose groups received oral Smecta Active control (n=43) groups matched for age all received only oral Smecta	3 days	No adverse reactions, No adverse reactions were observed in any group

Wang, 2012	To test the effectiveness of Smecta (a hydroscopic dioctahedral montmorillonite suspension) and Biostime (Probiokid) in infants with non-infectious diarrhea	Single center RDB active control trial	Children (3-36 mo, n=194) with non-infectious diarrhea	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071, plus FOS) < 12 mo (n=33) 5 x 10 ⁹ CFU 13-24 mo (n=43) 5 x 10 ⁹ CFU 25-36 mo (n=28) 15 x 10 ⁹ CFU All dose groups received oral Smecta Active control groups matched for age all received only oral Smecta	3 days	No adverse reactions. There was no report of adverse events.
Frech et al., 2011	To test whether gastrointestinal symptoms in systemic sclerosis patients with moderate bloating would improve with probiotic implementation.	Open label	10 Adult patients with systemic sclerosis and symptoms of bloating consumed B. infantis or L. rhamnosus	L. rhamnosus or B. infantis (10 ⁹ CFU/day)	2 months	No complication with probiotic use were reported.
Jenke et al., 2011	Case report of septicemia in ELBW infant	Case Study	VLBW Infant (27 wks gestation, 600 g)	Infloran® L. acidophilus and B. infantis; Dose not reported	10 days	Infant developed symptoms of septicaemia which were resolved with cessation of probiotic treatment, and antibiotics.
Cazzola et al., 2010	To evaluate the efficacy of a synbiotic in reducing common winter diseases in children.	Multicentre RDBPC trial	Healthy children (age 3-7 y) who reported at least 3 episodes of illness in previous winter received synbiotic (n=62) or placebo (n=73)	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071, and FOS (3x10 ⁹ CFU/day total)	3 months	Reported adverse events were not serious and did not differ between study groups. None were attributed to probiotic treatment.
Yang et al, 2010	To observe the effect of feeding with lactose-free milk powder plus Biostime (Probiokid) on infantile diarrhea	Randomized, DB, PC trial	Hospitalized children (6-30 mo) with rotaviral infection and diarrhea	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS) (n=58) 5 x 10 ⁹ CFU plus lactose-free milk powder formula or control (n=40 breast-fed or formula fed)	Until discharge (mean 8.5 ± 2.3 days)	No adverse events were reported
Jiang, 2008	To evaluate Biostime (Probiokid) in children with persistent diarrhea	RDB active control trial	Hospitalized or outpatient children (3-24 mo) with persistent diarrhea	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS)(n=32) < 6 mo 5 x 10 ⁹ CFU 12-24 mo 10 - 20 x 10 ⁹ CFU Golden Bifido control (n=20)	Until diarrhea resolved (mean 7.1 days)	No adverse events were reported

Mei & Chen, 2008	To evaluate the effect of Biostime (Probiokid) on pediatric diarrhea caused by rotavirus	RDB active control trial	Children (0-7 yr, n=78) diagnosed with rotavirus infection	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS)(n=32) 1 x 10 ¹⁰ CFU + Ribaviren vs Ribavirin only control (n=39)	7 days	There was no report of adverse events
Chen et al, 2007	To observe the effect of Biostime (Probiokid) on children with low secretory IgA levels	Control, parallel arm trial	Children (<1 - 4 years, n=28)	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS) (n=28, 1 x 10 ¹⁰ CFU) Age-matched healthy controls (n=8)	13 days	No adverse events were reported
Cui & Wure, 2007	To observe the effect of Biostime (Probiokid) on children with rotavirus gastroenteritis	RDB active control trial	Hospitalized children (6 - 24 mo, n=62) diagnosed with rotavirus infection who had diarrhea for less than 3 days	Probiokid a 1:1:1 mixture of L. helveticus R0052, B. infantis R0033, B. bifidum R0071 + FOS)(n=62) < 12 mo 5 x 10 ⁹ CFU 12 -24 mo 1 x 10 ¹⁰ CFU Lacidophilin control (n=60)	Until diarrhea resolved (at least 72 hrs)	There was no report of adverse events
Vivatvakin et al., 2006	To evaluate the effectiveness of probiotic blend of L. acidophilus and B. infantis in the treatment of acute watery diarrhea.	Open, randomized control trial	Infants admitted to hospital with acute watery diarrhea (age 1-24 months) received probiotic + ORS (n=35) or ORS only (n=36)	L. acidophilus and B. infantis (3x10 ⁹ CFU/day total)	2 days	No observed difference in baseline clinical symptoms between study and control groups. Probiotic shortened duration of diarrhea compared to control.
Whorwell et al., 2006	To confirm the efficacy of the probiotic bacteria B. infantis 35624 in a large-scale, multicenter, clinical trial of women with IBS and determine optimal dosage.	RDBPC parallel arm trial	IBS patients allocated to placebo, or probiotic at doses of 10 ⁶ , 10 ⁸ , or 10 ¹⁰ CFU (n=90 per group)	B. infantis 35624 (10 ⁶ , 10 ⁸ , or 10 ¹⁰ CFU/day)	4 weeks	No significant adverse events were recorded.
Lin, et al., 2005	To evaluate efficacy of probiotics in reducing incidence of NEC in VLBW infants.	Masked, randomized control trial	VLBW infants received probiotic (n=180) or control (n=187)	Infloran® L. acidophilus and B. infantis with breastmilk twice daily, dose not reported	Probiotics consumed for the duration of hospital stay, beginning at day 7 of life	Primary outcome was not safety, but no complications were observed related to probiotic treatment (such as Lactobacillus or Bifidobacterium sepsis)
O'Mahony et al., 2005	To compare the response of symptoms and cytokine ratios in IBS with ingestion of probiotic preparations.	RDBPC parallel arm trial	Adults with IBS (age 18-75) (n=77) randomized to receive B. infantis, L. salivarius, or placebo.	L. salivarius UCC4331 or B. infantis 35624 (10 ¹⁰ CFU/day)	8 weeks	Therapy was well tolerated and free of significant adverse events

Lee et al., 2001	To determine the effect of probiotic consumption on the course of acute diarrhea in hospitalized children.	Prospective clinical study	Children (6-60 months) allocated to receive probiotic (n=50) or rehydration alone (n=50)	L. acidophilus and B. infantis (10 ⁹ CFU/day each)	4 days	Other than primary outcome, no safety endpoints were discussed. Authors considered therapy to be safe and effective.
Hoyos, 1999	To test whether administration of L. acidophilus and B. infantis to newborns decreases the incidence of NEC.	Single center, open-label comparison study	Newborns admitted to hospital (average gestational age 35 weeks) during October 1994-October 1995 received probiotic (n=1237). Data was compared to previous year admissions receiving no probiotic (n=1282).	L. acidophilus and B. infantis (2.5x10 ⁸ CFU each) daily	Length of hospital stay (averages ranging from 5.5-8.5 days)	No complications attributed to the use of the probiotic preparation were observed.

GRAS Panel Report on the Generally Recognized as Safe (GRAS) Status of the Proposed Uses of *Bifidobacterium longum* subsp. *infantis* Bi-26™ in non-exempt infant and toddler formula

Introduction

DuPont Nutrition and Health convened a panel of independent scientists (the “GRAS Panel”), qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, to conduct a critical and comprehensive evaluation of the available pertinent data and information on *Bifidobacterium longum* subsp. *infantis* Bi-26™ (“*B. infantis* Bi-26™”) and to determine whether the proposed uses in non-exempt infant and toddler formula would be Generally Recognized as Safe (GRAS) based on scientific procedures. The GRAS Panel consisted of the below-signed qualified scientific experts: Michael W. Pariza, Ph.D. (University of Wisconsin) (Chair), Douglas L. Archer, Ph.D. (University of Florida), Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), and William C. MacLean, Jr. M.D., CM, FAAP (Ohio State University). Michael C. Falk, Ph.D. (LSRO Solutions LLC) served as technical advisor to the GRAS Panel.

The GRAS Panel, independently and collectively, critically evaluated a comprehensive package of scientific information and data compiled from the literature. The information was presented in a dossier provided by LSRO Solutions LLC (“Comprehensive GRAS Assessment of *Bifidobacteria longum* subsp. *infantis* Bi-26™: Food Usage Conditions for General Recognition of Safety”; July 17, 2019). To the best of our knowledge, this is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of this ingredient in non-exempt infant and toddler formulas.

Summary and Basis for GRAS

The GRAS Panel based its conclusions on the following information.

B. infantis Bi-26™ is intended to be added in non-exempt infant formulas and toddler formulas at a level of 1×10^8 CFU per g of powdered formula that is intended for consumption by term infants and toddler from the time of birth through 2 years of age. This level of *B. infantis* Bi-26™ is intended to ensure a minimum concentration of 10^6 CFU/g throughout the 12-18 month shelf life of the infant formula powder. With normal dilution of the infant formula powder in water according to label directions (i.e., 13.5 g/ 100 mL) and assuming an average daily formula intake of 800 milliliters, DuPont estimates that the daily intake of *B. infantis* Bi-26™ microorganism would be approximately 10^9 - 10^{10} CFU per day. *B. infantis* Bi-26™ is intended to serve as a probiotic microorganism. It will not proliferate in the foods and beverages to which it is added but will decline over the shelf-life of the formula.

B. infantis Bi-26™ has been sold worldwide, including in North America, China, South Africa, Middle East, Europe and Asia/Pacific countries. DuPont reports there have been no safety-related complaints related to *B. infantis* Bi-26™.

Various companies notified the U.S. Food and Drug Administration (FDA) that *Bifidobacterium* species were Generally Recognized as Safe (GRAS) for use in breads/baked goods, cereals, dairy

products, fruit products, functional beverages, nutritional powders, juices, bars, RTE breakfast cereals, chewing gum, and confections (GRN 49, 268, 377, 445, 453) and infant formula (GRN 454, 455 and 758). The FDA responded to these notifications that it had no objections. Several other lactic acid-producing species including *L. rhamnosus* GG (GRN 231), *L. rhamnosus* HN001 (GRN 281), *L. reuteri* (GRN 410), *L. fermentum* (GrN 531), and *L. helveticus* (GRN 758) were submitted to the FDA for use in infant formula and all received letters of no objection.

B. longum has been included in the list of microorganisms found to have a Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA). *B. longum* was included on this list continuously from 2007 through 2017. *B. longum* subsp. *infantis* has been approved for use as a medicinal ingredient in Natural Health Products by the Natural and Non-Prescription Health Product Directorate of Canada. Various *Bifidobacteria* were reviewed for use as food ingredients by FDA including *B. longum* and *B. infantis* and the FDA responded with letters of no objection. *Bifidobacterium longum* subsp. *infantis* has been documented as having a technical role in fermented food products. *Bifidobacterium* species have a long history of safe use when consumed as part of dairy food and supplement products.

B. infantis Bi-26™ is a human isolate, is well characterized, and has been deposited in the American Type Culture Collection as SD 6720.

Analysis of *B. infantis* Bi-26™ confirmed the identity of the strain to the *B. longum* subsp *infantis* species and absence of transferable antibiotic resistance elements, virulence factors, infectivity elements, and toxins. Further analysis of *B. infantis* Bi-26™ confirmed the lack of production of D(-)-lactic acid, histamines, and tyramine.

The *B. infantis* Bi-26™ strain is susceptible to various antibiotics but is neither pathogenic nor toxigenic.

B. infantis Bi-26™ is produced using standard, well-documented fermentation techniques under current GMP manufacturing conditions using approved food grade materials. The strain is produced reproducibly and meets standard food grade specifications.

The safety of *B. infantis* Bi-26™ was evaluated in an OECD compliant acute toxicity study in female CrI:CD(SD) rats. No treatment related deaths or signs of toxicity were reported after oral administration of 5000 mg/kg, which corresponded to an overall average dose of 1.94×10^{12} CFU/kg bw, the highest dose tested. The LD₅₀ is greater than 1.94×10^{12} CFU/kg bw under these conditions.

Forty eight relevant clinical studies of *B. longum* subsp. *infantis* were identified and reviewed. Of these 16 were randomized, blinded, placebo-control trials, while the remaining studies were either partially randomized/blinded trials, observational cohorts, uncontrolled, or open-label.

A total of 13,707 subjects were included in these studies and the total number of treatment days was 27.4×10^6 . The duration of treatment ranged from 2 days to 12 months. Doses ranged from 1×10^7 – 1.8×10^{11} CFU/day but the dose in most studies clustered around 10^9 – 10^{10} CFU/day. The median dose was 3×10^9 CFU/day. Stratified by health status, 12 studies were conducted on healthy subjects and 36 studies conducted on subjects compromised by such factors as low birth weight, premature birth, necrotizing enterocolitis, diarrhea, irritable bowel

syndrome, or other disorders. Stratified by age, studies on infants, children, and adults were the subject of 20, 8, and 10 studies, respectively. Four of the studies were case reports on 9 subjects. In the 20 studies on infants, the number of treated subjects was 12,410, the number of treatment days was 16.5×10^6 , and the median dose was 3×10^9 CFU/day.

Other than the case studies, the studies reported either no treatment-related adverse events, described the *B. infantis* treatment as well tolerated, or did not report any safety-related endpoints. In twelve of these studies the subjects were either very low birth weight, extremely low birthweight or premature infants. When adverse events were reported they were generally confined to gastrointestinal issues, were equally distributed between treatment and control groups, were typically considered mild and reversible, and were not considered related to *B. infantis* treatment. Bacteremia was reported in some preterm infants with extremely low birth weight or major gastrointestinal or immunocompromising disorders (i.e. bowel perforations, necrotizing enterocolitis, short bowel syndrome). These were all case reports and in each case the bacteremia was resolved on discontinuation of treatment (Bertelli et al. (2014), Zbinden et al. (2015), Esaiassen et al. (2016)).

Systematic reviews of the safety of lactic acid bacteria and *Lactobacillus* species used as probiotics concluded that these microbes are safe as long as they are devoid of any transferable antibiotic resistance genes.

The dose of 1×10^8 CFU per g of powdered infant formula powder will result in approximately 10^9 - 10^{10} CFU per day of *B. infantis* Bi-26™. This intake is consistent with the estimated intake of other Bifidobacteria currently in use in the U.S. market and falls within the range of levels without reported adverse effects in clinical trials.

The safety of *B. infantis* Bi-26™ was further evaluated using the decision tree analysis of Pariza et al. (2015). Based on the outcome of the decision tree for determining the safety of microbial cultures for consumption by humans and animals (Table 1), including strain characterization and genome sequencing, screening for undesirable attributes and metabolites, and experimental evidence of safety by appropriately designed safety evaluation studies, it was concluded that *B. infantis* Bi-26™ is not pathogenic and not toxigenic and is “deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.”

Common Knowledge Elements of GRAS Determinations

The first common knowledge element for a GRAS determination is that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing published, peer-reviewed scientific journals for the safety assessment. The human clinical studies that provided key evidence on which this GRAS determination was based were published in the peer-reviewed scientific literature.

The second common knowledge element required for a GRAS determination is consensus among qualified scientists that the safety of the proposed uses of the substance has been demonstrated. The GRAS Panel agrees there are adequate data in the scientific literature to

conclude that *B. infantis* is a common component of food sources for man and animals and that the weight of the available evidence demonstrates that the proposed uses are safe without any evidence of adverse effects.

B. infantis Bi-26™ is GRAS for use in powdered non-exempt infant formulas and toddler formulas at a level of 1×10^8 CFU per g of powder that is intended for consumption by term infants from the time of birth through 2 years of age. This level of *B. infantis* Bi-26™ is intended to ensure a minimum concentration of 10^6 cfu/g throughout the 12-18-month shelf life of the infant formula powder. With normal dilution of the infant formula powder in water according to label directions, DuPont estimates that the daily intake of *B. infantis* Bi-26™ microorganism would be approximately 10^9 - 10^{10} CFU per day.

Conclusion

We, the undersigned members of the GRAS Panel, are qualified by scientific education and experience to evaluate the safety of the addition of probiotic bacteria to conventional foods. We individually and collectively critically evaluated the materials on the safety of *B. infantis* Bi-26™ summarized above, and we unanimously conclude that DuPont's *B. infantis* Bi-26™, manufactured consistent with cGMP and meeting food grade specifications, is Generally Recognized As Safe (GRAS) based on scientific procedures for addition to powdered non-exempt infant formulas and toddler formulas at a level of 1×10^8 CFU per g of powder that is intended for consumption by term infants and toddlers from the time of birth through 2 years of age. This level of *B. infantis* Bi-26™ is intended to ensure a minimum concentration of 10^6 CFU/g throughout the 12-18 month shelf life of the powdered infant and toddler formulas.

It is our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusions.

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July 22, 2019

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Table 1: Decision Tree Analysis for Determining the Safety of Microbial Cultures for Consumption

1. Has the strain ⁱ been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? ⁱⁱ (If YES, go to 2. If NO, the strain must be characterized and unambiguously identified before proceeding).	YES
2. Has the strain genome been sequenced? (If YES, go to 3. If NO, the genome must be sequenced before proceeding to 3.) ⁱⁱⁱ	YES
3. Is the strain genome free of genetic elements ^{iv} encoding virulence factors ^v and/or toxins ^v associated with pathogenicity? ^{vi} (If YES, go to 4. If NO, go to 15.)	YES
4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? ^{vii} (If YES, go to 5. If NO, go to 15.)	YES
5. Does the strain produce antimicrobial substances? ^{viii} (If NO, go to 6. If YES, go to 15.)	NO
6. Has the strain been genetically modified using rDNA techniques? (If YES, go to 7. If NO, go to 8.)	NO
7. Do the expressed product(s) that are encoded by the introduced DNA have a history of safe use in food? ^{ix} (If YES, go to 8. If NO, the expressed product(s) must be shown to be safe before proceeding to 8.) ^x	NA
8. Was the strain isolated from a food that has a history of safe consumption for which the species , to which the strain belongs, is a substantial ^{xi} and characterizing ^{xii} component (not simply an 'incidental isolate')? (If YES, go to 9. If NO, go to 13.) ^{xiii}	NO
9. Has the species , to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been affirmed to be safe for food use by an authoritative group of qualified scientific experts? ^{xiv} (If YES, go to 10. If NO, go to 13.)	YES
10. Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9a continue to support the conclusion that the species , to which the strain belongs, is safe for use in food? (If YES, go to 11. If NO, go to 13.)	YES
11. Will the intended use of the strain expand exposure to the species beyond the group(s) that typically consume the species in "traditional" food(s) in which it is typically found (for example, will a strain that was isolated from a fermented food typically consumed by healthy adults be used in food intended for an 'at risk' group)? (If NO, go to 12. If YES, go to 13.)	YES

12. Will the intended use of the strain expand intake of the species (for example, increasing the number of foods beyond the traditional foods in which the species typically found, or using the strain as a probiotic rather than as a fermented food starter culture, which may significantly increase the single dose and/or chronic exposure)? (If NO, go to 14. If YES, go to 13.)	NA
13. Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? ^{xv} If yes, go to 15. If no, go to 14.)	NO
14. The strain is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.	YES
15. The strain is NOT APPROPRIATE for human or animal consumption. ^{xvi}	

ⁱ A strain is a “population of organisms that descends from a single organism or pure culture isolate.” P. 392, Prescott, Harley and Klein, 1996, Microbiology, Wiley. We recognize that the genotype and/or phenotype of a strain may change slightly when carried in culture, but such changes are irrelevant to safety considerations because there is no known mechanism or precedent for isolated strains in culture to begin spontaneously expressing pathogenic traits, unless that potential was already present in the genome at the time of isolation.

ⁱⁱ Whole Genome Sequencing provides distinct advantages for identification and characterization of microorganisms. In-depth analysis, including functional and comparative genomic studies, is afforded by sequencing the whole genome. This technology can provide a wealth of information that can be used for identification and characterization, including evidence of genetic evolution for adaptation of a species to a nutrient-rich environment, such as dairy products or the gastrointestinal tract (Pfeiler, EA, Klaenhammer, TR. 2007. The genomics of lactic acid bacteria. TRENDS in Microbiol, 15(12); 546-553). Less comprehensive molecular analysis, such as RAPD, FISH, and MLST, may also provide adequate information for identification, but the characterization ability is often times limited within a bacterial species (Gosiewski, T, Chmielarczyk, A, Strus M, Brzychczy-Wloch M, Heczko PB. 2012. The application of genetics methods to differentiation of three *Lactobacillus* species of human origin. Ann Microbiol 62:1437-1445).

ⁱⁱⁱ The genomic sequence provides the tools to mine the genome for a number of functions, uncovering information spanning from safety to host-cell interactions (Callanan, M. 2005. Mining the Probiotic Genome: Advanced Strategies, Enhanced Benefits, Perceived Obstacles. Current Pharmaceutical Design, 11: 25-36). From a regulatory perspective, the ability to show percentage/regions of similarity and differentiation between a new strain of interest in comparison with a *type strain*, or an accepted strain with history of safe use, is beneficial (U.S. FDA; July 2011. Draft Guidance for Industry: Dietary Supplements: New Dietary Ingredient Notifications and Related Issues). The genome sequence is analogous to a chemical specification for a food ingredient, that is, it defines precisely what is being evaluated and permits a genetic assessment of pathogenic and toxigenic potential. Isolates from a type-strain culture collection, or a strain collection held by a commercial culture manufacturer, may be considered to have the same safety characteristics as, and to be *substantially equivalent* to, the original source pure culture, so in these cases the requirement for genome sequencing may be satisfied by sequencing the genome of the original source pure culture.

^{iv} The term "genetic elements" refers to gene sequences encoded in the chromosome or extra-chromosomal DNA.

^v Known genetic element sequences for virulence factors and protein toxins are searchable, e.g. the MvirDb database of microbial virulence factors (<http://mvirdb.llnl.gov>) [ref Nucl. Acids Res.(2007) 35 (suppl 1): D391-D394.doi: 10.1093/nar/gkl791].

^{vi} In considering the issue of "pathogenicity" and the potential to produce an infection, it is important to distinguish between *true pathogens* (i.e., microbes that possess virulence factors and are therefore capable of crossing or evading non-compromised host barriers) versus *opportunistic pathogens* (i.e., microbes that do not possess the required virulence factors to produce an infection in a non-compromised host). Typically this can be accomplished via genome analysis for known virulence factors coupled with a comprehensive search of the peer-reviewed scientific literature for infectious potential.

^{vii} A functional antibiotic resistance gene results in an antibiotic resistance phenotype.

^{viii} In this context, the term 'antimicrobial substances' refers to antibiotics that are used in medical or veterinary applications, for example substances that are positive in the JECFA test (FAO. 1981. *FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives*, Appendix A, pp. 317–318, FAO/WHO, Geneva, Switzerland.)

^{ix} The use of the terms "food" and "feed" includes supplements, which are in most jurisdictions considered to be a subset of the general categories.

^x Demonstration of the safety of the expressed product may be accomplished by testing, e.g. toxicological testing as required by various regulatory bodies such as the US FDA Redbook 2000 (<http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditive/sGRASPackaging/ucm2006826.htm>) or by establishing a substantial equivalence of the test article to a substance with a safe history of use in food, or, in the case of animal feed additives, establishing a substantial equivalence of the test article to a substance with a history of safe use in target animal feeds.

^{xi} Food fermentations, e.g. Cheddar cheese or yogurt, commonly result in "substantial" microbial food culture populations of 10^6 - 10^8 colony forming units per gram of the food. Significance should be judged relative to the fermented food, i.e. numbers of different organisms in a microbial population may change during the course of the life of the fermented food, e.g. Lactobacilli counts in Cheddar cheese are routinely low in the initial stages of cheese maturation, but begin to increase in numbers while the Lactococci, responsible for initial acid production, count decreases as the cheese ripens and pH decrease. [Spatial and temporal distribution of non-starter lactic acid bacteria in Cheddar cheese. N.A. Fitzsimons, T.M. Cogan, S. Condon, T. Beresford. *Journal of Applied Microbiology* 90(4): 600–608, 2001; Kosikowski, F. V., and V. V. Mistry. *Cheese and Fermented Milk Foods*. 1997. 3rd Ed. F. V. Kosikowski, L. L. C. Westport, CT.]

^{xii} A species is a "characterizing" component of a food if it has a measurable impact on flavor, texture, stability or preservation properties that are characteristic of the food, e.g. typical color and flavor of "blue" cheeses derived from *Penicillium roqueforti*; or surface texture, flavor and odor of Limburger cheese resulting from *Brevibacterium linens* growth on the surface. The color and flavor of "blue" cheese and the aroma, flavor and texture of Limburger cheese are characteristic of the food and the microbial cultures that are responsible for these traits are characterizing components.

^{xiii} A strain that was isolated from a type-strain or a commercial culture, with a history of safe use in food fermentations, is deemed to have satisfied this requirement and may proceed to 9a.

^{xiv} For example, the Qualified Presumption of Safety list (<http://www.efsa.europa.eu/en/topics/topic/qps.htm>) prepared and periodically updated by the European Food Safety Authority is the output from a systematic safety review of the included microorganisms by qualified experts.

^{xv} Experimental evidence of safety is required. Such evidence may include, but is not necessarily limited to, studies in appropriate animal models, and clinical trials in humans.

^{xvi} In some cases, the strain may be shown to be appropriate by test and re-application of the decision tree, e.g., where an undesirable genetic element has been removed from a strain's genome.

AB-LIFE® has been marketed as a food supplement in various European countries since 2012.

July 7, 2021
Ellen Anderson
Division of Food Ingredients
Center of Food Safety and Applied Nutrition
US Food and Drug Administration



Where science
& creativity meet

Re: GRAS Notice No. GRN 000985

Dear Ms. Anderson,

Below please find Danisco's response to the remaining question from the FDA of June 3, 2021 regarding our GRAS Notice GRN 000985 for the intended use of *Bifidobacterium longum* subsp. *infantis* ATCC SD 6720 (*B. longum* subsp. *infantis* ATCC SD6720). The FDA's question is italicized followed by our response.

12. As the intended uses of B. longum subsp. infantis strain ATCC SD 6720 includes use in infant formula, please provide the following:
a. A specification for Cronobacter sakazakii, along with the respective analytical methods and results from three non-consecutive batch analyses to demonstrate that B. longum subsp. infantis ATCC SD 6720 meets the established specifications.

Response:

For this strain, the specification for *Cronobacter sakazakii* is Negative or Absence in 25 grams using ISO 22964:2017 method. This method has been validated for this intended use and sample size.

Results from three non-consecutive batch analyses are attached and demonstrates that *B. longum* subsp. *infantis* ATCC SD 6720 meets the established specifications.

200 Powder Mill Road, E320
Experimental Station
Wilmington, DE.
T 610.864.7219
iff.com

Please let me know if you have any additional questions about our GRAS notice.

Sincerely,

Jayne Chalfin Davies
Global Regulatory Affairs



Where science
& creativity meet

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First you add knowledge...

Certificate of Analysis

Date: 07 Jul 2021

Material: MSAMPCLINICAL

Material Name: Improve Active Bi-26

Batch No.: 1103702068

Production Date: Jun 2020

Best Before Date: Jun 2021

Test	Result	Specification	Unit	Reference
Viable Cell Count	4.24E+9	>5.0E+9	/unit	ISO 7889/IDF 117
Enterococcus	<100	<100	/g	SMEDP
Non-Lactic	<500	<500	/g	ISO 13559
Yeast & Mold	<50	<50	/g	USP
B. cereus per g	<100	<100	/g	AOAC
Enterobacteriaceae, negative in 10g	Negative	Negative	-	ISO 21528-1
Staph. aureus, neg. by test (<10/g)	Negative	Negative	-	AOAC
Salmonella, negative in 25g	Negative	Negative	-	AOAC
Listeria, negative in 25g	Negative	Negative	-	AOAC
Mesophilic Aerobic Bacteria	<500	<500	/g	SMEDP
Cronobacter spp, negative in 25g	Negative	Negative	/g	ISO 22964

Comments

The above product has been analyzed by Danisco and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

Best if used before the date listed above when stored at or below 4°C.

AOAC references above reflect the current edition of AOAC.


Phil Ihrke
Quality Control Department, Cultures
Dupont Nutrition and Health



Danisco USA-Madison Plant
3322 Agriculture Drive
Madison, WI 53716



CERTIFICATE OF ANALYSIS

SILLIKER, Inc.

Wisconsin Laboratory

3688 Kinsman Boulevard, Madison, WI 53704

Tel. 1-844-277-1738 Fax. 608-249-9886

Email: getresults8@mxns.com

COA No:	WIS-44890547-0
Supersedes:	None
COA Date	6/25/21
Page 1 of 2	

Received From:	Madison, WI
Received Date:	6/19/21
P.O.# / ID:	4501188687
Location of Test: (except where noted) Madison, WI	

TO:

Ms. Barbara Freeburg
Title: QC Manager
Danisco USA Inc
3322 Agriculture Drive
Madison, WI 53716

Analytical Results

Laboratory ID: 410855231 Condition Rec'd: NORMAL Temp Rec'd (°C): -20.0
 Desc. 1: KP-Bi-26 50B-20kg (A-B)
 Desc. 2: 1103956113
 Desc. 3: FD

Analyte	Result	Units	Method Reference	Test Date	Loc.
* Coliforms - 3 tube MPN	<0.3	/g	AOAC 966.24	6/22/21	
* Cronobacter spp	Negative	/25g	ISO 22964:2017	6/25/21	SNK
* E. coli - 3 tube MPN	<0.3	/g	AOAC 966.24	6/22/21	
Enterococci	<100	/g	SMEDP 17th ed.	6/21/21	
* Genus Listeria - ELFA	Negative	/25g	AOAC 2004.06	6/21/21	
* Salmonella - ELFA	Negative	/40g	AOAC 2004.03	6/21/21	
* Staphylococci - coag. positive	<10	/g	AOAC 975.55	6/21/21	

Laboratory ID: 410855239 Condition Rec'd: NORMAL Temp Rec'd (°C): -20.0
 Desc. 1: KP-GG 350B-20kg (A-B)
 Desc. 2: 1103952231
 Desc. 3: FD

Analyte	Result	Units	Method Reference	Test Date	Loc.
* Coliforms - 3 tube MPN	<0.3	/g	AOAC 966.24	6/22/21	
* E. coli - 3 tube MPN	<0.3	/g	AOAC 966.24	6/22/21	
Enterococci	<100	/g	SMEDP 17th ed.	6/21/21	
* Genus Listeria - ELFA	Negative	/25g	AOAC 2004.06	6/21/21	
* Salmonella - ELFA	Negative	/40g	AOAC 2004.03	6/21/21	
* Staphylococci - coag. positive	<10	/g	AOAC 975.55	6/21/21	

Results reported herein are provided "as is" and, unless otherwise indicated, are based solely upon samples as provided by client. This report may not be distributed or reproduced except in full. Client shall not at any time misrepresent the content of this report. These results are intended for use by persons having professional skill and training in the interpretation of testing results. Mérieux NutriSciences assumes no responsibility, and client hereby waives all claims against Mérieux NutriSciences, for interpretation of such results. If statements of conformity to client provided or regulatory specifications are made in this report, measurement of uncertainty has not been taken into account, except when requested by the client. While Mérieux NutriSciences reviews all results exceeding client specifications, the client is responsible for the compliance of its product and determining whether the results meet acceptance or other criteria. To the extent practicable, your company will give notice to, and consult with, Mérieux NutriSciences prior to implementing a withdrawal or recall of products based on any testing results. Except as otherwise stated, Merieux NutriSciences Terms and Conditions for Services apply.



CERTIFICATE OF ANALYSIS

SILLIKER, Inc.

Wisconsin Laboratory

3688 Kinsman Boulevard, Madison, WI 53704

Tel. 1-844-277-1738 Fax. 608-249-9886

Email: getresults8@mxns.com

COA No:	WIS-44890547-0
Supersedes:	None
COA Date	6/25/21
Page 2 of 2	

Received From:	Madison, WI
Received Date:	6/19/21
P.O.# / ID:	4501188687
Location of Test: (except where noted) Madison, WI	

TO:

Ms. Barbara Freeburg
 Title: QC Manager
 Danisco USA Inc
 3322 Agriculture Drive
 Madison, WI 53716

Analytical Results

Laboratory ID: 41085254 **Condition Rec'd:** NORMAL **Temp Rec'd (°C):** -20.0
Desc. 1: Nmade Advanced 76B (A-B)
Desc. 2: 1103953026
Desc. 3: FD

<u>Analyte</u>	<u>Result</u>	<u>Units</u>	<u>Method Reference</u>	<u>Test Date</u>	<u>Loc.</u>
* E. coli - 3 tube MPN	<0.3	/g	AOAC 966.24	6/22/21	
* Enterobacteriaceae	<10	/g	ISO 21528	6/20/21	
* Genus Listeria - ELFA	Negative	/25g	AOAC 2004.06	6/21/21	
* Salmonella - ELFA	Negative	/40g	AOAC 2004.03	6/21/21	
* Staphylococci - coag. positive	<10	/g	AOAC 975.55	6/21/21	

Mechelle Sneed

Laboratory Director

Noted Test Locations: SNK-Silliker, Inc. Northeast Laboratory, 6390 Hedgewood Drive, Allentown, PA 18106

I Customer supplied information * ISO17025 Accredited Analysis † Indicates reason for COA amendent when applicable

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CERTIFICATE OF ANALYSIS

SILLIKER, Inc.

Wisconsin Laboratory

3688 Kinsman Boulevard, Madison, WI 53704

Tel. 1-844-277-1738 Fax. 608-249-9886

Email: getresults8@mxns.com

COA No:	WIS-44923353-0
Supersedes:	None
COA Date	7/4/21
Page 1 of 1	

TO:
Ms. Barbara Freeburg
Title: QC Manager
Danisco USA Inc
3322 Agriculture Drive
Madison, WI 53716

Received From:	Madison, WI
Received Date:	6/30/21
P.O.#/ID:	4501188687
Location of Test: (except where noted) Madison, WI	

Analytical Results

Laboratory ID: 411109883 Condition Rec'd: NORMAL Temp Rec'd (°C): -5.9
Desc. 1: KP-Bi-26 50B-20kg (A-C)
Desc. 2: 1103961404
Desc. 3: FD

<u>Analyte</u>	<u>Result</u>	<u>Units</u>	<u>Method Reference</u>	<u>Test Date</u>	<u>Loc.</u>
* Coliforms - 3 tube MPN	<0.3	/g	AOAC 966.24	7/2/21	
* Cronobacter spp	Negative	/25g	ISO 22964:2017	7/4/21	SNK
* E. coli - 3 tube MPN	<0.3	/g	AOAC 966.24	7/2/21	
* Salmonella - ELFA	Negative	/40g	AOAC 2004.03	7/2/21	



Mehelle Sneed Laboratory Director

Noted Test Locations: SNK-Silliker, Inc. Northeast Laboratory, 6390 Hedgewood Drive, Allentown, PA 18106

I Customer supplied information * ISO17025 Accredited Analysis † Indicates reason for COA amendment when applicable

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From: [Jayne Davies](#)
To: [Anderson, Ellen](#)
Subject: RE: [EXTERNAL] RE: GRN 985
Date: Wednesday, September 01, 2021 12:11:08 PM
Attachments: [image002.png](#)
[image004.png](#)
[image006.png](#)
[GRN 985 Specs for Sept 1 response.pdf](#)

CAUTION: This email originated from outside of the organization. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Dear Ellen,
Thank you for the follow up question regarding GRN 985.

For the administrative record, Danisco clarifies that the specifications in Table 1 (p. 13) of the notice remain correct for all of the specified analytes. This includes viable cell count, non-lactic cell count, yeast and molds, as well as Salmonella serovars. I have attached this Table as confirmation.

I am happy to provide this response as an attachment if you prefer. Please let me know if you have additional questions.

Kind regards

Jayne

Jayne Chalfin Davies, MNS | Regulatory Affairs | IFF
Experimental Station 320 | 200 Powder Mill Road | Wilmington, DE 19803 | Mobile: 610-864-7219

From: Anderson, Ellen [mailto:Ellen.Anderson@fda.hhs.gov]
Sent: Tuesday, August 31, 2021 11:55 PM
To: Jayne Davies <Jayne.C.Davies@iff.com>
Subject: FW: [EXTERNAL] RE: GRN 985

External Warning: This email is from Ellen.Anderson@fda.hhs.gov - if this email address is unfamiliar, do not click links and forward as an attachment to SuspiciousEmail@iff.com

Dear Jayne,

We have a follow-up question regarding Danisco's response to question Q12 received on July 8, 2021. Our question is:

On page 3 of the July 8, 2021 amendment to the notice (attached for your reference), Danisco provides specifications for viable cell count, non-lactic cell count, yeast and molds, as well as *Salmonella* serovars that are not the

same as those listed in Table 1 (page 13) of the notice. For the administrative record, please clarify what the correct specifications are for these four microbial analyses.

Thank you in advance for your attention to this matter.

Sincerely,

Ellen

Ellen Anderson

Regulatory Review Scientist

Center for Food Safety and Applied Nutrition

Office of Food Additive Safety

U.S. Food and Drug Administration

Tel: 240-402-1309

ellen.anderson@fda.hhs.gov

Pronouns: she/her/hers



From: Jayne Davies <Jayne.C.Davies@iff.com>

Sent: Thursday, July 08, 2021 9:58 AM

To: Anderson, Ellen <Ellen.Anderson@fda.hhs.gov>

Subject: RE: [EXTERNAL] RE: GRN 985

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Dear Ellen,

Attached please find Danisco's response to the remaining question, Q12a from the FDA in regard to GRN 985. We appreciate having the additional time to answer this question.

Please let me know if you have any follow-up questions about our notice.

Kind regards

Jayne

Jayne Chalfin Davies, MNS | Regulatory Affairs | IFF

Experimental Station 320 | 200 Powder Mill Road | Wilmington, DE 19803 | Mobile: 610-864-7219

From: Jayne Davies

Sent: Wednesday, June 23, 2021 3:06 PM

To: Anderson, Ellen <Ellen.Anderson@fda.hhs.gov>

Subject: RE: [EXTERNAL] RE: GRN 985

Thanks, Ellen for the prompt follow up.

Kind regards

Jayne

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

Sent: Wednesday, June 23, 2021 2:47 PM

To: Jayne Davies <Jayne.C.Davies@iff.com>

Subject: RE: [EXTERNAL] RE: GRN 985

External Warning: This email is from Ellen.Anderson@fda.hhs.gov - if this email address is unfamiliar, do not click links and forward to SuspiciousEmail@iff.com

Hi Jayne,

Thanks very much for the responses. I will share them with the review team and circle back to you if we have any follow-up questions. We'll look forward to receiving the response to Question 12a later in July.

Sincerely,

Ellen

Ellen Anderson

Regulatory Review Scientist

Center for Food Safety and Applied Nutrition

Office of Food Additive Safety

U.S. Food and Drug Administration

Tel: 240-402-1309

ellen.anderson@fda.hhs.gov

Pronouns: she/her/hers



From: Jayne Davies <Jayne.C.Davies@iff.com>

Sent: Wednesday, June 23, 2021 1:24 PM

To: Anderson, Ellen <Ellen.Anderson@fda.hhs.gov>

Subject: [EXTERNAL] RE: GRN 985

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Dear Ellen,

Attached please find Danisco's response to the majority of the FDA's questions about GRN 985.

Appreciate your flexibility in allowing us a few additional days to compile our response. As discussed last week, response to Q 12a will be provided within a month (by July 23).

Please let me know if you have any questions about our response.

Kind regards

Jayne

Jayne Chalfin Davies, MNS | Regulatory Affairs | IFF
Experimental Station 320 | 200 Powder Mill Road | Wilmington, DE 19803 | Mobile: 610-864-7219

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

Sent: Thursday, June 3, 2021 9:07 AM

To: Jayne Davies <Jayne.C.Davies@iff.com>

Subject: GRN 985

External Warning: This email is from ellen.anderson@fda.hhs.gov - if this email address is unfamiliar, do not click links and forward to SuspiciousEmail@iff.com

Hello Jayne,

Please see the attached letter regarding GRAS notice 000985.

Sincerely,
Ellen

Ellen Anderson

Regulatory Review Scientist

Center for Food Safety and Applied Nutrition

Office of Food Additive Safety

U.S. Food and Drug Administration

Tel: 240-402-1309

ellen.anderson@fda.hhs.gov

Pronouns: she/her/hers



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