April 8, 2020

Dr. Mical Honigfort
Division of Biotechnology and GRAS Notice Review
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

Subject: GRAS Notification –
Bifidobacterium animalis subsp. *lactis* strain AD011 (*B. lactis* AD011) as a Food Ingredient

Dear Dr. Honigfort,

On behalf of BIFIDO, Co., Ltd. (BIFIDO), we are submitting a GRAS notification for *Bifidobacterium animalis* subsp. *lactis* strain AD011 (*B. lactis* AD011) as a food ingredient. The enclosed document provides the notice of a claim that a food ingredient, *B. lactis* AD011, described in the enclosed notification is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be generally recognized as safe (GRAS), based on scientific procedures, as a food ingredient. We believe that this determination and notification are in compliance with Pursuant to 21 C.F.R. Part 170, subpart E.

Please note that this is a resubmission of GRN 875. We enclose an original copy of this notification and a CD Rom for your review. Please feel free to contact me if additional information or clarification is needed as you proceed with the review. We would appreciate your kind attention to this matter.

Sincerely,

[redacted] 4/08/2020

Susan Cho, Ph.D.
Susanscho1@yahoo.com
Agent for BIFIDO

The Generally Recognized as Safe [GRAS] Determination of *Bifidobacterium animalis* subsp. *lactis* AD011 (*B. lactis* AD011) as a Food Ingredient

Prepared for BIFIDO CO., LTD.

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PART 1. SIGNED STATEMENTS AND A CERTIFICATION

Pursuant to 21 CFR Part 170, subpart E, BIFIDO Co., Ltd. (hereinafter referred to as 'BIFIDO') submits a Generally Recognized as Safe (GRAS) notice and claims that the use of *Bifidobacterium animalis* subsp. *lactis* strain AD011 (*B. lactis* AD011) in foods, as described in Parts 2 through 7 of this GRAS notice, is not subject to premarket approval requirements of the FD&C Act based on its conclusion that the substance is GRAS under the conditions of its intended use.

1.A. Name and Address of the Notifier

Contact: Myeong Soo Park, Ph.D.
Company: BIFIDO Co., Ltd.
Address: 23-16, Nonggongdanji-gil, Hongcheon-eup,
Hongcheon-gun, Gangwon-do, 25117
Republic of Korea

1.B. Common or Trade Name

Bifidobacterium animalis subsp. *lactis* strain AD011, *B. animalis* subsp. *lactis* AD011, *Bifidobacterium lactis* AD011, or *B. lactis* AD011.

1.C. Applicable Conditions of Use of the Notified Substance

1.C.1. Foods in Which the Substance is to be Used

B. lactis AD011 will be added to nonexempt term infant formulas (soy-, milk-, and/or whey-based) and selected conventional foods.

1.C.2. Levels of Use in Such Foods

Non-exempt Term Infant Formula Applications:

The use level is the same as those described in GRAS notices of other bifidobacteria (GRN 813 for *Bifidobacterium longum* BORI [*B. longum* BORI]; GRN 814 for *Bifidobacterium bifidum* BGN4 [*B. bifidum* BGN4]; and GRN 454 for *Bifidobacterium breve* MV-16 [*B. breve* MV-16]). Powdered non-exempt term infant formulas (milk-, soy-, or whey-based) will contain up to 10^8 colony forming units (cfu) of *B. lactis* AD011 per g of powdered formulas. *B. lactis* AD011 may be used alone or in combination with other safe and suitable *Bifidobacterium* or *Lactobacillus* strains.

Conventional Food Applications:

BIFIDO intends to add the nonpathogenic and non-toxigenic *B. lactis* AD011 strain to selected conventional food products (dairy products/dairy-based foods and dairy substitutes, including fermented milk, butter milk, and kefir; flavored milk beverage mixes, dried milk powder; imitation milk and yogurt; powdered baby cereals and foods; meal replacement and nutritional drink mix powders; and powdered sugar substitute) for the general population (Table 1). These target foods will contain up to 1×10^{10} cfu *B. lactis* AD011 per serving. *B. lactis* AD011

may be used alone or in combination with other safe and suitable *Bifidobacterium* or *Lactobacillus* strains.

Table 1. Proposed Food Categories for Conventional Food Applications

Dairy Products/dairy-based foods and diary substitutes
Fermented milk, including butter milk and kefir
Flavored milk beverages mix, dried milk powder
Imitation milk
Yogurt
Other foods
Baby cereals and foods, powder form
Meal replacement and nutritional drink mix powder
Sugar substitute, powder form

1.C.3. Purpose for Which the Substance is Used

The substance will be used to provide a dietary source of *B. lactis* AD011 as a food ingredient to non-exempt term infant formulas and selected conventional foods.

1.C.4. Description of the Population Expected to Consume the Substance

The population expected to consume the substance consists of term infants and members of the general population who consume at least one of the products described above.

1.D. Basis for the GRAS Determination

This GRAS conclusion is based on scientific procedures in accordance with 21 CFR 170.30(a) and 170.30(b).

1.E. Availability of Information

The data and information that are the basis for this GRAS conclusion will be made available to the FDA upon request by contacting Myeong Soo Park at BIFIDO. The data and information will be made available to the FDA in a form in accordance with that requested under 21 CFR 170.225(c)(7)(ii)(A) or 21 CFR 170.225(c)(7)(ii)(B).

1.F. Availability of FOIA Exemption

None of the data and information in Parts 2 through 7 of this GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. §552.

1.G. Certification

We certify that, to the best of our knowledge, our GRAS notice 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 the substance.

1.H. Name, Position/Title of Responsible Person Who Signs Dossier, and Signature

_____ 4/7/2020_____

Name: Myeong Soo Park, Ph.D. Date:
Title: Chief Technology Officer

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1.I. FSIS/USDA Statement

BIFIDO does not intend to add *B. lactis* AD011 to any meat and/or poultry products that come under USDA jurisdiction. Therefore, 21 CFR 170.270 does not apply.

PART 2. IDENTITY, MANUFACTURING, SPECIFICATIONS, AND TECHNICAL EFFECTS

2.A.1. Identity of the Notified Substance

2.A.1.1. Common Name

Bifidobacterium animalis subsp. *lactis* AD011, *Bifidobacterium lactis* AD011, *B. lactis* strain AD011, or *B. lactis* AD011.

2.A.1.2. Chemical Names of Main Component: Not applicable (NA)

Isolation and Identification of *B. lactis* AD011

The non-pathogenic and non-toxigenic *B. lactis* AD011 strain was isolated from infant stool. *B. lactis* AD011 is a non-spore forming, heterofermentative, gram-positive, anaerobic, non-genetically modified microorganism, and is a member of the lactic acid bacteria (LAB), a group characterized by the production of lactic acid as the major metabolic end product of carbohydrate metabolism. *Bifidobacterium* genus is an anaerobic, gram-positive bacterium that does not form spores. Bifidobacteria comprise up to 25% of the cultivatable fecal bacteria in adults and 80% in infants (Picard et al., 2005).

The whole genome sequence of *B. lactis* AD011 was published in GenBank (Accession no.: CP001213) in 2009 (Kim et al., 2009). The complete sequence of *B. lactis* AD011 consists of a 1,933,695-bp circular chromosome (60.49% G+C) with no plasmid capable of transmitting antibiotic resistances. The taxonomic classification of *Bifidobacterium lactis* AD011 is shown in Table 2.

Table 2. Taxonomic Classification of *Bifidobacterium lactis* AD011

Class	Scientific Classification
Domain	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Subclass	Actinobacteridae
Order	Bifidobacteriales
Family	Bifidobacteriaceae
Genus	*Bifidobacterium*
Species	*Bifidobacterium animalis*
Subspecies	*Bifidobacterium animalis* subsp. *lactis*
Strain	*Bifidobacterium animalis* subsp. *lactis* AD011

Strain Level Identification

B. lactis AD011 was identified by 16S rRNA sequence analysis. Chromosomal DNA from *B. lactis* AD011 strain was extracted from a single colony using Chelex®100 Resin matrix through a boiling protocol and the 16S rRNA gene was amplified using universal primers. The

PCR primer sequences were as follows: forward primer, 5′-AGAGTTTGATCCTGGCTCAG-3′; reverse primer, 5′-GGTTACCTTTGTTACGACTT-3′ (Bioneer, Korea). Sequence homologies were examined by comparing the obtained sequences with those in the DNA databases (http://www.ncbi.nlm.nih.gov/BLAST).

Primer Information:

PCR Primer Name Primer Sequences
27F 5' (AGA GTT TGA TCM TGG CTC AG) 3'
1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Sequencing Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3

The strain was identified as *Bifidobacterium lactis* and was named *Bifidobacterium lactis* AD011. Details of *B. lactis* AD011 identification are shown in Appendix A.

Similarity in 16S rRNA Genomic Sequences

Ribosomal RNA sequences, especially those of 16S ribosomal RNA, are the best single targets for defining phylogenetic relationships among bacteria. This genetic information provides a phylogenetic framework and is the basis for modern microbial taxonomy (Ludwig and Klenk, 2001). For the delineation of microorganisms at the species level, 97% similarity of 16S ribosomal RNA is a commonly applied conservative threshold in microbial phylogeny. Sequence homologies were examined by comparing the obtained sequences with those in the DNA database (http://www.ncbi.nlm.nih.gov/BLAST).

Table 3 shows the similarities of *B. lactis* AD011 in the genomic sequence of the 16S ribosomal RNA with those of other *B. lactis* strains. The 16S ribosomal RNA sequence of *B. lactis* AD011 has over 99.85% similarity with other GRAS strains of *B. lactis*, such as BB-12, Bi-07, Bl-04, and HN019. Details are shown in Appendix A.

Table 3. Homology of 16S rRNA Genomic Sequences between *B. lactis* AD011 and Other *B. lactis* Strains

Reference strain	Similarity, %
Bifidobacterium lactis BB-12	99.85%
Bifidobacterium lactis Bi-07	99.94%
Bifidobacterium lactis Bl-04	99.93%
Bifidobacterium lactis HN019	99.95%

Similarity in Whole Genomic Sequences

B. lactis AD011 has one circular chromosome of 1,933,695 bp (60.49% G+C), with no plasmid (Table 4; Kim et al., 2009). The *B. lactis* AD011's genome codes for 1,577 coding sequences, seven rRNA genes, and 52 tRNA genes. No functional prophages were identified from the genome sequence, except for a couple of phage-related genes, including integrases. The genome sequence of *B. lactis* AD011 has been deposited at GenBank under the accession number CP001213, and is also available from the Genome Encyclopedia of Microbes (GEM; http://www.gem.re.kr).

B. lactis strain AD011 and other GRAS strains, such as BB-12 (GRN 49 - FDA, 2002) and Bl-04 (GRN 445 - FDA 2013a), consist of one circular chromosome with 1,933,695-bp, 1,942,198-bp, and 1,938,709-bp, respectively, and have G+C content of 60.49%, 60.48%, and 60.48%, respectively. All three strains bear no plasmid capable of transferring antibiotic resistances (Table 4). *B. lactis* strains AD011, BB-12, and Bl-04 show over an 99.85% homology in genome sequences: 99.85% to 99.93% by average nucleotide identity (ANI) values and 99.99% by tetra-nucleotide analysis (TNA) values. Details are presented in Ku et al. (2019).

Table 4. Whole Genome Sequence of *B. lactis* AD011 in Comparison with Other *B. lactis* Strains

Original/User's Label	*B. lactis* AD011 (Current Notice)	*B. lactis* BB-12 (GRN 49)	*B. lactis* Bl-04 (GRN 445)
Project accession	GCA_000021425.1	GCA_000025245.1	GCA_000022705.1
Status	COMPLETE	COMPLETE	COMPLETE
No. of contigs	1	1	1
Plasmids	0	0	0
Genome size (bp)	1,933,695	1,942,198	1,938,709
DNA G+C content (%)	60.49	60.48	60.48
No. of CDSs	1,577	1,567	1,561
No. of rRNA genes	7	12	12
No. of tRNA genes	52	52	52
Mean of CDS lengths (bp)	1,067.5	1,074.5	1,076.8
Median of CDS lengths (bp)	936	948	951
Mean of intergenic lengths (bp)	159.9	159	159.1
Median of intergenic lengths (bp)	113	111	111
Homology with *B. lactis* AD011 by OrthoANI analysis		99.85%	99.93%
Homology with *B. lactis* AD011 by Tetra-nucleotide Analysis		99.99%	99.99%

Data source: Ku et al. (2019).

Abbreviations: ANI=average nucleotide identity; bp=base pair; C=cytosine; CDS=coding sequence; G=guanine.

2.A.1.3. Chemical Abstract Service (CAS) Registry Number: NA

2.A.1.4. Empirical Formula: NA

2.A.1.5. Structural Formula: NA

2.A.1.6. Molecular Weight: NA

2.A.2. Potential Toxicants in the Source of the Notified Substance

No toxicants are identified from *B. lactis* AD011.

2.A.3. Particle Size

NLT 99% pass 20 mesh and NLT 93% pass 50 mesh.

2.B. Method of Manufacture

A schematic diagram of the general manufacturing process used to produce the *B. lactis* AD011 ingredient is illustrated in Figure 1. Briefly, *B. lactis* AD011 is produced in a batch-type fermentation process with a medium composed of glucose, soy peptone, yeast extract, sodium acetate, sodium phosphate, L-cysteine HCl, and taurine. The medium is sterilized and then inoculated with *B. lactis* AD011, which is grown at 37°C for 10-20 h. After growth, the bacteria are centrifuged, washed, pelleted, mixed with maltodextrin (processing aid), freeze-dried, milled, and sieved. Corn starch, an excipient, is added to the concentrate to standardize the blends.

The first step involves fermentation of a starter culture of *B. lactis* AD011 using a food-grade culture medium composed of crystalline glucose, soy peptone, yeast extract, sodium acetate, sodium phosphate(mono), sodium phosphate(di), L-cysteine HCl, and taurine.

1. The medium is sterilized at 121°C for 30 minutes (min) and cooled to 37°C.
2. The medium is inoculated with *B. lactis* AD011 and the bacteria are precultured for 10~20 h at 37°C.
3. Additional medium is prepared for the main culture. The pH of the medium is adjusted to between 5.8 to 6.0. This culture medium is sterilized at 121°C for 20 min. The medium is cooled to 37°C and then inoculated with the starter culture from Step 2.
4. Culturing consists of six steps (from 10 mL to 2,000 L maximum), with incubation at 37°C for 10-20 h until the appropriate concentration is reached at each step.
5. After cultivation, the medium containing *B. lactis* AD011 is cooled to 10°C and then centrifuged at 7,500 rpm for 1 h.
6. The filtrate is then discarded, and the filtered "wet cake" containing the *B. lactis* AD011 cells is washed with water and recentrifuged at 7,500 rpm for 1 h.
7. The bacterial weight of *B. lactis* AD011 is measured and subjected to dilution with maltodextrin (cryoprotective agent). The ingredient is then freeze-dried and milled. The ratio of *B. lactis* AD011 and maltodextrin is 85:15 (w/w).
8. After milling, the excipient (corn starch) is added, and the ingredient is subjected to a metal separator (a standard process in South Korea) prior to packaging.

The number of *B. lactis* AD011 cells per one gram of the ingredient is estimated as 1.0×10^{11} cfu. The list of raw materials and their regulatory status are summarized in Table 5.

Table 5. List of Raw Materials and Their Regulatory Status

Raw material	CAS No.	Regulatory status
Fermentation medium		
Glucose	50-99-7	21 CFR 168.110; 21 CFR 184.1857
Soy peptone	73049-73-7	21 CFR 184.1553
Baker's yeast extract	8013-01-2	21 CFR 184.1983
Sodium acetate	127-09-3	21 CFR 184.1721
Sodium phosphate (monobasic)	7558-80-7	21 CFR 182.1778
Sodium phosphate (dibasic)	7558-79-4	21 CFR 182.1778
L-cysteine HCl	52-89-1	21 CFR 184.1272
Taurine*	107-35-7	No 21 CFR citation for the intended use
Processing aids/Excipients		
Maltodextrin from corn starch**	9590-36-6	No 21 CFR citation for the intended use
Corn Starch	9005-25-8 977050-51-3	SCOGS report 115

*GRN 586.

Abbreviations: CFR = Code of Federal Regulations; SCOGS = Select Committee on GRAS Substances, The raw materials used in fermentation are neither major allergens nor derived from major allergens.

** Maltodextrin from corn starch is approved as a direct food additive in 21 CFR 184.1444(b)(1) but not specifically as a cryoprotective agent. The addition of maltodextrin to the bacteria before freeze drying will result in the net effect as a direct food additive because it is not removed or destroyed during the processing.

Quality Assurance Procedure:

BIFIDO rigorously tests its final production batches to verify adherence to quality control specifications. BIFIDO observes the principles of a Hazard Analysis and Critical Control Point (HACCP)-controlled manufacturing process and current good manufacturing practices (cGMP) for food (21 CFR Part 110 and Part 117 Subpart B). The raw materials and processing aids used in the manufacturing process are food grade. BIFIDO routinely evaluates the quality of the *B. lactis* AD011 ingredient during the production process to ensure that the genetic identity is consistent with that of the original stock and the finished products are free of contaminants.

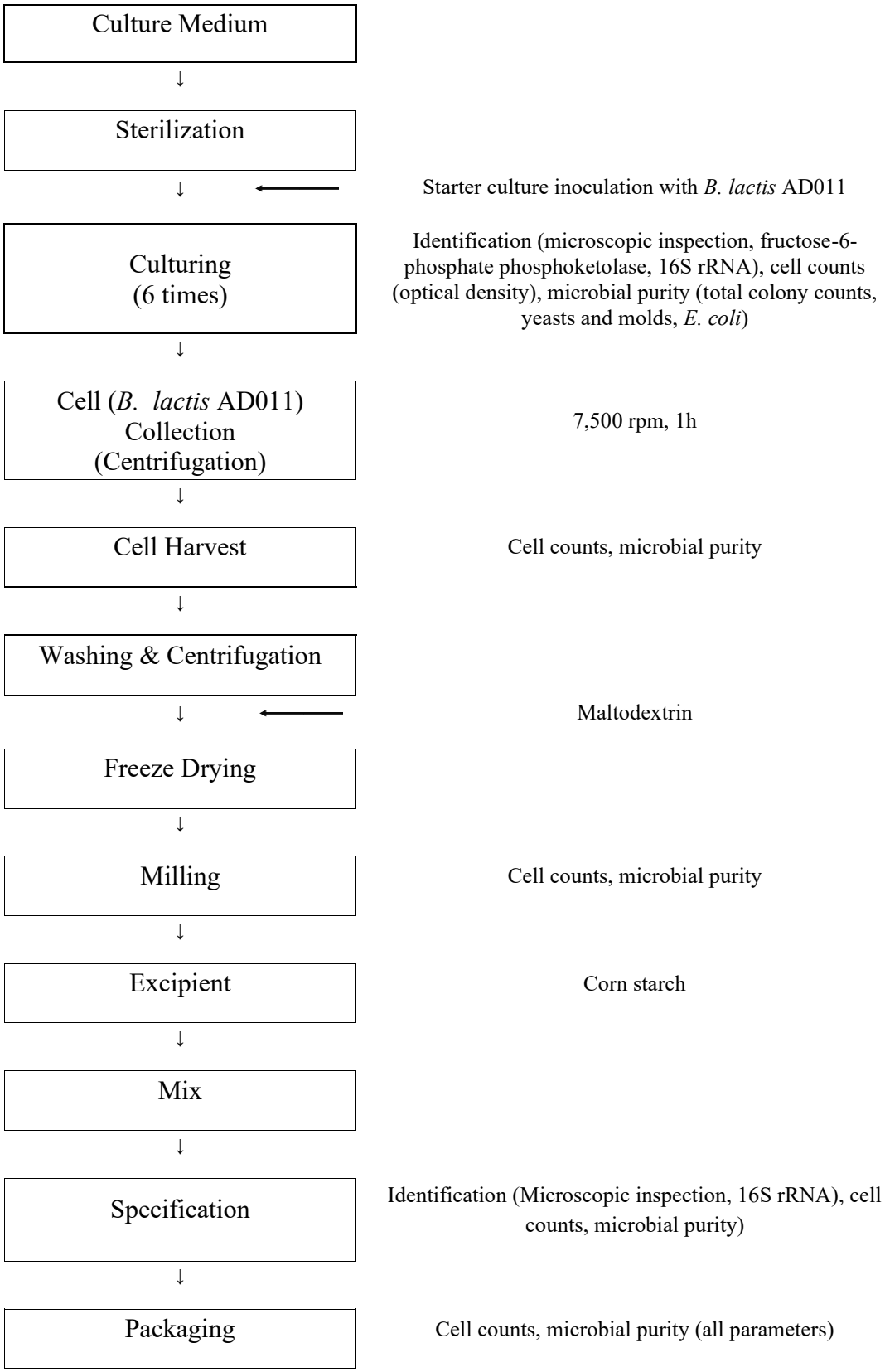

Figure 1. Schematic Overview of Manufacturing Process for *B. lactis* AD011

2.C. Specifications and Composition of *B. lactis* AD011

Table 6 presents the specifications of *B. lactis* AD011. Analyses of three non-consecutive lots of the *B. lactis* AD011 ingredient confirm that the material produced by the manufacturing process is consistent and complies with the product specifications, meeting appropriate food-grade specifications (Table 7; Appendix B).

Table 6. Specifications of *B. lactis* AD011 Stock Ingredient

Parameter	Specification	Typical composition*	Method of analysis
Appearance	No off-taste or off-flavor	Yellow white powder	
Cell Counts, cfu/g (as *B. lactis* AD011)	MT 1.00E+11	1.00E+11	ISO 29981:2010 or equivalent
Moisture, %	NMT 5.0	4.23%	AOAC 941.14 or equivalent
Heavy metals			
Lead (Pb), ppm	NMT 0.3	<0.01	AOAC 2013.06 or equivalent
Arsenic (As), ppm	NMT 0.3	<0.06	
Cadmium (Cd), ppm	NMT 0.1	<0.03	
Mercury (Hg), ppm	NMT 0.1	<0.04	
Microbiology			
Non-lactic acid bacteria, cfu/g	NMT 100	Negative	ISO 13559:2002 (IDF 153)
Total yeasts and molds, cfu/g	NMT 100	Negative	AOAC 2002.11 or equivalent
Escherichia coli, cfu/25 g	ND in 25 g	ND in 25 g	AOAC 991.14-petri *E.coli* count plate
Salmonella, cfu/25 g	ND in 25 g	ND in 25 g	AOAC 989.14 or equivalent
Listeria, cfu/25 g	ND in 25 g	ND in 25 g	AOAC 998.08 or equivalent
Cronobacter sakazakii, cfu/10 g	ND in 10 g	ND in 10 g	BAM - Chapter 29 *Cronobacter* cultural method
Ash, %	NA	5.99%	AOAC 900.02 or equivalent

*Average of 3 analytical values.

Abbreviations: AOAC = Association of Official Agricultural Chemists; ISO = International Standards Organization; MT = More Than; NA = Not Applicable; ND = Not Detected; NMT = Not More Than.

Table 7. Analytical Values of *B. lactis* AD011 (3 Non-Consecutive Lots)

Parameter	Samples					
	BL-R-190116	BL-R-190129	BL-R-190211	BL-R-190603-A	BL-R-190909	BL-R-200130-2
Sample collection date	2019.01.16	2019.01.29	2019.02.11	2019.06.03	2019.09.09	2020.01.30
Appearance	Yellow white powder	Yellow white powder	Yellow white powder			
Cell counts, cfu/g (as *B. lactis* AD011)	1.00E+11	1.00E+11	1.00E+11			
Moisture, %	4.3%	4.2%	4.2%			
Heavy metals						
Lead (Pb), ppm	<0.01	<0.01	<0.01			
Arsenic (As), ppm	<0.06	<0.06	<0.06			
Cadmium (Cd), ppm	<0.03	<0.03	<0.03			
Mercury (Hg), ppm	<0.04	<0.04	<0.04			
Microbial purity						
Non-lactic acid bacteria, cfu/g	ND	ND	ND			
Total yeasts and molds, cfu/g	ND	ND	ND			
Salmonella, cfu/25 g	ND in 25 g	ND in 25 g	ND in 25 g			
Listeria, cfu/25 g	ND in 25 g	ND in 25 g	ND in 25 g			
Escherichia coli,* cfu/25 g				ND in 25 g	ND in 25 g	ND in 25 g
Cronobacter sakazakii,* cfu/10 g				ND in 10 g	ND in 10 g	ND in 10 g
Ash, %	4.36%	5.69%	7.93%			

Abbreviations: ND= Not Detected. cfu = colony forming units; MPN = most probable number;
*In the initial analysis of the 3 non-consecutive samples (BL-R-190116, BL-R-190129, and BL-R-190211), *Escherichia coli* and *Cronobacter sakazakii* were not detected when employed the sample sizes of 200 and 60 g, respectively. To test the effects of sample sizes on the final results, 3 additional non-consecutive lot samples were tested using the sample sizes of 25 and 10 g, respectively. The sample sizes did not impact the final results. The analytical results based on the smaller sample sizes are reported in this table.

2.D. Stability of the *B. lactis* AD011

Observing that *B. lactis* strains are widely used as probiotic microorganisms, Briczinski et al. (2009) noted that the *B. lactis* is robust with regard to stressful conditions, such as acidity and oxygen. It is able to withstand the adverse conditions of product manufacture and storage, and can maintain viability and stability during the product's shelf life.

Bulk ingredient stability data indicated that *B. lactis* AD011 cells in the ingredient were stable for up to 18 months at 5°C and 25ºC when supplied in excess of 150% of the claim value at the time of shipment. Table 8 presents the stability of *B. lactis* AD011 at various temperatures.

Table 8. Stability of *B. lactis* AD011

Temperature /Month	5℃	25℃	40℃
0	1.50E+11	1.50E+11	1.30E+11
2	1.44E+11	1.30E+11	6.59E+10
4	1.38E+11	1.28E+11	1.01E+10
8	1.30E+11	1.11E+11	4.26E+09
10	1.25E+11	1.03E+11	1.30E+09
12	1.14E+11	9.72E+10	-
18	1.15E+11	9.51E+10	-
24	1.08E+11	8.85E+10	-
The viability of *B. lactis* AD011 at 18 months compared to the claim value (1.00E+11 cfu/g)	108%	95%	

2.E. Intended Technical Effects

The substance will be used to provide a dietary source of *B. lactis* AD011 as a food ingredient to non-exempt term infant formulas and selected conventional foods.

PART 3. DIETARY EXPOSURE

3.A. Estimated Dietary Intakes (EDIs) of *B. lactis* AD011 Under the Intended Use

3.A.1. Non-Exempt Term Infant Formula Applications

The use levels are the same as those for other *Bifidobacterium* species described in GRNs 454, 813, and 814. Powdered non-exempt term infant formulas (milk-, soy-, and/or whey-based) will contain up to 10^8 cfu *B. lactis* AD011 per g powdered formulas. The intended target intake level will be a minimum of 10^9 cfu *B. lactis* AD011 per day.

Based on 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, the EDIs of infant formula (as consumed, ready-to-drink or reconstituted formula prepared from powder) intakes by age were calculated (Tables 9-1 and 9-2). The mean and 90th percentile infant formula intakes of infants 0 to 11.9 months of age were estimated to be 770 and 1,188 g per person per day, respectively, in all user infants (Table 9-1). The mean and 90th percentile EDIs of infant formulas in the total infant population were 484 and 1,097 g formula per infant per day, respectively (Table 9-2).

Thus, the mean and 90th percentile intake of infants aged 0 - 11.9 months were estimated to be 1.04 x 10^{10} and 1.60 x 10^{10} cfu *B. lactis* AD011 per person per day, respectively, in all user infants. The EDIs of *B. lactis* AD011 cells were calculated based on the assumption that a typical infant formula contains an average of 13.5 g powdered formula per 100 mL and 1 g of powdered formula contains 10^8 cfu/g. The daily mean infant formula intake is 770 mL. Thus, the total number of cfu in 770 mL (mean intake) of formula can be calculated using the following formula: 10^8 cfu/g x 13.5 g/100 mL x 770 mL = 1.04 x10^{10} cfu. Using the same calculation method, the estimated mean and 90th percentile intake of infants aged 0 - 11.9 months were 6.53x10^9 and 1.48x10^{10} cfu per infant per day, respectively, in the total infant population.

Table 9-1. EDIs of *B. lactis* AD011 from the Proposed Use in Infant Formulas in All User Infants*

	N	Formula as consumed, g/day		*B. lactis* AD011, cfu/day	
		Mean	90th Pctl	Mean	90th Pctl
0-2.9 mo	61	805	1,120	1.09×10^{10}	1.51×10^{10}
3-5.9	59	813	1,239	1.10×10^{10}	1.67×10^{10}
6-8.9	73	768	1,197	1.04×10^{10}	1.62×10^{10}
9-11.9	54	695	1,097	9.38×10^{9}	1.48×10^{10}
0-11.9	247	770	1,188	1.04×10^{10}	1.60×10^{10}

**Based on the 2015-2016 National Health and Nutrition Examination Survey (NHANES) dataset; mo = months; pctl = percentile.

Table 9-2. EDIs of *B. lactis* AD011 from the Proposed Use in Infant Formulas in All Infant Population*

	N	Formula as consumed, g/day		*B. lactis* AD011, cfu/day	
		Mean	90th Pctl	Mean	90th Pctl
0-2.9 mo	92	491	1,000	6.63×10^9	1.35×10^{10}
3-5.9	89	417	1,084	5.63×10^9	1.46×10^{10}
6-8.9	96	549	1,184	7.41×10^9	1.60×10^{10}
9-11.9	82	460	1,018	6.21×10^9	1.37×10^{10}
0-11.9	359	484	1,097	6.53×10^9	1.48×10^{10}

*Based on the 2015-2016 National Health and Nutrition Examination Survey (NHANES) dataset; mo = months; pctl = percentile.

3.A.2. Conventional Food Applications

BIFIDO intends to add *B. lactis* AD011 to selected conventional food products for the general population (Table 1). Selected conventional foods will contain up to $1.0x10^{10}$ cfu per serving. The intended use of 1.0×10^{10} cfu *B. lactis* AD011 per serving in the target food categories would result in intakes in all users of 1.28×10^{10} and 2.71×10^{10} *B. lactis* AD011 cells per person per day in the mean and 90th percentile, respectively (Table 10-1). A maximum exposure would occur in adult females, with a 90th percentile EDI of 3.36×10^{10} cfu per person per day. In the total population, the mean and 90th percentile food intakes are estimated to be 4.01×10^9 and 1.16×10^{10} cfu per person per day, respectively (Table 10-2).

These estimates are amplified because it is not likely that *B. lactis* AD011 will be used at the maximum levels for all food categories under the intended uses.

Table 10-1. EDIs of *B. lactis* AD011 from Proposed Uses in Selected Conventional Foods in All Users*

	N	Food, serving/day		*B. lactis* AD011, cfu/day	
		Mean	90th Pctl	Mean	90th Pctl
Children, 1-5	278	0.67	1.37	0.67×10^{10}	1.37×10^{10}
Children, 6-12	229	0.56	0.99	0.56×10^{10}	0.99×10^{10}
Males, 13-18	53	0.95	1.65	0.95×10^{10}	1.65×10^{10}
Females, 13-18	68	0.66	1.11	0.66×10^{10}	1.11×10^{10}
Males, 19-99	526	1.39	2.54	1.39×10^{10}	2.54×10^{10}
Females, 19-99	733	1.47	3.36	1.47×10^{10}	3.36×10^{10}
All users	1,887	1.28	2.71	1.28×10^{10}	2.71×10^{10}

*Based on the 2015-2016 National Health and Nutrition Examination Survey (NHANES).

Table 10-2. EDIs of *B. lactis* AD011 from Proposed Uses in Selected Conventional Foods in All Population*

	N	Food, serving/day		*B. lactis* AD011, cfu/day	
		Mean	90th Pctl	Mean	90th Pctl
Children, 1-5	714	0.27	0.78	2.73×10^9	0.78×10^{10}
Children, 6-12	952	0.14	0.50	1.41×10^9	0.50×10^{10}
Males, 13-18	384	0.14	0.52	1.36×10^9	0.52×10^{10}
Females, 13-18	367	0.12	0.53	1.22×10^9	0.53×10^{10}
Males, 19-99	2,055	0.39	1.21	3.89×10^9	1.21×10^{10}
Females, 19-99	2,159	0.56	1.70	5.58×10^9	1.70×10^{10}
Total population	6,631	0.40	1.16	4.01×10^9	1.16×10^{10}

*Based on the 2015-2016 NHANES.

Summary of Consumption Data

Non-exempt term infant formula applications:

The intended target intake level will be a minimum of 10^9 cfu *B. lactis* AD011 per day because powdered term infant formulas will contain 10^8 cfu *B. lactis* AD011 per g powdered formulas.

Conventional food applications:

The intended use of 1.0×10^{10} cfu *B. lactis* AD011 per serving in the selected food categories will result in estimated mean and 90th percentile intakes of 1.28×10^{10} and 2.71×10^{10} cfu per person per day, respectively, in all users. In the total population, estimated mean and 90th percentile intakes are 4.01×10^9 and 1.16×10^{10} cfu per person per day, respectively. However, these EDIs are inflated because it is not expected that all food categories listed under the intended use will contain *B. lactis* AD011 at the maximum use level.

3.B. Food Sources of *B. lactis* AD011

Lactic acid bacteria, including bifidobacteria, are commonly consumed in fermented foods throughout the world. However, we could not find sufficient information to allow an estimate of the sources and EDIs of naturally occurring *B. lactis* AD011 from the diet.

3.C. EDIs of *B. lactis* AD011 from Diet

Not applicable.

3.D. Total EDIs of *B. lactis* AD011 from Diet and Under the Intended Use

Same as 3.A.

3.E. EDIs of Other Substances Under the Intended Use

Corn starch and maltodextrin are subjected to Select Committee on GRAS Substances (SCOGS) report 115 and 21 CFR 184.1444/§184.1(b)(1), respectively. Thus, EDIs of these carbohydrates from the intended use were not calculated.

PART 4. SELF LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with the *B. lactis* AD011 ingredient.

PART 5. HISTORY OF CONSUMPTION

The statutory basis for the conclusion of GRAS status of *B. lactis* AD011 in this document is not based on common use in food before 1958.

PART 6. NARRATIVE

6.A. Current Regulatory Status

In the United States, various *B. lactis* strains have been determined to be GRAS for use in conventional foods or infant formulas, including:

1) *B. lactis* BB-12 for use in infant formulas for children four months of age and older (GRN 49 [FDA, 2002]; 10^7-10^8 cfu/g infant formula);
2) *B. lactis* Bf-6 for use in selected foods (GRN 377 [FDA, 2011]; between 10^9 and 10^{11} cfu/serving of conventional foods, usually at less than 10^{10} cfu/serving); and
3) *B. lactis* HN019, Bi-07, Bl-04, and B420 strains (GRN 445 [FDA, 2013a]; up to 2×10^{11} cfu/serving of conventional foods).

In addition, various *Bifidobacterium* species have been determined to be GRAS for use in conventional foods or infant formulas, including

4) *B. longum* BB536 for use in selected foods and infant formulas (GRN 268 [FDA, 2009], up to 10^{10} cfu/serving of conventional foods, up to 10^{10} cfu/g of milk-based term infant formula for term infants aged 9 months and older);
5) *B. breve* M-16V for use in selected conventional foods (GRN 453, [FDA, 2013b], up to 5×10^9 cfu/serving of conventional foods);
6) *B. breve* M-16V for use in non-exempt powdered term infant formulas (milk- or soy-based) and exempt powdered term infant formulas containing partially hydrolyzed milk or soy proteins (GRN 454 [FDA, 2013c], at levels up to 10^8 cfu/g of infant formula powder);
7) *B. breve* M-16V for use in exempt term powdered amino acid-based infant formulas (GRN 455 [FDA, 2013d], up to 10^8 cfu/g of infant formula powder);
8) *B. longum* BORI for use in infant formulas (up to 10^8 cfu/g) and selected conventional foods (up to 10^9 cfu/serving) (GRN 813, FDA 2019a); and
9) *B. bifidum* BGN4 for use in infant formulas (up to 10^8 cfu/g) and selected conventional foods (up to 10^9 cfu/serving) (GRN 814, FDA 2019b).

The FDA did not have questions on the intended uses, use levels, and the summaries of safety of the above listed *Bifidobacterium* species.

The European Food Safety Agency (EFSA) considers the bacterial species *B. bifidum* suitable for the Qualified Presumption of Safety (QPS) approach for safety assessment (EFSA, 2007, 2010). The QPS approach is a generic assessment system used within EFSA to harmonize premarket safety assessments of selected groups of microorganisms used in food and food production (EFSA, 2007). The QPS approach establishes the safety of a defined taxon (genus or group of related species) based on four "pillars:" (a) established identity, (b) body of knowledge, (c) possible pathogenicity, and (d) end use. Exclusion or qualification of safety concerns should result in granting QPS status for a given taxonomic group (EFSA, 2007). Those applying for EFSA approval of such "new" strains are required to provide proof of the absence of transferable resistance to therapeutic antibiotics. Other primary criteria for functionality are a strain's ability

to survive passage through the upper gastrointestinal tract and its interaction under typical conditions in the small intestine. Therefore, it is generally believed that *B. lactis* strains do not require any specific demonstration of safety other than confirmed absence of any determinants of clinically significant resistance to antibiotics in humans and animals.

The EFSA Scientific Committee (EFSA, 2010) has noted that a variety of different *Lactobacillus* and *Bifidobacterium* species have occasionally been isolated from human clinical specimens. However, such occurrences have been rare and were mainly encountered in immune-compromised patients or in those with severe underlying illnesses. The Committee concluded that most *Lactobacillus* and *Bifidobacterium* species can be considered non-pathogenic to humans, and thus, pose no specific safety concerns.

In Korea, *B. lactis* AD011 has received the Korean FDA's approval as a functional food ingredient. The *B. lactis* AD011 ingredient has been marketed as a dietary supplement ingredient and as a dietary supplement in Korea since 2007. *B. lactis* AD011, at daily doses up to 1×10^{10} cells (or 1.5×10^{10} cfu at the time of shipment), has been safely used, and no adverse events or health-related complaints have been reported by consumers.

6.B. Review of Safety Data

Safety assessment tests included assessments of undesirable metabolic activities (e.g., biogenic amine production), determination of antimicrobial resistance factors, mucolytic or hemolytic activities, assessment of side effects in human studies, and assessment of post-market epidemiological surveillance of adverse effects/events in consumers. This review covers papers published until December 2019.

A non-pathogenic, non-toxigenic nature of *B. lactis* AD011 has been summarized in Ku et al. (2019):

1. The genome of *B. lactis* AD011 does not contain regions with significant homology to known toxigenic or pathogenic genes.
2. Functional assays indicate that *B. lactis* AD011 exhibits antibiotic susceptibility. The exception was tetracycline resistance for *B. lactis* AD011. The minimum inhibitory concentration (MIC) value of *B. lactis* AD011 for tetracycline was higher than that established by EFSA, but comparable to those of other GRAS strains, such as *B. lactis* BB-12, HN019, Bl-04, B420, and Bf-6 (GRN 49 - FDA, 2002; GRN 377 - FDA, 2011; GRN 445 - FDA, 2013a ; Kim et al., 2018) and *B. breve* M-16V (GRNs 453 to 455 - FDA, 2013b, 2013c, 2013d). These have received the U.S. FDA's 'no question' letters for use as ingredients in infant formulas and/or selected conventional foods.
3. *B. lactis* AD011 does not contain plasmid capable of transmitting antibiotic resistance genes.
4. *B. lactis* AD011 was not observed to have hemolytic and mucolytic activities.
5. *B. lactis* AD011 was not observed to produce clinically significant levels of biogenic amines and ammonia.

6. Human clinical studies found no adverse effects of *B. lactis* AD011.
7. No serious adverse effects/events were reported by consumers in the past 12 years.

Thus, it is reasonable to conclude that *B. lactis* AD011 be non-pathogenic and non-toxigenic. In addition, species of the genus *Bifidobacterium* are considered to be non-pathogenic and non-toxigenic, and have generally been considered safe for food use (Borriello et al., 2003).

6.B.1. Metabolism

Given that *B. lactis* AD011 retains its form, it is unlikely that *B. lactis* AD011 will enter organs or the systemic circulation from the gastrointestinal tract in normal, healthy individuals. Rather, the fate of *B. lactis* AD011 after ingestion is expected to be similar to that seen after consumption of live *Bifidobacterium* species. *B. lactis* AD011 is expected to transit through the gastrointestinal tract and be excreted in feces. It has also been shown that live *B. lactis* AD011, like other bifidobacteria, does not harbor the potential for translocation (Kim et al., 2018; Picard et al., 2005).

6.B.2. Genetic Stability Test

Genetic variation of edible bacteria presents a potential risk of indel (i.e., gene deletion and insertion) and mutation. A critical consideration for commercializing edible bacteria is whether it is possible to maintain the genetic safety over the long term. Theoretically, an evaluation of genetic stability requires the knowledge of the entire genome sequence of the strain.

The entire genome sequence of *B. lactis* AD011 has been published (Kim et al., 2009). *B. lactis* AD011 has one circular chromosome of 1,933,695 bp (60.49% G+C), without any plasmids (Kim et al., 2009). The genome sequence and annotation of the *B. lactis* AD011 chromosome, deposited in GenBank under accession number CP001213, are also available from the Genome Encyclopedia of Microbes (GEM; http://www.gem.re.kr). Park and Yang (2019) reported 99.99% similarity in the genomic comparison of the 1st and 25th generations via Orthologous Average Nucleotide Identity (OrthoANI) analysis. They reported small difference resulted from sequencing errors or spontaneous evolutionary mutations. These data indicate low genetic mutation, with no change in the genetic information during the process of cultivating 25 generations. Details are described in Appendix C.

There are no reports that present genetic instability of the *B. lactis* strains. Thus, the unpublished status of the Appendix C (Park and Yang, 2019) on genetic stability of *B. lactis* AD011 strain has no impact on the overall conclusion of this GRAS determination even if qualified experts do not have access to such data and information.

6.B.3. Absence of Virulence Genes

The search for virulence factors in *B. lactis* AD011 was completed using the VirulenceFinder 2.0Server, a publicly available web-based tool for whole-genome sequencing (WGS) analysis hosted by the Center for Genomic Epidemiology (CGE)

(www.genomicepidemiology.org) (Ku et al., 2019). The genome sequence of *B. lactis* AD011 was compared with the genome sequences of well-known pathogens, including *E. coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus* (*S. aureus*). The list of screened virulence genes can be found in Appendix D.

The results showed that the genomic sequence of *B. lactis* AD011 did not include any toxigenic or pathogenic genes.

6.B.4. Susceptibility of *B. lactis* AD011 to Antibiotics

The EFSA's Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has established MIC cut-off values for the antibiotic resistance of microorganisms used as food and/or feed additives (EFSA, 2012). The MIC cut-off values established by the EFSA were based on the distribution of the chosen antimicrobials' MICs in cell populations belonging to a single taxonomical unit. The MIC was defined as the lowest concentration of antibiotic giving a complete inhibition of visible growth in comparison to an antibiotic-free control well. The MIC values for all bacterial isolates were determined by the ISO 10932:2010 broth microdilution procedure, as described in Ku et al. (2019).

All *Bifidobacterium* spp. were susceptible to ampicillin, chloramphenicol, clindamycin, erythromycin, penicillin G, rifampicin, and vancomycin (MIC ranging from 0.01 to 4 μg/mL). They were generally resistant to aminoglycoside antibiotics, such as gentamicin, kanamycin and neomycin (Table 11).

In general, the MIC values of *B. lactis* AD011 were equal to or lower than the established cut-off values suggested by the EFSA. The MIC values of *B. lactis* AD011 for ampicillin sodium salt, streptomycin sulfate salt, erythromycin, vancomycin hydrochloride, chloramphenicol, and clindamycin hydrochloride were 0.5, 128, 0.063, <0.25, 2, and <0.032, respectively. The exceptions were gentamicin and tetracycline, with MIC values of *B. lactis* AD011 slightly higher than those established by EFSA cut-off points (*B. lactis* AD011 vs. EFSA cut-off values: gentamycin [sulfate]: 256 vs. 64 μg/mL; tetracycline: 16 vs. 8 μg/mL). However, it is noteworthy that the MIC value of *B. lactis* AD011 for gentamicin was equal to the proposed epidemiological cut-off (ECOFF) value (256 μg/mL; GRN 377-pages 29-30). The MIC value of *B. lactis* AD011 for tetracycline was comparable to those of other GRAS strains, such as *B. lactis* BB-12 (Kim et al., 2018), HN019, Bl-04, and B420 (GRN 445 - FDA, 2013a), Bf-6 (GRN 377 - FDA, 2011), and *B. breve* M-16V (Kim et al., 2018). These strains have received FDA 'no question' letters for use as ingredients for infant formulas and/or selected conventional foods. *B. lactis* BB-12 was the subject of GRN 49 (FDA, 2002).

As shown in Table 11, most *Bifidobacterium* species were shown to have resistance to tetracycline. Tetracycline resistance in *B. animalis* subsp. *lactis* is directly correlated with the presence of a single gene, *tet*(W) (Gueimonde et al., 2010). Resistance to tetracyclines is due to the presence of the *tet*(W) gene, which is widely distributed in *B. animalis* subsp. *lactis*. The studies by Gueimonde et al. (2010) and Aires et al. (2007) consistently found *tet*(W) in all strains tested. Noting the presence of the transposase gene, the authors concluded that there was no evidence that *tet*(W) in *B. animalis* subsp. *lactis* is transmissible. Aires et al. (2007) reported that

attempted parallel conjugation of *tet*(W) among *Bifidobacterium* isolates failed to produce any transconjugants. It is noteworthy that *B. lactis* AD011 has no plasmid capable of transmitting antibiotic resistance genes. Tetracycline is not a commonly used antibiotic in the U.S.

The MIC values of *B. lactis* AD011 for penicillin G, carbenicillin disodium salt, methicillin, dicloxacillin sodium salt hydrate, kanamycin sulfate, neomycin sulfate, cephalothin sodium salt, polymyxin B sulfate salt, metronidazole, rifampicin, phosphomycin disodium salt, mupirocin, and trimethoprim-sulfamethoxazol were 0.25, 2, 2, 8, 1,024, 512, 32, 256, 256, 2, 64, 32, and <0.5, respectively. EFSA cut-offs are not available for the above mentioned antibiotics. However, these values were comparable to or lower than the MICs for other GRAS strains (*B. lactis* BB-12 and *B. breve* M-16V). The MIC value of *B. lactis* AD011 for mupirocin was significantly lower than those for other GRAS strains such as *B. lactis* BB12 and *B. breve* 16V (32 vs. >128), while the value for metronidazole was significantly higher than those for other GRAS strains (256 vs. 4-31). The MIC value of *B. lactis* AD011 and BB-12 strains for polymixin B was significantly higher than those for the *B. breve* M-16V (256 vs. 1,024).

Ampicillin, vancomycin, gentamicin, and erythromycin are frequently used antibiotics in pediatric patients. For *B. lactis* AD011, none of these pediatric antibiotics had MIC values in excess of the EFSA or ECOFF break points.

Overall, the MIC values were comparable for *B. lactis* AD011 and other GRAS strains (*B. lactis* BB-12, HN019, Bl-04, B420, and Bf-6 strains, and *B. breve* M-16V).

Table 11. Antimicrobial Susceptibility of *B. lactis* AD011 and Other *Bifidobacterium* spp. (MIC values, ug/mL)

Data source	EFSA MIC Cut-off Values	ECO FF*	Current notice	*B. lactis* strains (GRN 445)				GRN 377	Kim et al., 2018		GRN 453
Antibiotic / Strains	*Bifidobac-terium*		*B.lactis* AD011	HN 019	Bl-04	Bi-07	B420	*B. lactis Bf-6*	*B. lactis* BB-12	*B. breve* M-16V	*B. breve* M-16V
Ampicillin sodium salt	2	0.5	0.5	0.12	0.5	0.5	0.25	0.25	0.125	0.25	0.125-<1.56
Gentamicin sulfate	64	256	256	64	64	256	64	64	128	128	15.6-64
Streptomycin sulfate salt	128	256	128	64	64	8	64	32-64	128	256	14-128
Tetracycline	8	2	16	32	16	0.12	16	4-16	16	16	0.5-2.0
Erythromycin	1	1	0.063	0.06	0.06	<0.03	0.06	0.032–0.5	0.125	0.125	<0.125-0.25
Vancomycin hydrochloride	2	1	<0.25	0.5	1	0.25	0.5	0.5-1	0.5	0.5	0.25-0.5
Chloramphenicol	4	4	2	2	2	2	2	1-2	2	2	1-2
Clindamycin hydrochloride	1	0.125	<0.032		<0.03	2	0.05	<0.032-0.06	<0.032	<0.032	0.032-0.125
Penicillin G	N/R	0.5	0.25					0.5	0.125	0.25	0.125-<1.52
Carbenicillin disodium salt	N/R		2						2	4	NA
Methicillin	N/R		2						2	8	NA
Dicloxacillin sodium salt hydrate	N/R		8						4	8	NA
Kanamycin sulfate	N/R	256	1024	256	512	64	256	256	1024	1024	250-512
Neomycin sulfate	N/R		512					64	512	1024	62.5 - >256
Cephalothin sodium salt	N/R		32						8	16	NA
Polymyxin B sulfate salt	N/R		256						256	1024	15.6-125
Metronidazole	N/R	16	256					8	4	8	15.6-31.3
Rifampicin	N/R	2	2					2	2	1	0.125
Phosphomycin disodium salt	N/R		64						64	32	NA
Mupirocin	N/R		32						>128	>128	NA

Trimethoprim-sulfamethoxazole	N/R		<0.5					<0.12-0.25	1	2	32-128

Data source: The proposed epidemiological cut-off (ECOFF)* was adopted from GRN 377 (pages 29-30). Other data sources include Ku et al. (2019), Kim et al. (2018), GRN 377 (pages 29-30), GRN 445 (stamped pages 28-30), and GRN 453 (pages 30-32); Abbreviations: NA= not applicable; N/R= not required. *B. lactis* BB12 was the subject of GRN 49 (FDA, 2002).

6.B.5. Antibiotic Resistance Transferability Test

The antimicrobial susceptibility test found that *B. lactis* AD011 was resistant to tetracycline (MIC of 16 μg/mL). Tetracycline resistance transferability test was conducted using *L. fermentum* AGBG1, a recipient strain that is highly susceptible to tetracycline. Conjugal transfer of antibiotic resistance was assessed via the 1987 Tannock method as described in Ku et al. (2019).

The tetracycline resistance of *B. lactis* AD011 was not transferred to the recipient, *L. fermentum* AGBG1. *L. fermentum* AGBG1, which is highly susceptible to tetracycline, grew well in normal MRS medium; however, it did not grow in the MRS medium containing tetracycline or the media that was co-cultured with *B. lactis* AD011. In contrast, *B. lactis* AD011 showed resistance to 16 μg/mL tetracycline in this study. The data indicate that *B. lactis* AD011's resistance to tetracycline was not transferred to the recipient strain under the test conditions.

Summary of Antibiotic Susceptibility

The available information on the antibiotic resistance pattern of *B. lactis* AD011 indicates that overall antibiotic susceptibilities of the strain are similar to patterns of other GRAS strains of bifidobacterial species, and that the strain is not likely to have transmissible antibiotic resistance genes. In addition, *B. lactis* AD011 does not contain a plasmid capable of transmitting antibiotic resistance genes. These findings indicate that the use of *B. lactis* AD011 in foods does not present concerns for antibiotic resistance.

6.B.6. Ammonia Production Test

Intestinal bacteria can degrade various nitrogen sources (e.g., proteins, peptides, and amino acids) present in the feces of the intestinal track (Kim et al., 2018). These naturally-occurring microbiota and artificially-administered flora have the potential to produce various toxic substances during the deamination stage via nitrogen derivatives. Multiple potentially toxic products (i.e., phenol, ammonia, and indole) are possible throughout the proteolytic process, especially in the large intestine. Thus, bacterial ammonia production is highly relevant to human intestinal health and is a necessary component of the safety evaluation of commercial bacteria intended for human consumption.

The ammonia production of *B. lactis* AD011 was assessed to verify the safety of these bacteria intended for human consumption (Ku et al., 2019). In this study, *B. lactis* AD011 did not produce ammonia. In contrast, *Enterococcus faecium* KCTC13225, the positive control, produced 109.3 ± 7 μg/mL of ammonia. Thus, it is concluded that *B. lactis* AD011 does not produce ammonia. Details are described in Ku et al. (2019).

6.B.7. Hemolytic Activity Test

Visualizing the physical changes caused by hemolytic activity by culturing the microorganisms on a medium containing animal or human blood is a commonly used tool to evaluate the hemolytic properties of pathogenic bacteria. In the study by Ku et al. (2019), the

potential hemolytic activity of *B. lactis* AD011 was assessed using the blood agar plating method.

Listeria ivanovii subsp. *ivanovii* ATCC 19119 (positive control) showed β-hemolysis colorless zones around the cell colonies, whereas *B. lactis* AD011 showed no hemolysis and no change of color in the periphery of the colonies. Thus, it is concluded that *B. lactis* AD011 is not hemolytic. Details are presented in Ku et al. (2019).

6.B.8. Biogenic Amine Production Test

To evaluate if *B. lactis* AD011 would produce biogenic amines, *B. lactis* AD011 was anaerobically cultured in whole milk or de Man-Rogosa-Sharpe (MRS) broth with supplementation of 0.05% (w/w) L-cysteine-HCl at 37°C for 15 h (Ku et al., 2019). The biogenic amines were extracted and analyzed by high performance liquid chromatography (HPLC). *B. lactis* AD011 did not produce cadaverine, histamine, tyramine, or putrescine. Details are described in Ku et al. (2019).

6.B.9. Mucin Degradation Test

The intestinal mucus gel layer is an important constituent of the intestinal barrier that consists of a glycoprotein family. Bacterial translocation can occur in infants and immunocompromised hosts even if the intestinal mucus acts as a biological shield from microbes. Bacterial translocation has the potential to cause sepsis and is one of the most serious safety concerns for edible bacteria. In Ku et al. (2019), the translocation capability of *B. lactis* AD011 was measured using *in vitro* mucolytic assays.

B. lactis AD011 did not use mucin as a carbon source for growth. *B. lactis* AD011 did not degrade mucin, indicating that the strain is not capable of damaging intestinal surfaces and does not have translocational abilities. Details are described in Ku et al. (2019).

6.B.10. Animal Toxicity Studies of *B. lactis* AD011

Due to the general consensus that bifidobacteria are considered safe for human consumption due to their long history of safe use, traditional safety studies of *B. lactis* AD011 have likely been considered unnecessary and have not been performed.

6.B.11. An Animal Efficacy Study of *B. lactis* AD011

One animal efficacy study of *B. lactis* AD011 was identified from the literature (Table 12). Although it was designed to investigate the anti-obesity or anti-allergic effects of *B. lactis* AD011, several safety-related endpoints were obtained during the experiment. Therefore, this study was reviewed as additional supporting information.

Kim et al. (2008) investigated if orally administered *B. lactis* AD011 and/or *Lactobacillus acidophilus* could suppress allergic responses in an ovalbumin (OVA)-induced allergy mouse model. Female C3H/HeJ mice were orally sensitized with OVA and cholera toxin for 4 weeks. They were fed the diet containing lyophilized *B. lactis* AD011 (1×10^{10} cfu/g), *L.*

acidophilus AD031 (1.5×10^{10} cfu/g), or the mixture of the two strains (*B. lactis* AD011 plus *L. acidophilus* AD031) via a diet pellet for 7 weeks starting from 2 weeks before the sensitization. Mice in the naive group did not receive OVA and cholera toxin and bacteria as a negative control. Mice in the sham group received OVA and cholera toxin but no bacteria, as a control. Measurements included body weight gain, serum OVA-specific IgE, IgG1, IgG2a, spleen levels of IL-6, IL-18, and IFN-γ, total and OVA-specific IgA in fecal samples, allergy symptoms on the tail, histology (mast cell degranulation during food allergy response), and hypersensitivity reaction scores. Daily intake of *B. lactis* AD011 at doses of 0.2% in the diet (or 1.0×10^{10} cfu/g), in combination with *L. acidophilus* AD031, did not cause any adverse effects on measured outcomes in mice.

Table 12. Animal Efficacy Studies of *B. lactis* AD011

Objective	Animal	Dose	Duration	Safety Endpoints	Reference
To investigate the effects of mixture of *B. lactis* AD011 and/or *L. acidophilus* AD031 on allergic responses	C3H/HeJ female mice, 6-wk old, sensitized with ovalbumin (OVA) and cholera-toxin (CT) for 4 wk; sham; and naïve (N= 6/group)	5 groups: a) *B. lactis* AD011 (1.0×10^{10} cfu/g), b) *L. acidophilus* AD031 (1.5×10^{10} cfu/g), c) the mixture of the two strains, d) sham, and e) naive	7 wk starting 2 wk before the initial sensitization	Changes in body weight; immune functions; histopathological changes of ear and small intestine. No adverse effects were reported on measured outcomes.	Kim et al., 2008

Abbreviations: wk = weeks

6.B.12. Human Clinical Studies

Human experience and the available scientific literature concerning the consumption of bifidobacteria by all age groups are remarkably free from any experiences of toxicity. There is no evidence that bifidobacteria produce any toxins or poisonous compounds.

As shown in Table 13, consumption of *B. lactis* AD011 (up to 1×10^{10} lyophilized cells/day), along with 2-3 other *Bifidobacterium* and *Lactobacillus* strains (total bacteria of up to 40 billion cfu/day), was tested for various parameters in pregnant women, infants, and adult subjects with irritable bowel syndrome (IBS).

The review was extended to the *B. lactis* BB-12 strain, which has over 99.85% whole genomic sequence similarity with the AD011 strain (Table 14). In all studies of *B. lactis* AD011 and BB-12 strains, there have been no reports of adverse effects on measured outcomes and/or treatment-related adverse events. For these studies, the dose levels represent the maximum doses administered, rather than the absolute safety endpoints.

Although these studies were designed to investigate the efficacy of *B. lactis* strains on various health parameters, several safety-related endpoints were obtained during the experiments. Therefore, these studies are reviewed as additional supporting information.

6.B.12.1. Human Clinical Studies of *B. lactis* AD011

In a randomized, double-blind, placebo-controlled trial, Kim et al. (2010) investigated whether supplementation with a mixture of Bifidobacteria and Lactobacilli can lower the risk of eczema development in infants at high risk (Table 13). Pregnant women with a family history of allergic diseases were randomized to receive a daily supplement of either a mixture composed of 4 viable lyophilized bacteria species (*B. lactis* AD011, *B. bifidum* BGN4, *L. acidophilus* AD031, and *L. casei* IBS04; 1.6×10^9 cfu each) or placebo, from 8 weeks before the expected delivery to 3 months after delivery. Infants were exclusively breastfed during the first 3 months. Subsequently, infants were fed the same Bifidobacteria and Lactobacilli mixture or placebo powder dissolved in breast milk, infant formula, or sterile water from 4 to 6 months of age. Measurements included the incidence of eczema in infants and six area six sign in atopic dermatitis (SASSAD) score at 3, 6, and 12 months of age, and total and specific IgE against food allergens at 12 months of age. In addition, the parents reported adverse effects were evaluated in this study. Authors stated that consumption of a mixture of Bifidobacteria and Lactobacilli did not result in serious adverse effects and that non-specific mild symptoms developed in some subjects were unlikely to have been related to the administration of *B. lactis* AD011 (page e389). However they did not define the criteria for 'serious adverse effects' and 'non-specific mild symptoms' and did not report the number of subjects who developed non-specific mild symptoms. Overall, no adverse effects of the mixture of *B. lactis* AD011 and other *Bifidobacterium* and *Lactobacillus* strains were reported on measured outcomes.

Hong et al. (2009) assessed the effects of the mixture of *Bifidobacterium* and *Lactobacillus* strains on IBS symptoms in adult patients. IBS patients who met Rome III criteria were randomly assigned to receive the mixture of *Bifidobacterium* and *Lactobacillus* strains (a mixture of *B. lactis* AD011, *B. bifidum* BGN4, *L. acidophilus* AD031, and *L. casei* IBS041; 1×10^{10} lyophilized cells/each; a total daily dose of 4×10^{10} cfu) or placebo for 8 weeks. Measurements included a daily diary of bowel habits (frequency and consistency), and questionnaires on IBS, quality of life, and symptom scores. No adverse effects of the mixture of *B. lactis* AD011 and other *Bifidobacterium* and *Lactobacillus* strains were reported on the measured outcomes.

Overall, daily doses of up to 10^{10} cells *B. lactis* AD011, in combination with other safe and suitable *Bifidobacterium* and *Lactobacillus* strains, resulted in neither adverse effects on the measured outcomes nor adverse events in humans.

Table 13. Human Clinical Studies of *B. lactis* AD011

Objective of the study	Subject	Dose	Duration	Safety endpoint	Reference
To investigate if supple-mentation of probiotics* prevents the development of eczema in infants at high risk	112 pregnant women; 68 infants completed a 1 year follow-up	The mixture of *B. lactis* AD011, *B. bifidum* BGN4, and *L. acidophilus* (1.6×10^9 cfu each)	Mothers, ~ 5 mo (from 8 wk before the expected delivery to 3 mo after delivery); Infants from 4 to 6 mo of age; measurements at 3, 6, and 12 mo of age	Adverse effects reported by parents; allergy-related endpoints; No treatment-related adverse effects were reported	Kim et al., 2010
To assess the effects of strains of probiotics* on irritable bowel syndrome (IBS) symptoms in adults	70 patients w/ presence of previous gastro-intestinal symptoms suggestive of IBS (19-75 y)	The mixture of *B. lactis* AD011, *B. bifidum* BGN4, *L. acidophilus* AD031, and *L. casei* IBS041 (total 1×10^{10} lyophilized cells/each; total 4×10^{10} cells)	8 wk	Gastrointesti-nal tolerance including bowel habits and IBS symptom score	Hong et al., 2009

*Probiotics is the term authors used in each paper. We did not modify their terminology in this table, although we use the term 'the mixture of *Bifidobacterium* and *Lactobacillus* strains' or a similar term in the text.

6.B.12.2. Human Clinical Studies of the *B. lactis* BB-12 Strain

Due to an abundance of literature, our review is limited to the published studies conducted on up to 4 strains of *Bifidobacterium* and *Lactobacillus*, including *B. lactis* BB-12. In the 24 studies of *B. lactis* BB-12 reviewed, no studies reported any side effects or adverse effects on the measured outcomes. Thus, we have summarized the efficacy studies using the highest doses tested in various populations (Table 14).

Adults:

Min et al. (2012) investigated whether composite yogurt (2 bottles a day) with *B. lactis* BB-12 ($\geq 10^{11}$ cfu/bottle) and acacia dietary fiber with two classic yogurt starter cultures, such as

Streptococcus thermophilus (≥ 3×10^9 cfu/bottle) and *L. acidophilus* (≥10^9 cfu/bottle) had additive effects in patients with IBS. A total of 130 patients (mean age, 35.8 years) were randomized to receive either the test or control yogurt products twice daily for 8 weeks. Measurements included IBS symptoms and improvement in bowel habits at baseline and after treatment. No adverse effects of *B. lactis* BB-12 ($2x10^{11}$ cfu a day) were reported on the measured outcomes.

Children:

Merenstein et al. (2010) investigated whether consumption of yogurt containing a high dose of *B. lactis* BB-12 ($1.2x10^{10}$ cfu/day) for 90 days improves health in 182 children aged 1-3 years attending daycare/school centers. A yogurt-based drink supplemented with or without *B. lactis* BB-12 was tested in children who attended daycare centers at least 3 days a week. Measurements included adverse events, compliance, missed days of school due to illness, parental satisfaction due to decreased absences from work, and overall health of the child. Six total adverse events, such as diarrhea, pyrexia, dermatitis (diaper), vomiting and cough, were reported, three in each group. There were no serious adverse events (SAEs) in either group reported throughout the entire study. No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

Tan et al. (2017) investigated the safety of *B. lactis* BB-12-supplemented yogurt when consumed by a generally healthy group of children. The primary outcomes were safety and tolerability, determined by the number of reported adverse events. The secondary outcome was gut microbiota. Sixty children aged 1 - 5 years were randomly assigned to consume four ounces of either BB-12-supplemented yogurt or non-supplemented control yogurt daily for 10 days. *B. lactis* BB-12-supplemented yogurt was safe and well tolerated when consumed by healthy children. No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

Pregnant women and/or offspring pairs:

In a prospective cohort by Schei et al. (2017), 298 pairs of healthy mothers (mean 29.6 years) and their offspring from 36 weeks of gestation until 2 years of age (1,516 samples) were followed. Pregnant mothers were randomized to drink milk containing *Bifidobacterium* and *Lactobacillus* strains or placebo milk during and after pregnancy, from 36 weeks of gestation until 3 months postpartum. The bacteria included *B. lactis* BB-12, *L. rhamnosus* GG (LGG), and *L. acidophilus* La-5 (5×10^{10} cfu/d each). Primary endpoint was gut mycobiota in maternal and offspring samples. No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

Infants:

Taipale et al. (2016) studied the impact of administration of *B. lactis* BB-12 on the risk of acute infectious diseases in healthy children. In this double-blind, placebo-controlled study, 109 1-month-old infants were randomly assigned to a *B. lactis* BB-12 group receiving a *B. lactis* BB-12-containing tablet or a placebo group. Daily dose of 10^{10} cfu *B. lactis* BB-12 was administered until the 2 years of age. Measurements included adverse effects/events, all signs and symptoms

of acute infections, and fecal recovery of *B. lactis* BB-12. Administration of *B. lactis* BB-12 in early childhood for 23 months did not result in adverse effects.

Kirjavainen et al. (2002) assessed whether the efficacy of bifidobacterial supplementation in the treatment of allergy could relate to modulation of intestinal microbiota. A total of 21 infants with early onset atopic eczema were included in the study. Of these, 13 infants tolerant to extensively hydrolysed whey formula were fed the formula with or without *B. lactis* BB-12 at daily dose of approximately 8×10^{10} cfu per kg body weight (bw; range 6 - $11x10^{10}$) for 4 months from 5.2 to 9.1 months of age. This level corresponds to 5.4 to 6.6×10^{11} cfu per infant per day. Total cfu per infant per day was calculated based on the typical weights of 5- and 9-month-old infants, 6.8 and 8.2 kg bw, respectively. The *B. lactis* cell intake/kg bw value was multiplied by the body weight to convert it to daily per capita cfu intake using the following calculation formula: 8×10^{10} cfu/kg bw × 6.8 kg bw = 5.4×10^{11} cfu; and 8×10^{10} cfu/kg bw × 8.2 kg bw = 6.6×10^{11} cfu. Measurements included gut microflora and the extent of allergic sensitization. No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

Overall, daily doses of up to 5.4 - 6.6×10^{11} cells *B. lactis* BB-12 resulted in neither adverse effects on the measured outcomes nor adverse events in humans.

Table 14. Human Clinical Studies of *B. lactis* BB-12

Objectives of the study	Subject	Dose	Duration	Measurements	Reference
Adult					
To investigate whether composite yogurt with acacia dietary fiber and *B. lactis* has additive effects in irritable bowel syndrome (IBS).	130 patients (mean age 35.8 y)	Yogurt (2 bottles a day) containing *B. lactis* BB-12 ($\geq 10^{11}$ cfu/bottle) acacia dietary fiber and yogurt starter cultures, *S. thermophilus* ($\geq 3\times10^{9}$ cfu/bottle) and *L. acidophilus* ($\geq10^{9}$ cfu/ bottle); control yogurt - *B. lactis* BB-12 ($\geq 10^{10}$ cfu/bottle)	8 wk	Abdominal symptoms and bowel habits; improvement of overall IBS symptoms	Min et al., 2012
Children					
To determine if consumption of yogurt containing a high dose of *B. lactis* BB-12 improves health in children attending daycare/school centers.	182 healthy children (aged 1-3 y)	Yogurt-based drink containing *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*; with or without *B. lactis* BB-12 (1.12×10^{10} cfu/d)	90 d	Adverse events; compliance; absences due to illnesses from daycare; overall parental satisfaction due to decreased absences from work and an overall healthier child. No adverse effects of *B. lactis* BB-12 were reported.	Meren-stein et al., 2010
To determine the safety of *B. lactis* BB-12-supplemented yogurt; to assess the effect of *B. lactis* BB-12-supplemented yogurt on the gut microbiota.	60 healthy children (aged 1-5 y)	Yogurt with *S. thermophilus* and *L. delbrueckii* subsp. *Bulgaricus*; with or without 1.12×10^{10} cfu/d *B. lactis* BB-12	10 d intervention; follow-ups at days up to 180	Safety and tolerability (frequency and severity of adverse events); compliance; fecal microbiota. No adverse effects of *B. lactis* BB-12 were reported.	Tan et al., 2017

Table 14. Human Clinical Studies of *B. lactis* BB-12, continued

Objectives of the study	Subject	Dose	Duration	Measurements	Reference
Pregnant Women and/or Offspring Pairs					
To describe gut mycobiota in pairs of healthy pregnant women and offspring from birth to 2 y of age	298 healthy mothers (gestational age 40.4 wk; mean age, 29.6 y at delivery) and offspring pairs	Placebo (heat-treated fermented skimmed milk); probiotic milk (5×10^{10} cfu/d each of *B. lactis* BB-12, *L. rhamnosus* GG, and *L. acidophilus* La-5)	Mothers - from 36 wk gestation until 3 mo after birth; offspring - up to 2 y of follow-up	Maternal and offspring fecal mycobiota (gut fungi)	Schei et al., 2017
Infants					
To study the effects of *B. lactis* BB-12 on the risk of acute infectious diseases in healthy children.	109 1-mo-old healthy infants	10^{10} cfu/d *B. lactis* BB-12 or placebo (tablet form)	From 1 mo to 2 y of life	Adverse effects/events, all signs and symptoms of acute infections (prevalence of respiratory tract infections, otitis media, fever, gastrointestinal infection), and fecal recovery of *B. lactis* BB-12. No serious adverse effects of *B. lactis* BB-12 were reported.	Taipale et al., 2016
To characterize the relationship between gut microbiota and the extent of allergic sensitization	21 infants with early onset atopic eczema; 8 infants were intolerant and 13 were tolerant to extensively hydrolyzed whey formula	13 tolerant infants - extensively hydrolyzed whey formula with or without ~8×10^{10} cfu/kg bw/d *B. lactis* BB-12 (or 5.4 - 6.6×10^{11} cfu/infant/d)	Before and after weaning (from 5.2 to 9.1 mo of age)	Fecal microbiota; the extent of allergic sensitization as measured by the total serum concentration of IgE. No adverse effects of *B. lactis* BB-12 were reported.	Kirjavai-nen et al., 2002

Abbreviations: bw = body weight; cfu = colony forming unit; d = day; IBS = irritable bowel syndrome; mo = months; wk = weeks; y = year.

6.B.12.3. Human Studies of Other *B. lactis* Strains

As described in GRNs 377 and 445 (FDA, 2011, 2013a), consumption of other strains of *B. lactis*, such as HN019, Bi-07, Bl-04, B420, and Bf-6, did not result in any serious adverse effects on the measured outcomes.

6.C. Potential Infection

Humans are exposed to bifidobacteria by eating fermented foods (e.g., yogurt, cheese, fermented vegetables, and olives), and in the host's own microflora. Even with these sources, bifidobacteria rarely cause infections in humans. This lack of pathogenicity extends to all age groups as well as immunocompromised patients (Boriello et al., 2003).

6.D. Safety Determination

Studies have demonstrated that the intended uses of *B. lactis* AD011 are safe based on the following facts:

1. *B. lactis* AD011 has a long history of safe consumption in humans. Several *B. lactis* strains are recognized as GRAS. Human clinical studies show that no *B. lactis* strains resulted in adverse effects in humans, regardless of age, gender, and health status of the subjects.

2. The information/data provided by BIFIDO (specifications, manufacturing process, intended use, and safety data) in this report, and supplemented by publicly available literature/safety data on *B. lactis* AD011 and other *B. lactis* strains, provide a sufficient basis for an assessment of the safety of *B. lactis* AD011 for the proposed use as a food ingredient prepared according to appropriate specifications.

   Key findings are summarized as follows:
   1) Animal and human studies showed no adverse effect of *B. lactis* AD011.
   2) Studies of another *B. lactis* strain (BB-12) with over 99.85% whole genomic sequence similarity with that of the AD011 strain also have shown no adverse effects in humans.
   3) *In vitro* studies show that the antibiotic susceptibility profiles of *B. lactis* AD011 are similar to those of other GRAS strains, which have been safely used in the U.S. for a decade. *B. lactis* AD011 has no hemolytic or mucolytic activities and does not produce biogenic amines or ammonia.
   4) The genomic sequence of *B. lactis* AD011 does not have homology with those of toxigenic or pathogenic genes.
   5) *B. lactis* AD011 does not have any plasmid capable of transmitting antibiotic resistance genes.
   6) *B. lactis* AD011 is genetically stable.

3. The *B. lactis* AD011 ingredient has been marketed as a dietary supplement ingredient and as a dietary supplement in Korea since 2007. *B. lactis* AD011, at daily doses up to 1×10^{10} cfu (or 1.5×10^{10} cfu at the time of shipment), has been safely used, with no adverse events or health-related complaints reported by consumers.

4. The intended use of *B. lactis* AD011 results in levels of exposure significantly below or within the historical human use levels and provides a reasonable certainty of safety.

5. *B. lactis* AD011 is well-characterized and is free from chemical or other microbial contamination.

Therefore, it is reasonable to conclude that *B. lactis* AD011 is non-pathogenic and non-toxigenic and that daily intakes of up to 10^8 cfu *B. lactis* AD011 per g powdered infant formulas and 1×10^{10} cfu *B. lactis* AD011 per serving in selected conventional foods are safe.

6.E. Conclusions and General Recognition of the Safety of *B. lactis* AD011

6.E.1. Common Knowledge Element of the GRAS Determination

B. lactis has been safely used as a food ingredient for a decade. As a result, comprehensive reviews of the safety of several strains of *B. lactis* and Bifidobacteria have been published. In addition, GRAS notices of several strains of *B. lactis* have received FDA 'no question' letters on their safety; such information is widely available. In the published literature, evidence for genetic similarity to other *B. lactis* strains is available for safety assessment of *B. lactis* AD011 (Ku et al., 2019). These facts meet the "common knowledge" element of the GRAS determination.

6.E.2. Technical Element of the GRAS Determination

Human and animal studies have reported benefits of *B. lactis* AD011 with no major adverse effects. BIFIDO rigorously tests its final production batches to verify adherence to quality control specifications, and thus, adheres to manufacturing standards consistent with cGMP for food (21 CFR Part 110 and Part 117 Subpart B). The raw materials and processing aids used in the manufacturing process are food grade. There is broad-based and widely disseminated knowledge concerning the safety of *B. lactis* AD011 and other *B. lactis* strains. The literature indicates that consumption of *B. lactis*, including *B. lactis* AD011 did not result in adverse effects/events. Thus, the intended uses of *B. lactis* AD011 have been determined to be safe though scientific procedures as set forth in 21 CFR 170.3(b), thus, satisfying the "technical" element of the GRAS determination.

BIFIDO has concluded that these uses of *B. lactis* AD011 are GRAS based on scientific procedures, and that other experts qualified to assess the safety of foods and food additives would concur with these conclusions. Therefore, the proposed use is safe within the terms of the Federal Food, Drug, and Cosmetic Act, meeting the standard of reasonable certainty of no harm. It is also Generally Recognized as Safe (GRAS) according to Title 21 Code of Federal Regulations (21 CFR).

BIFIDO is not aware of any information that would be inconsistent with the finding that the proposed use of *B. lactis* AD011 meets appropriate specifications, and its use according to cGMP, is GRAS. Recent reviews of the scientific literature revealed no potential adverse health concerns.

PART 7. REFERENCES

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7.B. References That Are Not Generally Available

Appendix C. Park and Yang, 2019. Evaluation of *Bifidobacterium animalis* subsp. *lactis* AD011

Appendix A. Identification of *B. lactis* AD011

Strain Level Identification

B. lactis AD011 was identified by 16S rDNA sequence analysis. Chromosomal DNA from each *B. lactis* AD011 strain were extracted and the 16S rRNA gene was amplified using universal primers. The PCR primer sequences were as follows: forward primer, 5′-AGAGTTTGATCCTGGCTCAG-3′; reverse primer, 5′-GGTTACCTTTGTTACGACTT-3′ (Bioneer, Korea). Sequence homologies were examined by comparing the obtained sequences with those in the DNA Databases (http://www.ncbi.nlm.nih.gov/BLAST).

Primer Information:

PCR Primer Name Primer Sequences

27F 5' (AGA GTT TGA TCM TGG CTC AG) 3'
1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Sequencing Primer Name Primer Sequences

785F 5' (GGA TTA GAT ACC CTG GTA) 3'
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3

The strain was identified as *B. lactis* and was named *B. lactis* AD011.

Standard ID

16S rRNA Full Sequencing

Humanizing Genomics macrogen

16S rRNA service report

Order Number : 180119KR-064
Sample name : B_lactis_AD011_contig_1

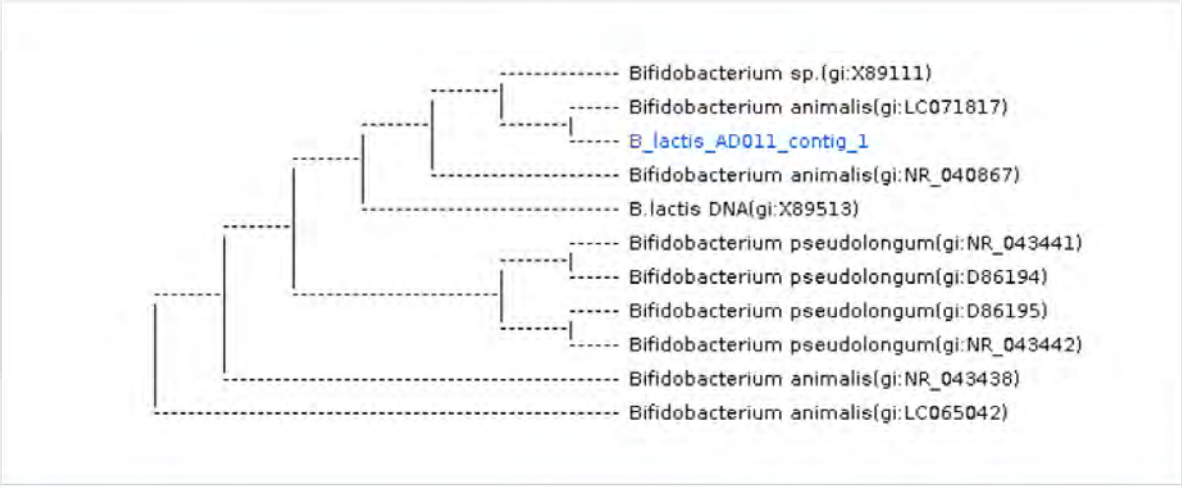
Information

Primer Information

Sequencing Primer Name Primer Sequences	PCR Primer Name Primer Sequences
785F 5' (GGA TTA GAT ACC CTG GTA) 3'	27F 5' (AGA GTT TGA TCM TGG CTC AG) 3
907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'	1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'

Subject						Score		Identities	
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct.(%)
CP001606.1	Bifidobacterium animalis	1938483	1476357	1474881	0	2706	0.0	1475/1479	99

Kingdom	Family	Genus	Species
Bacteria	Bifidobacteriaceae	Bifidobacterium	Bifidobacterium animalis



Characterization

Bifidobacterium is a genus of Gram-positive, nonmotile, often branched anaerobic bacteria. They are ubiquitous inhabitants of the gastrointestinal tract, vagina and mouth (B. dentium) of mammals, including humans. Bifidobacteria are one of the major genera of bacteria that make up the colon flora in mammals. Some bifidobacteria are used as probiotics.

Bifidobacterium animalis is a gram-positive, anaerobic, rod-shaped bacterium which can be found in the large intestines of most mammals, including humans. The manipulation of the gut flora is complex and may cause bacteria-host interactions.Although probiotics, in general, are considered safe, there are concerns about their use in certain cases.

Contig Summary

World Meridian Center 10F. 254 Beotkkot-ro, Geumcheon-gu, Seoul, Republic of Korea
Tel: 82-2-2180-7261 Fax: 02-2180-7100 Email: info@macrogen.com

Sample Name

Sample Name	B_lactis_AD011

Analysis Report

Name	Read Length (Normal)	Read Length (Q16)	Read Length (Q20)	GC Content
B_lactis_AD011	**1468**	**1386**	**1384**	**60.42234332425068**
B_lactis_AD011_R	724	724	720	60.0828729281768
B_lactis_AD011_F	939	934	932	60.70287539936102

Contig Sequence

CAGGATGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGGGATCCCTGGCAGCTTGCTGTCGGGGTGAGAGTGGCGAACGGGTGAGTAATG
CGTGACCAACCTGCCCTGTGCACCGGAATAGCTCCTGGAAACGGGTGGTAATACCGGATGCTCCGCTCCATCGCATGGTGGGGTGGGAAATGCTTT
TGCGGCATGGGATGGGGTCGCGTCCTATCAGCTTGTTGGCGGGGTGATGGCCCACCAAGGCGTTGACGGGTAGCCGGCCTGAGAGGGTGACCGGCC
ACATTGGGACTGAGATACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGCG
GGATGGAGGCCTTCGGGTTGTAAACCGCTTTTGTTCAAGGGCAAGGCACGGTTTCGGCCGTGTTGAGTGGATTGTTCGAATAAGCACCGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTATCCGGATTTATTGGGCGTAAAGGGCTCGTAGGCGGTTCGTCGCGTCCGGTGTGAA
AGTCCATCGCCTAACGGTGGATCTGCGCCGGGTACGGGCGGGCTGGAGTGCGGTAGGGGAGACTGGAATTCCCGGTGTAACGGTGGAATGTGTAGA
TATCGGGAAGAACACCAATGGCGAAGGCAGGTCTCTGGGCCGTCACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGG
TAGTCCACGCCGTAAACGGTGGATGCTGGATGTGGGGCCCTTTCCACGGGTCCCGTGTCGGAGCCAACGCGTTAAGCATCCCGCCTGGGGAGTACG
GCCGCAAGGCTAAAACTCAAAGAAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGC
TTGACATGTGCCGGATCGCCGTGGAGACACGGTTTCCCTTCGGGGCCGGTTCACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTCGCCGCATGTTGCCAGCGGGTGATGCCGGGAACTCATGTGGGACCGCCGGGGTCAACTCGGAGGAAGG
TGGGGATGACGTCAGATCATCATGCCCCTTACGTCCAGGGCTTCACGCATGCTACAATGGCCGGTACAACGCGGTGCGACACGGTGACGTGGGGCG
GATCGCTGAAAACCGGTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGGCGGAGTCGCTAGTAATCGCGGATCAGCAACGCCGCGGTG
AATGCGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCATGAAAGTGGGTAGCACCCGAAGCCGGTGGCCCGACCCTTGTGGGGGGGAGCCGTCT
AAGGTGAGACTCGTGATTGGGACTAAGT

BlastN Report

Query		Subject					Score			Identities		
Start	End	Description	AC	Leng	Start	End	Bit	Raw	EV	Matc	Total	Pct.(%)
1	1468	Bifidobacterium animalis subsp. lactis strain HN019 chromosome, complete genome	CP031154.1	1935423	1481783	1480317	2704	1464	0.0	1467	1468	99
1	1468	Bifidobacterium animalis subsp. lactis strain S7 16S ribosomal RNA gene, partial sequence	MH828367.1	1549	28	1494	2704	1464	0.0	1467	1468	99
1	1468	Bifidobacterium animalis subsp. lactis strain IDCC4301 chromosome, complete genome	CP031703.1	1944141	290541	292007	2704	1464	0.0	1467	1468	99
1	1468	Bifidobacterium animalis subsp. lactis strain S7 chromosome, complete genome	CP022724.1	1944072	39934	38468	2704	1464	0.0	1467	1468	99
1	1468	Bifidobacterium animalis strain BL3, complete genome	CP017098.1	1944323	1481926	1480460	2704	1464	0.0	1467	1468	99

Appendix B. Certificate of Analysis for *B. lactis* AD011

The three samples (lot numbers: BL-R-190116, BL-R-190129 and BL-R-190211) were analyzed at third party Korean laboratories using Korean Health functional Food Standards Codex (KHFSC) and Korean Food Standards Codex (KFSC) methods of analysis. The Korean methods of analysis and corresponding internationally recognized methods are listed in Table B.1.

For *Cronobacter sakazakii* and *Escherichia coli* (*E. coli*), 3 samples initially analyzed at a Korean laboratory were reanalyzed at Eurofins using the sample sizes of 10 g and 25 g, respectively. Thus, Korean methods of analysis for *Cronobacter sakazakii* and *E. coli* are not listed in Table B.1.

Table B.1. Methods of Analysis

Parameter	Method of analysis Method number	Corresponding internationally recognized method number
Appearance	Visual	
Cell Counts, cfu/g (as *B. lactis* AD011)	KHFSC 4/3/3-58	ISO 29981:2010
Moisture, %	KFSC 8/2/2.1/2.1.1	AOAC 941.14
Lead (Pb), ppm	KFSC 8/9/9.1/9.1.2	AOAC 2013.06
Arsenic (As), ppm	KFSC 8/9/9.1/9.1.4	AOAC 2013.06
Cadmium (Cd), ppm	KFSC 8/9/9.1/9.1.3	AOAC 2013.06
Mercury (Hg), ppm	KFSC 8/9/9.1/9.1.6	AOAC 2013.06
Non-Lactic acid bacteria, cfu/g	KFSC 8/4/4.5/4.5.1	ISO 13559:2002 (IDF 153)
Total yeasts and molds, cfu/g	KFSC 8/4/4.10	AOAC 2002.11
Salmonella, cfu/25 g	KFSC 8/4/4.11	AOAC 989.14
Listeria, cfu/25 g	KFSC 8/4/4.15	AOAC 998.08
Ash, %	KFSC 8/2/2.1/2.1.2	AOAC 900.02

Abbreviations: AOAC = Association of Official Agricultural Chemists; IDF = International Dairy Federation; ISO = International Standards Organization; KFSC = Korean Food Standards Codex; KHFSC = Korean Health functional Food Standards Codex (Available on http://www.foodsafetyk, orea.go.kr/portal/safefoodlife/food/foodRvlv/foodRvlv.do)

Soy allergen test was based on the Veratox soy allergen test sandwich ELISA kit (#8410) using polyclonal antibody against denatured protein (detection range 10 to 100 ppm). Detection of denatured protein was used as a proxy for the presence of soy allergen.

B I F I D O

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CERTIFICATE OF ANALYSIS

NAME OF PRODUCT			*Bifidobacterium lactis* AD011
LOT NO.			BL-R-190116
PRODUCTION DATE			2019.01.16
CERTIFICATED DATE			2019.01.20
EXPIRATION DATE			2021.01.15
ANALYSIS RESULT			
Parameter	BL-R-190116	Method of analysis/ Method number	Corresponding internationally recognized method number
Appearance	Yellow, White powder	Visual	
Cell Counts, cfu/g (as *B. lactis* AD011)	1.00E+11	KHFSC 4/3/3-58	ISO 29981:2010
Moisture, %	4.3 %	KFSC 8/2/2.1/2.1.1	AOAC 941.14
Heavy metals			
Lead (Pb), ppm	<0.01	KFSC 8/9/9.1/9.1.2	AOAC 2013.06
Arsenic (As), ppm	<0.06	KFSC 8/9/9.1/9.1.4	AOAC 2013.06
Cadmium (Cd), ppm	<0.03	KFSC 8/9/9.1/9.1.3	AOAC 2013.06
Mercury (Hg), ppm	<0.04	KFSC 8/9/9.1/9.1.6	AOAC 2013.06
Microbial purity			
Non-Lactic acid bacteria, cfu/g	Negative	KFSC 8/4/4.5/4.5.1	ISO 13559:2002(IDF153)
Total yeasts and molds, cfu/g	Negative	KFSC 8/4/4.10	AOAC 2002.11
Salmonella, cfu/25 g	Negative	KFSC 8/4/4.11	AOAC 989.14
Listeria, cfu/25 g	Negative	KFSC 8/4/4.15	AOAC 998.08
Proximate analysis			
Lipids, %	1.54 %	KFSC 8/2/2.1/2.1.5/2.1.5.1	AOAC 996.06
Protein, %	61.47 %	KFSC 8/2/2.1/2.1.3/2.1.3.1	AOAC 945.23
Carbohydrates, %	28.44 %	KFSC 8/2/2.1/2.1.4/2.1.4.1	AOAC 977.20
Ash, %	4.36 %	KFSC 8/2/2.1/2.1.2	AOAC 900.02

Q.C Manager Ji Yeong Shin _____

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CERTIFICATE OF ANALYSIS

NAME OF PRODUCT	*Bifidobacterium lactis* AD011		
LOT NO.	BL-R-190129		
PRODUCTION DATE	2019.01.29		
CERTIFICATED DATE	2019.02.02		
EXPIRATION DATE	2021.01.28		
ANALYSIS RESULT			
Parameter	BL-R-190129	Method of analysis/ Method number	Corresponding internationally recognized method number
Appearance	Yellow White powder	Visual	
Cell Counts, cfu/g (as *B. lactis* AD011)	1.00E+11	KHFSC 4/3/3-58	ISO 29981:2010
Moisture, %	4.2%	KFSC 8/2/2.1/2.1.1	AOAC 941.14
Heavy metals			
Lead (Pb), ppm	<0.01	KFSC 8/9/9.1/9.1.2	AOAC 2013.06
Arsenic (As), ppm	<0.06	KFSC 8/9/9.1/9.1.4	AOAC 2013.06
Cadmium (Cd), ppm	<0.03	KFSC 8/9/9.1/9.1.3	AOAC 2013.06
Mercury (Hg), ppm	<0.04	KFSC 8/9/9.1/9.1.6	AOAC 2013.06
Microbial purity			
Non-Lactic acid bacteria, cfu/g	Negative	KFSC 8/4/4.5/4.5.1	ISO 13559:2002(IDF153)
Total yeasts and molds, cfu/g	Negative	KFSC 8/4/4.10	AOAC 2002.11
Salmonella, cfu/25 g	Negative	KFSC 8/4/4.11	AOAC 989.14
Listeria, cfu/25 g	Negative	KFSC 8/4/4.15	AOAC 998.08
Proximate analysis			
Lipids, %	1.58%	KFSC 8/2/2.1/2.1.5/2.1.5.1	AOAC 996.06
Protein, %	57.52%	KFSC 8/2/2.1/2.1.3/2.1.3.1	AOAC 945.23
Carbohydrates, %	31.64%	KFSC 8/2/2.1/2.1.4/2.1.4.1	AOAC 977.20
Ash, %	5.69%	KFSC 8/2/2.1/2.1.2	AOAC 900.02

Q.C Manager Ji Yeong Shin _____

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Analytical Report

Analytical Report No. **AR-20-HX-002139-01** Date 19-Mar-2020

BIFIDO Co.,Ltd.

23-16, Nonggongdanji-gil, Hongcheon-eup,
Hongcheon-gun, Gangwon-do

Our reference:	EUKR01-00001724 / 984-2020-03000045		
Sample Description:	B.lactis AD011		
Test Purpose	Voluntary testing		
Reception Date:	12-Mar-2020		
Manufacturing Report Number	BL-R-190603-A		
Manufacture Date	03/06/2019	Sample Weight	200g
Sample Quantity	2		

Test Result(s):

		Results	Unit	Guidelines
HX051	Escherichia coli Method: Food code, Cultural technique (media film)			
	Escherichia coli	0	cfu/g	
HX0PM	Cronobacter spp. Method: FDA BAM Ch.29, Cultural technique (chromogenic media)			
	Cronobacter spp. (Enterobacter sakazakii)	negative		

SIGNATURE

Kevin Lee
Technical Manager

EXPLANATORY NOTE
Not Detected means not detected at or above the Limit of Quantification (LOQ)
This document can only be reproduced in full; it only concerns the submitted sample.
Results have been obtained and reported in accordance with our general sales conditions available on request.
When declaring compliance or non-compliance, the uncertainty associated with the result has been added or subtracted in order to obtain a result that can be compared to regulatory limits or specifications.The uncertainty has not been taken into account for standards that already include measurement uncertainty.
The tests are identified by a five-digit code, their description is available on request.

END OF REPORT

Eurofins Korea Analytic Service Co., Ltd.
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www.eurofins.co.kr

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Analytical Report

Analytical Report No. **AR-20-HX-002140-03** Date 25-Mar-2020

(*this report cancels and replaces the previous one, numbered AR-20-HX-002140-02/984-2020-03000046 dated 25/03/2020 which must be destroyed)

BIFIDO Co.,Ltd.

23-16, Nonggongdanji-gil, Hongcheon-eup,
Hongcheon-gun, Gangwon-do

Our reference:	EUKR01-00001724 / 984-2020-03000046		
Sample Description:	B.lactis AD011		
Test Purpose	Voluntary testing		
Reception Date:	12-Mar-2020		
Manufacturing Report Number	BL-R-190909		
Manufacture Date	09/09/2019	Sample Weight	200g
Sample Quantity	2		

Test Result(s):

		Results	Unit	Guidelines
HX051	Escherichia coli Method: Food code, Cultural technique (media film)			
Escherichia coli		0	cfu/g	
HX0PM	Cronobacter spp. Method: FDA BAM Ch.29, Cultural technique (chromogenic media)			
Cronobacter spp. (Enterobacter sakazakii)		negative		

SIGNATURE

Kevin Lee
Technical Manager

EXPLANATORY NOTE

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The tests are identified by a five-digit code, their description is available on request.

END OF REPORT

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Analytical Report

Analytical Report No. **AR-20-HX-002141-01** Date 19-Mar-2020

BIFIDO Co.,Ltd.

23-16, Nonggongdanji-gil, Hongcheon-eup,
Hongcheon-gun, Gangwon-do

Our reference:	EUKR01-00001724 / 984-2020-03000047		
Sample Description:	B.lactis AD011		
Test Purpose	Voluntary testing		
Reception Date:	12-Mar-2020		
Manufacturing Report Number	BL-R-200130-2		
Manufacture Date	30/01/2019	**Sample Weight**	200g
Sample Quantity	2		

Test Result(s):

		Results	Unit	Guidelines
HX051	Escherichia coli Method: Food code, Cultural technique (media film)			
	Escherichia coli	0	cfu/g	
HX0PM	Cronobacter spp. Method: FDA BAM Ch.29, Cultural technique (chromogenic media)			
	Cronobacter spp. (Enterobacter sakazakii)	negative		

SIGNATURE

Kevin Lee
Technical Manager

EXPLANATORY NOTE

Not Detected means not detected at or above the Limit of Quantification (LOQ)
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Results have been obtained and reported in accordance with our general sales conditions available on request.
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The tests are identified by a five-digit code, their description is available on request.

END OF REPORT

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SGS

Test Report No. F690101/LF-CTSAYFN19-22078

Issued Date : 2019. 07. 12 Page 1 of 1

BIFIDO CO.,Ltd
23-16 Nonggongdanji-gil, Hongchun-eup
Hongchun-gun, Kngwon-do
Korea

The following sample(s) was/were submitted and identified by/on behalf of the client as:-

SGS File No. : AYFN19-22078
Product Name : BL-R-190211
Item No./Lot No. : 2019.02.11, 2021.02.10
Test Period : 2019. 07. 03 to 2019. 07. 11
Purpose of Test Report : Data for reference

Test Results

Test Items	Unit	Test Method	LOQ	Results
Allergen Soybean	mg/kg	Veratox for Soy Allegen No. 8410	2.5	<2.5

NOTE: (1) Not detected = ≤ LOQ
(2) g/100g = %(w/w)
(3) LOQ = Limit Of Quantitation
(4) - = No regulation
(5) ** = Qualitative analysis (No Unit)

*** End of Report ***

ConardLee

Technical Manager / SGS KOREA

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Test Report No. F690101/LF-CTSAYFN19-22079

Issued Date : 2019. 07. 12 Page 1 of 1

BIFIDO CO.,Ltd
23-16 Nonggongdanji-gil, Hongchun-eup
Hongchun-gun, Kngwon-do
Korea

The following sample(s) was/were submitted and identified by/on behalf of the client as:-

SGS File No. : AYFN19-22079
Product Name : BL-R-190213
Item No./Lot No. : 2019.02.13, 2021.02.12
Test Period : 2019. 07. 03 to 2019. 07. 11
Purpose of Test Report : Data for reference

Test Results

Test Items	Unit	Test Method	LOQ	Results
Allergen Soybean	mg/kg	Veratox for Soy Allegen No. 8410	2.5	<2.5

NOTE: (1) Not detected = ≤ LOQ
(2) g/100g = %(w/w)
(3) LOQ = Limit Of Quantitation
(4) - = No regulation
(5) ** = Qualitative analysis (No Unit)

*** End of Report ***

[redacted]
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SGS

Test Report No. F690101/LF-CTSAYFN19-22080

Issued Date : 2019. 07. 12 Page 1 of 1

BIFIDO CO.,Ltd
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The following sample(s) was/were submitted and identified by/on behalf of the client as:-

SGS File No. : AYFN19-22080
Product Name : BL-R-190129
Item No./Lot No. : 2019.01.29, 2021.01.28
Test Period : 2019. 07. 03 to 2019. 07. 11
Purpose of Test Report : Data for reference

Test Results

Test Items	Unit	Test Method	LOQ	Results
Allergen Soybean	mg/kg	Veratox for Soy Allegen No. 8410	2.5	<2.5

NOTE: (1) Not detected = ≤ LOQ
(2) g/100g = %(w/w)
(3) LOQ = Limit Of Quantitation
(4) - = No regulation
(5) ** = Qualitative analysis (No Unit)

*** End of Report ***

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Appendix C.

Genetic Stability Evaluation of *Bifidobacterium animalis* subsp. *lactis* AD011

Prepared by
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Abstract

Over the past decade, a variety of lactic acid bacteria have been commercially available and steadily used by consumers. Since 2007, *Bifidobacterium animalis* subsp. *lactis* strain AD011 (herein after referred to as '*B. lactis* AD011') has been legally marketed with no side effects in Korea, Germany, Poland, Singapore, Thailand, Turkey, and Vietnam. A recent study by Ku et al. (2019) reported the safety of *B. lactis* AD011, indicating that *B. lactis* AD011 is non-pathogenic and non-toxigenic, and is suitable for human use. This genetic stability test found that there was little genetic mutation between the first and 25th generations of *B. lactis* AD011. The data showed that *B. lactis* AD011 is genetically stable.

Genetic Stability Test

1) Background

The 2007 method for determining a reference genome of *B. lactis AD011*

The whole genome sequence of *B. lactis* AD011 has been published (Kim et al., 2009). It was originally determined by the traditional Sanger pair-ended sequencing of plasmid and fosmid libraries. Shotgun sequences were base called and assembled into contigs using the Phred/Phrap/Consed software package (http://www.phrap.org). Sequencher (Gene Codes Corp., Ann Arbor, MI) was used for processing of the finishing reads from custom primer walks and manual validation. The complete sequence consists of a 1,933,695-bp circular chromosome (60.49% G+C) with no plasmid. From the nucleotide sequence, 1,577 coding sequences (CDSs), and 52 tRNAs were compiled.

The 2019 method used in the genetic stability test

The genetic stability of a bacteria reflects the susceptibility to genomic rearrangements in the course of its natural evolution. These may reflect small variations introduced at specific or random positions of the genome through mutations, deletions, and insertions. The genetic stability of *B. lactis* AD011 was investigated by comparing whole genome sequences determined at 1st and 25th generations using Illumina MiSeq sequencer. To test genetic stability, next generation sequencing (NGS) technologies, instead of the original sequence analysis method (Sanger sequencing), were utilized as sequencing technology evolved. The principles behind Sanger vs. NGS technologies are similar in that DNA polymerase adds fluorescent nucleotides one by one onto a growing DNA template strand in both methods. Each incorporated nucleotide is identified by its fluorescent tag. The critical difference between Sanger sequencing and NGS is sequencing volume. While the Sanger method only sequences a single DNA fragment at a time, NGS is massively parallel, sequencing millions of fragments simultaneously per run. This high-throughput process translates into sequencing hundreds to thousands of genes including bacterial genome at one time offering greater analysis power to comparative genomics.

2) Materials and Methods

2-1) Strains

B. lactis AD011 was plated on a MRS (de Man-Rogosa-Sharpe, CRITERION™ Lactobacilli MRS Broth, Hardy Diagnostics, USA) agar plate by streaking from a stock

stored in a -80°C deep freezer and incubated anaerobically at 37°C for 24 h to obtain a single colony. A single colony was inoculated into 10 mL of MRS broth supplemented with 0.05% L-cysteine hydrochloride. It was regarded as the first generation (about 10^6 CFU [colony forming unit]/mL) of *B. lactis* AD011.

It was incubated at 37°C for about 12 h under anaerobic conditions to reach about 10^9 CFU/mL to obtain the 10^{th} generation. In the second subculture, 0.01 mL (0.1% inoculation, about 10^6 CFU/mL) of the primary culture was inoculated into 10 mL of MRS broth and cultured under the same conditions to obtain the 20^{th} generation of *B. lactis* AD011. In the third subculture, 0.01 mL (0.1% inoculation, approximately 10^6 CFU/mL) of the secondary culture is inoculated into 10 mL of MRS broth and incubated to 10^7 or 10^8 CFU/mL to obtain the 25^{th} generation of *B. lactis* AD011. The number of bacteria was measured on the MRS agar plate during cultivation to confirm the generation.

2-2) DNA Extraction

The genomic DNA of pure culture bacteria was extracted using MG™ Cell SV (Doctor Protein, Korea). Extraction was performed according to the manufacturers' instructions and the total bacterial DNA was eluted with 200 μL of sterile water. The ratio value of absorbance at 260 nm to absorbance at 280 nm was checked to be 1.8-2.0. DNA extracts were aliquoted and stored at -20°C.

2-3) Whole Genome Sequencing Analysis for Genetic Stability Test

Sequencing was run on an Illumina MiSeq sequencer using the Nextera XT library preparation kit (Illumina, San Diego, CA, USA). Nextera XT library preparation workflow is divided into five steps: first, tagment genomic DNA; second, amplify tagmented DNA; third, cleanup amplified DNA; fourth, normalize libraries; fifth, pool libraries. Denature and dilute libraries used the Miseq reagent Kit V3 (Nextera XT library prep reference guide). Sequencing indices from the Nextera XT index kit were used for multiplexing; participants were free to choose any index combination for the samples. The run acceptance criteria were a sequencing output of 5.6 Gb (to achieve an average sequencing coverage of 100-fold for the 20 samples with genome sizes of 2.8 Mb) and a Q30 read quality score of 75% (Mellmann et al., 2017). For the similarity analysis between the whole genome sequences of the 1^{st} and 25^{th} generations, bioinformatics analysis and comparative genomics analysis were performed using software provided by ChunLab Co., Ltd (Seoul, Korea).

3) Results and Discussion

The whole genome sequence analysis showed 1,919,567-bp at 15 contigs for the 1^{st} generation and 1,919,618-bp at 25 contigs for the 25^{th} generation. Both genomes showed very similar characteristics for genome size, G+C contents, number of rRNA and tRNA genes, mean and median CDS length, and intergenic lengths (Table C.1).

Table C.1. Genetic Characteristics of Whole Genome Sequence of 1st and 25th Generations of *B. lactis* AD011

Taxon name	*B. animalis* subsp. *lactis*	
Strain name	1st G	25th G
No. of contigs	15	25
Genome size (bp)	1,919,567	1,919,618
DNA G+C content (%)	60.5	60.5
No. of CDSs	1,556	1,553
No. of tRNA genes	52	52
Mean of CDS lengths (bp)	1,077.4	1,077.2
Median of CDS lengths (bp)	948	948
Mean of intergenic lengths (bp)	158.5	160.2
Median of intergenic lengths (bp)	111	111

3-1) Phylogenomics by OrthoANI Analysis

OrthoANI (Orthologous Average Nucleotide Identity) value is a type of value that shows the similarity between two genome sequences. It is an improvement of the existing ANI (Average Nucleotide Identity), and it is a type of Overall Genome Relatedness Index (OGRI). OGRI is the first term introduced by Chun and Rainey (2014), which refers to all measurements indicating the similarity of two genomic sequences. Algorithms for calculating OGRI values vary, but the most widely used systematic study is the Average Nucleotide Identity (ANI). OrthoANI can be used for microbial classification and identification, and the boundary value suggested to distinguish species is about 95%.

As a result, the homology of the *B. lactis* AD011 1st and 25th generations' dielectrics was 99.99% via the OrthoANI value (Fig. C.1). When compared with the original genome sequence of *B. lactis* AD011 reported by Kim et al. (2009), the OrthoANI values were 99.96 % and 99.95% for the 1st and 25th generations, respectively.

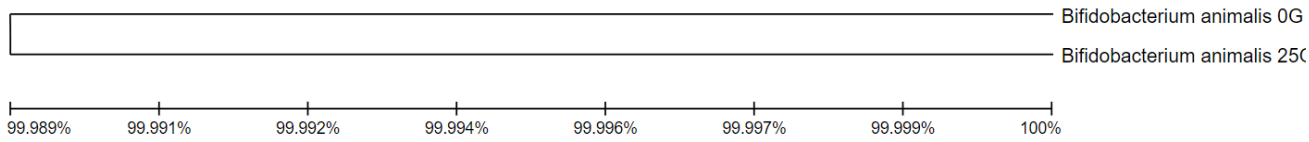

Figure C.1. ANI-derived UPGMA (Unweighted Pair Group Method with Arithmetic Mean) Dendrogram (Newick format)

3-2) Summary of Genetic Stability of *B. lactis* AD011 1st and 25th Generations

The difference under 0.01% is assumed to be due to sequencing errors or spontaneous evolutionary mutations. Therefore, it is concluded that there was little genetic mutation, and the genetic information did not change in the process of cultivating 25 generations.

4) References

Chun J, Rainey FA. Integrating genomics into the taxonomy and systematics of the Bacteria and Archaea. Int J Syst Evol Microbiol. 2014;64(Pt 2):316-24.

Kim JF, Jeong H, Yu DS, Choi SH, Hur CG, Park MS, Yoon SH, Kim DW, Ji GE, Park HS, Oh TK. Genome sequence of the probiotic bacterium *Bifidobacterium animalis* subsp. *lactis* AD011. J Bacteriol. 2009;191(2):678-9. Erratum in: J Bacteriol. 2009;191(6):1995.

Mellmann A, Andersen PS, Bletz S, Friedrich AW, Kohl TA, Lilje B, Niemann S, Prior K, Rossen JW, Harmsen D. High Interlaboratory Reproducibility and Accuracy of Next-Generation-Sequencing-Based Bacterial Genotyping in a Ring Trial. J Clin Microbiol. 2017;55(3):908-913.

Appendix D. List of Screened Virulence Toxins

All screened genes are listed on the website of Virulence Finder 2.0. (https://cge.cbs.dtu.dk/services/data.php)

K88ab: K88/F4 protein subunit
astA: Heat-stable enterotoxin 1
bfpA: Major subunit of bundle-forming pili
cba: Colicin B
ccI: Cloacin
cdtB: Cytolethal distending toxin B
celb: Endonuclease colicin E2
cfa_c: Colonisation factor antigen I
cma: Colicin M
cnf1: Cytotoxic necrotizing factor
cofA: Longus type IV pilus subunit
eae: Intimin
espB: Secreted protein B
ehxA: Enterohaemolysin
f17-A: Subunit A of F17 fimbrial protein
f17-G: Adhesin subunit of F17 fimbriae
fanA: Involved in biogenesis of K99/F5 fimbriae
fasA: Fimbriae 987P/F6 subunit
fedA: Fimbrial protein F107 subunit A
fedF: Fimbrial adhesin AC precursor
fim41a: Mature Fim41a/F41 protein
gad: Glutamate decarboxylase
hlyA: Haemolysin A
hlyE: Avian E.coli haemolysin
ipaD: Invasion protein Shigella flexneri
ipaH9.8: Invasion plasmid antigen
ireA: Siderophore receptor
iroN: Enterobactin siderophore receptor protein
iss: Increased serum survival
lngA: Longus type IV pilus
ltcA: Heat-labile enterotoxin A subunit
mchB: Microcin H47 part of colicin H
mchC: MchC protein
mchF: ABC transporter protein MchF
mcmA: Microcin M part of colicin H
nfaE: Diffuse adherence fibrillar adhesin gene
perA: EPEC adherence factor
pet: Autotransporter enterotoxin
senB: Plasmid-encoded enterotoxin
sfaS: S-fimbriae minor subunit
sta1: Heat-stabile enterotoxin ST-Ia
stb: Heat-stabile enterotoxin II
virF: VirF transcriptional activator
cif: Type III secreted effector

eatA: serine protease autotransporters of Enterobacteriaceae (SPATE)
efa1: EHEC factor for adherence
epeA: serine protease autotransporters of Enterobacteriaceae (SPATE)
espA: Type III secretions system
espC: serine protease autotransporters of Enterobacteriaceae (SPATE
espF: Type III secretion system
espI: serine protease autotransporters of Enterobacteriaceae (SPATE)
espJ: Prophage-encoded type III secretion system effector
espP: Putative exoprotein precursor
etpD: Type II secretion protein
iha: Adherence protein
katP: Plasmid-encoded catalase peroxidase
lpfA: Long polar fimbriae
nleA: Non-LEE encoded effector A
nleB: Non-LEE encoded effector B
nleC: Non-LEE encoded effector C
pic: serine protease autotransporters of Enterobacteriaceae (SPATE)
rpeA: serine protease autotransporters of Enterobacteriaceae (SPATE)
saa: Auto agglutinating adhesin
sat: serine protease autotransporters of Enterobacteriaceae (SPATE)
sepA: serine protease autotransporters of Enterobacteriaceae (SPATE)
sigA: serine protease autotransporters of Enterobacteriaceae (SPATE)
subA: Subtilase toxin subunit
tccP: Tir-cytoskeleton coupling protein
tir: Translocated intimin receptor protein
toxB: Toxin B
tsh: serine protease autotransporters of Enterobacteriaceae (SPATE)
vat: serine protease autotransporters of Enterobacteriaceae (SPATE)
stx1Aa - stx1Af: Shiga toxin 1, subunit A, variants a - f
stx1Ba - stx1Bf: Shiga toxin 1, subunit B, variants a - f
stx2Aa - stx2Ag: Shiga toxin 2, subunit A, variants a - g
stx2Ba - stx2Bg: Shiga toxin 2, subunit B, variants a - g
stx1A: Shiga toxin 1, subunit A
stx1B: Shiga toxin 1, subunit
stx2A: Shiga toxin 2, subunit A
stx2B: Shiga toxin 2, subunit B
ElrA: SrtA:ace:collagen adhesin precursor
acm
agg: aggregation substance
cCF10; cOB1; cad; camE:sex pheromone cAM373 precursor
cylA; cylB; cylL; cylM
ebpA; ebpB; ebpC; efaAfm; efaAfs; espfm; espfs
fsrB: biofilm formation
gelE
hylA; hylB; hylEfm; tpx
atl: bifunctional autolysin Atl
cap5A - cap5P: capsular polysaccharide biosynthesis protein cap5A – cap5P
capA - capP: capsular polysaccharide biosynthesis protein capA - capP
cap8A -cap8O: truncated capsular polysaccharide synthesis enzyme cap5A -cap8O

cap1A – cap1C: capsular polysaccharide biosynthesis protein cap1A -cap1C
clfA: fibrinogen-binding protein A, clumping factor
clfB: clumping factor ClfB, fibrinogen binding protein
cna: collagen adhesin precursor
coa: staphylocoagulase precursor
eap: extracellular adherence protein
eap/map: extracellular adherence protein
ebh: cell wall associated fibronectin-binding protein
ebpS: cell surface elastin binding protein
efb: extracelular fibrinogen-binding protein
fib: fibrinogen-binding protein
SEntA: putative enterotoxin type A
SEntB: enterotoxin B
SEntC: enterotoxin type C precursor
SEntG: extracellular enterotoxin type G precursor
SEntH: enterotoxin H
SEntI: extracellular enterotoxin type I precursor
SEntK: enterotoxin K
SEntL: extracellular enterotoxin L
SEntM: extracellular enterotoxin type I
SEntN - SEntQ: enterotoxin N - Q
SEnt: enterotoxin
SEnt-protiein: enterotoxin protein
SEnt-like: enterotoxin-like toxin
SEntyent1- 2: e nterotoxin yent1-2
eta: exfoliative toxin A
etd: exfoliative toxin A
set1 - 15: superantigen-like protein
set16 -17: exotoxin homolog
set18 -25: superantigen-like protein
set26: exotoxin homolog
set30: superanitgne-like protein
sal: superantigen-like protein
SExo: exotoxin
fnbA – B: fibronectin-binding protein A-B
geh: glycerol ester hydrolase
hla: alpha-hemolysin precursor
hlb: beta-hemolysin
hld: delta-hemolysin
hlgA: gamma-hemolysin chain II precursor
hlgB: gamma-hemolysin component B precursor
hlgC: gamma-hemolysin component C
hysA: hyaluronate lyase
icaA-D: intercellular adhesion protein A-D
icaR: intercellular adhesion regulator
lip: triacylglycerol lipase
lukF-PV: Panton Valentine leukocidin F component
lukS-PV: Panton Valentine leukocidin S component
lukM: LukM precursor

lukD: leukocidin D component
lukE: leukocidin E component
nuc: thermonuclease
sak: staphylokinase
isb: IgG-binding protein SBI
scn: staphylococcal complement inhibitor
sdrC: Ser-Asp rich fibrinogen-binding protein C
sdrD: Ser-Asp rich fibrinogen-binding protein D
sdrE: Ser-Asp rich fibrinogen-binding protein E
sdrH: Ser-Asp rich fibrinogen-binding portein H
splA - splF: serine protease splA - splF
sspA: serine V8 protease
spa: spa immunoglobulin G binding protein A
sspB: cysteine protease
sspB2: cysteine protease SspB
sspC: cysteine protease
tsst: toxic shock syndrome toxin-1
esxA: ESAT-6/WXG100 family secreted protein EsxA/YukE
esaA: type VII secretion protein EsaA
essA: protein secretion system EssA
esaB: Putative secretion accessory protein EsaB/YukD
essB: putative secretion system component EssB
essC: type VII secretion protein EssC
esaC: EsaC protein within ESAT-6 gene cluster
esxB: virulence factor EsxB family protein
vwb: von Willebrand factor-binding protein
stx11: S.dysenteriae 60R
stx12: ONTNA, variant a
stx13: S.dysenteriae 3818T
stx14: O111 PH, variant a
stx15: O111 CB168, variant a
stx16: O157 AI2001 52, variant a
stx17: O157 GPU96MM, variant a
stx18: O111 04-06263, variant a
stx19: O111 3385-00, variant a
stx110: S.sonnei CB7888
stx111: O48 94C, variant a
stx112: O157 FLY16, variant a
stx113: O157 EDL933, variant a
stx114: O165 HI-2, variant a
stx115: ONT HI-A, variant a
stx116: Out HI-N, variant c
stx117: ONT HI-B, variant c
stx118: ONT HI-C, variant c
stx119: ONT BCN26, variant c
stx120: O174 DG131-3, variant c
stx121: ONT 92-1251, variant d
stx122: ONT 92-1252, variant d
stx123: ONT AB8SF, variant d

stx124: ONT MHI813, variant d
stx21: O113 TS17-08, variant a
stx22: O22 EBC217, variant a
stx23: ONT 23765, variant a
stx24: O113 TS18-08, variant a
stx25: O88 N2688, variant a
stx26: ONT N5578, variant a
stx27: O153 TS20-08, variant a
stx28: O130 TS07-07, variant a
stx29: 178 TS02-07, variant a
stx210: O48 94C, variant a
stx211: OR TS05-07, variant a
stx212: E.cloacae 95MV2, variant a
stx213: O101 EBC201, variant a
stx214: O26 126814, variant a
stx215: O157 SF-258-98, variant a
stx216: O178 TS22-08, variant a
stx217: O178 TS24-08, variant a
stx218: O104 G5506, variant a
stx219: O8 VTB178, variant a
stx220: O83 N1135, variant a
stx221: ONT EK9900, variant a
stx222: ONT EBC210, variant a
stx223: O26 FD930, variant a
stx224: O157 SF-3573-98, variant a
stx225: O157 A397, variant a
stx226: O157 I6581, variant a
stx227: O136 VTB60, variant a
stx228: A.haemolyticus, variant a
stx229: O111 928-91, variant a
stx230: O157 93-111, variant a
stx231: O157 EDL933, variant a
stx232: O113 CL-3, variant a
stx233: O157 G5101, variant c
stx234: ONT pVTEC9, variant c
stx235: O174 031, variant c
stx236: O157 E32511, variant c
stx237: O177 CB7126, variant c
stx238: O174 EC1720a, variant d
stx239: ONT EBC219, variant c
stx240: O177 06-5121, variant c
stx241: O157 CB8028, variant c
stx242: O157 A580, variant c
stx243: O157 469, variant c
stx244: O157 A75, variant c
stx245: O157 310, variant c
stx246: O177 VTB323, variant c
stx247: O157 020324, variant c
stx248: O157 C394-03, variant c

stx249: O157 V20, variant c
stx250: O157 CS1718, variant c
stx251: O157 Y350, variant c
stx252: O157 FLY16, variant c
stx253: O171 EBC287, variant c
stx254: O171 VTB46, variant c
stx255: ONT EBC289, variant c
stx256: OR TS27-08, variant c
stx257: ONT EBC275, variant d
stx258: O91 B2F1, variant d1
stx259: O83 N15432, variant d
stx260: O113 VTB75, variant d
stx261: O77 VTB91, variant d
stx262: O103 pVTEC7, variant d
stx263: OR TS06-08, variant d
stx264: ONT EC1871a, variant d
stx265: O15 88 1509, variant d
stx266: O113 TS03-07, variant d
stx267: O174 EC173b, variant d
stx268: O8 C466-01B, variant d
stx269: O91 B2F1, variant d2
stx270: C.freundii LM76, variant d
stx271: O103 CVM9322, variant d
stx272: O157 7279, variant d
stx273: O55 5905, variant d
stx274: O55 06-5231, variant d
stx275: O6 NV206, variant d
stx276: O2 EC604a, variant d
stx277: O28 MT71, variant d
stx278: O73 C165-02, variant d
stx279: O22 KY-O19, variant d
stx280: O113 TS28-08, variant d
stx281: O113 TS21-08, variant d
stx282: Out S-4, variant b
stx283: O8 S-9, variant b
stx284: Out HI-N, variant b
stx285: O93 S-5, variant b
stx286: O111 S-3, variant b
stx287: O96 S-10, variant b
stx288: O111 S-1, variant b
stx289: O96 S-7, variant b
stx290: O96 S-6, variant b
stx291: O22 3143-97, variant b
stx292: O118 EH250, variant b
stx293: O40 5293-98, variant b
stx294: ONT I7606, variant b
stx295: O128 24196-97, variant b
stx296: O111 PH, variant b
stx297: O174 031, variant b

stx298: O8 FHI-1106-1092, variant e
stx299: Out S-8, variant g
stx2100: O2 7v, variant g
stx2101: O2 HI-11, variant g
stx2102: O2 S86, variant g
stx2103: O115 F08-101-31, variant f
stx2104: O128 T4-97, variant f
stx2105: O89 HI8, variant f
stx2106: O101 E-D42, variant e
stx2107: O22 3615-99, variant e
stx2108: OR TS09-07, variant e
stx2109: O26 R107, variant e
stx2110: O139 S1191, variant e
stx2111: O101 E-D43, variant e
stx2112: O101 E-D68, variant e
stx2113: O101 E-D53, variant e
stx2114: ONT TS29-08, variant e
stx2115: ONT TS03-08, variant e
stx2116: O138 NN, variant e
stx2117: O101 CB10394, variant e
stx2118: O100 TS01-07, variant e
stx2119: O121 NP9621, variant e
stx2120: ONT 26725-97, variant e
stx2121: ONT 2771, variant e
astA: EAST-1 heat-stable toxin
pet: Plasmid-encoded toxin
sigA: Shigella IgA-like protease homologue
Pic: Protease involved in intestinal colonization
sepA: Shigella extracellular protein A
tsh: Temperature-sensitive hemagglutinin
sat: Secreted autotransporter toxin
espC: EPEC secreted protein C
espP: Extracellular serine protease plasmid-encoded
pssA: Protease secreted by Shiga toxin-producing E. coli (STEC)
picU: Pic from uropathogenic E. coli (UPEC)
vat: Vacuolating autotransporter toxin
vat-EXPEC: Vacuolating autotransporter toxin from extra-intestinal pathogenic E. coli(EXPEC)
eatA: enterotoxigenic E. coli (ETEC) autotransporter A
epeA: enterohemorrhagic E. coli EHEC plasmid-encoded autotransporter
boa: Protease from Salmonella bongori
ORF3: Isoprenoid Biosynthesis
ORF4: Putative isopentenyl-diphosphate delta-isomerase
aap: Dispersin, antiaggregation protein
aaiC: Type VI secretion protein
aggR: AraC transcriptional activator
aatA: Dispersin transporter protein
agg4A: AAF/IV major fimbrial subunit
aggA: AAF/I major fimbrial subunit

aafA: AAF/II major fimbrial subunit
agg3A: AAF/III major fimbrial subunit
agg3C: Usher, AAF/III assembly unit
agg3D: Chaperone, AAF/III assembly unit
agg3B: AAF/III minor adhesin. Enterobacteria AfaD invasin protein
aafC: Usher, AAF/II assembly unit
aafD: Chaperone, AAF/II assembly unit
aafB: AAF/II minor adhesin. Enterobacteria AfaD invasin protein
agg4C: Usher, AAF/IV assembly unit
agg4D: Chaperone, AAF/IV assembly unit
agg4B: AAF/IV minor adhesin. Enterobacteria AfaD invasin protein
aggC: Usher, AAF/I assembly unit
aggD: Chaperone, AAF/I assembly unit
aggB: AAF/I minor adhesin. Enterobacteria AfaD invasin protein
aar: AggR-activated regulator
anr: AraC negative regulator
eilA: Salmonella HilA homolog
capU: Hexosyltransferase homolog
air: Enteroaggregative immunoglobulin repeat protein
clpK:
mrk:
ClpK:
tst: toxic shock syndrome toxin-1
ACME: arginine catabolic mobile element
aur: aureolysin
edinA: epidermal cell differentiation inhibitor A
edinB: epidermal cell differentiation inhibitor B
edinC: epidermal cell differentiation inhibitor C
seb: enterotoxin B
sea: enterotoxin A/P
sec: enterotoxin C
sed: enterotoxin D
see: enterotoxin E
seg: enterotoxin G
seh: enterotoxin H
sei: enterotoxin I
sej: enterotoxin J
sek: enterotoxin K
sel: enterotoxin L
sem: enterotoxin M
sen: enterotoxin N
seo: enterotoxin O
sep: enterotoxin P
seq: enterotoxin Q
ser: enterotoxin R
seu: enterotoxin U

Appendix E. Expert Panel Consensus Statement

Introduction

BIFIDO Co., Ltd (“BIFIDO”) convened a panel of independent scientists (the "Expert 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 animalis* subsp. *lactis* AD011 and to determine whether the proposed uses in food would be Generally Recognized as Safe (GRAS) based on scientific procedures. The Expert Panel consisted of the following qualified experts: Michael Falk, Ph.D. (LSRO Solutions, LLC), Roger A. Clemens, Ph.D. (Professor Emeritus, The University of Southern California), and Yong-Su Jin, Ph.D. (Professor, University of Illinois at Urbana-Champaign). Susan Cho, Ph.D. (NutraSource, Inc.) served as technical advisor to the Expert Panel.

The Expert 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 produced by NutraSource, Inc. ("The Generally Recognized As Safe [GRAS] Determination of *Bifidobacterium animalis* subsp. *lactis* AD011 (*B. lactis* AD011) as a Food Ingredient"). The Expert Panel evaluated other information deemed appropriate or necessary. To the best of our knowledge, this determination 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 for the uses of this ingredient in food.

Summary and Basis for GRAS

Introduction

The nonpathogenic and non-toxigenic *B. lactis* AD011 strain was isolated from infant stool. *B. lactis* AD011 is a non-spore forming, heterofermentative, gram-positive, anaerobic, non-genetically modified, nonpathogenic, and non-toxigenic microorganism. It is a member of the lactic acid bacteria (LAB), a group characterized by the production of lactic acid as the major metabolic end product of carbohydrate metabolism. *Bifidobacterium* genus is an anaerobic, gram-positive bacterium that does not form spores. Bifidobacteria comprise up to 25% of the cultivatable fecal bacteria in adults and 80% in infants (Picard et al., 2005).

Identification

The whole genome sequence of *B. lactis* AD011 was published in GenBank (Accession no.: CP001213) in 2009 (Kim et al., 2009). The complete sequence of *B. lactis* AD011 consists of a 1,933,695-bp circular chromosome (60.49% G+C) with no plasmid capable of transmitting antibiotic resistances. *B. lactis* strains AD011, BB-12, and Bl-04 share over 99.85% homology in genome sequences: 99.85 to 99.93% by average nucleotide identity (ANI) values and 99.99% by tetra-nucleotide analysis (TNA) values (Ku et al., 2019).

Manufacturing Process

B. lactis AD011 is produced in a batch-type fermentation process with medium composed of glucose, soy peptone, yeast extract, sodium acetate, sodium phosphate, L-cysteine HCl, and taurine. The medium is sterilized and then inoculated with *B. lactis*

AD011, which is grown at 37°C for 10-20 h. After growth, the bacteria are centrifuged, washed, re-centrifuged, pelleted, mixed with maltodextrin (processing aid), freeze-dried, and then milled and sieved. Corn starch, an excipient, is added to the concentrate to standardize the blends.

BIFIDO rigorously tests its final production batches to verify adherence to quality control specifications. BIFIDO observes the principles of a Hazard Analysis and Critical Control Point (HACCP)-controlled manufacturing process and current good manufacturing practices (cGMP) for food (21 CFR Part 110 and Part 117 Subpart B). The raw materials and processing aids used in the manufacturing process are food grade.

Specifications and Analytical values

The number of *B. lactis* AD011 cells per one gram of the ingredient is estimated as 1.0×10^{11} cells. Analyses of three non-consecutive lots of the *B. lactis* AD011 ingredient confirm that the material produced by the manufacturing process is consistent and complies with the product specifications, meeting appropriate food-grade specifications.

Table E.1. Specifications of *B. lactis* AD011 Stock Ingredient

Parameter	Specification	Typical composition*	Method of analysis
Appearance	No off-taste or off-flavor	Yellow white powder	
Cell Counts, cfu/g (as *B. lactis* AD011)	MT 1.00E+11	1.00E+11	ISO 29981:2010 or equivalent
Moisture, %	NMT 5.0	4.23%	AOAC 941.14 or equivalent
Heavy metals			
Lead (Pb), ppm	NMT 0.3	<0.01	AOAC 2013.06 or equivalent
Arsenic (As), ppm	NMT 0.3	<0.06	
Cadmium (Cd), ppm	NMT 0.1	<0.03	
Mercury (Hg), ppm	NMT 0.1	<0.04	
Microbiology			
Non-lactic acid bacteria, cfu/g	NMT 100	Negative	ISO 13559:2002 (IDF 153)
Total yeasts and molds, cfu/g	NMT 100	Negative	AOAC 2002.11 or equivalent
Escherichia coli, cfu/25 g	ND in 25 g	ND in 25 g	AOAC 991.14-petri *E.coli* count plate
Salmonella, cfu/25 g	ND in 25 g	ND in 25 g	AOAC 989.14 or equivalent
Listeria, cfu/25 g	ND in 25 g	ND in 25 g	AOAC 998.08 or equivalent
Cronobacter sakazakii, cfu/10 g	ND in 10 g	ND in 10 g	BAM - Chapter 29 *Cronobacter* cultural method
Ash, %	NA	5.99%	AOAC 900.02 or equivalent

*Average of 3 analytical values.

Abbreviations: AOAC = Association of Official Agricultural Chemists; ISO = International Standards Organization; MT = More Than; NA = Not Applicable; ND = ND: Not Detected; NMT = Not More Than.

Stability

Bulk ingredient stability data indicate that the number of *B. lactis* AD011 cells in the ingredient is stable for up to 18 months at 5°C and 25ºC when the cells are supplied in excess of 150% of the claim value at the time of shipment.

Intended Technical Effects

The substance will be used to provide a dietary source of *B. lactis* AD011 as a food ingredient to non-exempt term infant formulas and selected conventional foods.

Intended Uses and Exposure Estimates

B. lactis AD011 will be added to non-exempt infant formulas for term infants (soy-, milk-, and/or whey-based) and selected conventional foods. The use level is the same as those described in GRAS notices of other Bifidobacteria. Powdered non-exempt term infant formulas (milk-, soy-, or whey-based) will contain up to 10^8 colony forming units (cfu) of *B. lactis* AD011 per g of powdered formulas. *B. lactis* AD011 may be used alone or in combination with other safe and suitable *Bifidobacterium* or *Lactobacillus* strains. The addition of 10^8 cfu *B. lactis* AD011 per g infant formula will result in estimated mean and 90^{th} percentile daily intakes of 1.04×10^{10} and 1.6×0^{10} cfu per infant, respectively, in all users. These formulas will be supplemented appropriately to provide a minimum of 10^9 cfu *B. lactis* AD011 per day at the end of an 18-month shelf life at room temperature.

In addition, BIFIDO intends to add *B. lactis* AD011 strain to selected conventional food products (dairy products/dairy-based foods and dairy substitutes, including fermented milk, including butter milk and kefir; flavored milk beverage mixes, dried milk powder; imitation milk and yogurt; powdered baby cereals and foods; meal replacement and nutritional drink mix powders; and powdered sugar substitute) for the general population. These target foods will contain up to 1×10^{10} cfu *B. lactis* AD011 per serving. *B. lactis* AD011 may be used alone or in combination with other safe and suitable *Bifidobacterium* or *Lactobacillus* strains. The intended use of 1.0×10^{10} cfu *B. lactis* AD011 per serving in the target food categories would result in mean and 90^{th} percentile estimated daily intakes (EDI) of 1.28×10^{10} and 2.71×10^{10} *B. lactis* AD011 cfu per person per day, respectively, in all users. These estimates are amplified because it is not likely that *B. lactis* AD011 will be used at the maximum levels for all food categories under the intended uses.

Potential Infection

Humans are exposed to bifidobacteria by the use of eating fermented foods (e.g., yogurt, cheese, fermented vegetables, and olives), foods and/or dietary supplements containing bifidobacteria, and in the host's own microflora. Even with these sources, bifidobacteria rarely cause infections in humans. This lack of pathogenicity extends to all age groups as well as immunocompromised patients (Borriello et al., 2003).

Safety Determination

Studies have demonstrated that the intended uses of *B. lactis* AD011 are safe based on the following facts:

1. *B. lactis* AD011 and other *B. lactis* strains have a long history of safe consumption in humans. Several *B. lactis* strains are recognized as GRAS. Human studies showed

that no *B. lactis* strains resulted in adverse effects in humans, regardless of age, gender, or health status of the subjects.

2. The information/data provided by BIFIDO (specifications, manufacturing process, and intended use) in this report and supplemented by the publicly available literature/safety data on *B. lactis* AD011 and other *B. lactis* strains provide a sufficient basis for an assessment of the safety of *B. lactis* AD011 for the proposed use as a food ingredient prepared according to appropriate specifications.

   Key findings are summarized as follows:
   a) *In vitro* studies show that the antibiotic susceptibility profiles of *B. lactis* AD011 are similar to those of other GRAS strains, which have been safely used in the U.S. and Europe for over a decade. *B. lactis* AD011 has no hemolytic or mucolytic activities and does not produce biogenic amines and ammonia.
   b) The genomic sequence of *B. lactis* AD011 does not include toxigenic or pathogenic genes.
   c) *B. lactis* AD011 does not have any plasmid capable of transmitting antibiotic resistance genes.
   d) *B. lactis* AD011 is genetically stable.
   e) Animal and human studies showed no adverse effect of *B. lactis* AD011.
   f) Studies of another *B. lactis* strain (BB-12) whose whole genomic sequence has over 99.85% similarity with that of the AD011 strain also showed no adverse effects in humans.

3. The *B. lactis* AD011 ingredient has been marketed as a dietary supplement ingredient and as a dietary supplement in Korea since 2007. *B. lactis* AD011, at daily doses up to 1×10^{10} cfu (or 1.5×10^{10} cfu at the time of shipment), has been safely used; no adverse events or health-related complaints have been reported by consumers.

4. The intended use of *B. lactis* AD011 results in levels of exposure within historical human use levels and provides a reasonable certainty of safety.

5. *B. lactis* AD011 is well-characterized and is free from chemical or other microbial contamination.

Therefore, it is reasonable to conclude that *B. lactis* AD011is non-pathogenic and non-toxigenic and that intended uses of up to 10^8 cfu *B. lactis* AD011/g in powdered infant formulas and 1×10^{10} cfu *B. lactis* AD011/serving in selected conventional foods are safe.

Conclusions and General Recognition of the Safety of *B. lactis* AD011

Common Knowledge Element of the GRAS Determination

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 using published, peer-reviewed scientific journals for the safety assessment. The animal studies and human studies on which this GRAS determination is based have been published in the peer-reviewed scientific literature.

B. lactis has been safely used as a food ingredient for decades. As a result, comprehensive reviews of the safety of several strains of *B. lactis* have been published. In addition, GRAS notices of several strains of *B. lactis* have received FDA 'no question' letters on their safety, and such information is widely available. In the literature, evidence for genetic similarity to other *B. lactis* strains is available for safety assessment of *B. lactis* AD011. These facts meet the "common knowledge" element of the GRAS determination.

Technical Element of the GRAS Determination

Human and animal studies have reported that consumption of *B. lactis* AD011 and other *B. lactis* strains was not associated with any adverse effects/events. BIFIDO rigorously tests its final production batches to verify adherence to quality control specifications, and thus, manufacturing processes are consistent with cGMP for food (21 CFR Part 110 and Part 117 Subpart B). The raw materials and processing aids used in the manufacturing process are food grade. There is broad-based and widely disseminated knowledge concerning the safety of *B. lactis* AD011 and other *B. lactis* strains. The literature indicates that consumption of *B. lactis*, including *B. lactis* AD011, does not result in any adverse effects. Thus, the intended uses of *B. lactis* AD011 have been determined to be safe though scientific procedures as set forth in 21 CFR 170.3(b), thus, satisfying the "technical" element of the GRAS determination.

Conclusion of the Expert Panel

We, the undersigned members of the Expert Panel, have individually and collectively critically evaluated the materials summarized above on the safety of BIFIDO's *B. lactis* AD011 and other information deemed appropriate, and unanimously conclude that BIFIDO's *B. lactis* AD011, manufactured as described in the dossier and consistent with cGMP, and meeting appropriate food grade specifications, is Generally Recognized As Safe (GRAS) based on scientific procedures for use as an ingredient in term infant formulas and selected conventional foods at levels specified in the accompanying dossier. It is our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusions.

Expert Panel Members:

[redacted] 03/13/20

Michael Falk, Ph.D. Date
LSRO Solutions, Rockville, MD

[redacted] 3/16/2020

Roger A. Clemens, Ph.D. Date
Professor Emeritus, University of Southern California, Los Angeles, CA

[redacted] March 30, 2020

Yong-Su Jin, Ph.D. Date
Professor, University of Illinois at Urbana-Champaign, Urbana, IL

Technical Advisor to the Expert Panel:

[redacted] 3/27/2020

Susan Cho, Ph.D. Date
NutraSource, Inc., Clarksville, MD

References:

Borriello SP, Hammes WP, Holzapfel W, Marteau P, Schrezenmeir J, Vaara M, Valtonen V. Safety of probiotics that contain lactobacilli or bifidobacteria. Clin Infec Dis. 2003;36: 75-80.

Kim JF, Jeong H, Yu DS, Choi SH, Hur CG, Park MS, Yoon SH, Kim DW, Ji GE, Park HS, Oh TK. Genome sequence of the probiotic bacterium *Bifidobacterium animalis* subsp. *lactis* AD011. J Bacteriol. 2009;191:678-9. Erratum in: J Bacteriol. 2009;191:1995.

Ku S, Yang S, Lee HH, Choe D, Johnston TV, Ji GE, Park MS. Biosafety assessment of Bifidobacterium animalis subsp. lactis AD011 used for human consumption as a probiotic microorganism. Food Control. 2019. Online 12. Article no 106985.

Picard C, Fioramonti J, Francois A, Robinson T, Neant F, Matuchansky C. Review article: bifidobacteria as probiotic agents -- physiological effects and clinical benefits. Aliment Pharmacol Ther. 2005;22:495-512.

From:	Susan S Cho
To:	Honigfort, Mical
Cc:	Gaynor, Paulette M
Subject:	Bifidobacterium lactis AD011 GRAS
Date:	Sunday, April 19, 2020 9:13:44 PM

Dear Dr. Honigfort,

We sent a GRAS notice for *Bifidobacterium animalis* supsp. *lactis* AD011 on April 8th . I assume FDA received it on Aril 9th. This is a resubmission of GRN 875. I would appreciate your kind attention to our submission. We submittede this submission under the name of NutraSource, Inc. As written in the GRAS notice, please address correspondences to Dr. M.S. Park, at BIFIDO (the notifier's chief science officer). It is because of my company's name change.

Effective May 1, 2020, we will change our company name from NutraSource, Inc. to AceOne RS to avoid a confusion with Nutrasource Diagnostics, Inc. (NDI-- this company often shortens its name as Nutrasource), a Canadian company which acquired GRAS Associates two or three years ago. Future GRAS notices will be submitted under the name of AceOne RS.

Please take care and stay healthy!

Regards,
Susan
Susan Cho, Ph.D.
NutraSource, Inc. 6309 Morning Dew Ct Clarksville, MD 21029 +1-410-531-3336 (O) +1-301-875-6454 (C)

From: Gaynor, Paulette M
To: 박명수
Subject: RE: Reminder -> FW: GRN 952 - item for clarification
Date: Monday, December 7, 2020 9:00:00 AM
Attachments: image001.png
image002.png
image003.png
image004.png
image005.png
image006.png

Dear Dr. Park,

Thank you for the clarification that *B. lactis* AD011 is intended for use as an ingredient.

Sincerely,
Paulette Gaynor

From: 박명수 <bifidopark@bifido.com>
Sent: Sunday, December 6, 2020 7:28 PM
To: Gaynor, Paulette M <Paulette.Gaynor@fda.hhs.gov>
Subject: RE: Reminder -> FW: GRN 952 - item for clarification

Dear Paulette Gaynor,

We apologize for the delay and causing a confusion. We clarify that we intend to use *Bifidobacterium animalis* subsp. *lactis* strain AD011 (*B. lactis* AD011) as an ingredient in non-exempt infant formula (milk- and soy-based) for term infants at levels up to 10^8 colony forming units (CFU)/g of powdered formula; and, in fermented milk, including butter milk and kefir, flavored milk beverage mixes, dried milk powder, imitation milk, yogurt, powdered baby cereals and foods, meal replacement and nutritional drink mix powders, and powdered sugar substitutes at levels up to 10^{10} CFU *B. lactis* AD011/serving.

I deleted 'is' from the previous version. thank you.

Sincerely,

Park

MyeongSoo Park, PhD

Research Director/CTO
BIFIDO Co., Ltd
23-16 Nonggongdanji-gil, Hongchun
Kangwon, 25117, Korea
Tel) 82-33-435-4962/Fax) 82-33-435-4963
Mobile) 82-10-7311-0451
E-mail) bifidopark@bifido.com

From: Gaynor, Paulette M <Paulette.Gaynor@fda.hhs.gov>
Sent: Saturday, December 5, 2020 1:58 AM
To: 박명수 <bifidopark@bifido.com>
Subject: Reminder -> FW: GRN 952 - item for clarification

Myeong Soo Park, Ph.D.
BIFIDO Co., Ltd.
By email: Bifidopark@bifido.com

Dear Dr. Park,

I am sending this email as a reminder about our email of November 16, 2020, in which FDA identified an item that requires clarification as we continue with our evaluation of GRN 952. As a reminder, this is the item that requires clarification:

> In the cover letter, *B. lactis* AD011 is referred to as a food ingredient; however, in section 1.C.3. of the notice (page 6), there is a statement that "the substance will be used to provide a dietary source of *B. lactis* AD011 as a food ingredient". As such, we are seeking confirmation that the intended conditions of use (for the substance that is the subject of the notice) are as an ingredient. With the infant formula type as per the above paragraph, the subject and intended condition of use for the notice then would be as follows: *Bifidobacterium animalis* subsp. *lactis* strain AD011 (*B. lactis* AD011) for use as an ingredient in non-exempt infant formula (milk- and soy-based) for term infants at levels up to 10^8 colony forming units (CFU)/g of powdered formula; and, in fermented milk, including butter milk and kefir, flavored milk beverage mixes, dried milk powder, imitation milk, yogurt, powdered baby cereals and foods, meal replacement and nutritional drink mix powders, and powdered sugar substitutes at levels up to 10^{10} CFU *B. lactis* AD011/serving. Please clarify by confirming that *B. lactis* AD011 is intended for use as an ingredient.

Thank you,
Paulette Gaynor

From: Gaynor, Paulette M
Sent: Monday, November 16, 2020 10:06 AM
To: Bifidopark@bifido.com
Subject: GRN 952 - item for clarification

Myeong Soo Park, Ph.D.
BIFIDO Co., Ltd.
By email: Bifidopark@bifido.com

Dear Dr. Park,

For GRN 952, the subject of the notice is *Bifidobacterium animalis* subsp. *lactis* strain AD011 (*B. lactis* AD011). Please note that while our filing letter for GRN 952, which we sent to you in September 2020, refers to the infant formula as "non-exempt infant formula (milk-, soy-, and whey-based) for term infants at levels up to 10^8 colony forming units (CFU)/g of powdered formula" we are providing a update that we are now referring to the infant formula as "non-exempt infant formula (milk- and soy-based) for term infants at levels up to 10^8 colony forming units (CFU)/g of powdered formula" because 'whey-based' is considered to fall within the 'milk-based' category.

As we continue with our evaluation of GRNs 952, we have identified the following item that requires clarification.

> In the cover letter, *B. lactis* AD011 is referred to as a food ingredient; however, in section 1.C.3. of the notice (page 6), there is a statement that "the substance will be used to provide a dietary source of *B. lactis* AD011 as a food ingredient". As such, we are seeking confirmation that the intended conditions of use (for the substance that is the subject of the notice) are as an ingredient. With the infant formula type as per the above paragraph, the subject and intended condition of use for the notice then would be as follows: *Bifidobacterium animalis* subsp. *lactis* strain AD011 (*B. lactis* AD011) for use as an ingredient in non-exempt infant formula (milk- and soy-based) for term infants at levels up to 10^8 colony forming units (CFU)/g of powdered formula; and, in fermented milk, including butter milk and kefir, flavored milk beverage mixes, dried milk powder, imitation milk, yogurt, powdered baby cereals and foods, meal replacement and nutritional drink mix powders, and powdered sugar substitutes at levels up to 10^{10} CFU *B. lactis* AD011/serving. Please clarify by confirming that *B. lactis* AD011 is intended for use as an ingredient.

If you have any questions about the item that requires clarification, please let me know. FDA respectfully requests a response within 10 business days. If unable to complete the response within that timeframe, please contact me. Thank you.

Sincerely,
Paulette Gaynor

Paulette M. Gaynor, Ph.D.
Senior Policy Advisor

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety, Division of Food Ingredients
U.S. Food and Drug Administration
Tel: 240-402-1192
Paulette.Gaynor@fda.hhs.gov