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July 5, 2019

Dr. Paulette Gaynor
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

#875

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

Dear Dr. Gaynor,

On behalf of BIFIDO CO., LTD. (or BIFIDO), we are submitting a GRAS notification for the *Bifidobacterium animalis* subsp. *lactis* AD011 (or *B. lactis* AD011) as a food ingredient. The enclosed document provides the notice of a claim that a food ingredient, the *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.

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]

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

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF *BIFIDOBACTERIUM ANIMALIS* SSP. *LACTIS* AD011

Prepared for BIFIDO CO., LTD.

Prepared by:
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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 non-exempt term infant formulas (soy-, milk-, and 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.

Conventional Food Applications:

BIFIDO intends to add *B. lactis* AD011 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 (Table 1). These target foods will contain up to $1x10^{10}$ cfu *B. lactis* AD011 per serving.

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 as a food ingredient providing *B. lactis* AD011 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 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 FDA upon request by contacting Susan Cho at NutraSource, Inc. The data and information will be made available to 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

_____ _____7/5/2019_____

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

Address correspondence to
Susan S. Cho, Ph.D.
NutraSource, Inc., Agent for BIFIDO Co., Ltd.
Susanscho1@yahoo.com

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, *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 *B. lactis* AD011 strain was isolated from infant stool. *B. lactis* AD011 is a non-spore forming, heterofermentative, gram-positive, anaerobe, 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 and other physiological traits.

The whole genome sequence of *B. lactis* AD011 was published in GenBank (Accession no.: CP001213) in 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 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

Sequence homologies were examined by comparing the obtained sequences with those in the DNA databases (http://www.ncbi.nlm.nih.gov/BLAST). 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 databases (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 99.9% similarity with other GRAS strains of *B. lactis*, such as BB-12, Bi-07, and Bl-04. 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). This genome size is smaller than the other completely sequenced genomes in *Bifidobacteriales,* such as *Bifidobacterium adolescentis* (*B. adolescentis*)

ATCC 15703 (2.09 Mb; NC_008618), *Bifidobacterium longum* (*B. longum*) DJO10A (2.38 Mb; NC_010816), and *B. longum* NCC2705 (2.26 Mb; NC_004307). The *B. lactis* AD011's genome codes for 1,528 coding sequences, two rRNA operons, 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 Appendix B, Park and Yang, 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	1074.5	1076.8
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: EzBioCloud Comparative Genomics Database by ChunLab, Inc. (http://cg.ezbiocloud.net/)
Data set: *Bifidobacterium lactis strain*
Abbreviations: G=guanine; C=cytosine; CDS=coding sequence; bp=base pair; ANI=average nucleotide identity.

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 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 pelleted, mixed with a cryoprotectant, freeze-dried, and then 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, which is 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 from 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 to collect the cells.
6. The bacterial weight of *B. lactis* AD011 is measured and subjected to dilution with a cryoprotective agent (100% maltodextrin), which is 85% (w/w) *B. lactis* AD011 and 15% (w/w) maltodextrin. It is then freeze-dried and milled.
7. After milling, the excipient (100% corn starch) is added at a bacteria-to-weight ratio of 2:3, and the ingredient is subjected to a metal separator (a standard process in South Korea) prior to packaging.

The final stock of *B. lactis* AD011 ingredients are comprised of 51% *B. lactis* AD011 cells, 9% maltodextrin, and 40% corn starch. The number of *B. lactis* AD011 cells per one gram of the ingredient is estimated as $1.0x10^{11}$ cells. 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 §168.120
Soy peptone	73049-73-7	21 CFR §184.1553
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)	7782-85-6	21 CFR §182.1778
L-cysteine HCl	52-89-1	21 CFR §184.1272
Taurine	107-35-7	GRN 586
Processing aids/Excipients		
Maltodextrin	9590-36-6	21 CFR §184.1444
Corn Starch	9005-25-8	21 CFR §172.892

Quality Assurance Procedure:

BIFIDO rigorously tests its final production batches to verify adherence to quality control specifications and are manufactured consistent with current good manufacturing practice (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.

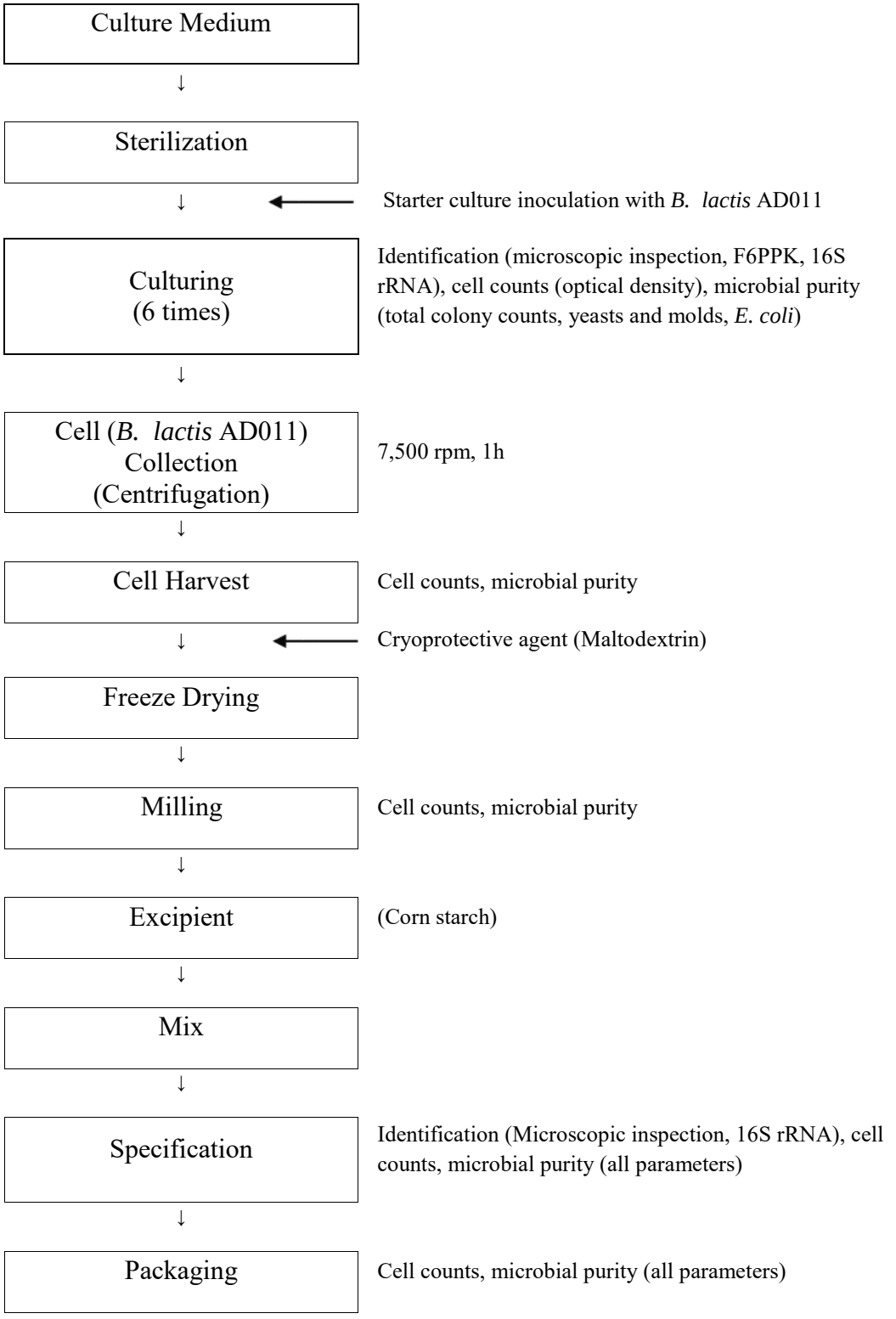

Figure 1. Schematic Overview of Manufacturing Process for *B. lactis* AD011

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

Table 6 presents the composition and 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 6; Appendix C). The analytical data also demonstrate the absence of any chemical impurities or microbiological contamination (Table 7).

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

Parameter	Specification	Typical composition*	Method of analysis Method number
Appearance	No off-taste and off-flavor	Yellow white powder	Visual
Cell Counts, cfu/g (as *B. lactis* AD011)	MT 1.00E+11	1.00E+11	KHFSC 4/3/3-58
Moisture, %	NMT 5.0	4.23%	KFSC 8/2/2.1/2.1.1
Heavy metals			
Lead (Pb), ppm	NMT 0.3	0.0305	KFSC 8/9/9.1/9.1.2
Arsenic (As), ppm	NMT 0.3	0.0085	KFSC 8/9/9.1/9.1.4
Cadmium (Cd), ppm	NMT 0.1	0.0179	KFSC 8/9/9.1/9.1.3
Mercury (Hg), ppm	NMT 0.1	ND	KFSC 8/9/9.1/9.1.6
Microbial purity			
Non-Lactic acid bacteria (Total Colony Counts)	NMT 100 cfu/g	Negative	Total Colony Counts KFSC 8/4/4.5/4.5.1
Total yeasts and molds	NMT 100 cfu/g	Negative	KFSC 8/4/4.10
Escherichia coli/ 200g	Negative	Negative	KFSC 8/4/4.8
Salmonella/25 g	Negative	Negative	KFSC 8/4/4.11
Listeria/25 g	Negative	Negative	KFSC 8/4/4.15
Enterobacter sakazakii (*Cronobacter* spp.)/60 g	Negative	Negative	KFSC 8/4/4.21
Proximate analysis			
Lipids, %	NA	1.54%	KFSC 8/2/2.1/2.1.5/2.1.5.1
Protein, %	NA	57.36%	KFSC 8/2/2.1/2.1.3/2.1.3.1
Carbohydrates, %	NA	31.38%	KFSC 8/2/2.1/2.1.4/2.1.4.1
Ash, %	NA	5.99%	KFSC 8/2/2.1/2.1.2

*Average of 3 analytical values. NA: Not Applicable.
KFSC: Korean Food Standards Codex, KHFSC: Korean Health Functional Food Standards Codex (Available on http://www.foodsafetykorea.go.kr/portal/safefoodlife/food/foodRvlv/foodRvlv.do)

KFSC's sample size requirements for microbiology tests: 200 g for *Escherichia coli;* 25 g for *Salmonella and Listeria;* 60 g for *Enterobacter sakazakii.*

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

Parameter	BL-R-190211	BL-R-190116	BL-R-190129
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, %	4.2%	4.3%	4.2%
Heavy metals			
Lead (Pb), ppm	0.0362	0.0046	0.0507
Arsenic (As), ppm	0.0073	0.0088	0.0093
Cadmium (Cd), ppm	0.0147	0.0178	0.0212
Mercury (Hg), ppm	ND	ND	ND
Microbial purity			
Non-Lactic acid bacteria	Negative	Negative	Negative
Total yeasts and molds	Negative	Negative	Negative
Escherichia coli	Negative in 200 g		
Salmonella	Negative in 25 g		
Listeria	Negative in 25 g		
Enterobacter sakazakii (*Cronobacter* spp.)	Negative in 60 g		
Proximate analysis			
Lipids, %	1.50%	1.54%	1.58%
Protein, %	53.11%	61.47%	57.52%
Carbohydrates, %	34.06%	28.44%	31.64%
Ash, %	7.93%	4.36%	5.69%

ND= Not Detected

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 subspecies is robust with regard to stressful conditions, such as acidity and oxygen, and is able to withstand the adverse conditions of product manufacture and storage and can maintain viability and stability during product shelf life.

Bulk ingredient stability data indicate that the number of *B. lactis* AD011 cells in the ingredient is stable for up to 2 years at 5°C and 25ºC when the cells are 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. The 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 24month compared to the claim value (1.00E+11 cfu/g)	108%	89%	

2.E. Intended Technical Effects

The intended effect is to provide probiotic *B. lactis* AD011 cells to non-exempt term infant formulas and/or selected conventional foods.

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). Probiotics, including *B. lactis*, are known to have several health benefits, including improved intestinal health and immune functions with no major side effects (Picard et al., 2005). In particular, *B. lactis* strain AD011 can also be used as a probiotic ingredient.

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 described for other *Bifidobacterium* species in GRNs 454, 813, and 814. Since the intended use level in this GRAS determination is the same as GRNs 454, 813, and 814, the EDI levels are consistent with those reported in these GRAS notices. Powdered non-exempt term infant formulas (milk-, soy-, or whey-based) will contain up to 10^8 cfu *B. lactis* AD011/g powdered formulas. The intended target intake level will be 10^9 - 10^{10} cfu *B. lactis* AD011/infant/day.

Infant formulas in the US market typically provide 0.67 kcal/mL (20 kcal/fluid oz.) (Martinez and Ballew, 2011). Assuming that these formulas are the sole source of nutrition, reconstituted at 14.1 g/100 mL with a caloric density of 0.67 kcal/mL, the caloric requirements for one-month-old and six-month-old infants are 472 kcal/day and 645 kcal/day, respectively (Institute of Medicine [IOM] Panel on Macronutrients and IOM Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 2005). The addition of 10^8 cfu *B. lactis* AD011/g infant formula will result in estimated daily intakes of 9.9 x 10 cfu for an one-month infant and 1.35 x 10 cfu *B. lactis* AD011 for a six-month infant. These formulas will be supplemented appropriately to provide a minimum of 10^9 cfu *B. lactis* AD011/day at the end of a 18-month shelf-life at room temperature.

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/serving.

The intended use of $1.0x10^{10}$ cfu *B. lactis* AD011 per serving in the target food categories would result in $1.36x10^{10}$ and $3.00x10^{10}$ *B. lactis* AD011 cells per person per day, respectively, in all users since the estimated mean and 90th percentile intakes of foods were 1.36 and 3.00 servings of foods per person per day, respectively (Table 9-1). A maximum exposure would occur in males aged 13 to 18 years of age, with a 90th percentile EDI of $3.5x10^{10}$ cfu/day. In the total population, the mean and 90th percentile food intakes are estimated to be 0.41 and 1.17 servings per day, providing $0.41x10^{10}$ and $1.17x10^{10}$ cfu/person/day, respectively (Table 9-2).

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

Table 9-1. EDIs of *B. lactis* AD011 from Proposed Uses in Selected Conventional Foods in All Users*

	N	% users	Food, serving/d		*B. lactis* AD011, cfu/day	
			Mean	90th Pctl	Mean	90th Pctl
Children 1-5	583	39.6	0.72	1.69	0.72×10^{10}	1.69×10^{10}
Children, 6-12	486	23.5	0.59	1.11	0.59×10^{10}	1.11×10^{10}
Males, 13-18	78	9.6	1.01	2.01	1.01×10^{10}	2.01×10^{10}
Females, 13-18	114	15.3	0.60	1.10	0.60×10^{10}	1.10×10^{10}
Males, 19-99	1,084	26.0	1.55	3.50	1.55×10^{10}	3.50×10^{10}
Females, 19-99	1,626	38.3	1.51	3.00	1.51×10^{10}	3.00×10^{10}
All-users	3,971	30.2	1.36	3.00	1.36×10^{10}	3.00×10^{10}

*Based on the 2011-2014 National Health and Nutrition Examination Survey (NHANES).

Table 9-2. EDIs of *B. lactis* AD011 from Proposed Uses in Selected Conventional Foods in All Population*

	N	% users	Food, serving/d		*B. lactis* AD011, cfu/day	
			Mean	90th Pctl	Mean	90th Pctl
Children 1-5	1,587	100	0.29	0.92	0.29×10^{10}	0.92×10^{10}
Children, 6-12	2206	100	0.14	0.50	0.14×10^{10}	0.50×10^{10}
Males, 13-18	822	100	0.10	NA	0.10×10^{10}	NA
Females, 13-18	838	100	0.09	0.41	0.09×10^{10}	0.41×10^{10}
Males, 19-99	4,294	100	0.40	1.32	0.40×10^{10}	1.32×10^{10}
Females, 19-99	4,587	100	0.58	1.67	0.58×10^{10}	1.67×10^{10}
Total population	14,334	100	0.41	1.17	0.41×10^{10}	1.17×10^{10}

*Based on the 2011-2014 NHANES.
NA-the 90th percentile intake was difficult to calculate due to insufficient number of subjects.

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/day since powdered term infant formulas will contain 10^8 cfu *B. lactis* AD011/g.

Conventional food applications:

The intended use of 1.0×10^{10} cfu *B. lactis* AD011/serving in the selected food categories would result in the mean and 90th percentile intakes were estimated to be 1.36×10^{10} and 3.00×10^{10} cfu/person/day, respectively, in all users. In the total population, the mean and 90th percentile intakes are estimated to be 0.41×10^{10} and 1.17×10^{10} cfu/person/day, respectively. However, these EDIs are overly inflated since 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, it is hard to estimate 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 Nutrients Under the Intended Use

Corn starch and maltodextrin are subjected to 21 CFR 172.892 and 21 CFR 184.1444, respectively. Thus, EDIs of these carbohydrates from the diet 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

Humans are exposed to bifidobacteria by the use of probiotics and eating fermented foods (e.g. yogurt, cheese, fermented vegetables, and olives) as well as in the host's own microflora. Even with these sources, bifidobacteria rarely cause infections in humans.

Since April 2007, *B. lactis* AD011 has been legally marketed in Korea as an ingredient for dietary supplements and as a part of dietary supplements and functional foods at the recommended daily dose of up to $5x10^8$~$1.0x10^{10}$ *B. lactis* AD011 cells per day. The use of *B. lactis* AD011 in functional foods and dietary supplements delivers daily doses up to $1.0x10^{10}$ *B. lactis* AD011 cells (or $0.5x10^9$ *B. lactis* AD011 cells per each of two servings per day) to the Korean population. No serious adverse effects/events were reported by consumers.

BIFIDO has been licensed to manufacture health functional foods on March 24, 2004 (No. 2004001507047; *B. lactis* AD011) and has been manufactured and sold as a health functional food ingredient since April 23, 2007.

In addition, *Bifidobacterium* species have a history of safe food use in dairy food and supplement products. There are eight (8) species (*B. longum*, *B. longum* subsp. *infantis*, *B. breve*, *B. bifidum*, *B. adolescents*, *B. pseudolongum*, *B. lactis*, and *B. animalis*) listed in the International Dairy Federation (IDF) Bulletin No. 377: Inventory of Microorganisms with a Documented History of Use in Food (Morgensen et al., 2002). No cases of clinical infection have been reported from such use.

PART 6. BASIS FOR GRAS DETERMINATION

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 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. animalis* ssp. *lactis* HN019, Bi-07, Bl-04, and B420 strains (GRN 445 [FDA, 2013a]; up to $2x10^{11}$ 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 infant formulas and selected conventional foods (GRN 453, [FDA, 2013b]; up to $5x10^9$ 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, *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 Scientific Committee concluded that most *Lactobacillus* and *Bifidobacterium* species can be considered nonpathogenic to humans and, therefore, pose no specific safety concerns.

In Korea, *B. lactis* AD011 has received the Korean FDA's approval as a functional food ingredient.

6.B. Review of Safety Data

Safety assessment tests required by FAO/WHO were considered when evaluating the safety of *B. lactis* AD011. These tests included assessment of undesirable metabolic activities (e.g., biogenic amine production), determination of antimicrobial resistance factors, mammalian toxin production or hemolytic activity (only if the strain belongs to a species known to be a mammalian toxin producer or to have hemolytic potential), assessment of side effects in human studies, and assessment of postmarket epidemiological surveillance of adverse effects in consumers. The general safety of the *B. lactis* strains, including AD011, has been confirmed on the basis of sensitivity to a range of antibiotics and the absence of hemolysis, mucolytic activity, and biogenic amine production (Park and Yang, 2019; Appendix B). This review covers papers published until June 30, 2019.

The following summarizes the studies of Park and Yang (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. However, 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, strains (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), which have received the US FDA's 'no question' letters for the use as ingredients for infant formulas and/or selected conventional foods.
3. *B. lactis* AD011 was not observed to 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. *B. lactis* AD011 is genetically stable for 25 generations.

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

Except the whole genomic sequence of B. lactis AD011, the above listed results from a study by Park and Yang (2019) are not in the public domain. Such outcomes confirmed the literature information that *Bifidobacterium* species does not pose safety concerns. Thus, the unpublished status of the Park and Yang study (2019) has no impact on the overall conclusion of this GRAS determination even if qualified experts do not have access to such data and information, especially since no animal and human clinical studies reported adverse effects of *B. lactis* AD011 and other *B. lactis* strains.

All *Bifidobacterium* species are listed as Biosafety Level 1 organisms by the American Type Culture Collection, indicating that they are not known to cause disease in healthy human adults (http://www.atcc.org/common/catalog/numSearch/numResults. cfm?atccN um=25527). Species of the genus *Bifidobacterium* are considered to be non-pathogenic and non-toxigenic, and have generally been considered safe for food use (Boriello 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 food-grade bacteria. *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 (AHRQ, 2011; Kim et al., 2018; Picard et al., 2005).

6.B.2. Genetic Stability Test

The genetic variation of edible microorganisms possibly results in indel (i.e., gene deletion and insertion) and mutation. A critical consideration for commercializing probiotics is whether it is possible to maintain genetic safety over the long term. Theoretically, an evaluation of genetic stability requires 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 AD011 chromosome, deposited in GenBank under accession number CP001213, are also available from the Genome Encyclopedia of Microbes (GEM; http://www.gem.re.kr). The study by Park and Yang (2019) showed that the similarity in the genomic comparison of the 1st and 25th generations of samples was 99.99% via the Orthologous Average Nucleotide Identity (OrthoANI) analysis. The difference is assumed to result 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 B.

6.B.3. Absence of Virulence Genes

The search for virulence factors in *B. lactis* AD011 was completed using the VirulenceFinder1.5 Server, which is a component of the publicly available web-based tool for whole-genome sequencing (WGS) analysis hosted by the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org).

The database detects homologous sequences for the virulence genes related to *E. coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus* in WGS data (Joensen et al., 2014). The output consists of best-matching genes from BLAST analysis of the selected database against the submitted genomes of *B. lactis* AD011. The selected %ID threshold was set at 90%, and the selected minimum length at 60%. In the event of a matching result, the output would show information on the predicted virulence gene, the %ID, the length of query and database gene, the position of the hit in the contig, and the accession number of the hit. The genome sequence of *B. lactis* AD011 was compared with the genome sequences of four well-known pathogens (*E. coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus* [*S. aureus*]). No virulence factors were found in the genomic sequence of *B. lactis* AD011. The results showed that the genomic sequence of *B. lactis* AD011 did not include toxigenic or pathogenic genes related to *E. coli*, *Enterococcus*, *Listeria*, or *S. aureus*.

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

To distinguish antibiotic resistance from antibiotic susceptible microorganisms, EFSA has established microbiological cut-off values for the antibiotic resistance of microorganisms used as food and/or feed additives. EFSA based these cut-off values on the distribution of the chosen antimicrobials' MICs in cell populations belonging to a single taxonomical unit (EFSA, 2012). 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. MIC values for all bacterial isolates were determined by the ISO 10932:2010 broth microdilution procedure, as described in Park and Yang (2019).

All *Bifidobacterium* spp. in the study by Kim et al. (2018) were susceptible to ampicillin, chloramphenicol, clindamycin, erythromycin, penicillin G, rifampicin, and vancomycin (MIC ranging from 0.01 to 4 μg/mL) and generally resistant to aminoglycoside antibiotics such as gentamicin, kanamycin, neomycin, and streptomycin (Table 10).

In general, the MIC values of *B. lactis* AD011 were equal to or lower than the established cut-off values suggested by the EFSA's Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (EFSA, 2012): the MIC values of *B. lactis* AD011 for ampicillin, streptomycin, erythromycin, vancomycin, chloramphenicol, and clindamycin were 0.5, 128, 0.063, <0.25, 2, and <0.032, respectively. The exceptions were gentamicin and tetracycline, whose MIC values of *B. lactis* AD011 were slightly higher than those established by EFSA cut off points (*B. lactis* AD011 vs. EFSA cutoff - gentamycin: 256 vs. 64; tetracycline: 16 vs. 8). It is noteworthy that the MIC values of *B. lactis* AD011 for gentamicin was equal to the established cut-off value suggested by the PROSAFE (256 μg/mL). The MIC value of *B. lactis* AD011 for

tetracycline was comparable to those of other GRAS strains, such as *B. lactis* BB-12, HN019, Bl-04, B420, and Bf-6 strains (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), which have received the US FDA's 'no question' letters for the use as ingredients for infant formulas and/or selected conventional foods.

As shown in Table 10, most *Bifidobacterium* species were shown to have resistance to tetracycline. Tetracycline resistance in *B. animalis* subsp. *lactis* has been shown to be 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), Masco et al. (2006), and Aires et al. (2007) consistently found *tet*(W) in all strains they tested. Gueimonde et al. (2010) also determined that "*tet*(W) is necessary and sufficient for the tetracycline resistance seen in *B. animalis* subsp. *lactis*." Noting the presence of the transposase gene, the authors, nevertheless, concluded that there is no evidence that *tet*(W) in *B. animalis* subsp. *lactis* is transmissible. The *tet*(W) is chromosomally located, and it is not associated with the conjugative transposon TnB1230, found in some other *tet*(W)-positive bacteria (Kastner et al., 2006; Masco et al., 2006; Matto et al., 2007). 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.

EFSA cutoffs are not available for the following 13 antibiotics: penicillin, carbenicillin, methicillin, dicloxacillin, kanamycin, neomycin, cephalothin, polymyxin B, metronidazole, rifampicin, phosphomycin, mupirocin, and trimethoprim-sulfamethoxazol. The MICs of *B. lactis* AD011 for penicillin, carbenicillin, methicillin, dicloxacillin, kanamycin, neomycin, cephalothin, metronidazole, rifampicin, phosphomycin, and trimethoprim-sulfamethoxazol were 0.25, 2, 2, 8, 1,024, 512, 32, 256, 256, 2, 64, and <0.5, respectively; these values were comparable to or lower than the MICs for other GRAS strains (*B. breve* M-16V and *B. lactis* BB-12). The MIC values of *B. lactis* AD011 for mupirocin was significantly lower than other GRAS strains (32 vs. >128) and that for metronidazole was significantly higher than those of other GRAS strains (256 vs. 4-8). The MIC value of *B. breve* M-16V for polymixin was significantly higher than *B. lactis* AD011 and BB-12 strains (1,024 vs. 256).

Ampicillin, vancomycin, gentamicin, and erythromycin are known as frequently used antibiotics in pediatric patients. For *B. longum* BORI, none of these pediatric antibiotics had MIC values exceeding EFSA/PROSAFE breakpoints.

Overall, the MIC values were comparable between *B. lactis* AD011 and other GRAS strains (*B. lactis* BB-12, HN019, Bl-04, B420, and Bf-6 strains, and *B. breve* M-16V), which received FDA's no question letters (FDA, 2002, 2011, 2013a, 2013b, 2013c, 2013d).

Table 10. Antimicrobial Susceptibility of *B. lactis* AD011 and Other *Bifidobacterium* spp. (MIC values, ug/mL)

Data source	EFSA MIC cut off	PRO-SAFE	Current notice	*B. lactis* strains (GRN 445)				GRN 377	Kim et al., 2018		GRN 453, 454, and 455
Antibiotic	*Bifidobac-terium*		*B.lactis* AD011	HN019	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	0.125-0.25
Gentamicin sulfate	64	256	256	64	64	256	64	64	128	128	32-128
Streptomycin sulfate salt	128	256	128	64	8	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	0.05	<0.03	0.05	0.032–0.5	0.125	0.125	0.016-0.25
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	<0.03-0.06	<0.032	0.063	0.032-0.125
Penicillin G	N/R	0.5	0.25					0.5	0.125	0.25	<1.52
Carbenicllin 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	
Neomycin sulfate	N/R		512						512	1024	>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						4	8	15.6-31.3
Rifampicin	N/R	2	2					2	2	1	
Phosphomycin disodium salt	N/R		64						64	32	NA
Mupirocin	N/R		32						>128	>128	NA
Trimethoprim-Sulfamethoxazole	N/R		<0.5						1	2	32-128

N/R= not required; NA= not applicable. GRN 49 and 268, FDA, 2002, 2009; GRN 377, FDA, 2011; GRN 445, 453-455, FDA, 2013a-2013d.

6.B.5. Antibiotic Resistance Transferability Test

Antibiotic resistance transferability studies were conducted to confirm the nature of this resistance. Conjugal transfer of antibiotic resistance was assessed via the 1987 Tannock method as described in Park and Yang (2019). Equal bacterial cell volumes (1 mL) of the donor and recipient strains were mixed and centrifuged at 7,000×g for 10 min. After disposing the supernatant, the bacterial cell pellet was resuspended in the de Man-Rogosa-Sharpe (MRS) broth medium and cultivated in an anaerobic chamber at 37°C for 12 h. The collected bacterial cells were filtered through a 0.45 μm micro-filter membrane. The membrane was placed on the surface of the MRS agar and incubated anaerobically at 37°C for 24 h. The bacterial cells were washed with 4 mL of 0.9% sterile saline, diluted to 10^{-3}, 10^{-4}, and 10^{-5}, respectively, and then plated on MRS agar-containing tetracycline. The plates were incubated aerobically or anaerobically at 37°C for 36 h.

Tetracycline resistance transferability tests were conducted using *L. fermentum* AGBG1, a recipient strain that is highly susceptible to tetracycline. The antimicrobial susceptibility test found that while *B. lactis* AD011 was resistant to tetracycline (MIC of 16 μg/mL). However, the tetracycline resistance of *B. lactis* AD011 was not transferred to the recipient, *L. fermentum* AGBG1, in this study. *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 the strain is not likely to have transmissible antibiotic resistance genes. In addition, *B. lactis* AD011 does not contain 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 probiotics. In this study, *B. lactis* AD011 and *Enterococcus faecium* KCTC13225 were anaerobically cultured in a Brain Heart Infusion (BHI) (BD BBL™, NJ, USA) medium at 37°C for 5 days.

The ammonia production of *B. lactis* AD011 was assessed to verify the safety of these probiotics. In this study, *B. lactis* AD011 and other probiotic strains did not produce ammonia. In contrast, *Enterococcus faecium* KCTC13225, used as the positive control, produced 109.3 ± 7 μg/mL of ammonia. The study found no indication of the ammonia production by *B. lactis* AD011. Details are described in Appendix B.

6.B.7. Hemolytic 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 this study, the potential hemolytic activity of *B. lactis* AD011 was assessed using the blood agar plating method.

B. lactis AD011 was anaerobically cultured in blood agar (BHI broth medium supplemented with 1.5% agar and 5% sheep blood) at 37°C for 2 days as described by Park and Yang (2019). *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. Details are presented in Appendix B.

6.B.8. Biogenic Amine Production Test

To evaluate if the *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. The biogenic amines were extracted and analyzed by high performance liquid chromatography (HPLC) as described by Kim et al. (2018). *B. lactis* AD011 did not produce cadaverine, histamine, tyramine, or putrescine. Details are described in Appendix B.

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. This bacterial translocation has the potential to cause sepsis and is one of the most serious probiotic safety concerns. In this study, the translocation capability of *B. lactis* AD011 was measured using *in vitro* mucolytic assays (Park and Yang 2019, Appendix B).

B. lactis AD011 did not use mucin as a carbon source for their growth. *B. lactis* AD011 did not degrade mucin, indicating that the strain is not capable of damaging intestinal surfaces and do not have translocational abilities. Details are described in Appendix B.

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

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

One animal efficacy study of *B. lactis* AD011 was identified from the literature (Table 11). Although this study 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. This review includes a study of live *B. lactis* AD011 strain, which has been published by June 30, 2019.

Kim et al. (2008) investigated if orally administrated probiotics could suppress allergic responses in an ovalbumin (OVA)-induced allergy mouse model. Thus, female C3H/HeJ mice were orally sensitized with OVA and cholera toxin for 4 weeks. Mice were fed 0.2% of each of lyophilized *B. lactis* AD011 ($1x10^{10}$ cfu/g), *Lactobacillus acidophilus* AD031 ($1.5x10^{10}$ 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. Daily intake of *B. lactis* AD011 at doses of 0.2% in diet (or $1.0x10^{10}$ cfu/g) did not cause any adverse effects in mice.

Table 11. Animal Efficacy Studies of *B. lactis* AD011

Objective	Animal	Dose	Duration	Measurements	Reference
To investigate if probiotic bacteria function as allergic immune modulators to prevent food allergy	30 C3H/HeJ mice, female, 6 wk old, sensitized with ov-albumin (OVA) and cholera-toxin (CT) for 4 wk	0.2% of lyophilized *B. lactis* AD011 (1.0×10^{10} cfu/g), *L. acidophilus* AD031 (1.5×10^{10} cfu/g), or the mixture of the 2 strains	Probiotics-7 wk starting 2 wk before (pre-treatment group) and 1 wk after (post-treatment group) the initial sensitization	Serum OVA-specific IgE, IgG1, IgG2a; spleen IL-6, IL-18 and IFN-γ levels; total and OVA-specific Ig A in fecal samples; allergy symptoms on the tail; histology (Mast cell degranulation during food allergy response); hypersensitivity reactions; allergic symptoms in the tail; body weight gain	Kim et al., 2008

6.B.12. Human Clinical Studies

As shown in Table 12, consumption of *B. lactis* AD011 (up to $1x10^{10}$ lyophilized cells/day) along with 2-3 other probiotics (total probiotics of up to 40 billion cfu/day) has been proven safe in pregnant women, infants, and adult subjects with irritable bowel syndrome (IBS).

Our review is extended to the *B. lactis* BB-12 strain, which has over 99.85% similarity in whole genomic sequences with the AD011 strain (Table 13). In the studies of *B. lactis* BB-12 in adults, a daily dose up to 10^{11} cfu for 8 weeks was well tolerated with no adverse effects (Min et al., 2012). In children, daily doses up to 10^{10} cfu for 90 days were well tolerated (Merenstein et al., 2010). In pregnant women and offspring pairs, a daily dose of $5x10^{10}$ cfu for 3-4 months was well tolerated with no side effects (Shei et al., 2017). In infants, daily doses up to 10 billion cfu for up to 2 years or approximately $4\text{-}6x10^{12}$ cfu for 4 months were well received with no adverse effects (Kirjavainen et al., 2002; Taipale et al., 2016).

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

In a randomized, double-blinded, placebo-controlled trial, Kim et al. (2010) investigated whether supplementation of probiotics prevented the development of eczema in infants at high-risk. Pregnant women with a family history of allergic diseases received a daily supplement of either a probiotic mixture composed of 4 viable lyophilized bacteria species (*B. lactis* AD011, *B. bifidum* BGN4, *L. acidophilus* AD031, and *Lactobacillus casei* IBS04; $1.6x10^{9}$ cfu each) or placebo, starting at 4-8 weeks before delivery and continuing until 6 months after delivery. Infants were fed the same powder dissolved in breast milk, infant formula, or sterile water from 4 to 6 months of age. Infants were exclusively breastfed during the first 3 months and were subsequently fed with breastmilk or cow's milk formula from 4 to 6 months of age. Mothers and infants in the placebo group took maltodextrin and alpha-corn without probiotic bacteria. The prevalence of eczema at 1 year in the probiotic group was significantly lower compare to the placebo group (40.0% vs 18.2%, P=0.048). The cumulative incidence of eczema during the first 12 months was reduced significantly in the probiotic group (62.9% vs 36.4%, P=0.029); however, there was no difference in total serum IgE level of the sensitization against food allergens between the two groups. Prenatal and postnatal supplementation with probiotics containing *B. lactis* AD011 was an effective approach in preventing the development of eczema in infants at high risk of allergy during the first year of life. No adverse effect of the probiotics, including *B. lactis* AD011, were reported.

Hong et al. (2009) assessed the immunomodulatory effects of probiotics in adults with IBS in a prospective double-blinded, randomized, placebo-controlled clinical study. IBS patients who met Rome III criteria were randomly assigned to receive probiotics with a total of 20 billion lyophilized bacteria (a mixture of *B. lactis* AD011, *B. bifidum* BGN4, and *L. acidophilus* AD031; twice daily [$1x10^{10}$ lyophilized cells/each; total $4x10^{10}$ cells]) or placebo for 8 weeks. Probiotics significantly reduced pain after 8 weeks of treatment compared to the placebo (-17.7 vs -31.9, P=0.045). No adverse effect of the probiotics, including *B. lactis* AD011, were reported.

Table 12. Human Clinical Studies of *B. lactis* AD011

Objective	Subject	Dose	Duration	Measurements	Results	Reference
To investigate if supple-mentation of probiotics prevents the development of eczema in infants at high risk	112 pregnant women and 68 infants	Probiotic mixture of *B. lactis* AD011, *B. bifidum* BGN4, and *L. acidophilus* (1.6 x 10^9 cfu each)	Mothers, ~ 5 mo (8 wk before expected delivery to 3 mo after delivery); Infants from 4 to 6 mo of age; FU of infants at 1 y	Occurrence of eczema in infants; Six Area Sis Sign in Atopic dermatitis score; total and specific IgE against food allergens	The prevalence of eczema at 1 y in the probiotic group was lower than in the placebo group (18.2% vs. 40.0%, p = 0.048). The cumulative incidence of eczema during the first 12 months was reduced in the probiotic group (36.4% vs. 62.9%, p = 0.029); however, there was no difference in serum total IgE level or the sensitization against food allergens between the two groups.	Kim et al., 2010
To assess the effects of strains of probiotics in Korean adults with irritable bowel syndrome (IBS)	70 patients w/ presence of previous gastro-intestinal symptoms suggestive of IBS (19-75 y)	Probiotic mixture of *B. lactis* AD011, *B. bifidum* BGN4, *L. acidophilus*, and *L. casei* (total 1x10^{10} lyophilized cells/each; total 4 x 10^{10} cells)	8 wk	Daily diary of bowel habits (frequency and consistency); Questionnaire on IBS; Questionnaire on quality-of-life; Symptoms score	Probiotic group had significant reductions in pain (abdominal pain, defecation discomfort, and sum of scores) after 8 weeks of treatment: probiotic vs. control: −31.9 vs. −17.7 (p=0.04). Probiotics containing *B. lactis* AD011, was safe and effective, especially in patients who excrete normal or loose stools.	Hong et al., 2009

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

Due to abundance of the literature, our review limited to the published studies conducted on up to 4 probiotic strains including *B. lactis* BB-12. Table 13 summarizes the efficacy or safety studies of the *B. lactis* BB-12 strain in various populations as listed below:

1) adults (Eskesen et al., 2015; Gueimonde et al., 2016; Kabeerdoss et al., 2011; Kekkonen et al., 2008; Lee et al., 2017a, b; Meng et al., 2016, 2017; Merenstein et al., 2015; Min et al., 2012),
2) Pregnant women (Dolatkhah et al., 2015; Schei et al., 2017),
3) children (Hojsak et al., 2015, 2016; Merenstein et al., 2010; Tan et al., 2017), and
4) infants (Holscher et al., 2012; Kirjavainen et al., 2002; Laursen et al., 2017; Mihatsch et al., 2010; Mohan et al., 2006, 2008; Taipale et al., 2011, 2012, 2016).

In the studies of *B. lactis* BB-12 in adults, a daily dose up to 10^{11} cfu for 8 weeks was tested with no adverse effects (Min et al., 2012). In children, daily doses up to 10^{10} cfu for 90 days were well tolerated (Merenstein et al., 2010). In pregnant women and offspring pairs, a daily dose of $5x10^{10}$ cfu for 3-4 months was well tolerated with no side effects (Schei et al., 2017). In infants, daily doses up to 10 billion cfu for up to 2 years or approximately 80 billion cfu/kg bw/day (corresponding to approximately 4 - 6 x 10^{12} cfu/person/day) for 4 months were well received in infants with no adverse effects (Kirjavainen et al., 2002; Taipale et al., 2016).

Overall, it is concluded that daily doses of up to 4 - 6 x 10^{12} cells *B. lactis* BB-12 resulted in no adverse effects on the measured outcomes in humans.

Table 13. Human Clinical Studies of *B. lactis* BB-12

Objective	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 containing high-dose *B. lactis* BB-12 ($\geq 10^{11}$ cfu/bottle), acacia dietary fiber, together with the two classic yogurt starter cultures, *S. thermophilus* ($\geq 3\times10^{9}$ cfu/bottle) and *L. acidophilus* ($\geq10^{9}$ cfu/ bottle); control	8 wk; P	Abdominal symptoms and bowel habits; improvement of overall IBS symptoms	Min et al., 2012
To evaluate the effects of three potentially anti-inflammatory probiotic bacteria on immune variables	62 healthy adults (mean age 44 y)	4 groups: Milk-based drink containing ~$3.5\text{x}10^{10}$ cfu/d *B. lactis* BB-12, ~$1.6\text{x}10^{10}$ cfu/d *L. rhamnosus* GG, or ~$3.3\text{x}10^{10}$ cfu/d *P. shermanii* JS; or placebo drink	3 wk of probiotic period followed by placebo for 3 wk	Blood cells (leukocytes, monocytes, and lymphocytes) and immunoglobulins (IgA, IgG, and IgM); serum hsCRP; serum cytokine (TNF-α, IL-6, IFN-γ, and IL-10); fecal microbiota	Kekkonen et al., 2008
To investigate the effect of *B. lactis* BB-12 on defecation frequency and gastrointestinal well-being.	1,248 healthy adults with low defecation frequency (3-4x/wk) and abdominal discomfort (mean 37.1-37.4 y)	3 groups: 1 or 10 billion cfu/d of *B. lactis* BB-12; placebo (capsule)	4 wk; P	Defecation frequency; gastrointestinal well-being; No obvious differences in adverse events and gastrointestinal symptoms between the treatment groups in the number of AE or the number of subjects with events, indicating that the *B. lactis* BB-12 is considered safe.	Eskesen et al., 2015

To evaluate the effect of *B. lactis* BB-12 on immune responses and whether the immune response to BB-12 differed depending on the delivery matrix.	30 healthy adults (mean 28.0 y)	$1x10^{10}$ cfu/d *B. lactis* BB-12 added before or after yogurt fermentation; yogurt smoothie	4 wk; X	Cytokine secretion in peripheral blood mononuclear cells (TNF-α and IL-6); incidence and severity of cold/flu infection; No adverse effects of probiotic yogurt containing *B. lactis* BB-12 were reported.	Meng et al., 2017
To investigate the effect of *B. lactis* BB-12, on natural killer (NK) and T-cell function in conjunction with self-reported cold/flu outcomes.	30 healthy adults (18-40 y)	$1x10^{10}$ cfu/d *B. lactis* BB-12 added before or after yogurt fermentation; $1x10^{10}$ cfu/d *B. lactis* BB-12 capsule; yogurt smoothie	4 wk; X	T-cell proliferation; cytokine secretion in peripheral blood mononuclear cells (IFN-γ, TNF-α, and IL-2); NK-cell cytotoxicity; No adverse effects of *B. lactis* BB-12 were reported.	Meng et al., 2016
To determine the safety of *B. lactis* strain BB-12 -supplemented yogurt; to assess the ability of BB-12 to affect the expression of whole blood immune markers associated with cell activation and inflammatory response.	40 generally healthy adults who were prescribed antibiotics for a respiratory infection (mean age, 31 y)	4 oz of *B. lactis* BB-12-supplemented yogurt; control yogurt	10 d; P	Adverse events; whole blood immune markers associated with cell activation and inflammatory response; fecal microbiota; *B. lactis* BB-12-supplemented yogurt was safe and well tolerated when consumed by healthy adults concurrently taking antibiotics.	Meren-stein et al., 2015
To examine the effects of *B. lactis* BB-12 on lipids, lipoproteins, and fecal excretion of SCFAs	30 healthy adults (mean 28.2 y)	4 tests: 1) Yogurt smoothie with no *B. lactis* BB-12; 2) & 3) yogurt with $3.16x10^{9}$ cfu/d *B. lactis* BB-12 pre- or post-fermentation; or 4) $3.16x10^{9}$ cfu/d *B. lactis* BB-12 capsule	4 wk; X	Serum concentrations of lipids, glucose, insulin, and CRP; fecal SCFA; dietary intakes and physical activity; No adverse effects of probiotic yogurt were observed.	Lee et al., 2017a

To investigate the impact of consuming dairy yogurt containing *L. paracasei*, *B. lactis*, and heat-treated *L. plantarum* on immune function	200 nondiabetic subjects (mean age 65.7 y)	120 mL/d dairy yogurt containing $1.2x10^9$ cfu/d each of *B. lactis* BB-12 and *L. casei* 431 and 0.0175% heat-treated *L. plantarum* nF1 or placebo (120 mL/d milk);	12 wk	Anthropometric measures; blood pressure; fasting serum concentrations of glucose and lipid profiles, albumin, white blood cells, hs-CRP, and Ig G1 and Ig G2; cytokines (TNF-α, IFN-γ, and IL-12); natural killer cell activity	Lee et al., 2017b
To test the effect of a probiotic yoghurt on fecal output of beta-defensin and immunoglobulin A in young healthy women eating a defined diet.	26 healthy women, (18-21 y, median 19 y)	200 mL/d normal yogurt for a wk, followed by probiotic yogurt containing 10^9 cfu/d *B. lactis* BB-12 for 3 wk, followed by normal yogurt for 4 wk	3 wk interven-tion	Fecal secretory IgA and beta-defensin 2; No side effects of probiotic yogurt were observed.	Kabeerd-oss et al., 2011
To investigate the impact of daily chewing of 2 different probiotic gums compared with placebo on saliva flow rate, saliva IgA levels, and saliva pH.	54 adult volunteers with hyposalivation, mean age 49.8 y	3 groups:1) 2.87×10^8 cfu *B. lactis* BB-12 per 2 gums; 2) total 3.35×10^8 cfu, equal amounts of *L. rhamnosus* LGG, *B. longum* 46, and *B. longum* 2C per 2 gums; or 3) placebo	12 wk; P	Salivary flow rate, pH, IgA, and bacteria; subjective symptoms. No side effects of probiotic or placebo chewing gums were observed.	Gueimon-de et al., 2016

Children					
To determine if consumption of yogurt containing a high dose of probiotics improves health in children attending daycare/school centers.	182 healthy children (aged 1-3 y)	Yogurt-based drink with or without *B. lactis* BB-12 (minimum of 10^{10} cfu/serving)	90 d; P	Missed days of school due to illness; presence of diarrhea; stool consistency; presence of respiratory infection; doctor visits; overall parental satisfaction; adverse events; 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 or without $1x10^{10}$ cfu/d *B. lactis* BB-12	10 d; P	Safety and tolerability (frequency and severity of adverse events); fecal microbiota; compliance; No adverse effects of *B. lactis* BB-12 were reported.	Tan et al., 2017
To investigate the role of *B. lactis* BB-12 in the prevention of common (gastrointestinal and respiratory) infections	210 healthy children who attend day care centers (mean age, 4.4 y)	10^9 cfu *B. lactis* BB-12 powder or placebo (maltodextrin powder)	3 mo; P	Number and duration of gastrointestinal symptoms and respiratory symptoms; No adverse effects of *B. lactis* BB-12 were reported.	Hojsak et al., 2016
To investigate the role of *B. lactis* in preventing nosocomial infections in the acute hospital setting	727 hospitalized children (aged 1–18 y; mean 10 y)	10^9 cfu/d *B. lactis* BB-12 powder or placebo (maltodextrin powder)	Duration of hospital stay; P	Number of children with common infections (gastrointestinal and respiratory infections); duration of symptoms; absence from day care due to infections; use of antibiotics; No adverse effects of *B. lactis* BB-12 were reported.	Hojsak et al., 2015

Pregnant women and/or Offspring Pairs					
To describe the 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 aged 29.6 y at delivery) and offspring pairs	Placebo (heat-treated fermented skimmed milk); probiotic milk ($5x10^{10}$ cfu/d each of *B. lactis* BB-12, *L. rhamnosus* GG, and *L. acidophilus* La-5)	From 36 gestation wk until 3 mo after birth, offspring follow up at 1 and 2 y; P	Maternal and offspring fecal mycobiota (gut fungi)	Schei et al., 2017
To assess the effect of a probiotic supplement containing four bacterial strains on glucose metabolism indices and weight changes.	64 women with gestational diabetes mellitus (26.5-28.2 y)	Probiotic capsule containing *B. lactis* BB-12, *L. acidophilus* LA-5, *S. thermophilus* STY-31, and *L. delbreudkii bulgaricus* LBY-27 (total > $4x10^9$ cfu); placebo	8 wk; P	Weight changes; glucose metabolism indices (fasting blood sugar, fasting serum insulin, HOMA-IR index, and quantitative insulin sensitivity check index)	Dolatkhah et al., 2015
Infants					
To study the impact of controlled administration of *B. lactis* BB-12 on the risk of acute infectious diseases.	109 healthy newborn infants (1 to <2 mo of life)	$1x10^{10}$ cfu/d *B. lactis* BB-12 or placebo tablets	Until 8 mo old; P	Fecal counts of *B. lactis* BB-12; cumulative incidence of acute respiratory infections and doctor-diagnosed acute otitis media, gastrointestinal symptoms, or use of antibiotics; No adverse effects of *B. lactis* BB-12 were reported.	Taipale et al., 2011
To study the effects of early administration of *B. lactis* BB-12	106 infants, 1 to 2 mo old	3 groups: 10^{10} cfu/d BB-12 or 2 types of polyols (200–600 mg of xylitol or sorbitol; tablet form)	From 1 mo to until 2 y of life; P	Oral colonization of *B. lactis* BB-12 and mutans streptococci; No adverse effects of *B. lactis* BB-12 were reported.	Taipale et al., 2012

on oral colonization of mutans streptococci and *B. lactis* BB-12.					
To study the impact of administration of *B. lactis* BB-12 on the risk of acute infectious diseases in healthy children.	109 1-mo-old healthy infants	10 billion cfu/d *B. lactis* BB-12 or placebo (tablet form)	From 1 mo to 2 y of life; P	Prevalence of respiratory tract infections, otitis media, fever, gastrointestinal infection; fecal recovery of *B. lactis* BB-12; adverse effects; No serious adverse effects of *B. lactis* BB-12 were reported.	Taipale et al., 2016
To investigate whether *B. lactis* reduces the incidence of nosocomial infections.	183 infants with very low birth weight (<1,500g, <30 wk of gestation)	Fortified human milk or preterm formula with or without *B. lactis* BB-12 ($1.2x10^{10}$ cfu/kg bw/d)	6 wk; P	Incidence and density of nosocomial infections; necrotizing enterocolitis; adverse events; No adverse effects of *B. lactis* BB-12 were reported.	Mihatsch et al., 2010
To investigate the role of *B. lactis* BB-12 supplementation in modifying the gut microbiota.	69 preterm infants (gestational age <37 wk), intervention from day 14 of life	Formula with or without $1.6x10^{9}$ cells of *B. lactis* BB-12 on days 1 to 3, then $4.8x10^{9}$ cells from day 4 onward; all infants with antibiotic therapy	21 d; P	Intestinal microbiota and bifidobacterial cell counts; occurrence of antibiotic-resistance bacterial groups; No adverse effects of *B. lactis* BB-12 were reported.	Mohan et al., 2006
To examine whether the oral application of *B. lactis* BB-12 may improve selected indicators of health status.	69 preterm infants (<37 gestation wk)	Formula with or without $1.6x10^{9}$ cells of *B. lactis* BB-12 on days 1-3, then $4.8x10^{9}$ cells on day 4-21	21 d; P	Weight gain; fecal concentrations of SCFAs, calprotectin, and IgA and pH; No adverse effects of *B. lactis* BB-12 were reported.	Mohan et al., 2008

To examine the effect of a combination of probiotics on absence from child care because of respiratory and gastrointestinal infections.	290 healthy infants (8-14 mo; mean 10.0 mo)	A mixture of 10^9 cfu/d *B. lactis* BB-12 and *L. rhamnosus* LGG or Placebo (maltodextrin powder)	6 mo; P	The number of days absence from child care due to respiratory and gastrointestinal infections and/or due to other illness; No adverse effects of *B. lactis* BB-12 were reported.	Laursen et al., 2017
To characterize the relationship between gut microbes and the extent of allergic sensitization	21 infants with early onset atopic eczema	Extensively hydrolyzed whey formula ± ~$8x10^{10}$ cfu/kg bw/d *B. lactis* BB-12	Weaning period (from 5.2 to 9.1 mo); P	Fecal microbiota (counts of *E. coli*, Bacteroides, Lactobacilli/enterococci, total cell counts, etc); No adverse effects of *B. lactis* BB-12 were reported.	Kirjavai-nen et al., 2002
To investigate the effect of infant starter formula containing *B. lactis* BB-12 on intestinal immunity and inflammation.	172 healthy, full-term infants (2-6 wk of age)	3 groups: Infant formula with or without providing 10^6 cfu *B. lactis* BB-12/g; breast- fed reference.	6 wk; P	Fecal secretory IgA, anti-poliovirus- and anti-rotavirus-specific IgA, calprotectin, lactate, and pH; No adverse effects of *B. lactis* BB-12 were reported.	Holscher et al., 2012

Study design: P= parallel; X= crossover study design

Bw=body weight; cfu= colony forming unit; CRP= C-reactive protein; d= days; HOMA-IR= homeostasis model assessment insulin resistance; hs-CRP= high sensitivity C-reactive protein; IFN= interferon; Ig= immunoglobulin; IBS=irritable bowel syndrome; IL= interleukin; mo= months; NK=natural killer; SCFAs= short chain fatty acids; TNF= tumor necrosis factor; wk= weeks; y= year.

Adults:

Min et al. (2012) investigated whether composite yogurt with *B. lactis* BB-12 ($\geq 10^{11}$ cfu/bottle) and acacia dietary fiber with two classic yogurt starter cultures, such as *Streptococcus thermophilus* ($\geq 3\times10^{9}$ cfu/bottle) and *L. acidophilus* ($\geq10^{9}$ cfu/bottle), has additive effects in IBS. A total of 130 patients (mean age 35.8 y) were randomly allocated to consume, twice daily for 8 weeks, either the probiotic composite yogurt or the control product. IBS symptoms and improvement in bowel habits were evaluated using the visual analog scale *via* a structured questionnaire administered at baseline and after treatment. No adverse effects of *B. lactis* BB-12 were reported.

Kekkonen et al. (2008) evaluated the effects of three potentially anti-inflammatory probiotic bacteria from three different genera on immune variables, such as blood cells including eukocytes, monocytes, neutrophils, basophils, and lymphocytes, immunoglobulins, and serum cytokines (TNF-α, IL-6, IFN-γ and IL-10), in healthy adults in a clinical setting. The 62 volunteers were randomized to receive one of three milk-based drinks containing *B. lactis* BB-12 (~$3.5x10^{10}$ cfu/d), *L. rhamnosus* GG (~$1.6x10^{10}$ cfu/d), or *P. shermanii* JS (~$3.3x10^{10}$ cfu/d) during the 3 week intervention period, which was followed by a placebo drink containing no probiotic bacteria for 3 weeks. Venous blood and saliva samples were taken at baseline and on days 1, 7, and 21. Fecal samples were collected at baseline and at the end of intervention. There were no differences between the groups during the intervention. No adverse effects of *B. lactis* BB-12 were reported.

Eskesen et al. (2015) investigated the effect of *B. lactis* BB-12 on two primary endpoints, defecation frequency and gastrointestinal well-being, in healthy, mildly constipated adults with low defecation frequency (2-4 times/week) and abdominal discomfort. After a 2-week run-in period, 1,248 subjects were randomized to 1 or 10 billion cfu/d of the probiotic strain Bb-12 or a matching placebo capsule once daily for 4 weeks. There were no obvious differences in adverse events and gastrointestinal symptoms between the treatment groups in the number of adverse events or the number of subjects with events. Based on these data, the Bb-12 probiotic strain is considered safe.

Meng et al. (2017) evaluated the effect of *B. lactis* BB-12 at a dose of $1x10^{10}$ cfu/day on immune responses in a randomized, partially blinded, 4-period crossover, free-living study, and whether the immune response to *B. lactis* BB-12 differed depending on the delivery matrix. Healthy adults (n=30), aged 18-40 years, were recruited and received four treatments in a random order: (A) yogurt smoothie alone, smoothie with *B. lactis* BB-12 added (B) before or (C) after yogurt fermentation, or (D) *B. lactis* BB-12 given in capsule form. At baseline and after each 4-week treatment, peripheral blood mononuclear cells were isolated, and functional and phenotypic marker expressions, including cytokine secretion in peripheral blood mononuclear cells (TNF-α and IL-6), and incidence and severity of cold/flu infection were assessed. No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

Meng et al. (2016) investigated the effect of *B. lactis* BB-12 on natural killer (NK) and T-cell function in conjunction with self-reported cold/flu outcomes in healthy adults. In a

randomized, partially blinded, four-period crossover study, healthy adults (n=30) were recruited, and received four treatments for 4 weeks in a random order: (i) yogurt smoothie alone (yogurt), smoothies with *B. lactis* BB-12 added (ii) before (PRE) or (iii) after (POST) yogurt fermentation, or (iv) *B. lactis* BB-12 capsule. Measurements included T-cell proliferation, cytokine secretion in peripheral blood mononuclear cells (IFN-γ, TNF-α, and IL-2), and NK-cell cytotoxicity. No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

Merenstein et al. (2015) determined the safety of *B. lactis* BB-12-supplemented yogurt when consumed by a generally healthy group of adults who were prescribed a 10-day course of antibiotics for a respiratory infection. Secondary aims were to assess the ability of *B. lactis* BB-12 to affect the expression of whole blood immune markers associated with cell activation and inflammatory response. Forty participants were randomly assigned to consume 4 ounces of either *B. lactis* BB-12-supplemented yogurt or non-supplemented control yogurt daily for 10 days. The primary outcome was to assess the safety and tolerability, assessed by the number of reported adverse events. A total of 165 non-serious adverse events were reported, with no differences between the control and the *B. lactis* BB-12 groups. When compared to the control group, *B. lactis* fecal levels were modestly higher in the *B. lactis* BB-12-supplemented group (0.017 vs. 0.001%, $p<0.05$). *B. lactis* BB-12-supplemented yogurt was safe and well tolerated when consumed by healthy adults concurrently taking antibiotics.

Lee et al. (2017a) examined the effects of *B. lactis* BB-12 ($3.16x10^9$ cfu/day) on lipids, lipoproteins, and fecal excretion of short chain fatty acids (SCFAs) in healthy adults. In a randomized, partially blinded, 4-period, crossover study, 30 adults (11 men, 19 women), aged 18-40 years, were randomly assigned to: 1) yogurt smoothie with no BB-12 (yogurt control), 2) yogurt smoothie with BB-12 added pre-fermentation (PRE), 3) yogurt smoothie with BB-12 added post-fermentation (POST), and 4) BB-12 containing capsule. Serum lipids/lipoproteins, glucose, insulin, C-reactive protein (CRP), and fecal SCFAs were measured at baseline and after each treatment period. No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

Lee et al. (2017b) investigated the impact of consuming dairy yogurt containing $1.2x10^9$ cfu/d each of *B. lactis* BB-12, *L. paracasei* ssp. *paracasei* (*L. paracasei*), and heat-treated *L. plantarum* on immune function in 200 nondiabetic subjects. Over a twelve-week period, the test group consumed dairy yogurt containing probiotics each day, whereas the placebo group consumed milk. Measurements included anthropometric measures, blood pressure, fasting serum concentrations of glucose, lipids, albumin, white blood cells, hs-CRP, Ig G1, Ig G2, and cytokines (TNF-α, IFN-γ, and IL-12), and natural killer cell activity. No adverse effects of probiotics were reported on the measured outcomes.

Kabeerdoss et al. (2011) tested the effect of a probiotic yogurt on fecal output of beta-defensin and immunoglobulin A in a group of young healthy women eating a defined diet. A total of 26 women, aged 18-21 (median 19) years, residing in a hostel were given 200 mL normal yogurt every day for a week, followed by probiotic yogurt containing *B. lactis* BB-12 (10^9 in 200 mL) for 3 weeks, followed again by normal yogurt for 4 weeks. Stool samples

were collected at 0, 4, and 8 weeks and assayed for immunoglobulin A and human beta-defensin-2 by ELISA. All participants tolerated both normal and probiotic yogurt well. No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

Gueimonde et al. (2016) investigated the impact of daily chewing for 12 weeks of 2 different probiotic gums compared with placebo on saliva flow rate, saliva IgA levels, and saliva pH. The intervention study included 54 adult volunteers with hyposalivation in a double-blinded, randomized, and placebo-controlled design with 3 parallel groups. Volunteers were randomly assigned to 3 different groups: subject in group A (n=19) were given placebo chewing gum, group B (n=17) received *B. lactis* BB-12 (ATCC 27536; 2.87×10^8 cfu per 2 gums), and group C (n=18) received a probiotic mixture (a total of 3.35×10^8 cfu per 2 gums), which is composed of equal amounts of *L. rhamnosus* LGG, *B. longum* 46, and *B. longum* 2C, for 3 months. Two volunteers from the BB-12 group left the study for personal reasons leaving 19, 15, and 18 volunteers, respectively, for analyses. Clinical examinations, personal interviews, and salivary analysis (flow rate, pH, and IgA concentration) were conducted at baseline and after 1, 2, 3, and 4 months. No side effects of the probiotic or placebo chewing gums were observed.

Children:

Merenstein et al. (2010) determined if consumption of yogurt containing a high dose of probiotics improves health in 182 children, aged 1-3 years, attending daycare/school centers. Yogurt-based drink supplemented with or without *B. lactis* BB-12 were tested in 182 children who attended daycare centers at least 3 days a week to determine if a probiotic-containing yogurt-based drink improves overall parental satisfaction due to decreased absences from work and an overall healthier child. Measurements included missed days of school due to illness, presence of diarrhea, stool consistency, presence of respiratory infection, doctor visits, overall parental satisfaction, and adverse events. No adverse effects of *B. lactis* BB-12 were reported.

Tan et al. (2017) determined the safety of *B. lactis* BB-12-supplemented yogurt when consumed by a generally healthy group of children. The secondary aim was to assess the effect of *B. lactis* BB-12-supplemented yogurt on the gut microbiota of children. 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. The primary outcome was to assess the safety and tolerability, as determined by the number of reported adverse events. The secondary outcome was the gut microbiota. *B. lactis* BB-12 supplemented yogurt is safe and well-tolerated when consumed by healthy children. No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

Hojsak et al. (2016) investigated the role of *B. lactis* BB-12 in the prevention of common (gastrointestinal and respiratory) infections in healthy children who attend day care centers. A randomized, double-blinded, placebo-controlled trial was conducted with 210 children who attend day care centers. They were randomly allocated to receive placebo (placebo group, n=106) or *B. lactis* BB-12 at a dose of 10^9 cfu/day (Intervention group, n=104) during the 3-month intervention period. Measurements included mean number of

infections per child, duration of symptoms, number of children with gastrointestinal and respiratory tract infections, absence from day care center due to infections, use of antibiotics, and exploratory infections (type of gastrointestinal and respiratory tract infection). No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

Hojsak et al. (2015) investigated the role of *B. lactis* BB-12 in preventing nosocomial infections in the acute hospital setting in a randomized, double-blinded, placebo-controlled trial with 727 hospitalized children (aged 1-18 years; mean 10 years). The children were randomly allocated to receive placebo (placebo group, n=365) or *B. lactis* BB-12 at a dose of 10^9 cfu/day (intervention group, n=362) once daily for the entire duration of the hospital stay. Nosocomial infections were defined as infections that occurred >48 hours after hospital admission and that were not present or incubating at the time of admission. Primary outcomes included incidence of common nosocomial gastrointestinal and respiratory tract infections, and duration of common nosocomial infections between groups. Secondary outcomes were incidence of gastrointestinal and respiratory tract infections separately, duration of gastrointestinal and respiratory infections, duration of hospitalization, and exploratory outcomes (such as gastrointestinal and respiratory symptoms, severity of gastrointestinal and respiratory tract infections, and the use of antibiotics). No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

Pregnant women and/or offspring pairs:

Schei et al. (2017) described the gut mycobiota in 298 pairs of healthy pregnant women and their offspring from birth to 2 years of age. In a prospective cohort, 298 pairs of healthy mothers (mean 29.6 years) and their offspring from 36 week of gestation until 2 years of age (1,516 samples) were followed and explored the gut mycobiota in maternal and offspring samples. Half of the pregnant mothers were randomized to drink probiotic milk during and after pregnancy: from 36 week of gestation until 3 months postpartum. The probiotic bacteria included *B. lactis* BB-12, *L. rhamnosus* GG (LGG), and *L. acidophilus* La-5 ($5x10^{10}$ cfu/d each). Maternal and offspring fecal mycobiota (gut fungi) were measured. No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

Dolatkhah et al. (2015) assessed the effect of a probiotic supplement capsule containing four bacterial strains on glucose metabolism indices and weight changes in 64 women with newly diagnosed gestational diabetes mellitus. They were randomly assigned to receive either a probiotic containing *B. lactis* BB-12, *L. acidophilus* LA-5, *S. thermophilus* STY-31, and *L. delbrueckii* ssp. *bulgaricus* LBY-27 (total $4x10^9$ cfu/d) or placebo capsule along with dietary advice for 8 weeks. The trend of weight gain along with glucose metabolism indices was assayed. No adverse effects of the probiotics were reported on the measured outcomes.

Infants:

Taipale et al. (2011) studied the impact of controlled administration of *B. lactis* BB-12 on the risk of acute infectious diseases in 109 healthy newborn (1 month old) infants who were assigned randomly to a probiotic group receiving a *B. lactis* BB-12-containing tablet or to a control group receiving a control tablet. Test tablets were administered to the infants

twice a day (daily dose of 10 billion cfu *B. lactis* BB-12) from the age of 1-2 months to 8 months with a novel slow-release pacifier or a spoon. Breastfeeding habits, pacifier use, dietary habits, medications, and all signs and symptoms of acute infections were registered. At the age of 8 months, fecal samples were collected for *B. lactis* BB-12 determination (quantitative PCR method). The primary outcome measures for the study were the reported cumulative incidence of acute respiratory infections and doctor-diagnosed acute otitis media occurring before the age of 8 months. Successful intestinal passage of BB-12 was chosen as the secondary outcome measure. No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

A randomized clinical trial by Taipale et al. (2012) studied the effects of early administration of *B. lactis* BB-12 on oral colonization of mutans streptococci and *B. lactis* BB-12. In this double-blinded, placebo-controlled study, 106 infants received *B. lactis* BB-12, xylitol (X group), or sorbitol (S group). Test tablets were administered twice a day (from the age of 1-2 months) with a novel slow-release pacifier or a spoon (daily dose of 10^{10} cfu *B. lactis* BB-12 and 200-600 mg polyol). The families were informed that they could receive tablets and pacifiers until the child was 24 months old. Measurements included oral colonization of *B. lactis* BB-12 and mutans streptococci. Samples were collected from mucosa/teeth at the age of 8 months and 2 years for *B. lactis* BB-12 determination (qPCR) and plate culturing of mutans streptococci, lactobacilli, and yeasts. The mutans streptococci levels of the mothers were determined. Mean duration of tablet delivery was 14.9 ± 6.7 months. No adverse effects of *B. lactis* BB-12 were reported on the measured outcomes.

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-blinded, placebo-controlled study, 109 1-month-old infants were assigned randomly to a probiotic group receiving a *B. lactis* BB-12-containing tablet (n=55) or a placebo (n=54). Test tablets were administered to the infants twice a day (daily dose of 10 billion cfu *B. lactis* BB-12) until the age of 2 years with a novel slow-release pacifier or a spoon. Measurements included prevalence of respiratory tract infections, otitis media, fever, gastrointestinal infection, fecal recovery of *B. lactis* BB-12, and adverse effects. The authors concluded that administration of *B. lactis* BB-12 in early childhood may reduce respiratory tract infections with no adverse effects.

Mihatsch et al. (2010) investigated whether *B. lactis* BB-12 reduces the incidence of nosocomial infections in infants with very low birth weight (VLBW; <1,500 g) and less than 30 weeks of gestation. In a randomized, controlled trial, 183 VLBW infants and <30 weeks of gestation were stratified according to gestational age (23-26 and 27-29 weeks) and early antibiotic therapy (days 1-3, yes or no), and randomly assigned to have their milk feedings supplemented with *B. lactis* BB-12 (12 billion cfu/kg bw/day) or placebo for the first 6 weeks of life. The primary outcome was the 'incidence density' of nosocomial infections defined as the period of elevated C-reactive protein (CRP>10 mg/L) from day 7 after initiation of milk feedings until the 42nd day of life (number of nosocomial infections/total number of patient days). The main secondary outcome was necrotizing enterocolitis (NEC; ≥stage 2). Adverse events were not noted.

A double-blinded, placebo-controlled, randomized clinical study by Mohan et al. (2006) was performed on 69 preterm infants to investigate the role of *B. lactis* BB-12 supplementation in modifying the gut microbiota. No adverse effects of *B. lactis* BB-12 were reported.

Mohan et al. (2008) examined whether the oral application of *B. lactis* BB-12 (probiotic) may improve selected indicators of health status in 69 preterm infants (<37 gestation weeks) in a double-blinded, placebo-controlled, randomized clinical study. Measurements included weight gain, fecal concentrations of SCFAs, calprotectin, IgA, and pH. No adverse effects of *B. lactis* BB-12 were reported.

Laursen et al. (2017) examined the effect of a combination of probiotics on absence from child care because of respiratory and gastrointestinal infections in healthy infants, aged 8 to 14 months at the time of enrollment in child care. A total of 290 infants were randomly allocated to receive a placebo or a combination of *B. lactis* and *L. rhamnosus* in a dose of 10^9 cfu of each daily for a 6-month intervention period. The primary endpoint was the number of days absent from child care because of respiratory or gastrointestinal infections, which are defined as symptoms related to the respiratory or gastrointestinal tracts. The secondary endpoints were the number of days absent from child care because of other illnesses (not infections); the number of infants with doctor-diagnosed upper and lower respiratory tract infections; the number of infants with at least 1 episode of diarrhea; the duration of diarrheal episodes, vomiting, fever, and common cold; the number of doctor visits because of infections or other illnesses; the number of antibiotic treatments; and the number of days caregivers were absent from work because of infant illnesses. No adverse effects of *B. lactis* BB-12 were reported.

Kirjavainen et al. (2002) characterized the relationship between gut microbes and the extent of allergic sensitization, and assessed whether the efficacy of bifidobacterial supplementation in the treatment of allergy could relate to modulation of the intestinal microbiota. This randomized study included 21 infants with early onset atopic eczema of whom 8 were intolerant (highly sensitized group) and 13 tolerant (sensitized group). Infants were fed extensively hydrolyzed whey formula with or without *B. lactis* BB-12 (~80 billion cells/kg bw/day for 4 months starting from 5.2 to 9.1 months of age). In the highly sensitized group, the fecal microflora of infants in the *B. lactis* BB-12 group was analyzed only before weaning, whereas in the sensitized group the fecal microflora was analyzed both before and after weaning. No adverse effects of *B. lactis* BB-12 were reported.

Holscher et al. (2012) investigated the effect of infant starter formula containing *B. lactis* BB-12 on intestinal immunity and inflammation. Six-week-old healthy, full-term infants were enrolled in a prospective, randomized, double-blinded, controlled clinical trial with 2 groups studied in parallel to a breastfed comparison group. Formula-fed infants were randomized to partially hydrolyzed whey formula with or without *B. lactis* BB-12 at a daily dose of 10^6 cfu/g for 6 weeks. Measurements included fecal secretory IgA, anti-poliovirus-

and anti-rotavirus-specific IgA, calprotectin, lactate, and pH. No adverse effects of *B. lactis* BB-12 were reported.

Overall, daily supplementation of *B. lactis* BB-12 at doses over 10^{12} cfu for the period of 8 weeks to 4 months in infants and adults (Kirjavainen et al., 2002; Min et al., 2012) or up to 10^{10} cfu/day for 2 years even in infants (Taipale et al., 2016) were not associated with any adverse effects.

6.B.12.3. Human Clinical 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, Bl-04, B420, and Bf-6, did not result in any serious adverse effects on measured outcomes. The studies of other strains published between January 2011 and June 30, 2019 also found that daily doses up to $5x10^{10}$ cfu *B. lactis* did not cause any adverse effects in adults, infants, and young children (Islek et al., 2014; Merenstein et al., 2014). Details are described in Appendix D.

6.C. Potential Infection

Humans are exposed to bifidobacteria by the use of probiotics and eating fermented foods (e.g., yogurt, cheese, fermented vegetables, and olives) as well as 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 is 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 showed 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, and intended use) in this report and supplemented by the publicly available literature/toxicity data on *B. lactis* AD011 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 and used according to cGMP.

   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) whose whole genomic sequence has an over 99.85% similarity with that of 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. and Europe for over a decade. *B. lactis* AD011 has no hemolytic or mucolytic activities and does not produce biogenic amines and ammonia.
   4) The genomic sequence of *B. lactis* AD011 does not include toxigenic or pathogenic genes related to *E. coli*, *Enterococcus*, *Listeria*, or *S. aureus.*
   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 x10^{10} cells, has been safely used and no serious adverse events have been reported by the 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 and other microbial contamination.

Therefore, it is reasonable to conclude that daily intakes of up to 10^8 cfu *B. lactis* AD011/g in powdered infant formulas and 1x10^{10} cfu *B. lactis* AD011/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 decades. 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's no question letters on their safety and such information is widely available. 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, are manufactured 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 *B. lactis*, including *B. lactis* AD011, offers consumers benefits without 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.

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

Part of Appendix B, Park and Yang, 2019. Safety Evaluation of *B. lactis* AD011.

Except the whole genomic sequence of B. lactis AD011, the above listed results from a study by Park and Yang (2019) are not in the public domain. Such outcomes confirmed the literature information that *Bifidobacterium* species does not pose safety concerns. Thus, the unpublished status of the Park and Yang study (2019) has no impact on the overall conclusion of this GRAS determination even if qualified experts do not have access to such data and information, especially since no animal and human clinical studies reported adverse effects of *B. lactis* AD011 and other *B. lactis* strains.

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

Sequence homologies were examined by comparing the obtained sequences with those in the DNA databases (http://www.ncbi.nlm.nih.gov/BLAST). The strain was identified as *B. lactis* and was named *B. lactis* AD011.

Standard ID

16S rRNA Full Sequencing

macrogen

16S rRNA service report

Order Number : 180119KR-064
Sample name : B_lactis_AD011_contig_1

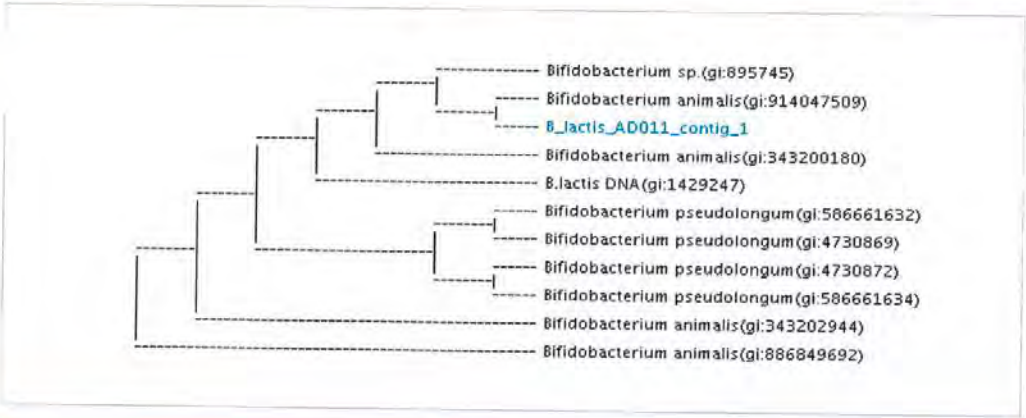
Information

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

Bifidobacterium은 운동성이 없고, 그람 양성이며 종종 가지가 있는 혐기성인 세균이다. 이들은 인간을 포함한 포유동물의 위장관, 질, 구강에서 볼 수 있는 흔한 균이다. Bifidobacteria는 포유류에서 colon flora를 형성하는 주요한 균 중 하나이고, 몇몇 Bifidobacteria는 활생균으로서 사용되곤 한다.

Bifidobacterium animalis는 그람 양성, 혐기성, 막대 모양 세균으로 인간을 포함한 포유 동물의 대장에서 발견 된다. B. animalis는 많은 식품 및 건강 보조 식품에 존재한다. 프로바이오틱은 주로 유제품에서 발견된다.

Characterization-Non-motile, gram-positive, anaerobic bacteria often with branches

3.2.2. 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	2905 41	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.

Safety Evaluation of *B. lactis* AD011

Prepared by
Myeong Soo Park, Ph.D., and SooYoung Yang
BIFIDO, Co., Ltd

Safety Evaluation of *B. animalis* subsp. *lactis* AD011

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 as a probiotic ingredient with no side effects in Korea, Germany, Poland, Singapore, Thailand, Turkey, and Vietnam.

However, given that the safety of some newly screened probiotic species has recently been debated, it is crucial that the consumer safety of each commercially utilized strain be confirmed. The aim of this study was to validate the safety of *B. lactis* AD011 in accordance with FAO/WHO guidance. The safety assessment included the analysis of whole genome sequence, ammonia production, hemolysis of blood cells, biogenic amine production, antimicrobial susceptibility pattern, antibiotic resistance transferability, mucin degradation, genome stability, and absence of virulence gene. The entire genomic sequence of *B. lactis* AD011 has been determined and published in GenBank (accession no.: CP001213.1), documenting the lack of retention of plasmids capable of transferring an antibiotic-resistant gene. Comparative genomics of AD011 showed very high sequence homology with other GRAS notified *B. lactis* strains BB-12 and Bl-04. This probiotic strain showed neither hemolytic activity nor mucin degradation activity, and it did not produce ammonia or biogenic amines (i.e., cadaverine, histamine putrescine, or tyramine). *B. lactis* AD011 showed higher resistance to gentamicin than the European Food Safety Authority (EFSA) cut-off. However, there was no related genes found in the genome of *B. lactis* AD011. Tetracycline-resistant genes are prevalent among *B. lactis* strains; *B. lactis* AD011 has a *tet*(W) gene on its chromosome DNA and has also shown resistance to tetracycline. However, this research shows that its tetracycline resistance was not transferred via conjugation with *L. fermentum* AGBG1, the latter of which is highly sensitive to tetracycline. Moreover, there was little genetic mutation between the first and 25th generations of *B. lactis* AD011, which shows its genetic stable characteristics. These findings support the continuous use of *B. lactis* AD011 as probiotics, of which has been reported as safe by several clinical studies, and has been used in food supplements for many years.

Objective and Methods

To evaluate the safety of *Bifidobacterium animalis* subsp. *lactis* strain AD011 (herein after referred to as '*B. lactis* AD011'), the following tests were conducted:

1. Whole Genomic Sequence to compare genomic sequences of *B. lactis* AD011 and other *B. lactis* strains, such as BB-12 and Bl-04,
2. Genetic Stability,
3. Absence of Virulence Genes,
4. Antimicrobial Susceptibility Test,
5. Antibiotics Resistance Transferability Test,
6. Hemolytic Activity Test,
7. Biogenic Amines Test,
8. Ammonia Production Test,
9. Mucin Degradation Test, and
10. Shelf-life of *B. lactis* AD011.

Results

It was found that *B. lactis* AD011, BB-12, and Bl-04 are very similar with genome sequence homology of 99.85% and 99.93% by ANI value and 99.99% by TNA value. The genome of *B. lactis* AD011 does not contain regions with significant homology to known antibiotic resistance, pathogenic, or toxigenic genes. Functional assays indicate that *B. lactis* AD011 exhibits antibiotic susceptibility; the exception was tetracycline resistance. However, the MIC values of *B. lactis* AD011 for tetracycline were higher than that established by EFSA but comparable to those of other GRAS strains, such as *B. lactis* BB-12 and *B. breve* M-16V. In addition, *B. lactis* AD011 was not observed to contain plasmids, have hemolytic and mucolytic activities, and produce clinically significant levels of biogenic amines and ammonia. *B. lactis* AD011 was shown to be genetically stable for 25 generations.

Conclusion

The data indicate that *B. lactis* AD011 is safe and is suitable for human use as a probiotic ingredient applicable to infant formulas, conventional foods, and/or dietary supplements.

1. Genetic Comparison of *B. lactis* AD011, *B. lactis* BB-12, and *B. lactis* Bl-04

1) Introduction

A complete genome sequence of an organism can be considered to be the ultimate genetic map, in the sense that the heritable characteristics are encoded within the DNA and that the order of all nucleotides along each chromosome is known. Identifying the genomic differences between two closely related strains of bacteria is important in order to establish potential probiotic characteristics (Delcenserie et al., 2007). Analysis of whole genome sequence is considered to be one of the gold standards to define taxonomy, phenotypic characteristics, and potential virulence. In this study, the whole genome sequence of *B. lactis* AD011 was obtained, and through comparative genomic analysis, the common features and phylogenetic differences among *B. lactis* strains AD011, BB-12, and Bl-04 have been pursued.

2) Method

B. lactis AD011 was obtained from BIFIDO Co., Ltd, and after properly cultured in BIFIDO R&D center, they were sent to Chunlab, Inc. to extract DNA and define whole genome sequence of *B. lactis* AD011 using PacBio_20K. The sequences were analyzed using CLgenomics™ program (http://www.chunlab.com/genomics/) for its comparison and annotation. The information is also available on EZBIOCLOUD (https://www.ezbiocloud.net/apps). Chunlab, Inc. (http://www.chunlab.com/) provides comprehensive genomics and bioinformatics solutions for genome sequencing, comparative genomics, transcriptomics, and microbial community analysis.

3) Result

3.1) Summary of Genome Information

B. lactis strains AD011, BB-12, and Bl-04 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 do not harbor a plasmid (Table 1).

B. lactis strains AD011, BB-12, and Bl-04 show over 98.5% homology in genome sequences: 99.85 to 99.93% by average nucleotide identity (ANI) values and 99.99% by tetra-nucleotide analysis (TNA) values. The analysis results imply that these strains share common characteristics and similar physiological function in the intestinal tract. The genome sequence of *B. lactis* AD011 has been deposited at GenBank under the accession number CP001213.1.

Table 1. Summary of Genome Information of *B. lactis* Strains

Strains	*B. lactis* AD011	*B. lactis* BB-12	*B. lactis* Bl-04
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	1074.5	1076.8
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: EzBioCloud Comparative Genomics Database by ChunLab, Inc. (http://cg.ezbiocloud.net/)
Data set: *Bifidobacterium animalis* subsp. *lactis strain*
Abbreviations: G=guanine; C=cytosine; CDS=coding sequence; bp=base pair; OrthoANI=orthologous average nucleotide identity.

The genome map of *B. lactis* strains AD011, BB-12, and Bl-04 are shown in Figures 1, 2, and 3.

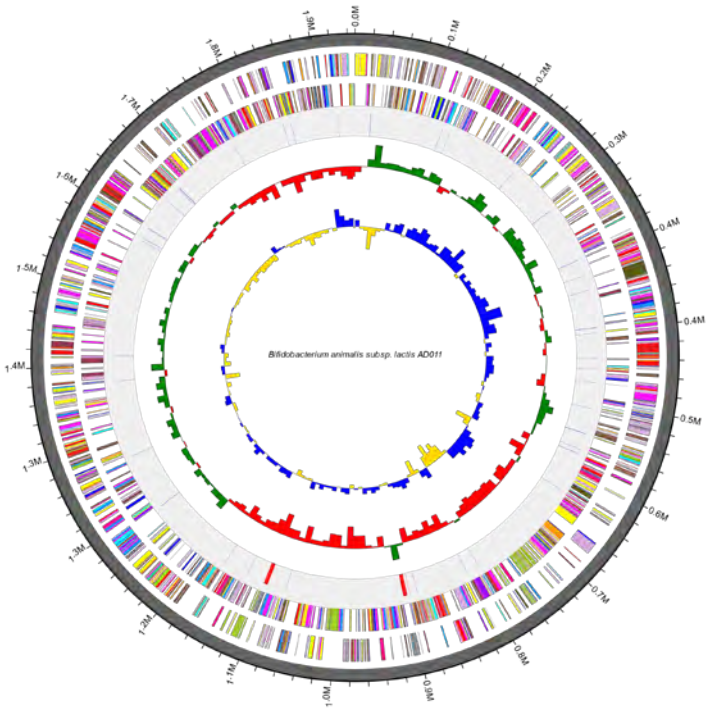


Figure 1. Genome Map of *B. lactis* AD011

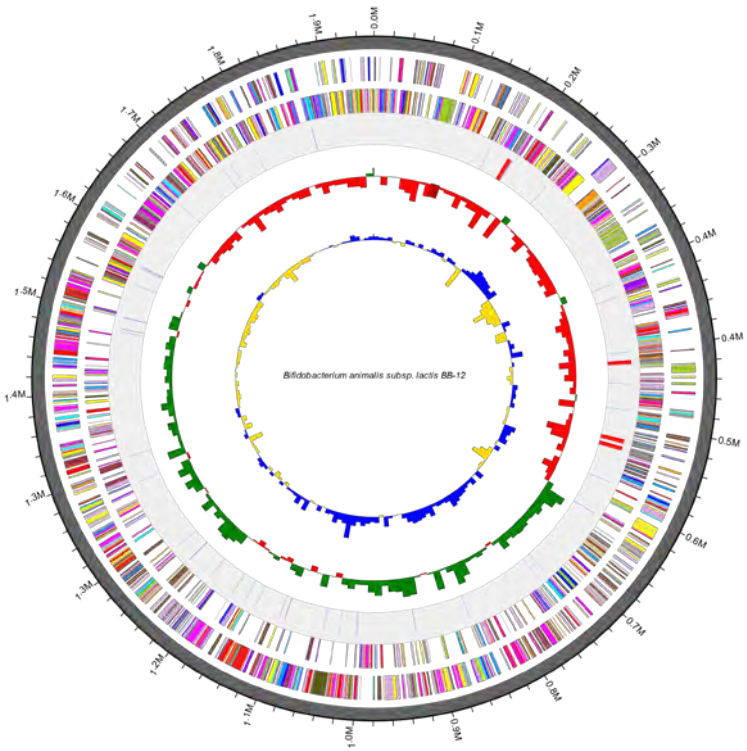


Figure 2. Genome Map of *B. lactis* BB-12

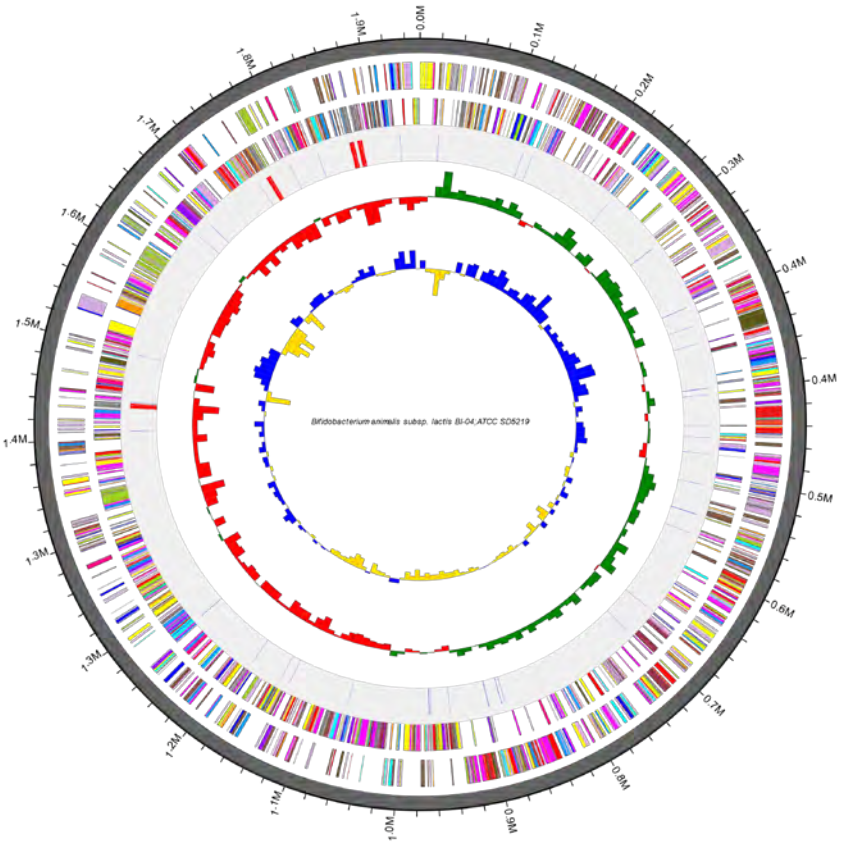


Figure 3. Genome Map of *B. lactis* Bl-04

3.2) Phylogenomics by OrthoANI analysis

OrthoANI (Orthologous Average Nucleotide Identity) is a type of similarity value between two genome sequences. It is an improved version of the original ANI (Average Nucleotide Identity) and is considered to represent the Overall Genome Relatedness Index (OGRIs). It can be used for classification and identification of bacteria, and the proposed cutoff for species boundary is 95~96%. The algorithmic scheme to calculate OrthoANI between two genomes is given in Fig. 4, which consists of three steps. First, both genome sequences of the strains were cut into fragments of 1,020 bp length, and any fragments less than 1,020 bp in size were omitted and ignored. Second, all fragments were searched, and nucleotide identities were calculated using the BLASTn program. Third, fragments between the two genomes were identified when they showed reciprocal best hit in BLASTn searches. Finally, the genome-wide nucleotide identity value was calculated between the two genomes.

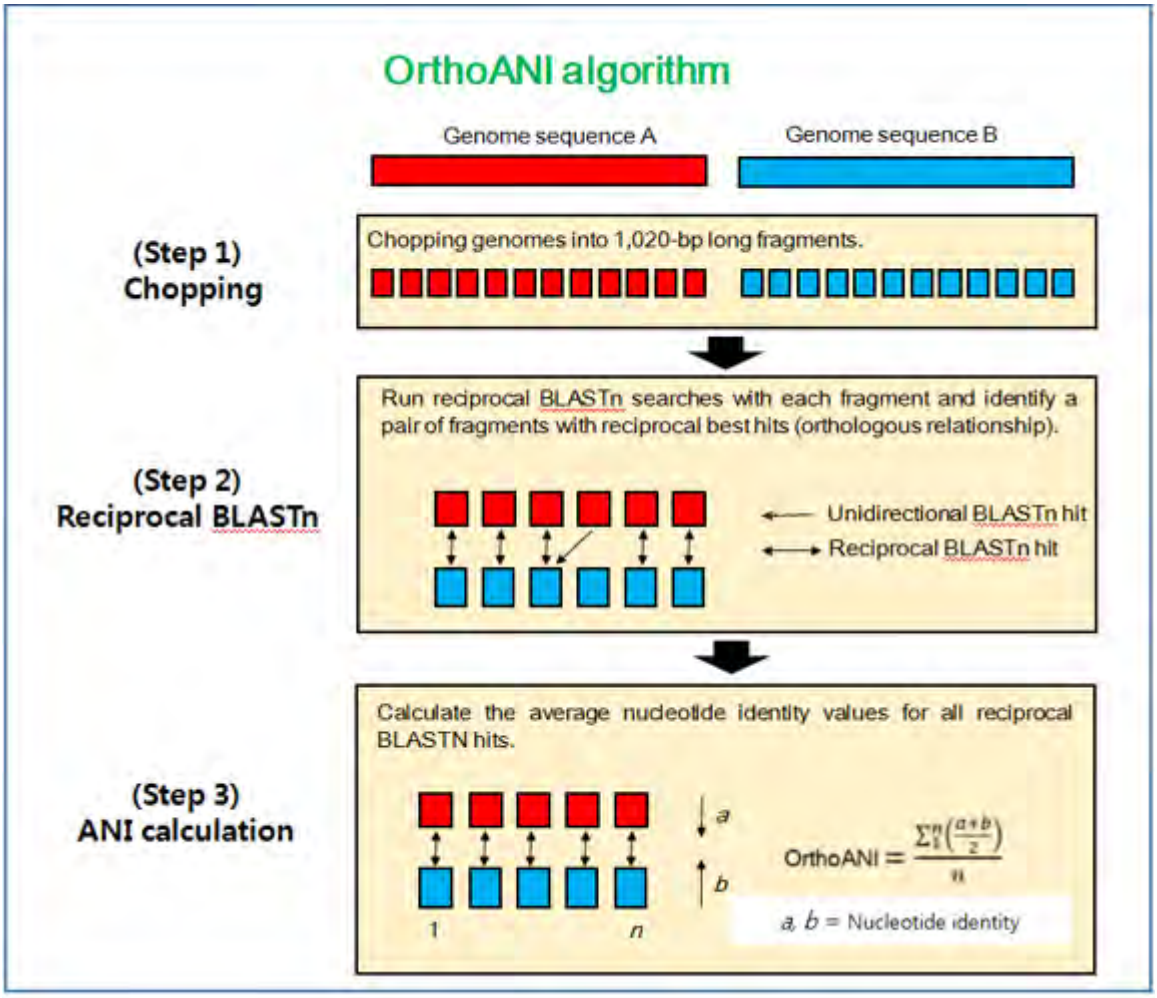


Figure 4. Schematic Diagram for OrthoANI Algorithm. The major differences between ANI and OrthoANI are: (1) in OrthoANI, both genomes are fragmented in silico; (2) OrthoANI does not use fragments of less than 1,020 bp; and (3) in OrthoANI, only when two fragments are reciprocally searched as best hits using BLASTn program are their nucleotide identity values included in the subsequent computation (Lee et al. 2016).

The relatedness measure (as %) between the two genomes of *B. lactis* strain AD011 and strains BB-12 and Bl-04 were analyzed by OrthoANI. *B. lactis* AD011 has over 99.85% similarity in whole genomic sequences with other reference strains of *B. lactis*, such as BB-12 and Bl-04 strains (Figure 5; Table 2).

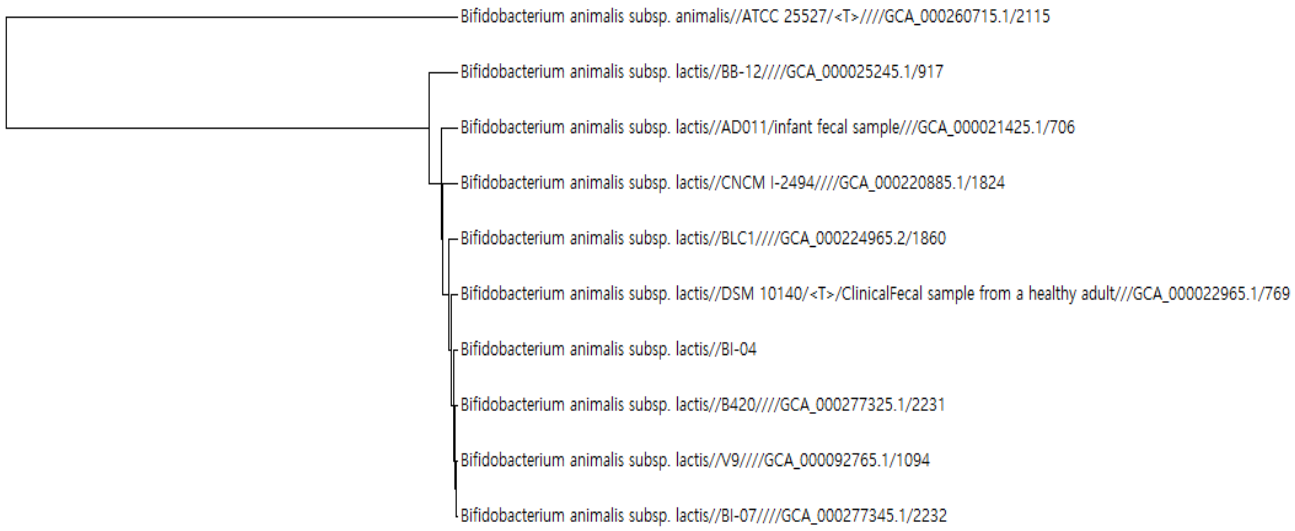


Figure 5. ANI-Derived UPGMA Dendrogram of *B. lactis* AD011, BB-12, and Bl-04 Strains

Table 2. OrthoANI Value between *B. lactis* Strains AD011, BB-12, and Bl-04

	B. lactis AD011	*B. lactis* BB-12	*B. lactis* Bl-04
B. lactis AD011	100	99.85	99.93
B. lactis BB-12	99.85	100	99.86
B. lactis Bl-04	99.93	99.86	100

3.3) Phylogenomics by Tetra-Nucleotide Analysis (TNA)

Tetra-nucleotide is a fragment of DNA sequence with 4 bases (e.g., AGTC or TTGG). Pride et al. (2003) showed that the frequency of tetra-nucleotides in bacterial genomes contain useful, albeit weak, phylogenetic signals. Even though tetra-nucleotide analysis (TNA) utilizes the information of the whole genome, it is evident that it cannot replace other alignment-based phylogenetic methods, such as OrthoANI or 16S rRNA phylogeny. However, TNA can be useful for phylogenetic characterization when the whole genome or 16S rRNA gene information is not available. For example, a partial genomic fragment obtained from a metagenome can be identified by TNA (Teeling et al., 2004).

In this analysis, AD011, BB-12, and Bl-04 showed over 99.99% similarity in TNA-values (Table 3).

Table 3. TNA Value between *B. lactis* Strains AD011, BB-12, and Bl-04

	B. lactis AD011	*B. lactis* BB-12	*B. lactis* Bi-04
B. lactis AD011	100	99.99	99.99
B. lactis BB-12	99.99	100	100
B. lactis Bi-04	99.99	100	100

4) Summary of Genetic Comparison of *B. lactis* AD011, *B. lactis* BB-12, and *B. lactis* Bl-04

The whole genome sequence of *B. lactis* AD011 showed very high genetic similarity (99.85%) with those of GRAS notified *B. lactis* BB-12 and *B. lactis* Bl-04.

5) Reference

Delcenserie V, Lessard MH, Lapointe G, Roy D. Genome comparison of Bifidobacterium longum strains NCC2705 and CRC-002 using suppression subtractive hybridization. FEMS Microbiol Lett. 2008;280:50-6.

Lee I, Ouk Kim Y, Park SC, Chun J. OrthoANI: An improved algorithm and software for calculating average nucleotide identity. Int J Syst Evol Microbiol. 2016;66(2):1100-3.

Pride DT, Meinersmann RJ, Wassenaar TM, Blaser M J. Evolutionary implications of microbial genome tetranucleotide frequency biases. Genome Res. 2003;13:145-58.

Teeling H, Meyerdierks A, Bauer M, Amann R, Glockner FO. Application of tetranucleotide frequencies for the assignment of genomic fragments. Environ Microbiol. 2004;6:938–47.

2. Genetic Stability Test

1) Background

The genetic stability of a probiotic strain 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 whole genome sequence of *B. lactis* AD011 was published (Kim et al., 2009). 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), 7 rRNA operons, and 52 tRNAs were compiled. We studied the genetic stability for 25 generations of *B. lactis* AD011.

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 and regarded it as the first generation (about 10^6 CFU [colony forming unit]/mL) of *B. lactis* AD011.

It is 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 is checked to be 1.8-2.0. DNA extracts were aliquoted and stored at -20℃.

2-3) Whole Genome Sequencing and Analysis:

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 using 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 1^{st} and 25^{th} generations, bioinformatics analysis and comparative genomics analysis were performed using a software provided by CunLab 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 4. Genetic Characteristics of Whole Genome Sequence of 1^{st} and 25^{th} 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 rRNA genes	5	4
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 OGRI (Overall Genome Relatedness Index). 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.

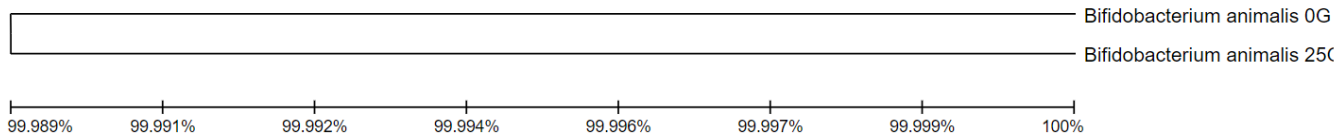


Figure 6. ANI-derived UPGMA (Unweighted Pair Group Method with Arithmetic Mean) Dendrogram (Newick format)

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.

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3. Absence of Virulence Genes

1) Introduction

Virulence factors are encoded in and translated from genes in the chromosomal DNA, bacteriophage DNA, or plasmids of bacteria. They can be readily transferred horizontally between bacteria (e.g., virulence factors for antibiotic resistance) via pathogenicity islands (PAIs) and/or virulence plasmids. Virulence plasmids are clusters of self-replicating extrachromosomal genes for virulence factors located in plasmids within the cytoplasm of the bacteria. In this study, we will verify the presence of virulence factors in the genetic information of *B. lactis* AD011.

2) Method

The search for virulence factors in *B. lactis* AD011 was completed using the VirulenceFinder 1.5 Server, which is a component of publicly available web-based tool for whole-genome sequencing (WGS) analysis hosted by the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org).

3) Results and Discussion

The genome sequences of *B. lactis* AD011 was compared with the genome sequences of four well-known pathogens (*E. coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus*). The virulence factors included *E. coli* Shiga toxin gene, *S. aureus* exoenzyme genes, host immune alteration or Evasion genes, and toxin genes.

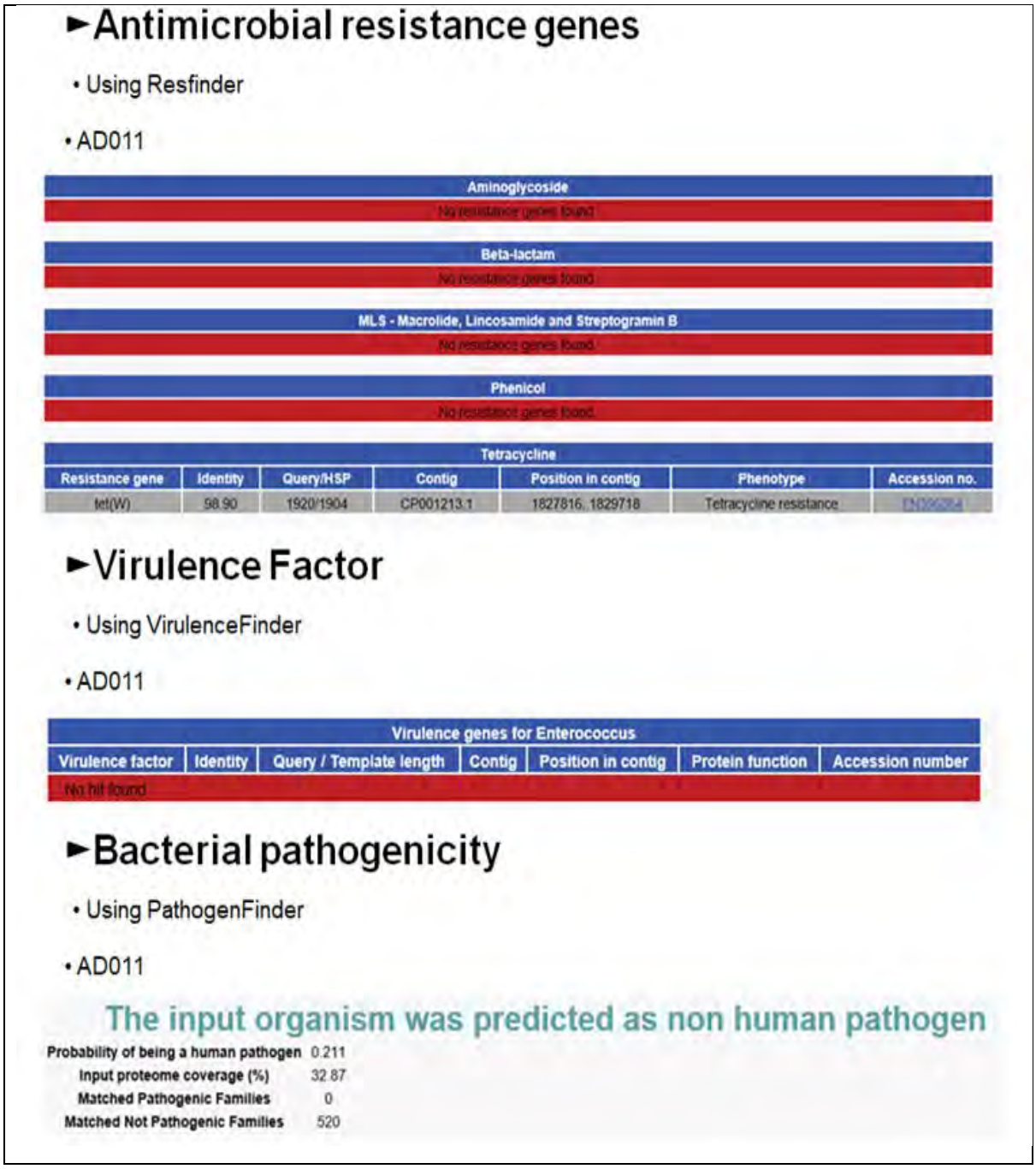

Figure 7. Virulence Factors, Antimicrobial Resistance Genes, and Bacterial Pathogenicity of *B. lactis* AD011

No virulence factors were found in the genomic sequence of *B. lactis* AD011. This result shows that the genomic sequence of *B. lactis* AD011 does not include toxic or pathogenic genes related to *E. coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus*. However, Resfinder analysis revealed a possible *tet*(W) gene in the genome of *B. lactis* AD011 so further study about tetracycline resistance test and tetracycline resistance transferability test were required for this strain.

4. Antimicrobial Susceptibility Test

1) Introduction

Boriello et al. (2003) reported that antimicrobial resistance might be considered as one of the criteria to assess the safety of strains used in food and feed because microorganisms may theoretically transfer antimicrobial resistant genes to pathogens. The transferable acquired genes have already been characterized in *Bifidobacteria* and *Lactobacilli* (Ammor et al., 2007). For the purpose of distinguishing resistant from susceptible strains, the EFSA's Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) defines the microbiological cut-off values for antimicrobial resistance assessment of a bacterial strain used as feed additive. Microbiological cut-off values are set by studying the distribution of MICs of the chosen antimicrobials in bacterial populations belonging to a single taxonomical unit (species or genus) (EFSA, 2012). Antibiotic susceptibility test methods are divided into dilution method and diffusion method. The advantage of the dilution method is that quantitative results (minimum inhibitory concentration; MIC) can be obtained, which are accurate comparing to that of disk diffusion method. The interpretation criteria for the antibiotic susceptibility test are based on the MIC, and the breakpoint can be decided based on the concentration of antibiotics that can reach the body.

2) Strains

The *B. lactis* AD011 strain was pre-cultivated twice in MRS (de Man-Rogosa-Sharpe) broth (CRITERION™ Lactobacilli MRS Broth, Hardy Diagnostics, USA) with or without 0.05% L-cysteine hydrochloride and incubated at 37℃ for 24h.

3) Antimicrobial Agents

The following 22 antimicrobial agents were used:

- ampicillin sodium salt (Sigma, Lot#BCB W1243),
- carbenicillin disodium salt (Sigma, Lot#116M4834V),
- cephalothin sodium salt (Sigma, Lot#056M4858),
- cephalothin sodium salt (Sigma, Lot#056 M4858V),
- chloramphenicol (Sigma, Lot#SLBR8869V),
- clindamycin hydrochloride (Sigma, Lot#021M1533),
- dicloxacillin sodium salt hydrate (Sigma, Lot#SZBD263XV),
- erythromycin (Sigma, Lot#WXBC4044V),
- gentamicin sulfate (Sigma, Lot#SLBP3082V),
- kanamycin sulfate (Sigma, Lot#066M4019V),
- metronidazole (Sigma, Lot#MKBZ3056V),
- mupirocin (Sigma, Lot#106M4733V),
- neomycin sulfate (Sigma, Lot#LRAB3300),
- penicillin G (Sigma, Lot#087M4834V),
- phosphomycin disodium salt (Sigma, Lot#096M4031V),
- polymyxin B sulfate salt (Sigma, Lot#027M4002V),
- rifampicin (Sigma, Lot#MKCC2435),
- streptomycin sulfate salt (Sigma, Lot#SLBT8451),
- tetracycline (Sigma, Lot#126M4769V),

- trimethoprim–sulfamethoxazole (Sigma, Lot#097M40 17V),
- sulfamethoxazole (Sigma, Lot#BCBT3855), and
- vancomycin hydrochloride (USP, Lot#R07250).

Each of the antibiotic powder was dissolved, diluted in appropriate diluents, and filter sterilized prior to addition to LSM-Cys broth medium, composed of 90% of IST and 10% of MRS broth medium. IST broth was purchased from KisanBio Co., Ltd. (Mbcell Iso-Sensitest Broth, Seoul, Korea) and MRS was purchased from Becton, Dickinson and Company (BD Difco™ MRS Lactobacilli broth, Franklin Lakes, NJ, USA). Serial dilutions of antimicrobial agents ranging from 1,024 to 0.0032g/mL were prepared.

4) Method

The MIC values for all bacterial isolates were determined according to the ISO 10932:2010 broth microdilution procedure. The LSM-Cys broth medium supplemented with 0.03% (w/v) L-cysteine HCl containing antibiotics at different concentrations was used to prepare each well of a microwell plate. The inoculum was adjusted to a turbidity equivalent to 0.16 to 0.2 at 625 nm as measured by a Hitachi Spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan). The solution corresponded to approximately $3x10^8$ CFU/mL. Each inoculum was added to a double strength LSM-Cys broth medium at a rate of 0.2%. A 50 uL diluted bacterial suspension was added to each well; no negative control well was employed. The microdilution plates were prepared with a series of twofold dilutions of antibiotics. The microdilution plates were incubated at 37°C for 48 h in an anaerobic (5% CO_2, 10% H_2, and 85% N_2) chamber. 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 experiments were replicated three times.

5) Results and Discussion

As shown in Table 5, *B. animalis* subsp. *lactis* AD011 was susceptible to ampicillin, clindamycin, erythromycin, chloramphenicol, and vancomycin comparing to those of the FEEDAP Panel suggested breakpoint values (EFSA, 2012).

Our study showed the MIC of gentamicin was 256 μg/mL, which is much higher than the breakpoint provided by EFSA (64 μg/mL), but it was equal to the breakpoint established by PROSAFE for *Bifidobacterium* species. Meanwhile, *B. lactis* AD011 was resistant to tetracycline; the MIC was 16 μg/mL in our study, which is higher than the cut-off by a single dilution (16 vs. 8 μg/mL). These values were comparable to those of other GRAS strains, such as *B. animalis* subsp. *lactis* BB-12 (FDA, 2002- GRN 49; FDA, 2009 – GRN 268; Kim et al., 2018) and *B. breve* M-16V (FDA, 2013a, 2013b, 2013c; GRN 453 to 455), which have received the US FDA's 'no question' letters for the use as ingredients for infant formulas and/or selected conventional foods. In addition, this is within the normal variation around the mean and, thus, does not raise concerns for safety.

Tetracycline resistance in *B. animalis* subsp. *lactis* has been shown to be 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), Masco et al. (2006), and Aires et al. (2007) consistently found *tet*(W) in all strains they tested. Gueimonde et al. (2010) also determined that "*tet*(W) is necessary and sufficient for the tetracycline resistance seen in *B. animalis* subsp. *lactis*." Noting the presence of the transposase gene, the authors, nevertheless, concluded that there is no evidence that *tet*(W) in *B. animalis* subsp. *lactis* is transmissible. The *tet*(W) is chromosomally located, and it is not associated with the conjugative transposon TnB1230, found in some other *tet*(W)-positive bacteria (Kastner et al., 2006; Masco et al., 2006; Matto et al., 2007). 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.

Table 5. Antimicrobial Susceptibility of *B. lactis* AD011 and Other *Bifidobacterium* spp. (MIC values, ug/mL)

Antibiotics	Cut off of *Bifidobacterium*		*B. lactis* strains						Kim et al., 2018		GRN 453, 454, and 455
				GRN 445				GRN 377			
	EFSA	PROSAFE	AD011	HN019	Bl-04	Bi-07	B420	*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	0.125-0.25
Gentamicin sulfate	64	256	256	64	64	256	64	64	128	128	32-128
Streptomycin sulfate salt	128	256	128	64	8	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	0.05	<0.03	0.05	0.032–0.5	0.125	0.125	0.016-0.25
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	<0.03-0.06	<0.032	0.063	0.032-0.125
Penicillin G	N/R	0.5	0.25					0.5	0.125	0.25	<1.52
Carbenicllin 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	1,024	256	512	64	256	256	1,024	1,024	
Neomycin sulfate	N/R		512						512	1,024	>256
Cephalothin sodium salt	N/R		32						8	16	NA
Polymyxin B sulfate salt	N/R		256						256	1,024	15.6-125
Metronidazole	N/R	16	256						4	8	15.6-31.3
Rifampicin	N/R	2	2					2	2	1	
Phosphomycin disodium salt	N/R		64						64	32	NA
Mupirocin	N/R		32						>128	>128	NA
Trimethoprim-Sulfamethoxazole	N/R		<0.5						1	2	32-128

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5. Antibiotics Resistance Transferability Test

1) Introduction

Antimicrobial resistance genes are already present in the gut bacterial population or otherwise increase the risk of transfer of drug resistance. When resistance to an antimicrobial is inherent to a bacterial species, it is generally referred to as 'intrinsic resistance' (sometimes called 'natural resistance') and is typical of all the strains of the bacterial species. In contrast, when a strain of a typically susceptible species becomes resistant to a given antimicrobial drug, it is considered to be 'acquired resistance.' Acquired resistance can be either due to added genes (genes acquired by the bacteria via gain of exogenous DNA) or to the mutation of indigenous genes (Ammor et al., 2007; Reenen and Dicks, 2011). The whole genome analyses of *B. lactis* AD011 showed that they contained no plasmid capable of transferring the antibiotics resistance gene. Apart from it, two strains showed high antibiotic resistance to gentamycin and tetracycline in the previous antimicrobial susceptibility test. In fact, *Lactobacillus* and *Bifidobacterium* generally showed high resistance to gentamycin in other studies (Zhou et al., 2005; D'Aimmo et al., 2007), and tetracycline resistance (tet) genes were widely distributed in *Bifidobacterium* genus, which is a protein to protect the ribosome from the action of tetracycline (Ammor et al, 2007; Gueimode et al., 2010). We studied the transferability of the tetracycline resistance of *B. lactis* AD011 using *Lactobacillus fermentum* AGBG1 (*L. fermentum* AGBG1) as a recipient strain, which is highly susceptible to tetracycline.

2) Materials and Methods

B. lactis AD011 and *L. fermentum* AGBG1 were obtained from BIFIDO Co., Ltd. All strains are cultivated in MRS broth with or without 0.05% L-cysteine-HCl (Sigma, USA). Conjugal transfer was conducted as described according to Tannock (1987) and modified. Briefly, equal bacterial cell volume (1 mL) of donor strains and recipient cells were mixed and centrifuged at 7,000 ×g for 10 min. After throwing away the supernatant, it was re-suspended in MRS broth medium again and cultivated at 37℃ for 12 h in an anaerobic jar. The collected bacterial cells were filtered on a 0.45 μm membrane filter. The filter was placed on the surface of MRS agar and was incubated anaerobically at 37℃ for 24 h. The bacterial cells were washed from the filter with 4 mL of 0.9% sterile saline and diluted to 10^{-5} and 10^{-6}, and then plated on MRS agar containing antibiotics, such as tetracycline. The plates were

incubated aerobically or anaerobically at 37℃ for 36 h. All experiments were conducted with three replicates.

3) Results and Discussions

Tetracycline resistance transferability test was conducted using *L. fermentum* AGBG1 as the recipient strain that is highly susceptible to tetracycline. The antimicrobial susceptibility test found that *B. lactis* AD011 was resistant to tetracycline (MIC of 16 μg/mL; Table 6). However, the tetracycline resistance of *B. lactis* AD011 was not transferred to the recipient, *L. fermentum* AGBG1, in this study. *L. fermentum* AGBG1, which is highly susceptible to tetracycline, grew well in normal MRS medium; however, *L. fermentum* AGBG1 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 the tetracycline resistance of *B. lactis* AD011 was not transferred to the recipient strains under the test conditions.

Table 6. Transferability of Tetracycline Resistance from Donor (*B. lactis* AD011) to Recipient (*L. fermentum* AGBG1) (CFU/mL)

Antibiotics	***L. fermentum* AGBG1 (Aerobic)**	***L. fermentum* AGBG1 + *B. lactis* AD011**		***B. lactis* AD011 (Anaerobic)**
		Aerobic	**Anaerobic**	
None	4.19E+09	4.12E+09	3.06E+09	2.51E+09
T12	0	0	6.13E+08	2.45E+09

None: No antibiotics were included in the counting agar medium. T12: Tetracycline (12 μg/mL) was included in the counting agar medium.

4) Reference

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6. Hemolytic Activity Test

1) Introduction

Hemolysis is a common virulence factor among pathogens that serves mainly to make iron available to the microbe and causes anemia and edema in the host. Lactic acid bacteria and bifidobacteria are generally known to be safe. *Bifidobacterium* is among the safest genera used as probiotics, and the risks of healthy consumers being seriously infected by eating dairy products containing *Bifidobacteria* are extremely low. Nevertheless, as *Bifidobacteria* are common members of the human intestinal microbiota, they may behave as opportunistic pathogens like other commensal bacteria. In fact, some lactic acid bacteria showed pathogenic potentiality in various studies. Therefore, a FAO/WHO working group recommended that probiotic strains are characterized by hemolytic activities (FAO/WHO, 2002). In this study, we assessed the potential hemolytic activity of *B. lactis* AD011.

2) Materials and Methods

B. lactis AD011 was cultivated in BL agar (BD Difco™ BL Agar, USA). *Listeria ivanovii* subsp. *ivanovii* ATCC 19119, a positive control for hemolysis, was purchased from American Type Culture Collection (USA) and cultivated in BHI medium. The strains were cultured under the condition of Table 7. The plates were then analyzed for the presence of hemolysis by holding the plate up to a light source (or use a colony counter) and view through both the back and the front of the plate. Strains that produced green-hued zones around the colonies (α-hemolysis) or did not produce any effect on the blood plates (γ-hemolysis) are considered non-hemolytic. Strains displaying blood lyses zones around the colonies are classified as hemolytic (β-hemolysis).

Table 7. Cultivation Condition

Strain	*B. lactis* AD011	*Listeria ivanovii* subsp. *ivanovii* ATCC 19119
Medium	BL Agar (BD Difco™ BL Agar, USA) supplemented 5% sheep blood	Brain Heart Infusion (BD BBL™ Brain Heart Infusion Broth, USA) supplemented 1.5% agar and 5% sheep blood
Incubation Condition	Anaerobic	Aerobic
Temperature	37℃	37℃
Time	48 h	48 h

3) Results and Discussion

In the experimental results, a positive control, *Listeria ivanovii* subsp. *ivanovii* ATCC 19119, showed β-hemolytic activity, whereas *B. lactis* AD011 resulted in no hemolysis and no change of color around the colonies (Figure 8).

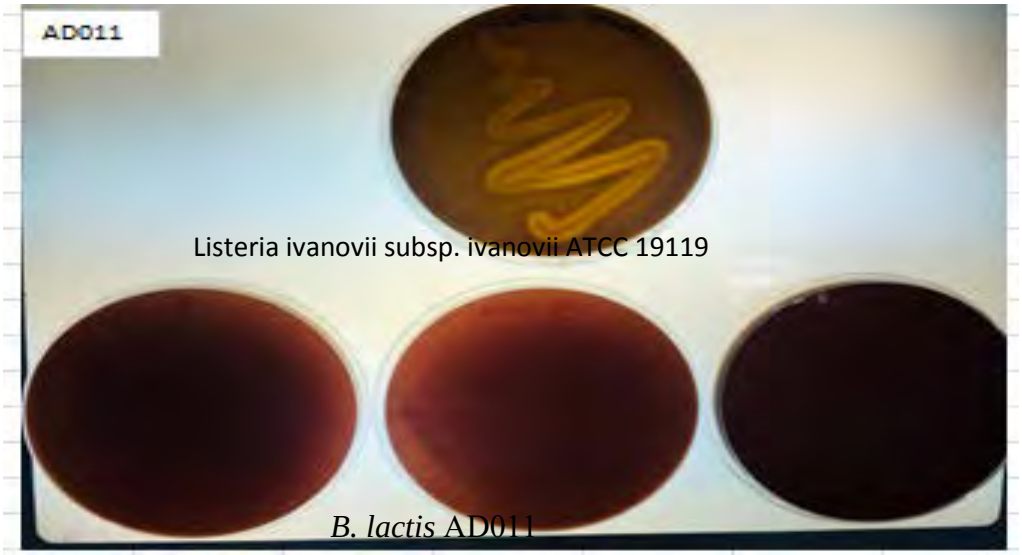

Figure 8. Hemolytic Activity of *B. lactis* AD011 and *Listeria ivanovii* subsp. *ivanovii* ATCC 19119

4) Reference

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7. Biogenic Amines Test

1) Introduction

Biogenic amines are naturally occurring in animals and humans. They are involved in natural biological processes, such as synaptic transmission, blood pressure control, allergic response, and cellular growth control. They are also contained in meat, vegetables, or cheese in various foods, often with significant contents. They are indicators of microbial activity and of food freshness because they are formed mainly by microbial decarboxylation of amino acids and transamination of aldehydes and ketones in foods that contain proteins or amino acids (Silla-Santos, 1996). Probiotic bacteria are added to food because of their beneficial dietary and therapeutic effect on human health. Nevertheless, the ability of some probiotic bacteria to produce biogenic amines has briefly been reported in literature. Some strains of *Lactobacillus* spp. and *Bifidobacterium* spp. might serve as examples of biogenic amines production (Burdychová, 2007; Deepika Priyadarshani and Rakshit, 2011; Lorencová et al., 2012). The aim of this study was to explore biogenic amines production of *B. lactis* AD011, regarding to the safety evaluation.

2) Materials and Method

B. lactis AD011 was cultivated each in milk (Seoul Milk, Korea) and in MRS broth medium (HARDY DIAGNOSTICS a culture of service™, USA) with 0.05% (w/w) L-cysteine-HCl at 37°C for 15 h. The four biogenic amine standards, cadaverine dihydrochloride (purity of 98.0%), histamine (approx. 97.0%), putrescine (98.5%), and tyramine (98.5%), were purchased from Sigma-Aldrich (USA). 1,7-diaminoheptane (internal standard; ISTD, 98.0%), dansyl chloride, and L-proline were purchased from Sigma-Aldrich (USA).

The extraction procedure for analysis of biogenic amines was carried out as described in Kim and Ji's study (2015). Briefly, each 5 g sample was weighed and vortexed with 25 mL of 0.1 N HCl for 5 min. After the resulting homogenate was centrifuged at 10,000 ×g for 15 min (4°C) (2236R high-speed centrifuge; Labogene Aps, Denmark), the aqueous layer was collected and the residue was re-extracted as described above. The collected extracts were filtered through Whatman No. 4 filter paper and, then, 1 mL of each extract was put in a glass test tube and added 0.1 mL of internal standard (1,7-diaminoheptane, 100 mg/L), 0.5 mL of saturated sodium carbonate, and 1 mL of 1% dansyl chloride in acetone. After thoroughly mixing, the test tubes were incubated in dark water bath (WBC 1510A; Jeio Tech. Co., Ltd., Korea) at 45°C for 60 min. Subsequently, 0.5 mL of 10% proline and 5 mL of ether were added to each sample and leaved for 5 min to remove residual dansyl chloride. The supernatants were suspended and evaporated (Scanvac Speed Vacuum Concentrator; Labogene Aps) at 20℃ until dry. The dry residue was diluted with 1 mL of acetonitrile (Sigma Chemical Co.). The reconstituted sample and standard were filtered through a 0.2 μm syringe filter for HPLC analysis.

3) Results and Discussions

The biogenic amine contents in *B. lactis* AD011 was shown in Table 8. The content of biogenic amines of strains was derived by subtracting the background content of biogenic

amines in each medium. *B. lactis* AD011 did not produce cadaverine, histamine, putrescine, and tyramine.

Table 8. Biogenic Amine Levels of *B. lactis* AD011

Strain	Cadaverine (μg/mL)	Histamine (μg/mL)	Putrescine (μg/mL)	Tyramine (μg/mL)	Medium
B. lactis AD011	N/D[1]	N/D[1]	N/D[1]	N/D[1]	Milk medium
	N/D[1]	N/D[1]	N/D[1]	N/D[1]	MRS broth medium

N/D[1] ; not detected

4) Reference

Burdychová R, Komprda T. Biogenic amine-forming microbial communities in cheese. FEMS Microbiol Lett. 2007; 276(2):149-55.

Deepika Priyadarshani WM, Rakshit SK. Screening selected strains of probiotic lactic acid bacteria for their ability to produce biogenic amines (histamine and tyramine). Intl J Food Sci Technol. 2011; 46(10): 2062–29.

Lorencová, E.; Buňková, L.; Matoulková, D.; Dráb, V.; Pleva, P.; Kubáň, V.; Buňka, F. Production of biogenic amines by lactic acid bacteria and bifidobacteria isolated from dairy products and beer. Int'l J Food Sci Technol. 2012;47(10), 2086-91.

Kim NY, Ji GE. Characterization of the production of biogenic amines and gamma-aminobutyric acid in the soybean pastes fermented by *Aspergillus oryzae* and *Lactobacillus brevis*. J Microbiol Biotechnol. 2015; 25(4):464-8.

Silla Santos MH. Biogenic amines: Their importance in foods. Intl J Food Microbiol. 1996; 29: 213–31.

8. Ammonia Production Test

1) Introduction

Bacteria produce ammonia from proteins and their derivatives by several processes, such as proteolysis, peptide degradation, deamination, and deamination. Potentially toxic products of protein breakdown in the large intestine include phenols, ammonia, and indoles (Smith and Macfarlane, 1997). Therefore, the production of ammonia by bacteria is highly relevant to human gut health. Vince and Burridge (1980) reported that the considerable amounts of ammonia were generated by gram-negative anaerobes, clostridia (including *Clostridium perfringens*), *Enterobacter*, and *Bacillus* spp. Some strains of *Streptococci*, *Micrococci*, and the gram-positive, non-sporing, anaerobes produced moderate concentrations of ammonia, whereas the gram-positive, aerobic rods, mostly *lactobacilli*,

produced very little ammonia. We assessed the ammonia production of *B. lactis* AD011 for the safety aspect.

2) Materials and Method

2.1) Strains

B. lactis AD011, *Bifidobacterium bifidum* BGN4 (*B. bifidum* BGN4), *Bifidobacterium longum* BORI (*B. longum* BORI), and *Enterococcus faecium* KCTC13225 strains were cultivated aerobically or anaerobically in Brain Heart Infusion (BD BBL™ Brain Heart Infusion Broth, USA) at 37℃ for 5 days. The supernatants of each strain were obtained by centrifuging at 10,000 g for 30 min under 4℃ and, then, were adjusted to pH 7 by using 1 N NaOH.

2.2) Determination of Ammonia

The production of ammonia by catalyzed indophenol reaction was determined according to Chaney and Marbach (1962). Solution 1 (2 g of phenol and 0.01 g of sodium nitroferricyanide dehydrate were dissolved in 200 mL of distilled water) and solution 2 (1 g of sodium hydroxide and 0.08 g of sodium hypochlorite were dissolved in 200 mL of distilled water) are prepared. In 96 well plates, 10 μL of samples and each 100 μL of solution 1 and 2 were added. The test was conducted on three replicates. The plates were placed at room temperature for one hour, and the absorbance was taken at 625 nm. BHI medium is used as a negative control, and the ammonia concentration was calculated using a standard curve.

3) Result

B. bifidum BGN4, *B. longum* BORI, and *B. lactis* AD011 did not produce ammonia. In contrast, *E. faecium* KCTC13225 produced 109.3 ± 7 μg/mL of ammonia (Table 9).

Table 9. Concentrations of Ammonia Produced by Bacteria Strains Ammonia (μg/mL)

Strain	Ammonia (μg/mL)
Bifidobacterium animalis subsp. *lactis* AD011	negative
Bifidobacterium bifidum BGN4	negative
Bifidobacterium longum BORI	negative
Enterococcus faecium KCTC13225	109.3 ± 7

4) Reference

Chaney AL, Marbach EP. Modified reagents for determination of urea and ammonia. Clinical Chemistry, 1962;8(2): 130-2.

Smith EA, Macfarlane GT. Formation of phenolic and indolic compounds by anaerobic bacteria in the human large intestine. Microbial Ecology, 1997; 33: 180-8.

Vince A J, Burridge SM. Ammonia production by intestinal bacteria: the effects of lactose, lactulose and glucose. Journal of Medical Microbiology, 1980;13(2):177-91.

9. Mucin Degradation Test

1) Introduction

The intestinal mucus gel layer is an important constituent of the intestinal barrier that consists of a glycoprotein family. Multiple groups have reported that bacterial translocation can occur in infants and immunocompromised hosts, even if the intestinal mucus acts as a biological shield from microbes. This bacterial translocation has the potential to cause sepsis, and is one of the most serious probiotic safety concerns. Some scientists have also reported the possibility of bacteremia—endocarditis due to the administration of probiotic strains (De Groote et al., 2005; Liong et al., 2008). For microbial safety, it is necessary to evaluate the translocation ability via mucolytic capacity analysis of each strain. We assessed the mucin degradation of *B. lactis* AD011 for the safety aspect.

2) Materials and Method

In this study, the translocation capabilities of *B. lactis* AD011 was measured using *in vitro* mucolytic assays. The cell growth rates after incubation were examined in five types of modified MRS media by measuring their absorbances at 550 nm: basal medium (glucose-free MRS,3) with or without 0.5% mucin, 1.0% mucin, or 0.5% or 1.0% glucose.

Partially purified mucin from porcine stomach (Type III) was purchased from Sigma (St. Louis, MO, USA). A MRS broth medium without a carbon source (i.e., basal medium) contained 0.75% (w/v) yeast extract , 0.25% (w/v) soy peptone, 0.25% (w/v) fish extract, 0.25% (w/v) sodium acetate, 0.1% (w/v) ammonium citrate, 0.05% (w/v) sodium phosphate monobasic, 0.025% (w/v) sodium phosphate dibasic, 0.05% (w/v) Tween 80, 0.05% (w/v) L-cysteine HCl, 0.005% (w/v) maleic acid, 0.00625% (w/v) taurine, 0.005% (w/v) magnesium sulfate, and 0.0025% (w/v) manganese sulfate. Distilled water (98.2% [v/v]) was used as a negative control. To each of the four MRS broth media, 0.5% (w/v) mucin, 1.0% (w/v) mucin, or 0.5% or 1% (w/v) glucose were added. After the inoculation of the microorganisms in each MRS medium, the samples were cultured at 37°C for 48 h under anaerobic conditions. After incubation, the bacterial growth was assessed by measuring absorbance at 550 nm at 12, 24, 36, and 48 h. The initial optical density value of the media was subtracted from the final value for each test sample.

3) Result

As shown in Fig 9, no growth was observed with *B. lactis* AD011 when mucin was added instead of glucose. These observations clearly indicate that *B. lactis* AD011 did not use mucin as a carbon source for their growth. This study shows that *B. lactis* AD011 did not degrade mucin, indicating that the strains are not capable of damaging intestinal surfaces and do not have trans locational abilities.

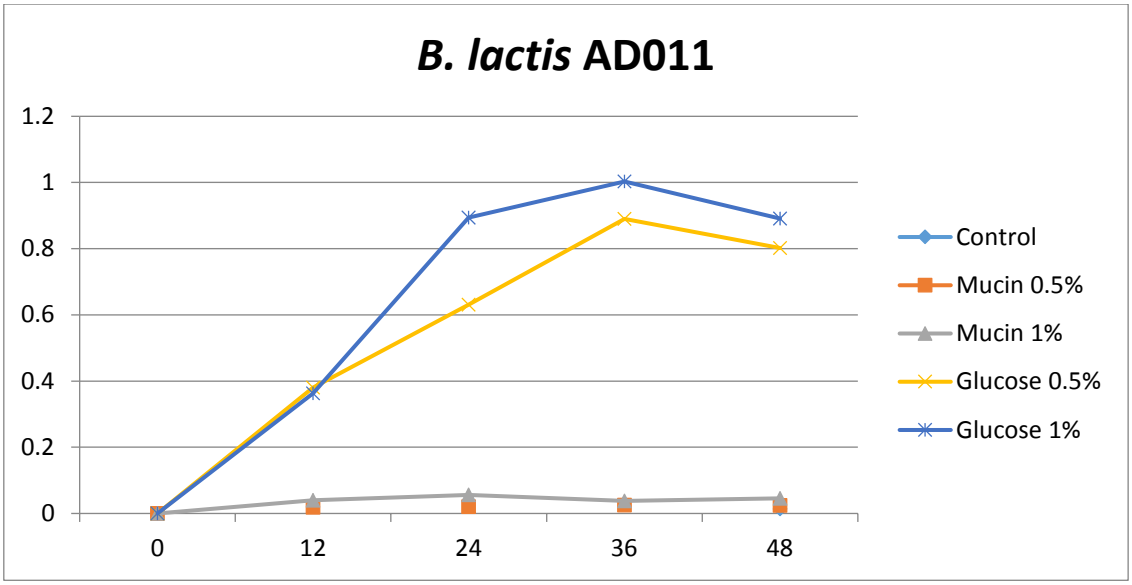

Figure 9. Mucin Degradation Test of *B. animalis* subsp. *lactis* AD011

In general, when simple sugars (glucose, fructose, maltose, and sucrose) are added, mucinase production can be inhibited due to catabolic repression. A false negative result can be obtained despite the microorganisms' potential to produce mucinolytic enzymes. Therefore, to obtain accurate data, glucose, which is generally used as a carbon source in the MRS medium, was intentionally removed from the medium in which the experimental cells were cultivated. If *B. lactis* AD011 was able to produce mucinase, it would be able to source carbon and grow actively through mucin digestion, and the growth of both probiotic strains was actively induced when glucose was added as a carbon source.

4) Reference

De Groote MA, Frank DN, Dowell E, Glode MP, Pace NR. *Lactobacillus rhamnosus* GG bacteremia associated with probiotic use in a child with short gut syndrome. Pediatr Infect Dis J. 2005; 24: 278-80.

Liong MT. Safety of probiotics: Translocation and infection. Nutr Rev. 2008; 66: 192–202.

10. Shelf-Life of *B. lactis* AD011

1) Introduction

Stability of probiotics is particularly important for supplements as they are often stored at room temperature for long periods compared to dairy products that are chilled and consumed much more quickly. In this experiment, viability of *B. lactis* AD011 was measured during storage at various temperature conditions, including room temperature.

2) Materials and Method

The lyophilized powder of *B. lactis* AD011 was used for this experiment, and the storage conditions are as follows.

Table 10. Stability Test Conditions

Temperature	25℃	5℃	40℃
Test period	24 months	24 months	8 months
Interval of surviving cell number measurement	2~6 months	2~6 months	2~6 months

3) Results and Discussions

Bulk ingredient stability data indicate that the number of *B. lactis* AD011 cells in the ingredient is stable for up to 2 years at 5℃ and 25℃ when the cells are supplied in excess of 150% of the claim value at the time of shipment. Table 11 presents the stability of *B. lactis* AD011 at various temperatures.

Table 11. 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 24 months compared to the claim value (1.00E+11 CFU/g)	108%	89%	1.3%

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

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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
Appearance	Yellow white powder	Visual
Cell Counts (as B. *lactis*), cfu/g	1.00E+11	KHFSC 4/3/3-58
Moisture, %	4.3%	KFSC 8/2/2.1/2.1.1
Heavy metals		
Lead (Pb), ppm	0.00461	KFSC 8/9/9.1/9.1.2
Arsenic (As), ppm	0.00881	KFSC 8/9/9.1/9.1.4
Cadmium (Cd)	0.01780	KFSC 8/9/9.1/9.1.3
Mercury (Hg)	0.0	KFSC 8/9/9.1/9.1.6
Microbial purity		
Non-Lactic acid bacteria	Negative	KFSC 8/4/4.5/4.5.1
Total yeasts and molds	Negative	KFSC 8/4/4.10
Escherichia coil	Negative	KFSC 8/4/4.8
Salmonella	Negative	KFSC 8/4/4.11
Listeria	Negative	KFSC 8/4/4.15
Enterobacter sakazakii (Cronobacter spp/)	Negative	KFSC 8/4/4.21
Proximate analysis		
Lipids, %	1.54%	KFSC 8/2/2.1/2.1.5/2.1.5.1
Protein, %	61.47	KFSC 8/2/2.1/2.1.3/2.1.3.1
Carbohydrates, %	28.44%	KFSC 8/2/2.1/2.1.4/2.1.4.1
Ash, %	4.36%	KFSC 8/2/2.1/2.1.2

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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
Appearance	Yellow white powder	Visual
Cell Counts (as B. *lactis*), cfu/g	1.00E+11	KHFSC 4/3/3-58
Moisture, %	4.2%	KFSC 8/2/2.1/2.1.1
Heavy metals		
Lead (Pb), ppm	0.0507	KFSC 8/9/9.1/9.1.2
Arsenic (As), ppm	0.0093	KFSC 8/9/9.1/9.1.4
Cadmium (Cd)	0.0212	KFSC 8/9/9.1/9.1.3
Mercury (Hg)	0.0	KFSC 8/9/9.1/9.1.6
Microbial purity		
Non-Lactic acid bacteria	Negative	KFSC 8/4/4.5/4.5.1
Total yeasts and molds	Negative	KFSC 8/4/4.10
Escherichia coil	Negative	KFSC 8/4/4.8
Salmonella	Negative	KFSC 8/4/4.11
Listeria	Negative	KFSC 8/4/4.15
Enterobacter sakazakii (Cronobacter spp/)	Negative	KFSC 8/4/4.21
Proximate analysis		
Lipids, %	1.58%	KFSC 8/2/2.1/2.1.5/2.1.5.1
Protein, %	57.52%	KFSC 8/2/2.1/2.1.3/2.1.3.1
Carbohydrates, %	31.64%	KFSC 8/2/2.1/2.1.4/2.1.4.1
Ash, %	5.69%	KFSC 8/2/2.1/2.1.2

QC Manager Kwon Bin

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CERTIFICATE OF ANALYSIS

NAME OF PRODUCT	Bifidobacterium Lactis AD011	
LOT NO.	BL-R-190211	
PRODUCTION DATE	2019.02.11	
CERTIFICATED DATE	2019.02.15	
EXPIRATION DATE	2021.02.10	
ANALYSIS RESULT		
Parameter	BL-R-190211	Method of analysis/Method number
Appearance	Yellow white powder	Visual
Cell Counts (as B. *lactis*), cfu/g	1.00E+11	KHFSC 4/3/3-58
Moisture, %	4.2%	KFSC 8/2/2.1/2.1.1
Heavy metals		
Lead (Pb), ppm	0.0362	KFSC 8/9/9.1/9.1.2
Arsenic (As), ppm	0.0073	KFSC 8/9/9.1/9.1.4
Cadmium (Cd)	0.0147	KFSC 8/9/9.1/9.1.3
Mercury (Hg)	0.0	KFSC 8/9/9.1/9.1.6
Microbial purity		
Non-Lactic acid bacteria	Negative	KFSC 8/4/4.5/4.5.1
Total yeasts and molds	Negative	KFSC 8/4/4.10
Escherichia coil	Negative	KFSC 8/4/4.8
Salmonella	Negative	KFSC 8/4/4.11
Listeria	Negative	KFSC 8/4/4.15
Enterobacter sakazakii (Cronobacter spp/)	Negative	KFSC 8/4/4.21
Proximate analysis		
Lipids, %	1.50%	KFSC 8/2/2.1/2.1.5/2.1.5.1
Protein, %	53.11%	KFSC 8/2/2.1/2.1.3/2.1.3.1
Carbohydrates, %	34.06%	KFSC 8/2/2.1/2.1.4/2.1.4.1
Ash, %	7.93%	KFSC 8/2/2.1/2.1.2

QC Manager Kwon Bin

Appendix D. Human Clinical Studies of Other *B. lactis* Strains

The summary table below shows that no *B. lactis* strains resulted in adverse effects in humans, regardless of subjects, daily doses, and the duration of the study. Due to an abundance of the studies reporting no adverse effects of *B. lactis*, our summary is limited to the studies with the test duration of over 2 weeks with a maximum number of 4 strains or 4 active components (such as prebiotic oligosaccharides) including *B. lactis*. Our literature search covers papers published until June 30, 2019. These studies are presented to show that no *B. lactis* strains have shown adverse effects in humans and animals.

Human studies evaluating the effect of other *B. lactis* strains than AD011 and BB-12 on various measured outcomes showed no adverse effects (Baglatzi et al., 2016; Baştürk et al., 2016, 2017; Bernini et al., 2016; Bocquet et al., 2013; Çekin et al., 2017; Childs et al., 2014; Chisari et al., 2017; Cox et al., 2014; Dilli et al., 2015; Favretto et al., 2013; Hays et al., 2016; Ishizuka et al., 2012; Islek et al., 2014; Maneerat et al., 2013; Marteau et al., 2013; Matsumoto et al., 2014; Merenstein et al., 2014; Nozari et al., 2015; Pinto et al., 2014; Roberts et al., 2016; Simeoni et al., 2016; Singh et al., 2013; Strasser et al., 2016; Tabbers et al., 2011; Tanaka et al., 2015; Ustundag et al., 2017; Waller et al., 2011; West et al., 2014; Wibowo et al., 2016; Yazar et al., 2016).

This review covers research reports published between January 2011 and May 2019, which were not included in GRN 377 (FDA, 2011) and GRN 455 (FDA, 2013). The studies included in this review found that a daily dose up to $5x10^{10}$ cfu *B. lactis* did not cause any adverse effects in adults, infants, and young children (Islek et al., 2014; Merenstein et al., 2014).

Although a few studies on *B. lactis* CNCM I-3446 (Gibson et al., 2009; Gore et al., 2012; Radke et al., 2017) and *B. lactis* HN019 (Ibarra et al., 2018) reported adverse effects during the studies, adverse effects were similarly distributed among the test and control groups. For example, Gibson et al. (2009) reported a total of 403 adverse effects in 124 infants; 60 in the experimental and 64 in the control groups during the study period. The most common adverse effects were infections, dermatitis, digestive problems, and feeding problems (vomiting during or right after feeding). In the study by Gore et al. (2012), infants were switched to extensively hydrolyzed formula together with either *B. lactis* CNCM I-3446 or placebo-sachet. At the 4-week visit, 42/137 (30.7%) parents reported some difficulties (e.g., green loose stools, increased vomiting, feed-refusal, or colic) related to the change in formula and 24/137 (17.5%) stopped the study formula. However, the authors did not report any treatment-related abnormalities. In the study by Radke et al. (2017), the proportion of infants with adverse effects related to infections was comparable between the groups. Ibarra et al. (2018) reported 3 unlikely treatment-related adverse effects (2 in the low-dose HN019 and 1 in the placebo).

Overall, studies reported no adverse effects of any of *B. lactis* strains tested.

Table AD 1. Human Studies of Other *B. lactis* Strains

Objective	Subject	Dose	Duration	Measurements	Reference
Bi-07 Strain (GRN 445, FDA, 2013a)					
To identify the effects of *B. lactis* Bi-07 and xylooligosaccharide (XOS) on bowel habits, self-reported mood, composition of the gut microbiota, blood lipid concentrations and immune function.	44 healthy adults (25–65 years) BMI 20–30 kg/m^2	3 groups - 1) 8 g/d XOS; 2) *B. lactis* Bi-07 10^9 cfu/d; 3) combination of the two	21 d; X	Fecal microbiota and SCFA; bowel habit and mood; plasma lipids (TC, HDL-C, LDL-C, TAG, and non-esterified fatty acids); and fecal and salivary IgA	Childs et al., 2014
To study the effects of *B. lactis* Bi-07 galacto-oligosaccharides (GOS) on immune function and the gut microbiota	37 healthy elderly adults (mean age ~67.2 y)	4 groups - 1) *B. lactis* Bi-07 10^9 cfu/d; 2) 8 g/d GOS; 3) Combination; 4) Control (8 g/d maltodextrin);	3 wk; X	Phagocytosis and oxidative burst by monocytes and granulocytes; whole-blood response to lipopolysaccharide, plasma chemokine concentrations; salivary IgA levels; and fecal microbiota and SCFA	Maneerat et al., 2013
To evaluate de effect of the consumption of a fresh cheese, enriched with *B. lactis* Bi-07 on the symptoms of constipated women.	30 constipated women (median age 37.5-40.8 y)	30 g Minas Frescal cheese with and without *B. lactis* Bi-07 (10^8 cfu/serving)	30 d; P	Changes in constipation symptoms (symptoms of Rome III criteria)	Favretto et al., 2013
Bl-04 and Bi-07 Strains					
To report the effects of supplementation with either a single- or a double-strain probiotic on routine hematology and clinical chemistry measures.	125 physically active healthy adults; 18–60 y	3 groups -1) *B. lactis* Bl-04 (2.0×10^9 cfu/d); 2) *B. lactis* Bi-07 and *L. acidophilus* NCFM; 3) placebo (5×10^9 CFU/d each)	150 d; P	Serum hematology and clinical chemistry (electrolytes, ALT, AST, ALP, LDH, total bilirubin, urea, bicarbonate, uric acid, total protein, albumin, lipid	Cox et al., 2014

				profile, insulin, thyroid-stimulating hormone, and C-reactive protein)	
To examine the effect of supplementation with probiotics on respiratory and gastrointestinal illness in healthy active men and women.	465 subjects (241 males, mean age 35 y, and 224 females; mean age 36 y)	3 groups -1) *B. lactis* Bl-04 (2.0 x 10^9 cfu/d); 2) *L. acidophilus* NCFM and *B. lactis* Bi-07 (5x10^9 cfu/d each); 3) placebo mixed in a drink	150 d; P	Episodes of upper respiratory tract and gastrointestinal illness; duration of illness; physical activity patterns; medication usage and doctor visits; emotional resilience scores; delay in the median time to an illness episode	West et al., 2014
BF-6 Strain					
To investigate the effect of *B. lactis* Bf-6 (LMG 24 384)-supplemented yogurt on colonic transit time.	68 generally healthy women with a history of straining during bowel movements or hard or lumpy stools in the past 2 years, 18-65 y	2 groups -1) *B. lactis* Bf-6 ranged from 5.6x10^{10} cfu/serving at the beginning to 2.0x10^{10} cfu/serving at the end of the intervention period; 2) placebo	14 d; X	Colonic transit time; quality of life; frequencies of bowel movements and constipated stools	Merenstein et al., 2014
B-94 Strain					
To investigate the synbiotic effects of *B. lactis* B94 plus inulin on acute infectious diarrhea.	156 children with acute diarrhea, 2-60 mo old	2 groups - 1) *B. lactis* B94 (5×10^{10} cfu) plus 900 mg inulin; 2) placebo	5 d; P	Diarrhea duration; stool microbiota	Islek et al., 2014
To investigate the efficacy of synbiotic, probiotic, and	71 children with IBS	3 groups - 1) *B. lactis* B94 (1x10^{10} cfu/d); 2) 1,800	4 wk; P	Symptoms of IBS	Baştürk et al., 2016

prebiotic treatment for irritable bowel syndrome (IBS)	(mean age 10.08-12.33 y)	mg/d inulin (synbiotic); 3) combination			
To evaluate the effect of probiotics administered as an adjuvant to sequential *H. pylori* eradication therapy on treatment outcome and patient compliance	159 patients with *H. pylori* infection (mean age 46.8 y)	*B. lactis* B94 ($7x10^9$ cfu/d) or placebo; all subjects had sequential *H. pylori* eradication therapy with antibiotics	2 wk; P	Treatment outcome (eradication rate, compliance, reasons for treatment discontinuation), and symptoms related to side effects of eradication therapy	Çekin et al., 2017
To evaluate the effects of the synbiotic *B. lactis* B94 plus inulin addition to the standard triple therapy on *H. pylori* infection eradication rates	69 children with *H. pylori* infection (mean age 11.2 y)	Standard triple therapy (amoxicillin + clarithromycin + omeprazole) ± synbiotic consists of *B. lactis* B94 ($5x10^9$ cfu/d) and 900 mg/d inulin	14 d; P	*H. pylori* eradication as measured by ^{14}C-urea breath test after 4-6 wk after therapy discontinuation	Ustundag et al., 2017
CNCM I-2494 Strain					
To confirm the findings that the probiotic fermented milk (PFM) containing *B. lactis* CNCM I-2494 improved gastrointestinal (GI) well-being and digestive symptoms in a previous trial involving women reporting minor digestive symptoms.	388 subjects with no IBS or functional GI disorders, (18-60 y)	2 groups -1) *B. lactis* CNCM I-2494 (same as DN-17310; $1.25x10^{10}$ cfu/cup) + *S. thermophilus* and *L. bulgaricus* ($1.2x10^9$ cfu/cup each); 2) control	4 wk; P	Gastrointestinal well-being; composite score of digestive symptoms; nutrient intakes	Marteau et al., 2013
CNCM I-3446 Strain					
To investigate whether dietary supplementation of infants with eczema at age	208 infants with eczema	*B. lactis* CNCM I-3446 (10^{10} cfu/d),	12 wk; P	Eczema severity (SCORing Atopic Dermatitis, SCORAD) at 3 12 wk; SCORAD; infant	Gore et al., 2012

3–6 mo with *B. lactis* CNCM I-3446 or *L. paracasei* CNCM I-2116 had a treatment effect or altered allergic disease progression.	(3-6 mo of life)	*L. paracasei* CNCM I-2116 (10^{10} cfu/d), or placebo while receiving extensively hydrolyzed whey-formula (dairy-free diet)		dermatitis quality of life; gastrointestinal permeability; urinary eosinophilic protein X; allergen-sensitization; allergic symptoms at age 12, 18, 36 mo	
To assess whether immune-related beneficial effects of regular dose (10^7 cfu/g of powder) of the probiotic *B. lactis* CNCM I-3446 in starter infant formula can be maintained with starter formula containing a low dose (10^4 cfu/g of powder) of *B. lactis*.	77 infants delivered by C-section per intervention group, 44 infants in reference group	3 groups - 1) -2) *B. lactis* CNCM I-3446 at 10^7 or 10^4 cfu/g of powder; 3) breastfed reference	From birth to 6 mo of age, with a 12 mo follow-up; P	Incidence, duration, and severity of diarrhea; immune maturation (fecal IgA); gut maturation (fecal calprotectin and 1-antitrypsin); immune responses to vaccines; anthropometry; adverse events	Baglatzi et al., 2016
To compare the effect of *B. lactis* alone or with a blend of GOS and fructo-oligosaccharide (FOS) on infections in infants.	528 newborn infants, less than 42 days of life at the time of enrollment	2 groups- 1) formula *B. lactis* CNCM I-3446 with10^7 cfu/g + GOS/FOS (0.4 g/100 mL; GOS to FOS ration = 9:1); or 2) *B. lactis* alone	12 mo; P	Mean number of infectious episodes; anthropometry; formula tolerance and acceptability of formula; frequency and duration of antibiotic use	Bocquet et al., 2013
To evaluate the efficacy and safety of an infant formula containing bovine milk-derived oligosaccharides (BMOS) and *B. lactis* CNCM I-3446 on incidence of diarrhea and febrile infections during the first year of life	413 healthy full-term infants (aged 0-14 d)	3 groups – 1)-2) formula with and without BMOS (5.8 g/100 g of powder formula) + *B. lactis* CNCM I-3446 ($1x10^7$ cfu/g of powder formula) for 6 mo; 3) breastfed reference group. All infants had the same follow up formula without	12 mo total; P	Incidence of diarrhea and febrile infections at 6 and 12 mo; digestive tolerance; gut microbiota; fecal immune measurements (total protein, salivary IgA, and α-1 antitrypsin); anthropometry	Radke et al., 2017

		pre- and probiotics for another 6 mo			
To investigate the impact of a synbiotic formula (BMOS plus *B. lactis* CNCM I-3446) on gut microbiota composition	115 healthy full-term infants (mean age 5 d)	3 groups - 1) -2) formula with or without BMOS (5.7 g/100 g of powder formula) plus *B. lactis* CNCM I-3446 ($1x10^7$ cfu/g of powder formula); 3) breastfed reference group	12 wk; P	Fecal microbiota; stool characteristics and infant behavior; detection of the added probiotic in stools	Simeoni et al., 2016
DN-173 010 Strain					
To assess the effects of a fermented dairy product containing *B. lactis* DN-173010 in constipated children	159 constipated children (defecation frequency <3 times a wk; mean age 6.5-7.0 y)	2 groups – 1) fermented dairy product containing $8.5x10^9$ cfu/d *B. lactis* DN-173 010 or 2) placebo control	3 wk; P	Stool frequency; stool consistency; rate of success and responders; frequency of fecal incontinence; frequency of pain during defecation; frequency of digestive symptoms (abdominal pain and flatulence), frequency of bisacodyl use	Tabbers et al., 2011
To assess how consumption of yogurt containing *B. lactis* DN-173010 probiotic affects salivary and dental plaque levels of mutans streptococci and lactobacilli in patients undergoing orthodontic treatment.	30 patients undergoing orthodontic or bimaxillar fixed orthodontic treatment and had good oral health	2 groups - 1) 200 g of probiotic-containing yogurt (*B. lactis* DN-173010 dose not specified); or 2) control yogurt	2 wk; X	Counts of S*treptococcus mutans*, lactobacilli, and total cultivable microorganisms in saliva and dental plaque	Pinto et al., 2014
GCL-2505 Strain					
To evaluate the changes in endogenous bifidobacteria	17 females with	2 groups - *B. lactis* GCL2505 (10^{10} cfu/ 100	2 wk; X	Intestinal bifidobacterial counts; the number of	Ishizuka et al., 2012

and administered *B. lactis* GCL2505 in the intestine after administration of *B. lactis* GCL2505 in humans	constipation (20-23 y)	mL) in a milk-like drink; placebo		defecations, number of days with defecation and stool quantity	
	17 healthy males with no *B. lactis* in their feces (26-40 y)	2 groups - 1) *B. lactis* GCL2505 in 100 mL milk; high dose -$10^{10.3}$ cfu or 2) low dose-$10^{9.3}$ cfu	7 d; P	Fecal *B. lactis* counts	
To investigate the daily dynamics of intestinal bifidobacterial and the effects of long-term ingestion of probiotics on the intestinal microbiota.	53 healthy females (mean 20.2 y)	3 groups - 1) *B. lactis* GCL2505 ($1.5x10^{10}$ cfu) plus *B. bifidum* ($2.6x10^{10}$ cfu); 2) *L. bulgaricus* ($3.0x10^{10}$ cfu) plus *S. thermophilus* ($3.0x10^{10}$ cfu) in test beverage; or 3) control	2 wk; P	Changes in fecal 10 bifidobacterial counts (*B. bifidum*, *B. longum* subsp. *longum*, *B. adolescentis*, *B. breve*, *B. catenulatum*, *B. pseudocatenulatum*, *B. longum* subsp. *infantis*, *B. anglatum*, *B. dentium*, and *B. lactis*).	Tanaka et al., 2015
	38 subjects with mild constipation (mean 40.8-42.8 y)	2 groups -1) *B. lactis* GCL2505 ($1.5x10^{10}$ cfu) in test beverage; or 2) placebo control	8 wk; P		
HN-019 Strain					
To assess the impact of *B. lactis* HN019 supplementation on whole gut transit time and frequency of functional gastrointestinal (GI) symptoms in adults.	100 subjects with functional GI symptoms (25-65 y, mean 44 y; 64% female)	3 groups - 1) *B. lactis* HN019 -high dose - 17.2; 2) low dose - 1.8 billion cfu; or 3) placebo capsule (each type of capsule was added to yogurt containing no probiotics)	14 d; P	Whole gut transit time; frequency of functional gastrointestinal symptoms	Waller et al., 2011
To determine the efficacy and safety of *B. lactis* HN019 for constipation	228 adults with functional	3 groups - *B. lactis* HN019 (high dose - $1x10^{10}$ or low	28 d; P	Colonic transit time; other constipation related	Ibarra et al., 2018

	constipation (mean 38.1-41.7 y)	dose - $1x10^9$ cfu/d); or placebo capsules		Parameters (patient assessment of constipation symptoms; quality of life; bowel function index); adverse effects	
To evaluate the effect of consumption of milk containing *B. lactis* HN019 on the classical parameters of metabolic syndrome and other related cardiovascular risk factors	51 patients with metabolic syndrome	2 groups - Milk containing *B. lactis* HN019 (~$2.72x10^{10}$ cfu/d); control milk	45 d; P	Anthropometric measures; blood pressure; fasting blood biochemistry (serum or plasma conc of glucose, plasma insulin, lipid profile, HOMA-IR, proinflammatory cytokines [TNF-α, and IL-6])	Bernini et al., 2016
To evaluate the effect of milk powder fortified with micronutrients, docosahexaenoic acid (DHA), a prebiotic, and probiotic *B. lactis* HN019 on the micronutrient status, as well as the presence of fecal probiotic and immune markers	104 pregnant women 8–12 weeks (mean 9.8 wk) of gestation (18-35 y, mean 29.6 y)	2 groups - fortified milk with micronutrients (folic-acid and iron) with or without a composite of *B. lactis* HN019 (DR10™; $1x10^7$ cfu/d), DHA, and inulin (5 g/d)	Until 36-38 wk of gestation; P	Maternal micronutrient level (hemoglobin, transferrin, complete blood count, serum ferritin, retinol, 25-OH-vitamin D, vitamin B, DHA); maternal fecal microbiota (*B. lactis* HN019); inflammation biomarker level; maternal and fetal wellbeing during pregnancy	Wibowo et al., 2016
LKM-512 Strain					
To examine the effects of the probiotic *B. lactis* LKM512 on adult-type atopic dermatitis and the expression of metabolites that are known to be influenced by gut microbiota in fecal samples.	44 Japanese adults with moderate or severe AD (mean 33.8 y)	2 groups - *B. lactis* LKM 512 capsule ($6x10^9$ cfu/d); placebo capsule	8 wk; P	Severity of atopic dermatitis; dermatology-specific quality of life; fecal microbiota; fecal metabolome in patients whose symptoms were prominently improved by LKM512; tolerability and adverse events	Matsumoto et al., 2014
NCC-2818 Strain					

To evaluate the effect of orally administering the probiotic *B. lactis* NCC2818 on immune parameters and nasal symptom scores in subjects suffering from seasonal allergic rhinitis (SAR).	20 adults with clinical history of seasonal allergic rhinitis and positive skin prick test to grass pollen (30.2-41.2 y)	2 groups - *B. lactis* NCC2818 ($4x10^9$ cfu/d); or maltodextrin placebo powder	8 wk during the peak of the pollen season; P	Total nasal symptom scores; whole blood cell cytokines (IL-5, IL-10, IL-13, IL-1β, IFN-γ, and TNF-α); basophile activation test in peripheral blood	Singh et al., 2013
DSM 25566					
To evaluate the effects of supplementation with mixture (*B. lactis* and *B. bifido*) on the tear film in subjects with dry eye syndrome	40 patients with Dry Eye Syndrome (mean 57.5 y)	2 groups - Substitute tear with and without synbiotic (*B. lactis* DSM 25566, and *B. bifido* DSM 25565, and FOS, dosage not specified)	30 d; P	Dry eye symptoms (tear film and ocular surface tera function; tear composition, and ocular surface alterations); incidence of culture positive bacterial tests and total number of aerobic and anaerobic isolates in conjunctiva swab samples	Chisari et al., 2017
Not Specified Strains					
To demonstrate the efficacy of synbiotic treatment in children with functional constipation	146 children with functional constipation (4-18 y, mean 9.2 y)	A mixture of *B. lactis*, *L. casei*, *L. rhamnosus*, and *L. plantarum* ($4x10^9$ cfu/d) + 2.0 g/d prebiotics (fiber, polydextrose, fructo-oligosaccharides, and galacto-oligosaccharides)	4 wk; P	Symptoms of functional constipation	Baştürk et al., 2017
To test the efficacy of probiotic and prebiotic, alone or combined	400 VLBW (<1500g) infants <32 wk	4 groups - 1) *B. lactis* ($5x10^9$ cfu/d); 2) inulin (900 mg/d); 3) combination;	≤ 8 wk; P	NEC; time to reach full enteral feeding; late-onset sepsis; length of neonatal intensive care unit stay; death	Dilli et al., 2015

(synbiotic), on the prevention of necrotizing enterocolitis (NEC) in very low birth weight infants.		4) placebo with breast milk or unsupplemented formula			
To evaluate postnatal growth in preterm infants who received different probiotic supplements; to assess the safety of probiotic administration	199 preterm infants (mean gestation 29.1 wk; mean birth wt. 1,173 g)	4 groups – 1) *B. lactis;* 2) *B. longum;* 3) combination (10^9 cfu/d each); or 4) control (maltodextrin) added to milk	4-6 wk; P	Growth and body composition (body weight, length, head circumference, bone mineral content, and soft tissue composition); nutrient intakes and gastrointestinal tolerance; gut microbiota	Hays et al., 2016
To evaluate the effect of consumption of probiotic yogurt on the children's salivary cariogenic microflora.	49 healthy children (6-12y, mean 9.2 y)	200 g yogurt containing *B. lactis* (1×10^6 cfu/d); or control	2 wk; X	Salivary *Lactobacilli* and *Streptococcus mutans* counts	Nozari et al., 2015

Study design: P= parallel; X= crossover study design

ALP= alkaline phosphatase; ALT= alanine transaminase; AST= aspartate aminotransferase; BMI= body mass index; cfu= colony forming unit; d= days; DHA=docosahexaenoic acid; FOS= fructooligosaccharides; GOS=galactooligosaccharides HDL-C= high density lipoprotein cholesterol; HOMA-IR= homeostasis model assessment insulin resistance; IBS=irritable bowel syndrome; IFN= interferon; Ig= immunoglobulin; IL= interleukin; LDH= lactate dehydrogenase; LDL-C= low density lipoprotein cholesterol; mo= months; NEC=necrotizing enterocolitis; SCFA=short chain fatty acids; TNF= tumor necrosis factor; TAG= triacylglycerol; TC= total cholesterol; TG= triglyceride; TGF= transforming growth factor; XOS=xylooligosaccharides; wk= weeks.

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From: Susan S Cho
To: Zhang, Janet
Subject: Re: Acknowledgement letter for GRN 000875
Date: Monday, August 19, 2019 11:14:21 AM
Attachments: image001.png

Dear Dr. Zhang,

Thank you very much. Have a nice day!

Sincerely,
Susan

Sent from Yahoo Mail for iPhone

On Monday, August 19, 2019, 11:07 AM, Zhang, Janet <Janet.Zhang@fda.hhs.gov> wrote:

> Dear Ms. Cho, attached is the acknowledgement letter for GRN 000875. Thanks
>
> *Jianrong (Janet) Zhang, Ph.D.*
>
> FDA/OFVM/CFSAN/OFAS/DST
>
> College Park, MD 20740
>
> Phone: 240-402-1327
>
> janet.zhang@fda.hhs.gov

From:	Susan S Cho
To:	Gaynor, Paulette M
Subject:	Re: Information regarding GRN 000875 - response requested
Date:	Wednesday, October 23, 2019 5:27:52 PM
Attachments:	image001.png image003.png image002.png image005.png image004.png image006.png

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Dear Dr. Gaynor,

On behalf of Bifido, Ltd., we ask you to cease evaluation. We would appreciate it if you would let us know deficiencies. Thank you very much.

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

On Wednesday, October 23, 2019, 04:26:13 PM EDT, Gaynor, Paulette M <Paulette.Gaynor@fda.hhs.gov> wrote:

Susan Cho, Ph.D.

NutraSource, Inc.

Dear Dr. Cho,

To let you know, the project management responsibilities for GRN 000875 have passed from Dr. Jianrong (Janet) Zhang to me.

This email is to inform you that after evaluating BIFIDO Co., Ltd. (BIFIDO)'s GRAS notice for *Bifidobacterium animalis* subsp *lactis* strain AD011, GRN 000875, our review team has identified a number of issues and deficiencies or inconsistencies with this notice.

These issues focus on the context and text emphasizing the purported health benefit aspects (e.g., "probiotic" in multiple sections and Appendices) of the subject of the notice, the notifier's view that the subject of the notice is a nutrient under the intended use (e.g., header for Section 3.E), as well as the lack of a definitive statement that the subject of the notice is non-pathogenic and non-toxigenic. We consider the safety of the intended use of a substance in our evaluation of a GRAS notice rather than purported health benefit aspects.

Additional deficiencies include: lack of clarity whether there is a wash step after the microorganism is harvested from the soy-peptone fermentation medium, lack of an English translation for the non-English text in Appendix A, lack of clarity about the use of this microorganism with other microorganisms (i.e., substitutional for or additive to), inconsistent information about the excipient because some text states corn starch and some text refers to FDA's regulation for modified food starch in 21 CFR 172.892 (and if, the excipient is a modified corn starch, then what is the applicable modification under this regulation), lack of clarity whether the maltodextrin is from one of the three starches identified in 21 CFR 184.1444.

Further, please note that part 5 of a GRAS notice (experience based on common use in food before 1958) relates to a statutory basis for a conclusion of GRAS status based on common use in food (please see 21 CFR 170.245). Please also note there are other inconsistencies (e.g., part 1 of a GRAS notice in terms of the text as compared to 21 CFR 170.225, maltodextrin is referred to as cryoprotective agent or as excipient), as well as that regulatory status applies to conditions of use of a substance rather than to the substance itself.

FDA does not accept re-written parts of a GRAS notice. Due to the quality of this submission, we offer BIFIDO the opportunity to request that we cease our evaluation of GRN 000875. BIFIDO is welcome to resubmit a new notice after the issues and deficiencies/inconsistencies are addressed. Prior to the submission of a new GRAS notice, we suggest that BIFIDO request a pre-submission meeting with FDA.

We want to give you time to discuss the issues and deficiencies with BIFIDO. Thus, we ask to you provide your response within 10 business days (before COB November 6, 2019).

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
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