JHeimbach LLC

August 30, 2022

Susan J. Carlson, Ph.D., Director
Office of Food Additive Safety (HFS-200),
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Dr., College Park, MD 20740

Dear Dr. Carlson:

Pursuant to 21 CFR Part 170, Subpart E, Nestle Nutrition, through me as its agent, hereby provides notice of a claim that the addition of *Bifidobacterium longum* ssp. *infantis* LMG 11588 to non-exempt infant formula is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because Nestle Nutrition has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

A virus-free CD is enclosed containing Form 3667, the GRAS monograph, and the signatures of members of the GRAS panel in a zip directory produced through COSM.

If you have any questions regarding this notification, please feel free to contact me at 202-320-3063 or jh@jheimbach.com.

Sincerely,

[redacted]

James T. Heimbach, Ph.D., F.A.C.N.
President

Encl.

1205 Prince Edward Street, Fredericksburg Virginia 22535, USA
tel. (+1) 804-742-5548 cell (+1) 202-320-3063 jh@jheimbach.com

Generally Recognized as Safe (GRAS) Determination for the Intended Use of *Bifidobacterium longum* ssp. *infantis* LMG 11588 in Non-Exempt Infant Formula

Prepared by Nestle Nutrition and JHeimbach LLC
August 29, 2022

Table of Contents

List of Tables

List of Figures

Part 1. Signed Statements and Certification

1.1 GRAS Notice Submission

Nestlé Nutrition hereby submits this Generally Recognized as Safe (GRAS) notification through its agent James T. Heimbach, president of the consulting firm JHeimbach LLC, in accordance with the requirements of 21 CFR Part 170, Subpart E.

1.2 Name and Address of Notifier

Notifier Contact
Cheryl Callen
Senior Director, Regulatory Affairs
Nestlé Nutrition
1812 North Moore Street
Arlington, VA 22209
Cheryl.Callen@us.nestle.com
Tel: 201-650-1561

Agent Contact
James T. Heimbach, Ph.D., F.A.C.N.
President, JHeimbach LLC
923 Water Street, P.O. Box 66
Port Royal, VA 22535
jh@jheimbach.com
Tel: 804-742-5543

1.3 Name of Notified Substance

The subject of this GRAS notice is *Bifidobacterium longum* ssp. *infantis* LMG 11588. *Bifidobacterium longum* ssp. *infantis* LMG 11588 is deposited under several culture collections, including the German Collection of Microorganisms (DSMZ) under DSM 20218, the American Type Culture Collection (ATCC) under ATCC 17930, and the Belgian Coordinated Collection of Microorganisms (BCCM/LMG) under LMG 11588. It is deposited in the internal Nestlé Culture Collection (NCC) under NCC 3089.

1.4 Intended Conditions of Use

Bifidobacterium longum ssp. *infantis* LMG 11588 is intended to be added to powder non-exempt infant formula and toddler drinks for healthy children up to 3 years of age. The addition level will not exceed 1.2 x 10^8 CFU/g powder.

1.5 Statutory Basis for GRAS Status

Nestlé Nutrition's GRAS determination for the intended use of *Bifidobacterium longum* ssp. *infantis* LMG 11588 is based on scientific procedures in accordance with 21 CFR §170.30(a) and §170.30(b).

Determination of the safety and GRAS status of the intended use of *Bifidobacterium longum* ssp. *infantis* LMG 11588 has been made by a GRAS panel consisting of Dr. Douwe van Sinderen, Dr. Colin Hill, and Dr. Dan O'Sullivan[1]. These individuals are qualified by scientific training and experience to evaluate the safety of food ingredients intended for addition to powder non-exempt infant formula and toddler drinks. They independently critically reviewed and evaluated the publicly available information and the potential infant and toddler exposure to *Bifidobacterium longum* ssp. *infantis* LMG 11588 anticipated to result from its intended use, and individually and collectively determined that no evidence exists in the available information on *Bifidobacterium longum* ssp. *infantis* LMG 11588 that demonstrates, or suggests reasonable grounds to suspect, a hazard to infants or toddlers under the intended conditions of use.

It is the GRAS Panel's opinion that other qualified scientists reviewing the same publicly available information would reach a similar conclusion regarding the safety of the substance under its intended conditions of use. Therefore, the intended use of *Bifidobacterium longum* ssp. *infantis* LMG 11588 in powder non-exempt infant formula and toddler drinks for consumption by infants and toddlers is GRAS by scientific procedures.

1.6 Premarket Exempt Status

The intended use of *Bifidobacterium longum* ssp. *infantis* LMG 11588 is not subject to the premarket approval requirements of the Federal Food, Drug and Cosmetic Act based on Nestlé Nutrition's determination that it is GRAS.

[1]Douwe van Sinderen, APC Microbiome Ireland and School of Microbiology, University College Cork
Colin Hill, APC Microbiome Ireland and School of Microbiology, University College Cork
Dan O'Sullivan, Department of Food Science and Nutrition, University of Minnesota

1.7 Data Availability

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. FDA upon request, or will be available for review and copying at reasonable times at the offices of:

Nestlé Nutrition
1812 North Moore Street
Arlington, VA 22209

Should the U.S. FDA have any questions or additional information requests regarding this notification, Nestlé Nutrition will supply these data and information.

1.8 Freedom of Information Act Statement

None of the information in this GRAS notice is exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

1.9 Certification

To the best of Nestlé Nutrition's knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to Nestlé Nutrition and pertinent to the evaluation of the safety and GRAS status of the intended use of *Bifidobacterium longum* ssp. *infantis* LMG 11588.

1.10 Name, Position, and Signature of Notifier

James T. Heimbach, Ph.D., F.A.C.N.
President, JHeimbach LLC
Agent to Nestlé Nutrition

1.11 FSIS Statement

Not applicable.

Part 2. Identity, Methods of Manufacture, Specifications, and Physical or Technical Effect

2.1 Name of the Organism

The subject of this GRAS notification is *Bifidobacterium longum* ssp. *infantis* LMG 11588.

2.2 Source of the Organism

Bifidobacterium longum ssp. *infantis* LMG 11588 was initially isolated in 1950 from a 2-month-old breast-fed infant under the isolation name "Timberlain" (Norris, 1950). The same strain is also referred to as "308" in the 1956 publication of Pine & Howell (Howel, 1956). The strain is deposited in several culture collections, including the German Collection of Microorganisms (DSMZ) under the DSM 20218, the American Type Culture Collection (ATCC) under ATCC 17930, and the Belgian Coordinated Collection of Microorganisms (BCCM/LMG) under LMG 11588. The strain was obtained from the BCCM/LMG and deposited in the internal Nestlé Culture Collection as NCC 3089.

2.3 Description of the Organism

Bifidobacteria have been used in food products and dietary supplements for decades, with a compelling record of safe consumption (Kocian, 1994; FAO/WHO, 2002). The organism that is the subject of this GRAS notice is a thoroughly characterized strain belonging to this genus. It has not been genetically modified, is non-pathogenic and non-toxigenic, and does not produce antibiotics.

2.3.1 *Bifidobacterium longum* ssp. *infantis* LMG 11588

Bifidobacteria predominate in the intestinal tract of infants shortly after birth. They are important and normal constituents of the human gastrointestinal microbiota and occur at concentrations of 10^9 to 10^{10} CFU/g of feces (Tanaka et al., 2000). *Bifidobacterium longum* ssp. *infantis* is a natural inhabitant of the intestinal tract microbiota.

In 2002, the International Dairy Federation (IDF), in collaboration with the European Food and Feed Cultures Association (EFFCA), assembled a list of microorganisms with a documented history of safe use in food (Mogensen et al., 2002). The species *Bifidobacterium infantis* was listed in this initial inventory and was further included in the revised version of this inventory as *Bifidobacterium longum* ssp. *infantis* in 2012 (Bourdichon et al., 2012). *Bifidobacterium longum*

ssp. *infantis* has also been included in the Qualified Presumption of Safety (QPS) list established by the European Food Safety Authorities (EFSA, 2017).

2.3.1.1 Phenotypic identification of *Bifidobacterium longum* ssp. *infantis* LMG 11588

Morphology

- Irregular rod shaped (see Figure 1)
- Non-motile
- Non sporulating
- Gram-positive
- On RCM agar, *Bifidobacterium longum* ssp. *infantis* LMG 11588 forms small white and smooth colonies (see Figure 2 below)

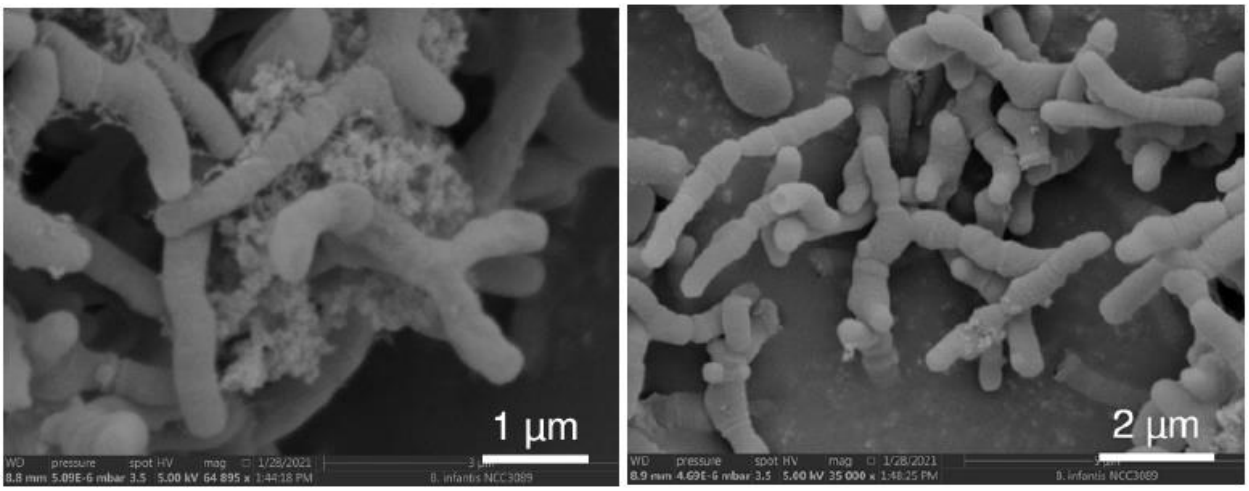

Figure 1: Scanning Electron Microscopy picture of *Bifidobacterium longum* ssp. *infantis* LMG 11588 (Magnification 64'895 and 35,000x). Scanning Electron Micrograph photo by Nestlé Research (Lausanne, Switzerland) by Carine Meyer.

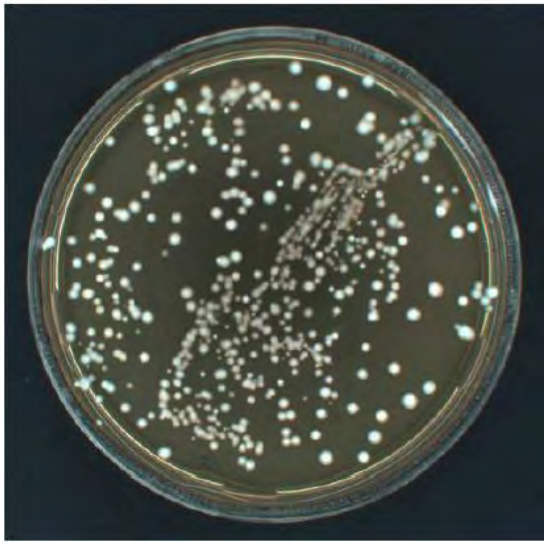

Figure 2: Colonies of *Bifidobacterium longum* ssp. *infantis* LMG 11588 grown on RCM Agar (24h on RCM agar, grown at 37°C anaerobically)

Biochemical Testing

Bifidobacterium longum ssp. *infantis* LMG 11588 is heterofermentative and produces lactic acid and acetic acid in a 1.74:3 molar ratio, which is close to the 2:3 theoretical ratio produced through the bifid shunt (Sánchez et al., 2004). The strain is catalase-negative and harbors bile salt deconjugase activity (Table 1). It predominantly produces L-lactic acid and is therefore classified as a L-lactic acid -producing strain. *Bifidobacterium longum* ssp. *infantis* LMG 11588 is classified as a urease positive strain, as its urease enzyme activity has been shown to be induced when growing in a synthetic medium comprising urea as the sole nitrogen source (Schimmel, 2021).

Table 1: General Biochemical Characteristics of *Bifidobacterium longum* ssp. *infantis* LMG 11588

Biochemical Characteristic	Result
Heterofermentation (MRS broth, 18h at 37°C anaerobically)	Lactic acid (2.32 g/L; 0.026 mol/L) Acetic acid (2.21 g/L; 0.045 mol/L)
Catalase (24h colonies on RCM agar, grown at 37°C anaerobically)	Negative
Bile salt deconjugase (RCM agar suppl. 0.5% w/v taurodeoxycholic acid, 5 days at 37°C anaerobically)	Positive

Lactic Acid type (D/L-lactic acid enzymatic kit MRS broth, 18h at 37°C anaerobically)	L-Lactic acid ≥ 90% D-Lactic acid ≤ 10%
Urease (Schimmel, 2021)	Positive
Optimal Growth Temperature	37°C
Oxygen requirement	Strict anaerobe

The carbohydrate consumption capacity of the strain was evaluated using API 50 CHL (Biomérieux), which is a micro-method evaluating the acidification capacity of the strain on 49 different carbohydrates (Table 2). The API 50 CHL gallery (Biomérieux) is an identification tool employed here to characterize the strain *Bifidobacterium longum* ssp. *infantis* LMG 11588 and its metabolic activity, not as a means of identification.

Table 2: Carbohydrate Consumption Profile of *Bifidobacterium longum* ssp. *infantis* LMG 11588, as determined using API 50 CHL.

API 50 CHL (37°C, 48 hours)									
Control	-	Galactose	+	a-Methyl-D-mannoside	-	Melibiose	+	D-Turanose	-
Glycerol	-	D-Glucose	+	a-Methyl-D-glucoside	-	Sucrose	+	D-Lyxose	+/-
Erythritol	-	D-Fructose	+	N-Acetylglucosamine	+	Trehalose	-	D-Tagatose	-
D-Arabinose	-	D-Mannose	+	Amygdalin	-	Inulin	-	D-Fucose	-
L-Arabinose	-	L-Sorbose	-	Arbutin	-	Melezitose	-	L-Fucose	+/-
Ribose	+	Rhamnose	-	Esculin	-	D-Raffinose	+	D-Arabitol	-
D-Xylose	+	Dulcitol	-	Salicin	-	Starch	-	L-Arabitol	-
L-Xylose	-	Inositol	+	Cellobiose	-	Glycogen	-	Gluconate	-
Adonitol	-	Mannitol	-	Maltose	+	Xylitol	-	2-Ketogluconate	-
B Methylxyloside	-	Sorbitol	-	Lactose	+	b-Gentiobiose	-	5-Ketogluconate	+/-

Note: The strain was incubated for 48h at 37°C under anaerobiosis.

Bifidobacterium longum ssp. *infantis* LMG 11588 was also characterized using API ZYM (Biomérieux) which is a micro-method designed for testing of 19 enzymatic activities. The following enzymatic activities were detected:

- leucine arylamidase
- alpha-galactosidase
- beta-galactosidase
- alpha-glucosidase

2.3.1.2 Genotypic Identification of *Bifidobacterium longum* ssp. *infantis* LMG 11588

The genotypic characteristics of *Bifidobacterium longum* ssp. *infantis* LMG 11588 are described below.

2.3.1.2.1 16S rDNA Sequence

16S rDNA-based identification of *Bifidobacterium longum* ssp. *infantis* LMG 11588 was performed in an ISO 17025 accredited laboratory (Accugenix, Charles River, Ecully, France). The 16S rDNA sequence obtained was compared to a reference database and showed the closest similarity to sequences of *B. longum* strains, confirming its identity at the species level.

2.3.1.2.2 MALDI-ToF Profiling

Matrix Assisted Laser Desorption Ionization - Time of Flight based identification was performed in an ISO 17025 accredited laboratory (Accugenix, Charles River, Ecully, France). The obtained overall protein profile of *Bifidobacterium longum* ssp. *infantis* LMG 11588 was compared to a reference database and showed the closest similarity to different strains of *Bifidobacterium longum* ssp. *infantis*, including the type strain DSM 20088, confirming its identity at the subspecies level.

2.4 Genomic Analysis

The genome of *Bifidobacterium longum* ssp. *infantis* LMG 11588 has been sequenced and annotated to assure that the strain does not harbor known virulence genes, potentially transferable antibiotic resistance genes, or the capability to synthesize biogenic amines.

2.4.1 *Bifidobacterium longum* ssp. *infantis* LMG 11588

2.4.1.1 Sequencing

The whole genome sequence of *Bifidobacterium longum* ssp. *infantis* LMG 11588 was sequenced in 2021 using PacBio technology according to the supplier's recommendation. The DNA library preparation was performed following the recommended protocol from PacBio: "Preparing multiplexed microbial library using the SMRTbell Express Template Prep Kit 2.0" (Part Number 101-696-100 Version 07 (July 2020)). DNA quality was checked along the library preparation using the Fragment Analyzer (Agilent Technologies, Inc., Santa Clara, CA) and quantified by QuBit dsDNA protocol (Thermo Fisher Scientific AG, Basel, Switzerland). Sequencing was performed on a Sequel platform with 10 hours movies on LR SMRT cell. The loading was performed by diffusion at 8 pM with 2 hours of pre-extension time. The sequencing data were further assembled using the Hierarchical Genome Assembly Process (HGAP4) *de novo* assembly analysis application available through the SMRT Link portal (Pacific Biosciences, Menlo Park, CA), resulting in a single closed contig (Table 3). The obtained genome, together with its annotation (see below) were deposited at the Joint Genome Institute (JGI) (https://img.jgi.doe.gov; Project ID Ga0526375).

Table 3: *Bifidobacterium longum* ssp. *infantis* LMG 11588 Genome Sequencing Statistics

Element	Quantity
Total genome size (bp)	2,608,122
Contig	1
GC content (%)	59

2.4.1.2 Annotation of the Genome

The LMG 11588 contigs were further annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). A total of 2301 CDS were predicted, among which 2150 have been annotated. A total of 2078 genes were found to code for proteins. In addition, 57 tRNAs, 12 rRNAs, and 3 ncRNA were predicted (Table 4).

Table 4: *Bifidobacterium longum* ssp. *infantis* LMG 11588 Genome Annotation Statistics

Element	Quantity
Genes (total)	2,301
CDSs (total)	2,229
Genes (coding)	2,078
CDSs (with protein)	2,078
Genes (RNA)	72
rRNAs	4, 4, 4 (5S, 16S, 23S)
complete rRNAs	4, 4, 4 (5S, 16S, 23S)
partial rRNAs	0
tRNAs	57
ncRNAs	3
Pseudo Genes (total)	151
CDSs (without protein)	151
Pseudo Genes (ambiguous residues)	0 of 151
Pseudo Genes (frameshifted)	79 of 151
Pseudo Genes (incomplete)	87 of 151
Pseudo Genes (internal stop)	21 of 151
Pseudo Genes (multiple problems)	31 of 151
CRISPR Arrays	2

2.4.1.3 Annotation of Plasmids

The *Bifidobacterium longum* ssp. *infantis* strain LMG 11588 does not contain any plasmids.

2.4.1.4 Whole Genome-Based Identification of the Microorganism

A recent phylogenetic comparison of *Bifidobacterium longum* species based on 500 core proteins was obtained using the Phylogenetic Tree Building Service of the PATRIC database. This analysis showed that the strain ATCC 17930 (LMG 11588) belongs to *Bifidobacterium longum* ssp. *infantis*

and is undistinguishable from three other strains: *Bifidobacterium longum* ssp. *infantis* Bi-26, EK3, and Rossell-33 (Zabel et al., 2020).

To further confirm the identity of the strain using its whole genome, its sequence was compared to a similar set of public *Bifidobacterium* genomes by average nucleotide identity (ANI). All genomes from the above-mentioned work that could be found on the PATRIC database were downloaded (Table 5). ANI was computed using the OrthoANIu software (Yoon et al., 2017) with default parameters. ANI analysis enabled to confirm that LMG 11588 belongs to *Bifidobacterium longum* ssp. *infantis*, as its genome clustered with other strains of the same subspecies and showed an ANI identity of 98.36 % to the type strain *Bifidobacterium longum* ssp. *infantis* ATCC 15697 (Figure 3). This was confirmed using a similar analysis performed on a set of 23 *Bifidobacterium longum* ssp. *infantis* genomes as LMG 11588 showed an ANI identity of 98.28 % to the typestrain ATCC 15697 (Duboux et al., 2022).

LMG 11588 is closely related to other strains of *Bifidobacterium longum* ssp. *infantis* (e.g., Rossell-33) displaying over 99.9 % of ANI identity to those genomes (rounded to 100.0 in Figure 3) (Duboux et al., 2022).

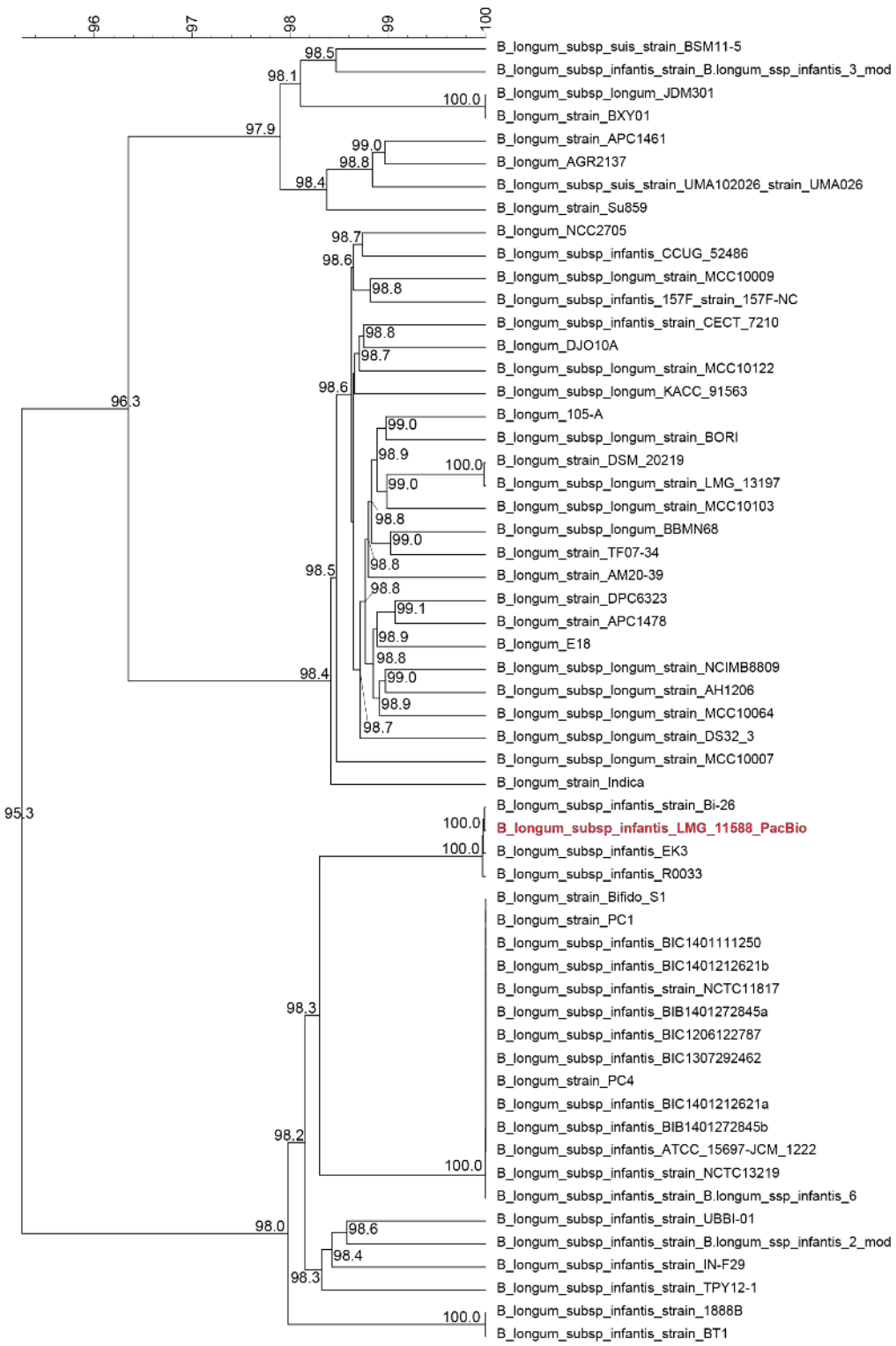

Figure 3: Whole Genome-Based ANI Phylogeny Analysis of *Bifidobacterium longum* ssp. *infantis* LMG 11588 and Other Publicly Available Genomes

Table 5: List of Bifidobacterium longum Genomes Used for the ANI Phylogenetic Analysis

Genome strain name	
Bifidobacterium longum strain APC1461	*Bifidobacterium longum* strain DPC6323
Bifidobacterium longum subsp *infantis* ATCC 15697=JCM 1222	*Bifidobacterium longum* subsp *longum* strain LMG 13197
Bifidobacterium longum strain Bifido S1	*Bifidobacterium longum* subsp *infantis* strain 3 mod
Bifidobacterium longum NCC2705	*Bifidobacterium longum* DJO10A
Bifidobacterium longum subsp *longum* strain MCC10064	*Bifidobacterium longum* subsp *infantis* strain 1888B
Bifidobacterium longum E18	*Bifidobacterium longum* subsp *infantis* strain NCTC13219
Bifidobacterium longum subsp *infantis* strain UBBI-01	*Bifidobacterium longum* subsp *longum* strain BORI
Bifidobacterium longum subsp *longum* BBMN68	*Bifidobacterium longum* subsp *infantis* strain IN-F29
Bifidobacterium longum strain DSM 20219	*Bifidobacterium longum* subsp *infantis* strain 2 mod
Bifidobacterium longum subsp *infantis* BIC1401212621b	*Bifidobacterium longum* subsp *infantis* R0033
Bifidobacterium longum subsp *longum* strain NCIMB8809	*Bifidobacterium longum* subsp *longum* strain MCC10122
Bifidobacterium longum subsp *longum* strain AH1206	*Bifidobacterium longum* subsp *infantis* strain BT1
Bifidobacterium longum subsp *longum* KACC 91563	*Bifidobacterium longum* strain APC1478
Bifidobacterium longum subsp *longum* strain MCC10009	*Bifidobacterium longum* subsp *infantis* BIC1206122787
Bifidobacterium longum subsp *infantis* 157F strain 157F-NC	*Bifidobacterium longum* AGR2137
Bifidobacterium longum subsp *infantis* strain CECT 7210	*Bifidobacterium longum* subsp *infantis* BIC1307292462
Bifidobacterium longum subsp *infantis* EK3	*Bifidobacterium longum* subsp *suis* strain BSM11-5
Bifidobacterium longum strain PC1	*Bifidobacterium longum* strain AM20-39
Bifidobacterium longum subsp *longum* strain MCC10007	*Bifidobacterium longum* subsp *infantis* BIC1401111250
Bifidobacterium longum subsp *infantis* strain TPY12-1	*Bifidobacterium longum* strain TF07-34
Bifidobacterium longum subsp *longum* strain MCC10103	*Bifidobacterium longum* strain PC4
Bifidobacterium longum subsp *infantis* CCUG 52486	*Bifidobacterium longum* subsp *infantis* BIC1401212621a
Bifidobacterium longum subsp *infantis* strain NCTC11817	*Bifidobacterium longum* subsp *infantis* LMG 11588 (NCC 3089)
Bifidobacterium longum subsp *infantis* strain 6	*Bifidobacterium longum* subsp *suis* strain UMA102026 strain UMA026
Bifidobacterium longum subsp *longum* JDM301	*Bifidobacterium longum* subsp *infantis* strain Bi-26
Bifidobacterium longum 105-A	*Bifidobacterium longum* strain BXY01
Bifidobacterium longum subsp *infantis* BIB1401272845a	*Bifidobacterium longum* strain Su859
	Bifidobacterium longum subsp *longum* strain DS32 3
	Bifidobacterium longum subsp *infantis* BIB1401272845b
	Bifidobacterium longum strain *Indica*

Note: Tree was obtained using BioNumerics UPGMA method. ANI similarity value represented at each node is rounded to 1 decimal point.

2.4.1.5 Antibiotic Resistance

To identify potentially acquired antimicrobial resistance, *Bifidobacterium longum* ssp. *infantis* LMG 11588 genome was screened for the presence of antimicrobial resistance genes using two different software programs: ResFinder-PointFinder (Zankari et al., 2012) and AMR-Finder (https://github.com/ncbi/amr/wiki). Analysis was performed at the nucleotide level, and when appropriate, identity and coverage thresholds were set to 70% and 60%, respectively. Using those thresholds, no hit was found in the LMG 11588 genome using ResFinder. Using the same software, no specific point mutation conferring antimicrobial resistance was found in the *Bifidobacterium longum* ssp. *infantis* LMG 11588 genome. The LMG 11588 genome was further screened with AMR-Finder for the presence of genes or point mutations known to be involved in antimicrobial resistance. Results show a positive hit with a *Campylobacter* streptomycin resistant rpsL gene coding for the 30S ribosomal protein S12 (Figure 4).

Contig id	Start	Stop	Strand	Gene symbol	Sequence name	Scope	Element type	Element subtype	Class	Subclass	Method	Target length	Reference sequence length	% Coverage of reference sequence	% Identity to reference sequence	Alignment length	Accession of closest sequence	Name of closest sequence
Contig_24_113.547	96972	97340	+	rpsL_K43R	Campylobacter streptomycin resistant RpsL [NO_CALL]	core	AMR	POINT	AMINOGLYCOSIDE	STREPTOMYCIN	POINTX	123	128	96.09	77.24	123	WP_057042458.1	30S ribosomal protein S12 RpsL
Contig_24_113.547	96972	97340	+	rpsL_K88E	Campylobacter streptomycin resistant RpsL [NO_CALL]	core	AMR	POINT	AMINOGLYCOSIDE	STREPTOMYCIN	POINTX	123	128	96.09	77.24	123	WP_057042458.1	30S ribosomal protein S12 RpsL
Contig_24_113.547	96972	97340	+	rpsL_K88Q	Campylobacter streptomycin resistant RpsL [NO_CALL]	core	AMR	POINT	AMINOGLYCOSIDE	STREPTOMYCIN	POINTX	123	128	96.09	77.24	123	WP_057042458.1	30S ribosomal protein S12 RpsL
Contig_24_113.547	96972	97340	+	rpsL_K88R	Campylobacter streptomycin resistant RpsL [NO_CALL]	core	AMR	POINT	AMINOGLYCOSIDE	STREPTOMYCIN	POINTX	123	128	96.09	77.24	123	WP_057042458.1	30S ribosomal protein S12 RpsL

Figure 4: Potential Antibiotic Resistance Found in the Genome of *Bifidobacterium longum* ssp. *infantis* LMG 11588, Identified by the AMR-Finder Software and Database

It has been previously described that a mutation in the rpsL gene (A to G replacement occurring at nucleotide position 128 of the rpsL gene) can confer streptomycin resistance in bifidobacteria (Xiao et al., 2010; Kiwaki and Sato., 2009). This mutation is, however, different from the one described in *Campylobacter* and the LMG 11588 strain does not harbor it. Furthermore, it was shown that the LMG 11588 strain is sensitive to streptomycin (Table 9), confirming that the sequence of the LMG 11588 rpsL gene does not encode a resistance to that antibiotic.

2.4.1.6 Synthesis of Biogenic Amines

It is also worth noting that LMG 11588 genome annotation did not reveal the presence of genes encoding tyrosine decarboxylase (EC 4.1.1.25), ornithine decarboxylase (EC 4.1.1.17), histidine decarboxylase (EC 4.1.1.22), or lysine decarboxylase (EC 4.1.1.18), which are responsible for the

formation of biogenic amines (respectively tyramine, putrescine, histamine, and cadaverine) through amino acid decarboxylation.

2.4.1.7 Virulence/Infectivity

The *Bifidobacterium longum* ssp. *infantis* LMG 11588 genome sequence was screened for the presence of virulence and toxigenic genes using sequence similarity search (BLAST (Altschul et al., 1990)) against the reference database VFDB (Chen et al., 2005). Proteic and nucleic versions of the core dataset (includes genes associated with experimentally verified VFs only) were downloaded from VFDB (http://www.mgc.ac.cn/VFs/download.htm). BlastN was used to search the complete LMG 11588 genome sequence against the nucleic version of the downloaded VFDB, and BlastP was used to search all LMG 11588 proteins against the proteic version of the downloaded VFDB. In both searches, results were filtered to identify hits with more than 70% identity and 60% subject coverage. Following these thresholds, no hit was found for both searches.

2.4.2 *Bifidobacterium longum* ssp. *infantis* LMG 11588 & Rosell®-33 (R0033) Are Two Isolates of the Same Strain

The previous Average Nucleotide Based analysis revealed a close relationship between the two R0033 and LMG 11588 strains, overall sharing an ANI of 99.98% (Duboux et al., 2022). Furthermore, the GRAS dossier (GRAS notice 758) of Rosell-33 (R0033) is referring to this strain as being ATCC 17930 (page 172 of the GRAS notice), obtained by Lallemand Health Solutions (formerly known as the Institut Rosell) in 1988.

We have previously demonstrated using a *Bifidobacterium longum* ssp. *infantis* strain that different isolates obtained from different culture collections, could harbor up to 15 SNP of differences over the entire strain genome, representing the standard genetic diversity that can be expected in different isolates of the same *Bifidobacterium longum* ssp. *infantis* strain (Duboux et al., 2022). With the below analysis, we clarify the level of potential genetic differences observed between the two LMG 11588 and R0033 isolates.

2.4.2.1 Detailed Genetic Comparison Between *Bifidobacterium longum* ssp. *infantis* LMG 11588 & Rosell®-33 (R0033)

Both LMG 11588 & R0033 sequences were further compared using a dotplot (Figure 5), which confirmed their close relationship: the R0033 sequence is fully covered by the LMG 11588 sequence demonstrating that LMG 11588 has no additional nor lacking sequence as compared

to R0033. Further analysis was performed to reveal Single Nucleotide Polymorphisms (SNP) that would differentiate the two strains. The raw Illumina reads obtained for LMG 11588 were mapped on the Rossell-33 (R0033) genome using the OrthoANI v1.2 software (Yoon et al., 2017). A low number of SNP (16) was found between the two genomes. Using this analysis pipeline, a threshold of <21 Single Nucleotide Polymorphisms (SNPs), associated with a monophyletic phylogenetic tree topology, was suggested to define that pathogenic isolates are from "the same origin" (Pightling et al., 2018). Overall, our data confirm a clonal relationship between the LMG 11588 and the Rossell-33 (R0033) strain, as the number of SNP observed between the two isolates is within the standard genetic diversity that can be expected in different isolates of the same *Bifidobacterium longum* ssp. *infantis* strain (Duboux et al., 2022).

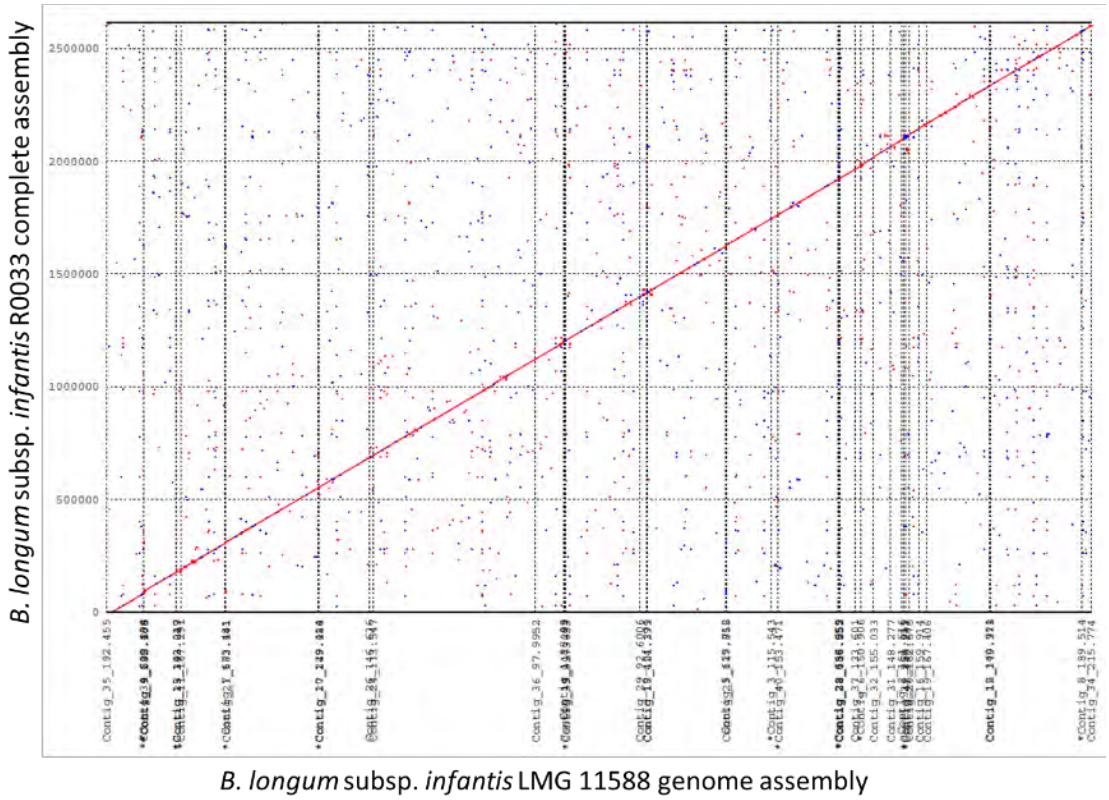

Figure 5: Doptplot Comparison of *Bifidobacterium longum* ssp. *infantis* R0033 (vertical) and *Bifidobacterium longum* ssp. *infantis* LMG 11588 (horizontal) Genomes

Further analysis of the 16 SNP differentiating LMG 11588 and Rosell-33 (R0033) revealed that 3 of them were found in intergenic regions and 4 within a repeated region. Furthermore, 3 additional SNP were shown to be silent, keeping the amino acid sequence of the target proteins unchanged. The 5 last SNPs were observed respectively in an uncharacterized peptidase, a GntR family regulator, a lacI family regulator, a sucrose permease and a metalloendopeptidase membrane protein (Table 6).

Table 6: Single Nucleotide Polymorphism (SNPs) Observed in LMG 11588 as compared to R0033 (reference)

position on reference	Ref base	LMG11588 base	Annotation on ref (locus_tag;product)	AA outcome	Change in aa sequence
100455	G	T	fig|1678.111.peg.93; product=FIG00672241: Uncharacterized peptidase	CGG [R] -> CTG [L]	Yes
365775	A	C	fig|1678.111.repeat.5; product=repeat region		
365776	A	C	fig|1678.111.repeat.5; product=repeat region		
365777	G	C	fig|1678.111.repeat.5; product=repeat region		
365779	C	-	fig|1678.111.repeat.5; product=repeat region		
434380	T	G	intergenic. Upstream of gene fig|1678.111.peg.412; product=Glutamine amidotransferase, class I		
765009	T	G	fig|1678.111.peg.718; product=Transcriptional regulator, GntR family	CTC [L] -> CGC [R]	Yes
773809	A	G	fig|1678.111.peg.725; product=Oligopeptide/dipeptide ABC transporter, permease protein / Oligopeptide/dipeptide ABC transporter, ATP-binding protein	GCA [A] -> GCG [A]	No
797786	C	T	fig|1678.111.peg.741; product=hypothetical protein	ACG [T] -> ACA [T]	No
956918	A	G	fig|1678.111.peg.893; product=Sucrose permease, major facilitator superfamily	CTT [L] -> CCT [P]	Yes
958703	A	C	fig|1678.111.peg.894; product=Transcriptional regulator, LacI family	GTG [V] -> GCG [A]	Yes
1253918	T	G	fig|1678.111.peg.1169; product=Membrane proteins related to metalloendopeptidases	TGG [W] -> GGG [G]	Yes
1429307	C	A	intergenic. Upstream of gene fig|1678.111.peg.1372; product=hypothetical protein (1429376..1430452)		
1628768	A	G	fig|1678.111.peg.1564; product=LOG family protein	GGT [G] -> GGC [G]	No
1817802	A	G	intergenic. Upstream of gene fig|1678.111.peg.1738; product=Transcriptional regulator, LysR family		
2535874	A	C	fig|1678.111.peg.2354;EC=4.3.1.19,4.3.1.17; product=Threonine dehydratase, catabolic (EC 4.3.1.19) @ L-serine dehydratase, (PLP)-dependent (EC 4.3.1.17)	GCT [A] -> GCG [A]	No

2.4.3 Assessment of the Safety of *Bifidobacterium longum* ssp. *infantis* LMG 11588 by a Panel of Experts

The above phenotypic and genomic data were evaluated by a panel of experts[2], who concluded that *Bifidobacterium longum* ssp. *infantis* LMG 11588 is essentially identical to *Bifidobacterium longum* ssp. *infantis* R0033 in terms of its genetic composition and functional characteristics. The panel of experts supported the conclusion that the safety data on strain R0033 is equally applicable to strain LMG 11588 and therefore supports its safety for the intended use (Appendix A).

2.5 Production Process

Bifidobacterium longum ssp. *infantis* LMG 11588 is manufactured according to the principles of current good manufacturing practices (cGMP) at the Nestlé Konolfingen plant, Switzerland.

The manufacturing process is subject to a thorough quality management system. Sterility of the equipment and safety and suitability of the process is ensured by a hazard analysis and critical control point system (HACCP). The plant is certified according to the Food Safety System Certification (FSSC) 22000.

The master stock deposited in the Nestle Culture Collection is the stock from which any new culture ampoule is produced to avoid genetic drift. It is also the one on which all characterizations at the genotypic and phenotypic levels are initially made. To guarantee the identity of each new NCC ampoule batch produced, the identity to the master stock is verified using a set of strain-level typing methods based on repetitive DNA sequences (Versalovic et al., 1991).

All materials used in the production process are safe and suitable food grade materials and Kosher and Halal certified. Raw material qualification and quality assurance programs are in place to verify compliance and absence of microbial contaminations or allergens. All raw materials are approved by Quality Control prior to use. No solvents, other than water, are used in the manufacturing process. All necessary equipment is subject to cleaning-in-place (CIP) and steam sterilization prior to use.

[2] Douwe van Sinderen, APC Microbiome Ireland and School of Microbiology, University College Cork
Colin Hill, APC Microbiome Ireland and School of Microbiology, University College Cork
Dan O'Sullivan, Department of Food Science and Nutrition, University of Minnesota

2.5.1 Manufacturing Process of the Bacterial Culture Powder

Fermentation Media Preparation

Culture media ingredients are dissolved in water and subjected to a heat treatment of time and temperature adequate to destroy any microorganisms present. The media ingredients used in the manufacturing process consist mainly of yeast derived nutrients, carbohydrates, vitamins, and minerals.

Starter and Main Fermentation

The culture media is inoculated with concentrated culture originating from an ampoule of lyophilized *Bifidobacterium longum* ssp. *infantis* LMG 11588. Fermentation takes place to create the starter culture. The starter culture is then used to inoculate sterile growth medium to perform the starter fermentation. The fermentate of the starter fermentation is used to inoculate the larger volumes of sterile growth medium for the main fermentation. Each fermentation takes place under controlled conditions (pH and T°C) and under anaerobic conditions enabling optimal growth and bacterial yield of *Bifidobacterium infantis*.

Centrifugation and Spray Drying

Following the main fermentation, the culture is subjected to centrifugation to separate the bacterial biomass from the fermentation medium. At this point, protective agents (patented composition; WO/2017/001590[3]) and base for pH adjustment are added. All protective agents are either GRAS or approved Food Additives. The biomass is then spray-dried. The obtained culture powder is stored under cool conditions.

A graphic depiction of the manufacturing process is provided in Figure 6. Regular and systematic sampling is in place during production and in finished product for sensory, chemical, physical and/or microbiological analyses. Finished product is released only after all analytical results have been evaluated in comparison to established specifications and assessed to be compliant.

[3] WO/2017/001590A1

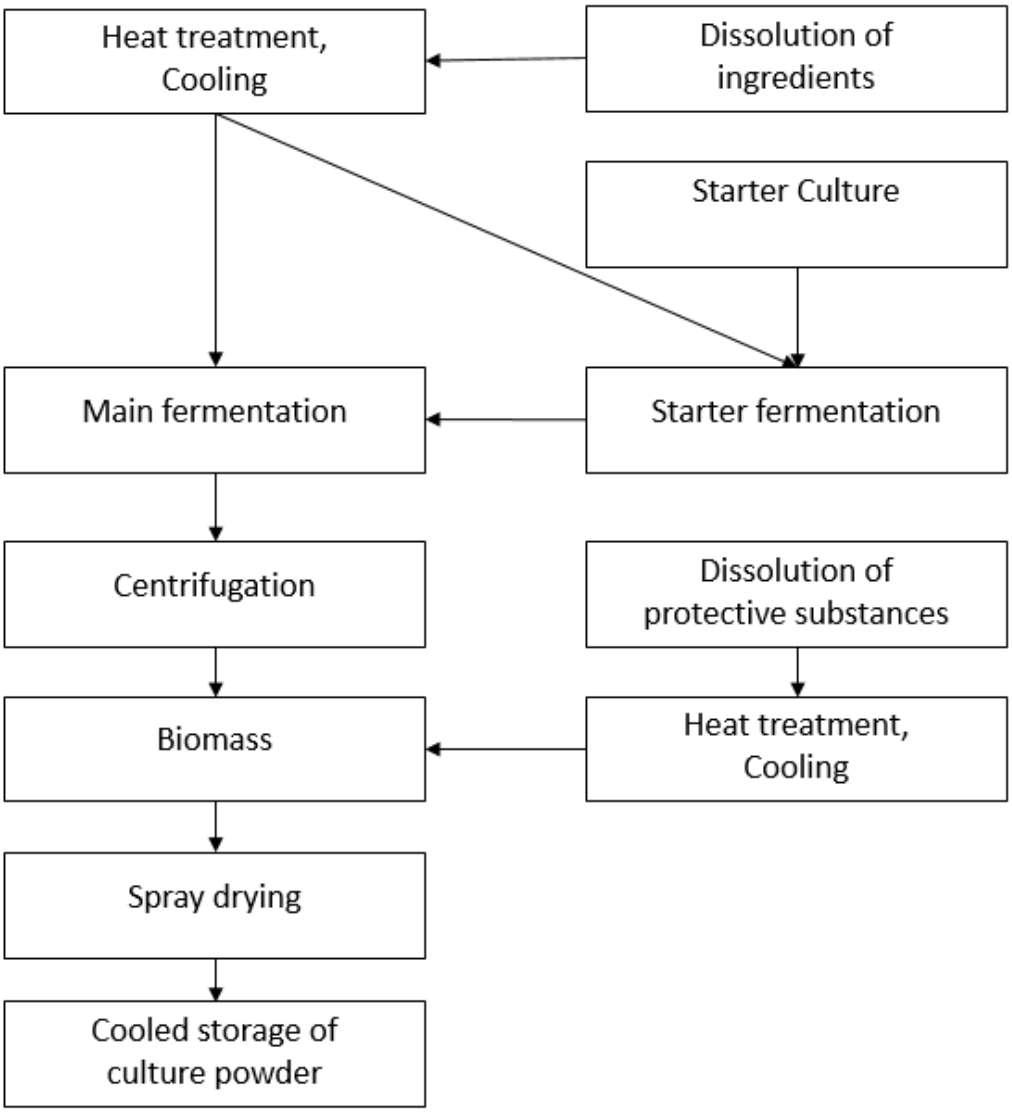

Figure 6: Flow Diagram of the Manufacturing Process of *Bifidobacterium longum* ssp. *infantis* LMG 11588

2.6 Specifications

2.6.1 Specifications of the Bacterial Powder

All batches of *Bifidobacterium longum* ssp. *infantis* LMG 11588 meet the specifications set forth in Table 7.

Table 7: Specification for *Bifidobacterium longum* ssp. *infantis* LMG 11588 Spray Dried Powder

Test	Acceptance Criterion	Methods/Based on
Physical aspect	Free flowing particles, creamy beige powder	Visual observation
Bifidobacterium longum ssp. *infantis*	> 4.0E+10 CFU/g	Bacteriological enumeration – in-house method
	Identity correct	Identity check by MALDI-ToF
Yeast and Molds	< 1000 CFU/g	ISO 21527-2
Aerobic mesophilic microorganisms	< 500 CFU/g	In-house method based on ISO 13559:2002/IDF 153:2002
Enterobacteriaceae	Negative in 10 g	ISO 21528
Coagulase positive staphylococci	< 10 CFU/g or negative in 1 g	ISO 6888-1 or ISO 6888-3
Salmonella spp.	Negative in 10 g or negative in 25 g	AOAC 2011.03
Cronobacter spp.	Negative in 10 g	ISO 22964
Lead	< 0.10 mg/kg	ICP-MS, similar to AOAC 2013.06
Arsenic	< 0.10 mg/kg	ICP-MS, similar to AOAC 2013.06
Mercury	< 0.05 mg/kg	ICP-MS, similar to AOAC 2013.06
Cadmium	< 0.50 mg/kg	ICP-MS, similar to AOAC 2013.06

ISO or AOAC methods are used where they are applicable, appropriate, and fit for purpose. Validated in-house methods are used when no suitable ISO or AOAC method is available.

Table 8 summarizes analytical results of three batches of culture powder of *Bifidobacterium longum* ssp. *infantis* LMG 11588 produced in three different weeks in 2020 and 2021.

Table 8: Analysis Results of 3 Batches of *Bifidobacterium longum* ssp. *infantis* LMG 11588

Test	Acceptance Criterion	Batch no. 869251	Batch no. 874186	Batch no. 00171099V1
Physical aspect	Free flowing particles, creamy beige powder	Conform	Conform	Conform
Bifidobacterium longum ssp. *infantis*	> 4.0E+10 CFU/g	1.3E+11 CFU/g	8.2E+10 CFU/g	1.1E+11 CFU/g
	Identity correct	Identity correct	Identity correct	Identity correct
Yeast and molds	< 1000 CFU/g	< 100 CFU/g	< 100 CFU/g	< 10 CFU/g
Aerobic mesophilic microorganisms	< 500 CFU/g	< 500 CFU/g	< 100 CFU/g	< 10 CFU/g
Entero-bacteriaceae	Negative in 10 g	Negative	Negative	Negative
Coagulase positive staphylococci	< 10 CFU/g in	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g
Salmonella ssp.	Negative in 10 g or negative in 25 g	Negative (25 g)	Negative (25 g)	Negative (10 g)
Cronobacter ssp.	Negative in 10 g	Negative	Negative	Negative
Lead	< 0.10 mg/kg	< 0.004 mg/kg	< 0.004 mg/kg	< 0.004 mg/kg
Arsenic	< 0.10 mg/kg	0.023 mg/kg	0.023 mg/kg	0.025 mg/kg
Mercury	< 0.05 mg/kg	< 0.005 mg/kg	< 0.005 mg/kg	< 0.005 mg/kg
Cadmium	< 0.5 mg/kg	0.043 mg/kg	0.049 mg/kg	0.015 mg/kg

The results show that culture powder of *Bifidobacterium longum* ssp. *infantis* LMG 11588 fulfils the specifications.

2.6.2 Allergens

The fermentation media and the culture powder of *Bifidobacterium longum* ssp. *infantis* LMG 11588 produced by Nestlé is not produced with nor does it contain ingredients that are considered major food allergens including milk/lactose, egg, soy, sesame, peanuts, tree nuts, wheat, fish, or crustacean shellfish.

2.7 Stability

The culture powder of *Bifidobacterium longum* ssp. *infantis* LMG 11588 is stored for up to 1 year at around 12°C (53.6°F). The cell count of the culture powder is stable during this time (see Figure 7).

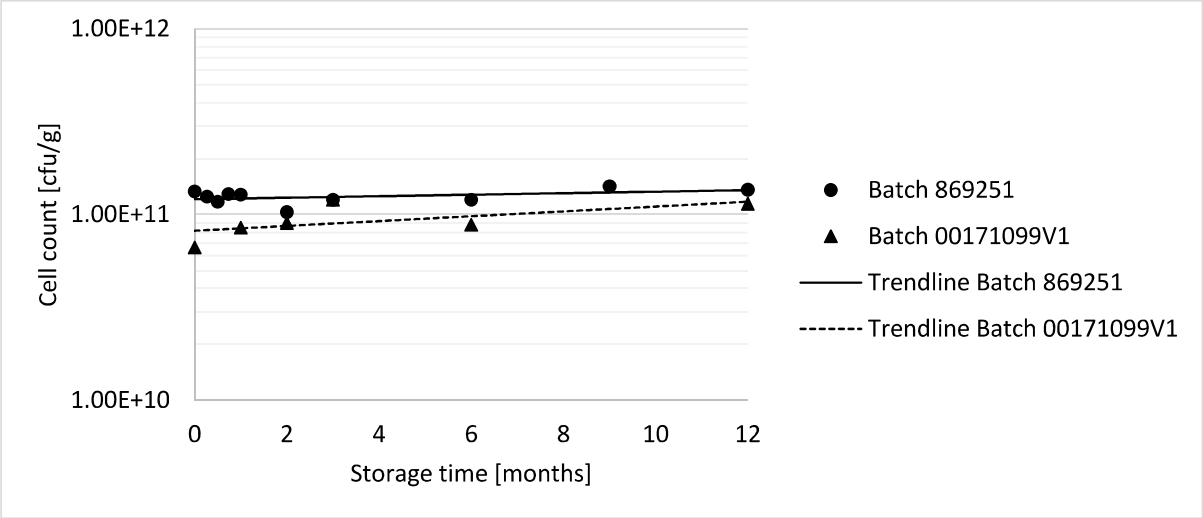

Figure 7: Stability of Two Batches of *Bifidobacterium longum* ssp. *infantis* LMG 11588 Culture Powder at 12°C (53.6°F) and 0.23 Water Activity for 1 Year

For use in infant formula, the culture powder is typically mixed with maltodextrin into a preblend with a standardized cell count. The preblend is stored at around 4°C (39.2°F) for up to 8 months before being mixed into infant formula. Cell counts of *Bifidobacterium longum* ssp. *infantis* LMG 11588 in a preblend are stable during storage, even well beyond 8 months (Figure 8).

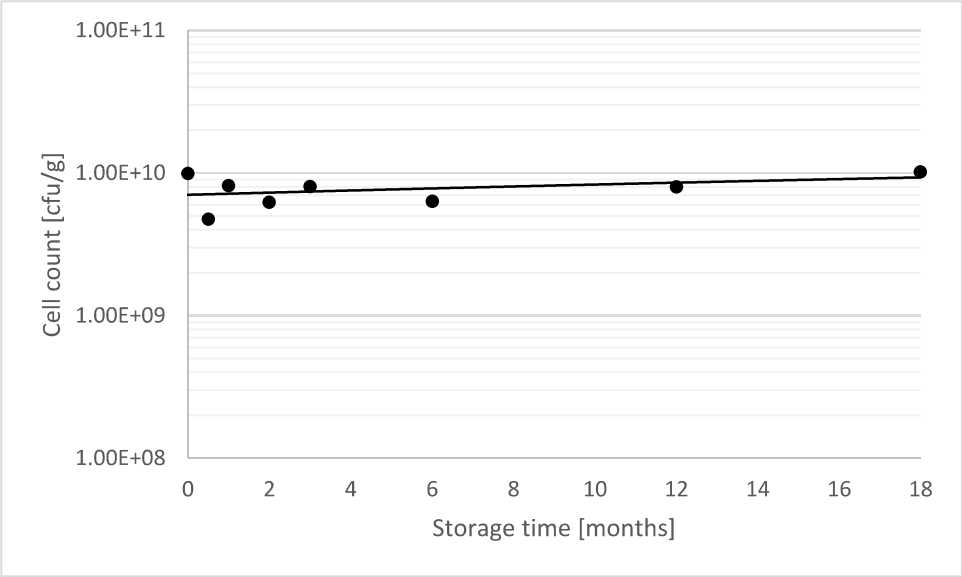

Figure 8: Stability of *Bifidobacterium longum* ssp. *infantis* LMG 11588 in a Maltodextrin Preblend at 4°C and 0.16 Water Activity for 1.5 years

Figure 9 shows that the *Bifidobacterium longum* ssp. *infantis* LMG 11588 strain is stable in infant formula at 4°C (39.2°F) and at room temperature (25°C (77°F)) for 18 months. Even storage at 37°C (98.6°F) for up to 3 months leads to only minor losses in viability (Figure 10).

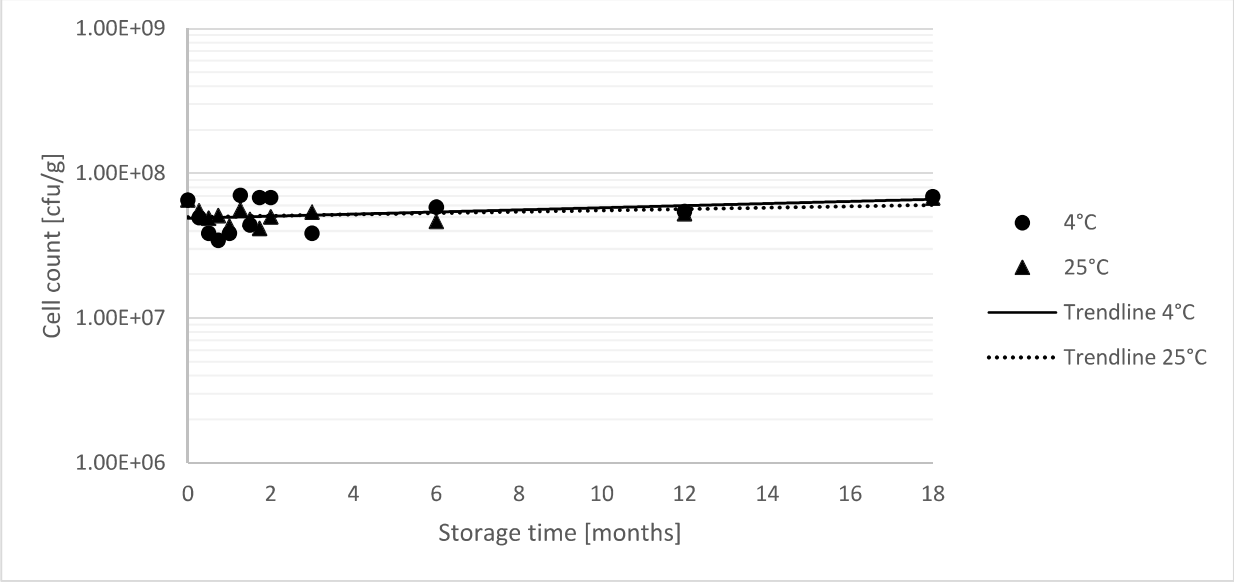

Figure 9: Stability of *Bifidobacterium longum* ssp. *infantis* LMG 11588 in Infant Formula at 4°C and 25°C (77°F) at 0.15 Water Activity for 1.5 Years

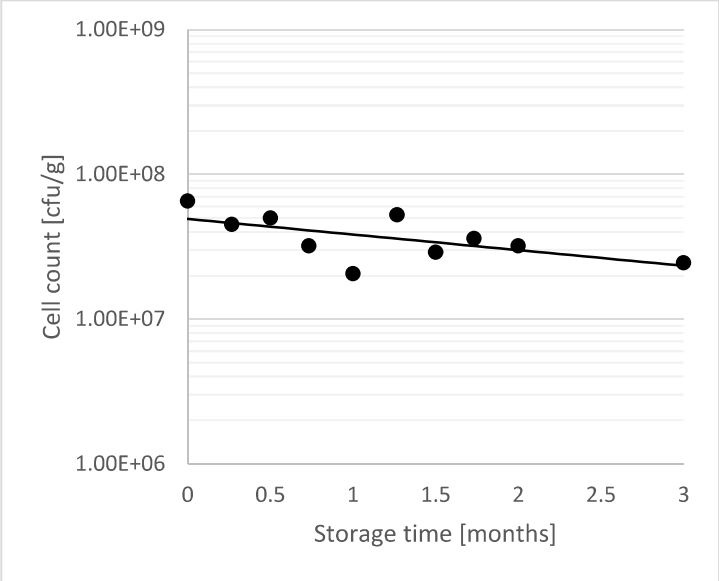

Figure 10: Stability Challenge Test of *Bifidobacterium longum* ssp. *infantis* LMG 11588 in Infant Formula at 37°C (98.6°F) and 0.15 Water Activity for 3 Months

The stability of *Bifidobacterium longum* ssp. *infantis* LMG 11588 is thus suitable for infant formula applications. We would expect differences in the stability of *Bifidobacterium longum* ssp. *infantis* LMG 11588 in powder toddler drinks, when compared to powder infant formula, to be negligible.

Part 3. Dietary Exposure

3.1 Estimated Daily Intake

3.1.1 Assessment of *Bifidobacterium longum* ssp. *infantis* LMG 11588 Use in Infant Formula

Bifidobacterium longum ssp. *infantis* LMG 11588 is intended for use in powder non-exempt infant formula up to 1.2 x 10^8 CFU/g powder. Powdered infant formulas are generally reconstituted by adding 8.7 g powder to 2 fl oz water (i.e., 147 g powder/L). This dilution will result in an intake of 1.76 x 10^{10} CFU/L.

According to tables of daily energy intake by formula-fed infants provided by Fomon (1993), boys aged 14-27 days have the highest intake per kg body weight (i.e., mean intake of 121.1 kcal/kg bw/day and 90th percentile intake of 141.3 kcal/kg bw/day). Likewise, this same age group has the highest intake among girls (i.e., the mean and 90th percentile intake is 117.8 and 138.9 kcal/kg bw/day, respectively). Therefore, to achieve an energy intake of 141.3 kcal/kg bw/day from an infant formula with a caloric density of 20 kcal/fl oz (i.e., 676 kcal/L), an infant boy must consume 209 mL formula/kg bw/day. To reach an energy intake of 138.9 kcal/kg bw/day, an infant girl must consume 205 mL formula/kg bw/day. Assuming a 90th percentile of formula intake for males and females in this age group of 207 mL/kg bw/day, the maximum estimated dietary intake of *Bifidobacterium longum* ssp. *infantis* LMG 11588 from infant formula is 3.6 x 10^9 CFU/kg bw/day.

3.1.2 Assessment of *Bifidobacterium longum* ssp. *infantis* LMG 11588 Use in Toddler Drinks

Bifidobacterium longum ssp. *infantis* LMG 11588 is intended for use in powder toddler drinks up to 1.2 x 10^8 CFU/g powder. Toddler drinks refer to powder-based products for children 1-3 years of age intended to replace liquid milk in the diet.

As noted in the most recent report of Nestle's Feeding Infants and Toddlers Study (Feeding Infants and Toddlers Study (FITS), 2016; unpublished), children 1-2 years of age (n=966) consumed 153-179 g milk per eating occasion (median lower 95% confidence interval to median upper 95% confidence interval) and children 2-3 years of age (n=257) consumed 144-184 g milk per eating occasion (median lower 95% confidence interval to median upper 95% confidence interval). Milk consumption reflects combined intake from low-fat milk, reduced-fat milk, and whole milk. Based on three eating occasions/day, the estimated intake for children 1-2 years old and 2-3 years old is 459-537 g milk/day and 432-552 g milk/day, respectively. Assuming the

caloric density of milk is approximately 1.03 g/mL and 1 fl oz is equivalent to 29.57 mL, this intake is equivalent to 15.1-17.6 fl oz milk/day and 14.2-18.1 fl oz milk/day, respectively.

According to the reconstitution instructions for many toddler drinks (for children 1-3 years of age), about 36 g powder is to be added to 8 fl oz water. The amount of powder in 18.1 fl oz, the upper range of milk consumption reported in FITS (2016, unpublished) for this age group, is 81.5 g. Based on the intended use of *Bifidobacterium longum* ssp. *infantis* LMG 11588, the maximum estimated dietary intake of *Bifidobacterium longum* ssp. *infantis* LMG 11588 from toddler drinks for children 1-3 years of age is 9.8×10^9 CFU/day. Assuming an average weight of 8.1 kg (3rd percentile weight of 8.4 kg and 7.8 kg for 1 year old boys and girls, respectively; Kuczmarski et al., 2000), the maximum estimated dietary intake of *Bifidobacterium longum* ssp. *infantis* LMG 11588 from toddler drinks for children 1-3 years of age is 1.2×10^9 CFU/kg bw/day.

3.1.3 Summary

It isn't expected that healthy infants and toddlers aged 0-3 years of age will have other dietary sources of *Bifidobacterium longum* ssp. *infantis* LMG 11588. Therefore, the estimated exposure estimates represent intake from all sources. If an older infant were to consume a toddler drink with *Bifidobacterium longum* ssp. *infantis* LMG 11588, it is assumed that consumption of such product would displace consumption of infant formula and any related *Bifidobacterium longum* ssp. *infantis* LMG 11588 exposure. The estimated daily intake is consistent with the levels reported for very similar *Bifidobacterium longum* ssp. *infantis* strains reported in GRN 758 and GRN 985.

Part 4. Self-limiting Levels of Use

There are no known self-limiting levels of use associated with Nestlé Nutrition's *Bifidobacterium longum* ssp. *infantis* LMG 11588.

Part 5. Experiences Based on Common Use in Food

The conclusion that the intended use of Nestlé Nutrition's *Bifidobacterium longum* ssp. *infantis* LMG 11588 is GRAS is based on scientific procedures rather than experience based on common use in food prior to 1958.

Part 6. Narrative

6.1 Recognized Safety of bifidobacteria

The intestinal microbiota is incredibly complex and includes an estimated 10^{13} - 10^{14} or more bacteria representing over 400 different species (Zetterström et al., 1994; Edwards and Parrett, 2002) or more than 2000 phylotypes (McFall-Ngai, 2006). These bacteria help process food into absorbable components (Edwards and Parrett, 2002), support local immune responses (Zetterström et al., 1994), and contribute to an environment that keep potential pathogens from colonizing the gut (Heavey and Rowland, 1999). Probiotic strains impart beneficial effects on the host and on the intestinal microbiota without causing adverse effect on the host.

Over 15 types of bifidobacteria have been notified as GRAS with no objection by FDA and four of these GRAS notices have been for *Bifidobacterium longum* ssp. *infantis* strains (GRN 758, GRN 950, GRN 985, and GRN 1003).

6.2 History of Consumption of *Bifidobacterium longum* ssp. *infantis* LMG 11588

Bifidobacterium longum ssp. *infantis* LMG 11588 is not yet available for commercial use. However as discussed, this strain is closely related to both R0033 (GRN 758) and Bi-26 (GRN 985). In a review of the phylogenetic, functional, and safety features of *Bifidobacterium longum* ssp. *infantis* strains, Duboux et al. (2022) obtained isolates from three culture collections. They constructed pairwise comparisons of Average Nucleotide Identities (ANI) and of single nucleotide polymorphisms (SNP). These analyses showed that LMG 11588, R0033, Bi-26, and EK3, share a mean homology of >99.96% ANI. Additionally, a GRAS Panel reviewed the comparisons and concluded that the safety of *Bifidobacterium longum* ssp. *infantis* LMG 11588 would be equivalent to that of R0033 (Appendix A).

<u>*Bifidobacterium longum* ssp. *infantis* R0033 and Bi-26</u>

Bifidobacterium longum ssp. *infantis* Rosell®-33 (R0033) has been sold worldwide for many years as a powder or as a part of Probiokid®, a blend providing $3x10^9$ CFU/sachet, corresponding to $3x10^8$ CFU of *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033). Probiokid® is used in infants, toddlers, and children. Probiokid® was first launched as a health food in China beginning in October 2002 under the trade name Biostime. Since that time, Probiokid® has been sold in more than ten countries, including: Australia, Canada, France, South Africa, Ukraine, and United Kingdom.

Additionally, *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) has also been extensively marketed by Lallemand Health Solutions as a combination (with other strains) in more than 40 other formulas with no reports of adverse effects.

According to GRN 985, "*B. infantis* strain Bi-26 is a human isolate, identified according to standard taxonomic guidelines. *B. infantis* Bi-26™ has been in commercial use since 2014 and is a lyophilized bacteria fermentation product that is produced in accordance with cGMP as provided for in 21 CFR 111 and 21 CFR 117. DuPont sells *B. infantis* Bi-26™ for inclusion in food and supplement products globally. *B. infantis* Bi-26™ has been sold worldwide, including in North America, China, South Africa, Middle East, Europe, and Asia/Pacific countries. Over 23,000 Kg of *B. infantis* Bi-26™ has been sold since 2012; DuPont affirms that no safety-related complaints related to *B. infantis* Bi-26™ have been received."

6.3 Safety Parameters

6.3.1 Infectivity

The *Bifidobacterium infantis* taxonomic groups are not known to contain toxin producers or strains that possess virulence factors (Gasser, 1994). Therefore, their pathogenic potential is extremely low. Infection cases reported invariably concern individuals in a fragile state with underlying conditions (Salminen et al., 1998; Esaiassen et al., 2017).

6.3.2 Undesirable Metabolic Activity

6.3.2.1 D-Lactate Production

The lactate dehydrogenase (LDH) enzyme of *Bifidobacterium longum* is one of the first bifidobacterial enzymes that has been cloned and characterized. These works demonstrated the LDH to be an allosteric enzyme, producing L-lactate from pyruvate, which is activated by the presence of fructose 1,6-biphosphate, hence classifying this enzyme as a L-lactate dehydrogenase (L-LDH; EC 1.1.1.27) (Iwata et al., 1989; Minowa et al., 1989).

Bifidobacterium longum ssp. *infantis* LMG 11588 contains a L-lactate dehydrogenase homologue that shares a high DNA homology with the above-mentioned L-LDH (100% coverage, 96.57% identity). This is supported by phenotypic data which demonstrated that *Bifidobacterium longum* ssp. *infantis* LMG 11588 produces predominantly (>90%) L(+)-lactate (Table 1). There are no reports in the literature of D-lactate production by genus *Bifidobacterium*.

6.3.3 Presence of Antibiotic Resistance Genes and Likelihood of Transference

6.3.3.1 Minimal Inhibitory Concentrations

Antibiotic susceptibility of *Bifidobacterium longum* ssp. *infantis* LMG 11588 was assessed according to ISO 10932:2010 method. Phenotypic testing demonstrated that the strain is considered sensitive to antibiotics of human and veterinary importance (gentamycin, kanamycin, streptomycin, tetracyclin, erythromycin, clindamycin, chloramphenicol, and ampicillin) as the obtained Minimal Inhibitory Concentrations (MIC) are all below or equivalent to the breakpoints determined by EFSA (EFSA, 2012) (Table 9).

Table 9. Phenotypic Antibiotic Resistance Profile of *Bifidobacterium longum* ssp. *infantis* LMG 11588 Determined by Microdilution Following the ISO 10932:2010 Standard

Antibiotic	Minimal Inhibitory Concentration (mg/L)	Applicable EFSA Breakpoint (Mg/L)	Sensitive (S) / Resistant (R)
Gentamycin	32.00	64.00	S
Streptomycin	16.00	128.00	S
Tetracyclin	2.00	8.00	S
Erythromycin	0.50	1.00	S
Clindamycin	0.13	1.00	S
Chloramphenicol	2.00	4.00	S
Ampicilin	0.25	2.00	S
Vancomycin	0.50	2.00	S

It is worth noting that strains belonging to *Bifidobacterium longum* ssp. *infantis* have been previously demonstrated to be resistant to streptomycin (Xiao et al., 2010). LMG 11588 is, however, sensitive to streptomycin, as is the Rossell-33 strain (GRN 758).

6.4 *In Vivo* Safety Studies

Bifidobacterium longum ssp. *infantis* strains have been extensively studied and shown to be inherently safe in animals and humans (see GRNs 758, 950, 985, and 1003). Further, GRNs 758,

950, 985, and 1003 all included non-exempt infant formula as an intended use for the subject *B. longum* ssp. *infantis* strain(s) and received a No Further Questions (NFQ) response from FDA following their review of the respective GRAS determination.

In a review of the phylogenetic, functional, and safety features of *Bifidobacterium longum* ssp. *infantis* strains, (Duboux et al., 2022) obtained isolates from three culture collections. They constructed pairwise comparisons of Average Nucleotide Identities (ANI) and of single nucleotide polymorphisms (SNP). These analyses showed that the LMG 11588, R0033, Bi-26, and EK3 strains are closely related, as they displayed not more than 16 SNPs (as compared to LMG 11588) and share a mean homology of >99.96% ANI.

In recognition of these high degrees of similarity, and as supported by the similarities between *Bifidobacterium longum* ssp. *infantis* strains LMG 11588 and R0033 described above, available *in vivo* studies of these *Bifidobacterium longum* ssp. *infantis* strains are reported in this order. It's important to note that strains R0033 and Bi-26 were the subject of GRN 758 (intended for use in non-exempt infant formula) and GRN 985 (intended for use in non-exempt infant formula and toddler products), respectively. As mentioned above, each GRAS determination was reviewed by and received a NFQ response from the FDA.

6.4.1 Animal Studies

6.4.1.1 *Bifidobacterium longum* ssp. *infantis* R0033

The following table (Table 10) is incorporated by reference from GRN 758 (Table 43; page 152). Additional study details are described in section 6.4.3.1 of GRN 758 (page 152). For reference, Probiokid® is a combination of *L. helveticus* Rosell®-52 (R0052) (80%), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) (10%), and *B. bifidum* Rosell®-71 (R0071) (10%).

Table 10: Animal Safety Studies with *Bifidobacterium longum* ssp. *infantis* R0033

Reference	Objectives	Study Design	Animal Model	Dose/Day	Duration	Safety-Related results
Cazzola et al. (2010a)	Investigate the impact of Probiokid® on the immune system regulation in rats in T_h1 (cellular immune	First experiment: 18 male Wistar rats divided in 3 groups (n=6/group): first with saline solution (vehicle), second with vehicle and induced with E. coli on day 14,	Male Wistar Rats	Experiment 1: 0.67 billion CFU/day Experiment 2: 1 billion CFU/day	Experiment 1: 17 days Experiment 2: 14 days	In the two experiments the mean serum levels of pro-inflammatory modulators was significantly lower in the symbiotic group than in the control group ($p \leq 0.01$). In addition, in the first experiments, mean serum levels of the

	system) and T_h2 (humoral immune system) models.	and the third with Probiokid® and induced with E. coli on day 14. <u>Second experiment</u>: 6 groups of n=5 male Wistar rats were orally administered saline solution (group 1 and 2) or Probiokid® (group 3 to 6) for 10 days, then injected with either saline (group 1) or 3000 third-stage infective larvae of N. *brasiliensis* (groups 2 to 6)				anti-inflammatory modulators were significantly increased in the Probiokid® group vs placebo group ($p \leq 0.004$). The decrease in mass body weight of rats in the Probiokid® group was significantly smaller than in the control group ($p \leq 0.02$). In the second experiment, Probiokid® reduced the level of circulating pro-inflammatory immune factors in T_h1 and T_h2 models of infection.
Huang et al. (2011)	Observe the effect of Probiokid® (Biostime [Probiokid®]) probiotics on mice intestinal microflora.	2 groups (n=10/group): negative control group (saline)/ probiotic group (1 g/ kg Biostime [Probiokid®]) once/day	20 healthy specific pathogen free BALB/c mice	1 g/kg/day or approx. 3.3×10^9 CFU/kg/day	14 days	Significant increase in levels of bifidobacteria after gavage with the symbiotic ($p \leq 0.05$). But no significant differences for *Enterobacteriaceae*, *Clostridium perfringens*, *Enterococci*, or *Lactobacilli* levels.

6.4.1.2 *Bifidobacterium longum* ssp. *infantis* Bi-26

The following is incorporated by reference from GRN 985 (part 6.A.3.a; page 19).

<u>Acute toxicity</u>

Bifidobacterium longum ssp. *infantis* Bi-26™ was administered by gavage to five fasted female Crl:CD(SD) rats at a dose of 5000 mg/kg, which corresponded to an overall dose of 1.94×10^{12} CFU/kg body weight consistent with OECD 425. The rats were then observed for mortality, body weight effects, and clinical signs for 15 days after dosing. The rats were necropsied to detect grossly observable evidence of organ or tissue damage. There was no incidence of mortality, clinical abnormalities, or overall body weight losses. No gross lesions were reported at necropsy.

It was concluded that under the conditions of this study, *Bifidobacterium infantis* Bi-26™ was not considered acutely toxic via the oral route of exposure in female rats (GRN 985).

In summary, given the similarity between *Bifidobacterium longum* ssp. *infantis* strains R0033, Bi-26, and LMG 11588, these animal studies also support the safety of *Bifidobacterium longum* ssp. *infantis* strain LMG 11588.

6.4.2 Human Studies

6.4.2.1 *Bifidobacterium longum* ssp. *infantis* LMG 11588

Capeding et al. (2022) reported on a prospective, randomized, double-blind, placebo-controlled trial in which healthy breastfed and formula-fed infants aged 14-21 days were assigned to a control group (n = 77), a low-dose group (n =75) receiving 10^8 CFU *Bifidobacterium longum* ssp. *infantis* LMG 11588/day, or a high-dose group (n = 76) receiving 1.8 x 10^{10} CFU *Bifidobacterium longum* ssp. *infantis* LMG 11588/day for 8 weeks. The primary objective of the study was to assess safety and tolerability of the intervention. The primary endpoint was weight gain, with secondary endpoints including adverse events (AEs), GI tolerance indications, illness symptoms, additional anthropometrics, and stooling patterns.

There were no differences in weight gain or other anthropometric measures, stooling, crying/fussiness, or AEs. Vomiting was significantly lower among infants receiving the *Bifidobacterium longum* ssp. *infantis*. The authors concluded that, "The *B. infantis* LMG 11588 supplement supports normal infant growth, is safe and well-tolerated."

6.4.2.2 *Bifidobacterium longum* ssp. *infantis* R0033

The following table (Table 11) includes information incorporated by reference from GRN 758 (Table 34, page 102 and Table 38, pages 110-114). For reference, Probiokid® is a combination of *L. helveticus* Rosell®-52 (R0052) (80%), *Bifidobacterium longum* ssp. *infantis* Rosell®-33 (R0033) (10%), and *B. bifidum* Rosell®-71 (R0071) (10%).

Table 11: Infant and Children Safety Studies on *Bifidobacterium longum* ssp. *infantis* R0033

Reference	Objectives	Study Design	Subjects	Strain Dose/Day	Duration	Safety-Related Results
Manzano et al. (2017)	Investigate the safety and tolerance of three probiotic strains *B. longum* ssp. *infantis* Rosell®33 (R0033); *L. helveticus* Rosell®-52 (R0052) and *B. bifidum* Rosell®71 (R0071)	Multi center randomized double-blind placebo-controlled 12-week study with 4 treatment groups; *B. longum* ssp. *infantis* Rosell®-33 (R0033); *L. helveticus* Rosell®-52 (R0052); *B. bifidum* Rosell®-71 (R0071) and placebo	202 infants (3-12 months)	$3x10^9$ CFU	12 Weeks	The data related to the primary outcome, growth, showed that all participants grew similarly independent of the group where they were allocated. Regarding safety variables, none of the participants suffered a Serious Adverse Event during the study and all groups were equivalent in the number of Adverse Events. The number of episodes of fever and the number of unscheduled visits to the doctor were equivalent in all groups of the study. None of the participating infants showed any signs of D-lactic acidosis. The changes in sleeping and crying habits show that all 4 groups were homogenous in their responses. The consumption of *B. longum ssp. infantis* R0033, *L. helveticus* R0052, B. *bifidum* R0071 was well tolerated and safe for infants from 3 to 12 months of age, at a dose of 3 billion CFU per day.
Cui and Wure (2003)	Evaluate Biostime (Probiokid®) for the treatment of 62 cases of pediatric rotavirus	Randomized, controlled Biostime (Probiokid®) group (n=62) Age <12mo: 5 B CFU QD; Age 12-24 mo: 5B CFU	122 children (624 mo) who had diarrhea for less than 3 days and who	<12 months: 5 billion CFU/day; 12-24 months: 10 billion CFU/day	Not stated (but evaluated treatment effects for at	Biostime (Probiokid®) group duration of diarrhea was 39.3±17.1h while that in the Lacidophilin group was 63.8±22.9h. Biostime (Probiokid®) group: the total effective rate is 93.5%

	gastroenteritis	BID Lacidophilin group (n=60) Both groups also received Ribavirin. Intervention continued until diarrhea resolved.	tested positive for Rotavirus antigen in their feces		least 72 h)	(58/62). Lacidophilin group, the total effective rate was 61.7% (37/60). The difference was significantly different (p≤0.01). There was no report of adverse events.
Chen et al. (2007)	Evaluate the impact of Probiokid® on IgA level	Randomized, controlled 1 Biostime (Probiokid®) sachet (5B CFU), BID, for 13 days	28 children less than 4 years, divided into 4 groups by age. Control was 2 children from each age group (8 children)	10 billion CFU/day	13 Days	For the children who had low sIgA level before taking the sachet, the sIgA level increased to and was maintained at a normal level after they took the sachets. No adverse events were reported.
Jiang (2008)	Clinical evaluation of Biostime (Probiokid®) in the treatment of children with persistent diarrhea	Randomized, active control Biostime (Probiokid®) group (n=32) <6mo: 2.5B CFU BID; 6-12 mo: 5B CFU BID; 12-24 mo: 5-10B CFU BID Golden Bifido group (n=20). Intervention continued until diarrhea resolved	52 children (3 to 24 mo) in hospital or outpatient clinic with persistent diarrhea	<6 months: 5 billion CFU/day; 6-12 months: 10 billion CFU/day; 12-24 months: 10-20 billion CFU/day	Treated until diarrhea resolved	After 6 days of treatment, Biostime (Probiokid®) group had normalized the number of defecations per day whereas the Golden Bifido group remained high (p≤0.05). Treatment time (7.1 vs 12.6 days) and cost (652 vs 843 Yuan) was significantly (P≤0.001) better in Biostime (Probiokid®) group compared to Golden Bifido. Clinically effective rate in Biostime (Probiokid®) was 91% vs 65% (p≤0.01). No adverse events were reported.
Mei and Chen (2008)	Evaluate the therapeutic effect of Biostime (Probiokid®	Randomized, active control Biostime (Probiokid®) group: (n=39) 1 Biostime	78 children (0-5 yrs) with rotavirus infection	10 billion CFU/day	7 days	Difference in treatment effective rate between the two groups was significant, in favor of the probiotic group (94.9 vs. 74.3%;

	) product on pediatric diarrhea caused by rotavirus infection	(Probiokid®) sachet (5B CFU) BID, for 7 days + Ribaviren Control group: (n=39) Ribaviren only				p≤0.05). There was no report of adverse events.
Cazzola et al. (2010b)	Investigate the effects of a synbiotic supplementation in reducing common winter diseases in children	Double-blind, randomized, placebo controlled Probiokid® group (n=62): 3B CFU/day for 3 months, Placebo group (n=73)	135 school-age children (3 to 7 years old); suffered at least 3 physician diagnosed episodes of ENT, respiratory or GI infection last winter	3 billion CFU/day	3 months	Decrease in the % of children who suffered from at least one health problem during the 3-month study compared with placebo. Relative risk reduction is 24.7% (P≤0.045) Decrease in the % of children suffering from at least one episode characterized by an ear, nose and throat (ENT), respiratory tract or gastrointestinal symptom compared with placebo (50% vs. 67.1% ; P≤0.044) Decrease in the % of children with at least one health problem including one or more day school loss compared with placebo (25.8% vs. 42.5%; P≤0.043). Investigators reported a total of 24 adverse events in 20 children. None were serious events. Most of these events were expected ENT, respiratory tract or gastrointestinal problems. In two cases the intensity of the event was noted as severe. Two adverse events with digestive problems were considered by investigators as possibly related to the study

						medication in the placebo group and none in the Probiokid® group.
Yang et al. (2010)	Observe the therapeutic effects of supplemental feeding with lactose-free milk powder combined with Biostime (Probiokid®) on the infantile diarrhea	Randomized, controlled Biostime (Probiokid®) Group: (n=58) 1 Biostime (Probiokid®) sachet (5B CFU) + lactose-free milk powder formula. Control group (n=40) + breast-fed or formula fed.	98 infants (6-30 mo) admitted to inpatient clinic between Jan 2008-Oct 2009 with diarrhea due to rotavirus infection	5 billion CFU/day	Not stated	Significant improvement (p≤0.01) in the disappearance of diarrhea symptom (2.8±1.1 days vs. 4.9±2.6 days) and duration of hospital stay (5.5±1.7 days vs 8.5±2.3 days). Clinically effective rate in Biostime (Probiokid®) was 94.8% vs 77.5% 2(p≤0.05, analyzed by χ test). No adverse events were reported.
Wang (2012)	Evaluate the effectiveness of Smecta and Probiokid® versus Smecta alone in infants diagnosed with non-infectious diarrhea	Randomized, active control Observation group (n=104): oral Smecta + Biostime (Probiokid®) <12 mo (n=33): 1.7B CFU TID 13-24 mo (n=43): 2.5 B CFU BID 25-36 mo (n=28): 5B CFU BID	194 children (aged 3-36 months) with non-infectious diarrhea	<12 months: 5 billion CFU/day 13-24 months: 5 billion CFU/day 25-36 months: 10 billion CFU/day	3 days	No adverse reactions. Analyzed by X^2 test. Observation group effective rate was 90.7-92.9% vs control group effective rate of 87.1-88.6% (Not significant). However, very effective rates are statistically significant: 78.882.1% for treatment group vs. 74.275% for control. (p≤0.05). There was no report of adverse events.

		Active Control (n=90): oral Smecta only 0-12 mo (n=31); 13-24 mo (n=35): 25-36 mo (n=24). For 3 days.				
Gao (2013)	Evaluate the effectiveness of Smecta and Probiokid® versus Smecta alone in infants diagnosed with non-infectious diarrhea	Randomized, active control Observation group (n=43): oral Smecta + Biostime (Probiokid®) 0-12 mo: 1.7B CFU TID 13-24 mo: 2.5B CFU BID 25--36 mo: 5B CFU TID Active Control (n=43): oral Smecta only	86 hospitalized children (0-36 mo) with non-infectious diarrhea	0-12 months: 5 billion CFU/day 13-24 months: 5 billion CFU/day 25-36 months: 15 billion CFU/day	3 days	No adverse reactions. Analyzed by X^2 test. Observation group effective rate was 90.7% vs control group effective rate of 62.8% ($p \leq 0.05$). No adverse reactions were observed in either group.
Pantovic (2013)	Investigate the effectiveness and the optimal time of supplementation with Probiokid® in atopic children with common respiratory and/or ear infections	Uncontrolled before and after study 3B CFU/day for 6 months	31 atopic children (6 to 42 mo) hospitalized with common respiratory and/or ear infections and low sIgA.	3 billion CFU/day	6 months	After 3 months level of IgA increased for 1.8 times up from 0.33±3.42 g/l to 0.6±0.78 in 35% children and after 6 months increased for 3.9 times up to 1.3±1.76 in 81% children ($t=0.43$, $p \leq 0.05$). At least 6 months is the optimal duration of supplementation with synbiotic to reduce the risk of common infectious disease. No adverse events were reported.
Wu (2013)	Evaluate the effectiveness of Smecta and	Randomized, active control Observation group (n=84): oral Smecta +	148 hospitalized children (2-36	<12 months: 5 billion CFU/day 13-24 months: 5 billion CFU/day 25-36 months:	3 days	Analyzed by X^2 test. Intervention group had a significantly more effective rate that the control for all groups.

	Probiokid® versus Smecta alone in infants diagnosed with non-infectious diarrhea	Biostime (Probiokid®) <12 mo (n=32): 1.7B CFU TID 13-24 mo (n=35): 2.5 B CFU BID 25-36 mo (n=17): 5B CFU BID Active Control (n=64): oral Smecta only 0-12 mo (n=21); 13-24 mo (n=33): 25-36 mo (n=10).	mo) with non-infectious diarrhea.	10 billion CFU/day		<12 mo: 93.8% vs 76.1% 13-24 mo: 91.4% vs 78.8% 25-36 mo: 82.3% vs 60.0% p≤0.05 in all groups There was no report of adverse events.
Xi et al. (2013)	Examine the impact of Probiokid® on oral thrush	Randomized, active control Experimental group (n=35): 2% sodium bicarb + nystatin + 1 sachet Biostime (Probiokid®) (5B CFU) BID Active Control (n=35): 2% sodium bicarb + nystatin. After 3 days, effective rate. For 14 days, follow up after 30 days for recurrence rate.	70 children (42M/28 F; aged 1-36 mo) diagnosed with oral thrush.	10 billion CFU/day	17 days	No adverse reactions were reported. Experimental group vs Control group: Total effective rate: 94.3% vs 77.1%, p≤0.05 Recurrence rate: 2.9% vs 17.1%, p≤0.01
Stojkovic et al. (2016)	Determine optimal time efficiency of Probiokid® in controlling	Children were classified into 3 groups; Group I with respiratory infection and wheezing; Group II with	78 children (1.5 months to 5 years)	5 billion CFU/day	9 months	Synbiotic is effective for immunomodulation, controlling frequency of respiratory infections by 3 months and wheezing by 6 months. No side effects of synbiotic were

	respiratory infections and wheezing disease	respiratory infection without wheezing; Group III - with wheezing but without accompanying respiratory infection. No control group				identified in the examined children and it was well tolerated.

In addition to the studies in the table above (Table 11), a recently conducted literature search for infant-related safety evaluations of *Bifidobacterium longum* ssp. *infantis* strains Bi-26 and R0033 resulted in one additional publication. Briefly, healthy infants (3.5-6 months old) were randomized to receive either probiotic- (n=66) or placebo-supplemented (n=66) formula once a day for four weeks. One probiotics sachet contained 1.425×10^8 CFU of each *Bifidobacterium infantis* R0033 and *Bifidobacterium bifidum* R0071, with 9.6×10^9 CFU of *Lactobacillus helveticus* R0052. No serious adverse events were reported, and all adverse events were mild and unrelated to the product or study. This study demonstrates the safety of this probiotic formulation in infants (Xiao et al., 2019).

In summary, a clinical study assessing the use of *Bifidobacterium longum* ssp. *infantis* strain LMG 11588 in infants together with multiple clinical evaluations of the very closely related *Bifidobacterium longum* ssp. *infantis* strain R0033 in both infants and children provide significant evidence in support of the safety of *Bifidobacterium longum* ssp. *infantis* strain LMG 11588 for the intended use.

6.5 Authoritative Evaluations of *Bifidobacterium longum* ssp. *infantis*

First evidence of bifidobacteria presence in the infant gastrointestinal tract dates from 1899 (Tissier, 1899). Today, bifidobacteria are known to predominate in the intestinal tract shortly after birth (O'Callaghan and van Sinderen, 2016) and are therefore important normal constituents of the human gastrointestinal microflora, occurring at concentrations of 10^9 to 10^{10} cells/g in feces (Tanaka et al. 2000).

As noted above, in 2002 the IDF, in collaboration with the European Food and Feed Cultures Association (EFFCA), assembled a list of microorganisms with a documented history of safe use in food (Mogensen et al., 2002). The species *Bifidobacterium infantis* was listed in this initial inventory and was further included in the revised version of this inventory as *Bifidobacterium longum* ssp. *infantis* in 2012 (Bourdichon et al., 2012). *Bifidobacterium longum* ssp. *infantis* has

also been included in the Qualified Presumption of Safety (QPS) list established by the European Food Safety Authorities (EFSA, 2017).

Additional review of approvals for *Bifidobacterium longum* ssp. *infantis* strains are summarized in GRN 785, GRN 950 and GRN 1003. These include countries such as Canada, Australia, Indonesia, China, and France.

6.6 Summary

Nestlé Nutrition has determined that *Bifidobacterium longum* ssp. *infantis* LMG 11588 is GRAS for use in powder non-exempt infant formula and toddler drinks as described in section 1.4 on the basis of scientific procedures. Nestlé Nutrition has reviewed the available data and information and are not aware of any data and information that are, or may appear to be, inconsistent with this conclusion. This GRAS determination is based on data generally available in the public domain pertaining to the safety of *Bifidobacterium longum* ssp. *infantis* LMG 11588, as discussed herein, and on consensus among a panel of experts (the GRAS Panel) who are qualified by scientific training and experience to evaluate the safety of infant formula ingredients and food ingredients. The GRAS Panel consisted of the following qualified scientific experts: Dr. Douwe van Sinderen, Dr. Colin Hill, and Dr. Dan O'Sullivan.

The GRAS Panel, convened by Nestlé Nutrition, independently and critically evaluated all data and information presented herein, and concluded that *Bifidobacterium longum* ssp. *infantis* LMG 11588 is GRAS for use in powder non-exempt infant formula and toddler drinks as described in section 1.4 based on scientific procedures.

6.6. Statement Regarding Information Inconsistent with GRAS

I have reviewed the available data and information and am not aware of any data or information that are, or may appear to be, inconsistent with our conclusion of GRAS status of the intended use of *Bifidobacterium longum* ssp. *infantis* LMG 11588.

[redacted]

James A. Heimbach, Ph.D., F.A.C.N.

6.7 GRAS Panel Evaluation and Conclusion

We, the undersigned members of the GRAS Panel, are qualified by scientific education and experience to evaluate the safety of substances intended for infants and toddlers. We have critically evaluated the publicly available information on *Bifidobacterium longum* ssp. *infantis* LMG 11588 summarized herein and have individually and collectively determined that no evidence exists in the available information on *Bifidobacterium longum* ssp. *infantis* LMG 11588 that demonstrates, or suggests reasonable grounds to suspect, a hazard to infants or toddlers under the intended conditions of use in infant formula and toddler drinks.

We unanimously conclude that the addition of *Bifidobacterium longum* ssp. *infantis* LMG 11588, produced by Nestlé Nutrition consistent with current Good Manufacturing Practices and meeting the specifications in this monograph, up to 1.2 x 10^8 CFU/g in powder non-exempt infant formula and toddler drinks is safe and is GRAS by scientific procedures.

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

Douwe van Sinderen APC Microbiome Ireland and School of Microbiology, University College Cork	Date
Colin Hill APC Microbiome Ireland and School of Microbiology, University College Cork	Date
Dan O'Sullivan Department of Food Science and Nutrition, University of Minnesota	Date

Part 7. List of Supporting Data and Information

7.1 References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol. 215:403-410.

Bongaerts G, Bakkeren J, Severijnen R, Sperl W, Willems H, Naber T, Wevers R, vanMeurs A, Tolboom J. 2000. Lactobacilli and acidosis in children with short small bowel. J Pediat Gastrointent Nutr. 30:288-293.

Borriello SP, Hammes WP, Holzapfel W, Marteau P, Schrezenmeir J, Vaara M, Valtonen V. 2003. Safety of probiotics that contain lactobacilli or bifidobacteria. Clin Dis. 36:775-780.

Bourdichon F, Casaregola S, Farrokh C, Frisvad JC, Gerds ML, Hammes WP, Harnett J, Huys G, Laulund S, Ouwehand A, Powell IB, Prajapati JB, Seto Y, Ter Schure E, Van Boven A, Vankerckhoven V, Zgoda A, Tuijtelaars S, Hansen EB. 2012. Food fermentations: microorganisms with technological beneficial use. Int J Food Microbiol. 154:87-97.

Capeding, MRW, Phee LCM, Ming C, Cercamondi CI. 2022. Safety and tolerability of *Bifidobacterium longum* subspecies *infantis* LMG11588 supplementation in healthy term infants: A double-blind, randomized placebo-controlled trial. 9th International Conference on Nutrition and Growth, 17-19 March.

Cazzola M, Tompkins TA, Matera MG. 2010a. Immunomodulatory impact of a synbiotic in TH1 and TH2 models of infection. Ther Adv Respir Dis. 4:259-270.

Cazzola M, Pham-Thi N, Kerihuel JC, Durand H, Bohbot S. 2010b. Efficacy of a synbiotic supplementation in the prevention of common winter diseases in children: a randomized, double-blind, placebo-controlled pilot study. Ther Adv Respir Dis. 4:271-278.

Chen L, Yang J, Yu J, Yao Z, Sun L, Shen Y, Jin Q. 2005. VFDB: a reference database for bacterial virulence factors. Nucleic Acids Res. 33:D325–D328

Chen L, Ouyang L-J, Liao W-J, Zhang W-H. 2007. Mucous membrane immunity enhanced by taking Biostime probiotics. Chin J Ecol 19:137-141.

Cui X, Wure G. 2003. The treatment of 62 cases of rotavirus gastroenteritis by Biostime probiotics. Chin J Gen Med. 5:53-54.

Duboux S, Ngom-Bru C, De Bruyn F, Bogicevic B. 2022. Phylogenetic, functional and safety features of 1950s B. infantis strains. Microorganisms. 10:203.

Edwards CA, Parrett AM. 2002. Intestinal flora during the first months of life: new perspectives. Brit J Nutr. 88 (Suppl l):S11-S18.

EFSA. 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J. 10(6):2740.

EFSA. 2017. The 2016 updated list of QPS Status recommended biological agents in support of EFSA risk assessments. EFSA J. 15:4884.

Esaiassen E, Hjerde E, Cavanagh JP, Simonsen GS, Klingenberg C. 2017. Norwegian study group on invasive bifidobacterial infections. Bifidobacterium bacteremia: clinical characteristics and a genomic approach to assess pathogenicity. J Clin Microbiol. 55(7):2234-2248.

FAO/WHO. 2002. Guidelines for the Evaluation of Probiotics in Food. Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation in Food, Apr. 30-May, 1, 2002, London, Ont. Geneva, Switz.: Food and Agriculture Organization of the United Nations (FAO) / World Health Organization (WHO). Available at: http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf.

Feeding Infants and Toddlers Study (FITS). 2016. Unpublished.

Fomon SJ. 1993. Energy intake by normal infants. In Nutrition of Normal Infants, p. 104–111. Baltimore, MD: Mosby.

Gao M. 2013. The efficacy analysis of the combination of Smecta and Biostime probiotics in the treatment of non-infectious diarrhea in children. Clin Res. 164-166.

Gasser F. 1994. Safety of lactic acid bacteria and their occurrence in human clinical infections. Bull Inst Pasteur. 92:145-167.

Heavey PM, Rowland IR. 1999. The gut microflora of the developing infant: microbiology and metabolism. Microb Ecol Healfh Dis. 11:75-83.

Hove H, Mortensen PB. 1995. Colonic lactate metabolism and D-lactic acidosis. Dig Dis Sci. 40:320-330.

Howel AP. 1956. Comparison of physiological and biochemical characters of actinomyces spp. with those of Lactobacillus Bifidus. J Gen Microbiol. 428-445.

Huang X-Q, Liu S-L, Zheng J-L, Huang H-K, Huang X-H, Rao Z-L, Zhong Z-Y, Tang XJ. 2011. The study of the effects of Biostime probiotics on mice intestinal microflora. Chin J Comp Med. 21:127-130.

Iwata S, Minowa T, Sakai H, Ohta T. 1989. Amino acid residues in the allosteric site of l-Lactate dehydrogenase from Bifidobacterium longum. Agricultural and Biological Chemistry. 53:3365–3366.

Jiang X. 2008. Clinical evaluation of Biostime in the treatment of children with persistent diarrhea. The 6th Chin National Conf on Pediatr Microecol. 142-145.

Kaneko T, Bando Y, Kurihara H, Satomi K, Nonoyama K, Matsuura N. 1997. Fecal microflora in a patient with short-bowel syndrome and identification of dominant lactobacilli. J Clin Microbial. 35:3181-3185.

Kiwaki M, Sato T. 2009. Antimicrobial susceptibility of Bifidobacterium breve strains and genetic analysis of streptomycin resistance of probiotic B. breve strain Yakult. Int J Food Microbiol. 134(3):211-215.

Knarreborg A, Engberg RM, Jensen SK, Jensen BB. 2002. Quantitative determination of bile salt hydrolase activity in bacteria isolated from the small intestine of chickens. Appl Environ Microbiol. 68:6425-6428.

Kocian J. 1994. Further possibilities in the treatment of lactose intolerance-lactobacilli. Prakticky Lekar. 74:212-214.

Kuczmarski RJ, Ogden CL, Grummer-Strawn LM, Flegal KM, Guo SS, Wei R, Mei Z, Curtin LR, Roche AF, Johnson CL. 2000. CDC growth charts: United States. Adv Data. 314:1-28.

Iino T, Uchimura T, Komagata K. 2003. The effect of sodium acetate on the activity of Land D-lactate dethydrogenases in Lactobacihs sakei NRIC 1071(T) and other lactic acid bacteria. J Gen and App Microbial. 49:51-58.

Manzano S, De Andrés J, Castro I, Rodríguez JM, Jiménez E, Espinosa-Martos I. 2017. Safety and tolerance of three probiotic strains in healthy infants: a multi-centre randomized, double-blind, placebo-controlled trial. Benef Microbes. 8(4):569–578.

McFall-Ngai M. 2006. Love the one you're with: vertebrate guts shape their microbiota. Cell. 127(2):247-9.

Mei LX, Chen Z. 2008. Evaluation of the efficacy of Biostime on rotaviral infection in children. Med Inform. 21:893-895.

Minowa T, Iwata S, Sakai H, Masaki H, Ohta T. 1989. Sequence and characteristics of the Bifidobacterium longum gene encoding L-lactate dehydrogenase and the primary structure of the enzyme: a new feature of the allosteric site. Gene. 85(1):161-8.

Mogensen G, Salminen S, O'Brien J, Ouwenhand A, Holzapfel W, Shortt C, Fonden R, Miller GD, Donohue D, Playne MJ, Crittenden RG, Biannchi-Salvadori B, Zink R. 2002. Inventory of microorganisms with a documented history of use in food. Bulletin of the International Dairy Federation. 377:10-19.

Moser SA, Savage DC. 2001. Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in lactobacilli. Appl Environ Microbiol. 67:3476-3480.

Norris RF. 1950. The isolation and cultivation of Lactobacillus; A comparison of branched and unbranched strains. J Bacteriol. 681-696.

O'Callaghan A, van Sinderen D. 2016. Bifidobacteria and their role as members of the human gut microbiota. Front Microbiol. 7:925.

Pantovic F. 2013. Serum immunoglobulin levels in children with respiratory infections who used a synbiotic dietary supplement. PONS Med J. 10:7-11.

Pightling AW, Pettengill JB, Luo Y, Baugher JD, Rand H, Strain E. 2018. Interpreting whole-genome sequence analyses of foodborne bacteria for regulatory applications and outbreak investigations. Front Microbiol. 9:1482.

Reid G. 2002. Safety of Lactobacillus strains as probiotic agents. Clin Infect Dis 35:349- 350.
Rhee YK, Han MJ, Choi EC, Kim DH. 2002. Hypocholesterolemic activity of bifidobacteria isolated from a healthy Korean. Arch Pharm Res. 25:681-684.

Salminen S, von Wright A, Morelli L, Marteau P, Brassart D, de Vos WM, Fondén R, Saxelin M, Collins K, Mogensen G, Birkeland SE, Mattila-Sandholm T. 1998. Demonstration of safety of probiotics-a review. Int J Food Microbiol. 44:93-106.

Sanchez B, Noriega L, Ruas-Madiedo P, de los Reyes-Gavilan CG, Margolles A. 2004. Acquired resistance to bile increases fructose-6-phosphate phosphoketolase activity in Bifidobacterium. FEMS Microbiol Lett. 235:35-41.

Schimmel P, Kleinjans L, Bongers RS, Knol J, Belzer C. 2021. Breast milk urea as a nitrogen source for urease positive Bifidobacterium infantis. FEMS Microbiol Ecol. 97(3).

Stojkovic A, Simovic A, Bogdanovic Z, Bankovic D, Poskurica M. 2016. Optimal time period to achieve the effects on synbiotic-controlled wheezing and respiratory infections in young children. Srp Arh Celok Lek. 144:38-45.

Tanaka H, Hashiba H, Kok J, Mierau I. 2000. Bile salt hydrolase of Bifidobacterium longum. Biochemical and genetic characterization. Appl Environ Microbiol. 66:2502-2512.

Tissier H. 1899. *La reaction cromophile d'Escherichia et le bacterium coli.* Com rend Soc Biol. 943-945.

Versalovic J, Koeuth T, Lupski JR. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. 19(24):6823-31.

Wang F. 2012. The effectiveness analysis of the combination of Smecta and Biostime probiotics in the treatment of infantile non-infectious diarrhea. Modern Prevent Med. 39:2726-2727.

Wu X. 2013. The efficacy analysis of the combination of Smecta and Biostime probiotics in the treatment of non-infectious diarrhea in children. Road to Health 12.

Xi Y, Ding S, Guo JZ, Ma S, Kong X, Pan L. 2013. Observation of clinical effects of Biostime probiotics on thrush. Chinese J Microecol 25:830-831.

Xiao, JZ, Takahashi S, Odamaki T, Yaeshima T, Iwatsuki, K. 2010. Antibiotic susceptibility of bifidobacterial strains distributed in the Japanese market. Bioscience, Biotechnology, and Biochemistry. 74:336-342.

Xiao L, Gong C, Ding Y, Ding G, Xu X, Deng C, Ze X, Malard P, Ben X. 2019. Probiotics maintain intestinal secretory immunoglobulin A levels in healthy formula-fed infants: a randomised, double-blind, placebo-controlled study. Benef Microbes. 10(7):729-739.

Yang J, Tian X, Chen Y, Li P, Lin Y, Wang Y, Xu Z, Zhao W. 2010. Clinical observation of different feeding methods in adjuvant treatment of infantile diarrhea. Occupa Health 26:2807-2808.

Yoon SH, Ha SM, Lim J, Kwon S, Chun J. 2017. A large-scale evaluation of algorithms to calculate average nucleotide identity. Antonie Van Leeuwenhoek. 110:1281-1286.

Zabel BE, Gerdes S, Evans KC, Nedveck D, Singles SK, Volk B, Budinoff C. 2020. Strain-specific strategies of 2'-fucosyllactose, 3-fucosyllactose, and difucosyllactose assimilation by Bifidobacterium longum subsp. infantis Bi-26 and ATCC 15697. Sci Rep. 10:15919.

Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 67:2640-2644.

Zetterström R, Bennet R, Nord KE. 1994. Early infant feeding and micro-ecology of the gut. Acta Paediatr Jpn. 36(5):562-71.

7.2 Appendices

Appendix A: An assessment of the safety of *Bifidobacterium longum* subsp. *infantis* 11588

Form Approved: OMB No. 0910-0342; Expiration Date: 07/31/2022
(See last page for OMB Statement)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
001107	Sep 7, 2022
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

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

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

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

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

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

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person: Cheryl Callen		Position or Title: Senior Director, Regulatory Affairs	
Organization *(if applicable)*: Nestle Nutrition			
Mailing Address *(number and street)*: 1812 North Moore Street			
City: Arlington	State or Province: Virginia	Zip Code/Postal Code: 22209	Country: United States of America
Telephone Number: 201-650-1561	Fax Number:	E-Mail Address: cheryl.callen@us.nestle.com	

1b. Agent or Attorney *(if applicable)*

Name of Contact Person: James Heimbach		Position or Title: President	
Organization *(if applicable)*: JHeimbach LLC			
Mailing Address *(number and street)*: 923 Water Street #66			
City: Port Royal	State or Province: Virginia	Zip Code/Postal Code: 22535	Country: United States of America
Telephone Number: 8047425543	Fax Number:	E-Mail Address: JH@JHEIMBACH.COM	

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

Bifidobacterium longum ssp. infantis LMG 11588

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

☒ Electronic Submission Gateway

☐ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

_____

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

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

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

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

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

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

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

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

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

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

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

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

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

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

Addition to non-exempt infant formula powder and powdered toddler drinks for healthy children up to 3 years of age at up to 1.2x10E8 cfu/g powder

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

(Check one)

☐ Yes ☒ No

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

(Check one)

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

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

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

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

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

☐ Yes ☒ No

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

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Nestle Nutrition

(name of notifier)

has concluded that the intended use*(s)* of Bifidobacterium longum ssp. infantis LMG 11588

(name of notified substance)

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

2. Nestle Nutrition agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

(name of notifier)

1812 North Moore Street, Arlington VA 22209

(address of notifier or other location)

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

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
	James T. Heimbach, Ph.D., President, JHeimbach LLC	08/30/2022

SECTION G – LIST OF ATTACHMENTS

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

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form3667.pdf	Administrative
	NestleBinfantisCoverLetter20220830.pdf	Administrative
	NestleLMG11588GRAS.pdf	Administrative
	NestleGRASReportfromtheExpertPanel.pdf	Administrative
	SignatureColinHill.pdf	Administrative
	SignatureDanOSullivan.pdf	Administrative
	SignatureDouwevanSinderen.pdf	Administrative

From: Callen,Cheryl,US-Arlington
To: James Heimbach; Anderson, Ellen
Cc: Craig Hadley
Subject: [EXTERNAL] RE: Nestle Nutrition GRAS notice
Date: Wednesday, February 22, 2023 5:23:52 PM
Attachments: image001.png

Hi Ellen and Jim,
First of all- I am so sorry to hear about your start to the New Year Jim! I will reach out separately to catch up.

Regarding the use of *B. Infantis* – it is intended for use in powder non-exempt infant formulas for term infants.

Let me know of any other questions.

Regards,
Cheryl

From: James Heimbach <jheimbach@va.metrocast.net>
Sent: Wednesday, February 22, 2023 4:45 PM
To: Callen,Cheryl,US-Arlington <Cheryl.Callen@us.nestle.com>; Ellen Anderson <ellen.anderson@fda.hhs.gov>
Cc: Craig Hadley <hadley.craig1@gmail.com>
Subject: FW: Nestle Nutrition GRAS notice

Dear Ellen—

I'm forwarding this to Cheryl Callen at Nestle to respond. (b) (6) [redacted]

(b) (6) [redacted] So, let's rely on Cheryl to respond to your note.

Regards,
Jim

James T. Heimbach, Ph.D., F.A.C.N.
JHeimbach LLC
923 Water Street #66
Port Royal VA 22535
USA
Tel: (+1) 804-742-5543
Cell: (+1) 202-320-3063
Email: jh@jheimbach.com

From: Anderson, Ellen <Ellen.Anderson@fda.hhs.gov>
Sent: Wednesday, February 22, 2023 4:15 PM
To: jh@jheimbach.com
Subject: Nestle Nutrition GRAS notice

Hello Dr. Heimbach,

A belated Happy 2023 to you; I hope this email finds you well.

I am facilitating the review of the GRAS notice you submitted on behalf of Nestle Nutrition for the use of *Bifidobacterium longum* ssp. *infantis* LMG 11588. Before filing the notice, I'd like to get some clarity on the intended use of the substance. On page 30, the GRAS notice states that the substance is "intended for use in powder non-exempt infant formula." For infant formula uses, we typically ask notifiers to specify if the use is for term or pre-term infants. Could you please confirm that the substance is intended for use as an ingredient in powder non-exempt infant formula for term infants?

Sincerely,
Ellen

Ellen Anderson (she/her/hers)
Regulatory Review Scientist

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 240-402-1309
ellen.anderson@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

Nestlé Nutrition RESPONSES: LMG 11588 **6/29/2023**

1. Please provide a statement that all processing aids used in the manufacture of B. longum ssp. infantis strain LMG 11588 are used in accordance with applicable U.S. regulations, were concluded to be GRAS for their respective uses, or are the subject of an effective food contact notification.

Nestlé Response:

The use of amino acids is addressed in the response to question 5. In addition, two other ingredients are used as processing aids. Both ingredients are GRAS for their respective uses and have no limitation other than Good Manufacturing Practices (GMP).

2. Table 7 provides the specifications for B. longum ssp. infantis strain LMG 11588, and the results from the analyses of three nonconsecutive batches are provided in Table 8. We note that the specifications for lead, arsenic, mercury, and cadmium are higher than the results from your batch analyses. For example, the specification for cadmium is < 0.5 mg/kg and the results of the batch analyses are between 0.015 mg/kg and 0.049 mg/kg. We request that specifications for ingredients, particularly those consumed by infants and young children, are as low as possible and reflect the results of the batch analyses for an ingredient produced in accordance with current good manufacturing practices. Please consider reducing these specifications to ensure that dietary exposure to heavy metals is as low as possible.

Nestlé Response:

This ingredient is proposed to be used in infant formula at a very low level and therefore would not be a significant contributor to heavy metals in infant formula. That said, we appreciate your comment and agree with your suggestion to modify the specification limits based on our batch testing results. However, given that relatively few samples have been produced and tested, we are recommending the levels be established with sufficient overage to allow for potential raw material and production variability.

A modified Table 7 is included below with the revised specifications for lead, arsenic, cadmium, and mercury.

Table 7: Specification for *Bifidobacterium longum* ssp. *infantis* LMG 11588 Spray Dried Powder

Test	Acceptance Criterion	Methods/Based on
Physical aspect	Free flowing particles, creamy beige powder	Visual observation
Bifidobacterium longum ssp. *infantis*	> 4.0E+10 CFU/g	Bacteriological enumeration – in-house method
	Identity correct	Identity check by MALDI-ToF
Yeast and Molds	< 1000 CFU/g	ISO 21527-2
Aerobic mesophilic microorganisms	< 500 CFU/g	In-house method based on ISO 13559:2002/IDF 153:2002

Enterobacteriaceae	Negative in 10 g	ISO 21528
Coagulase positive staphylococci	< 10 CFU/g or negative in 1 g	ISO 6888-1 or ISO 6888-3
Salmonella spp.	Negative in 10 g or negative in 25 g	AOAC 2011.03
Cronobacter spp.	Negative in 10 g	ISO 22964
Lead	< 0.01 mg/kg	ICP-MS, similar to AOAC 2013.06
Arsenic	< 0.05 mg/kg	ICP-MS, similar to AOAC 2013.06
Mercury	< 0.03 mg/kg	ICP-MS, similar to AOAC 2013.06
Cadmium	< 0.10 mg/kg	ICP-MS, similar to AOAC 2013.06

3. Also in Table 7, the following specifications are provided:

- Coagulase positive staphylococci: <10 cfu/g or negative in 1 g
- Salmonella spp.: Negative in 10 g or Negative in 25 g

For the administrative record, please explain why there are two specifications for these microbial tests.

Nestlé Response:

Salmonella spp can be analyzed in different sample sizes (10g or 25 g). When the sample size is reduced, the number of samples analyzed is increased accordingly. For on-going production, we will be using a negative in 10 x 10 g sampling program.

For coagulase positive staphylococci, there are 2 methods available - quantitative or qualitative. For on-going production, we intend to use the qualitative method (negative in 1 g) which is more stringent than the quantitative method (<10 cfu/g).

In developing our GRAS submission, we reviewed the previous *B. infantis* GRAS notices on file and accepted by FDA with no objection. In those notices (GRN 758; GRN 950; GRN 985; GRN 1003) all included staphylococci and salmonella as part of the ingredient specification. The comparison of these specifications is below for your reference (Table 12).

Table 12: Prior *B. infantis* GRAS notices on file and accepted by FDA with no objection.

GRN	Salmonella		Staphylococci	
	Sample Description	Method	Sample Description	Method
758	Neg in 25 g (60 samples)	ISO 6579	<10 cfu/g	ISO 6888-1
950	Neg 10 X 10g	ISO 6579 (modified)	<10 cfu/g Not detected in 0.1g	Ph.Eur. 2.6.13 (modified)

985	Neg in 40 g	AOAC 2004.03	<10 /g	AOAC 975.55
1003	Neg in 25 g	ISO 6579	Neg in 0.01g	ISO 6888-1

4. We note that a specification for Listeria was not provided in Table 7. Listeria monocytogenes can cause life-threatening infections in neonates and FDA requires testing for L. monocytogenes for infant formula ingredients. We request that the notifier incorporate testing for L. monocytogenes and provide the analytical method used, specification, and batch analyses data for three non-consecutive batches.

Nestlé Response:

In developing our GRAS submission, we reviewed the previous *B. infantis* GRAS notices on file and accepted by FDA with no objection. In those notices, two included Listeria as part of the ingredient specification (GRN 985; GRN 1003) and two did not (GRN 758; GRN 950). As we did not consider this ingredient to be a risk for listeria contamination, this parameter was not included in our specification.

Based on the FDA comment, we have incorporated Listeria into our specification. An updated Table 7 is included below (including Listeria as well as the updated criteria for heavy metals, as addressed in response to question 2). Batch testing results demonstrating compliance with the specifications are also provided (Table 8a).

Table 7: Specification for *Bifidobacterium longum* ssp. *infantis* LMG 11588 Spray Dried Powder

Test	Acceptance Criterion	Methods/Based on
Physical aspect	Free flowing particles, creamy beige powder	Visual observation
Bifidobacterium longum ssp. *Infantis*	> 4.0E+10 CFU/g	Bacteriological enumeration – in-house method
	Identity correct	Identity check by MALDI-ToF
Yeast and Molds	< 1000 CFU/g	ISO 21527-2
Aerobic mesophilic microorganisms	< 500 CFU/g	In-house method based on ISO 13559:2002/IDF 153:2002
Enterobacteriaceae	Negative in 10 g	ISO 21528
Coagulase positive staphylococci	< 10 CFU/g or negative in 1 g	ISO 6888-1 or ISO 6888-3
Salmonella spp.	Negative in 10 g or negative in 25 g	AOAC 2011.03
Cronobacter spp.	Negative in 10 g	ISO 22964
Listeria monocytogenes	Negative in 25 g	VIDAS® Listeria Monocytogenes II (LMO2) (validated against ISO 11290-1)

Lead	< 0.01 mg/kg	ICP-MS, similar to AOAC 2013.06
Arsenic	< 0.05 mg/kg	ICP-MS, similar to AOAC 2013.06
Mercury	< 0.03 mg/kg	ICP-MS, similar to AOAC 2013.06
Cadmium	< 0.10 mg/kg	ICP-MS, similar to AOAC 2013.06

Table 8a: Analysis Results for Listeria for Three Batches of *Bifidobacterium longum* ssp. *infantis* LMG 11588

Test	Acceptance Criteria	Batch no. 00172189V1	Batch no. 00172210V1	Batch no. 00172210V2
Listeria monocytogenes	Negative in 25g	Absent /25 g	Absent /25 g	Absent /25 g

5. On page 23 of the notice, it states that "all protective agents are either GRAS or approved Food Additives" and a number is provided: WO/2017/001590. When researching the number, we located the following link to a patent: https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2017001590. This patent states that the protective agent contains "at least one amino acid selected from cysteine, lysine, alanine, and arginine." We note that 21 CFR 172.320 authorizes the use of amino acids as nutrients added to food and is not applicable to their use as cryoprotectants. Additionally, we have not evaluated the use of these amino acids as cryoprotectants under our GRAS notification program. Therefore, we request that you provide a statement indicating that you will remove the use of cysteine, lysine, alanine, and arginine as cryoprotectants. If an alternative cryoprotectant will be used, please indicate the identity of this ingredient and confirm that it is safe and suitable for its intended use.

Nestlé Response:

The manufacturing process for *Bifidobacterium longum* ssp. *Infantis* LMG 11588, described in section 2.5.1 of the GRAS notice, includes a spray drying step. Since a freeze drying step is not used, the protective agents are not functioning as cryoprotectants.

In spray-drying, the cells may be damaged from heat, oxidation and/or dehydration. Heat damage to the cells can be avoided, to a large extent, by controlling heat exposure (time and temperature) during the drying process. To minimize hot air induced oxidation and dehydration effects, adding a suspension medium to the biomass consisting, in part, of amino acids has been shown to offer a favorable and protective environment for cells during the drying process. This is why we refer to them as "protective agents".

In reviewing previously submitted and accepted GRAS notices for bifidobacteria and lactobacilli, we have seen that amino acids are commonly used to help ensure cell viability during processing. We note many of these are freeze dried applications and use the term "cryoprotectant".

GRN 950 *Bifidobacterium longum subsp. infantis DSM 33361*

"The bacterial cells are harvested and concentrated by centrifugation using a separator. The concentrated bacterial cells are then mixed with cryoprotectants. The cryoprotectants used are mainly carbohydrates and amino acids that are safe and suitable for human consumption."

GRN 1002 *Bifidobacterium breve MCC1274*

"The manufacturing of the subject of this Notice involves two processes, a culturing and non-culturing process. During the culturing process, working stocks are thawed and expanded in sterilized medium in two phases to produce the manufacturing culture. During the non-culturing process, the manufacturing culture is then cooled, concentrated via centrifugation, washed with sterilized water, and reconcentrated via centrifugation. The concentrated B. breve MCC1274 biomass is then resuspended in a sterilized resuspension medium, which is used as a cryoprotectant, is composed of carbohydrates and amino acids, and has no technical function other than to ensure the viability of the bifidobacterium. All ingredients of the resuspension medium comply with the specifications listed in the Food Chemicals Codex (FCC) monographs for each ingredient and are therefore safe and suitable for their intended use."

GRN 1013 *Lactobacillus rhamnosus* DSM 33156

"Concentration and mixing with cryoprotectants. The bacterial cells are harvested and concentrated by centrifugation using a separator. The concentrated bacterial cells are then mixed with cryoprotectants. The cryoprotectants used are mainly carbohydrates and amino acids that are safe and suitable for human consumption."

GRN 1003 *Bifidobacterium longum* subsp. *infantis* M-63

Does the resuspension medium contain buffer and cryoprotectant or any other ingredients?

"The resuspension medium, which has no technical function other than to ensure the viability of the bifidobacterium, is composed of carbohydrates, amino acids, phosphate, and a vitamin, all of which comply with the specifications listed in the FCC monographs for each ingredient."

GRN 950 *Bifidobacterium longum* subsp. *infantis* DSM 33361

"The bacterial cells are harvested and concentrated by centrifugation using a separator. The concentrated bacterial cells are then mixed with cryoprotectants. The cryoprotectants used are mainly carbohydrates and amino acids that are safe and suitable for human consumption"

Additionally, there are GRAS notices for amino acids as processing aids as shown below (Table 13).

Table 13: Amino Acid GRAS Notices – Processing Aids

Amino acid	GRAS citation	GRAS function	FA Citation	FA function
L- alanine	----	------	172.320	nutrient
L- Cysteine	184.1271	Dough strenthener	172.320	nutrient
L- Cysteine hydrochloride	184.1272	Dough Strenthener	172.320	nutrient

L- Lysine monohydrochloride	GRN 414	Reduce acrylamide formation	172.320	Nutrient
L- arginine	GRN 290 GRN 317	Processing aid	172.320	Nutrient

At the proposed levels of use of for *Bifidobacterium longum* ssp. *Infantis* LMG 11588 in infant formula products, the amino acids present in the probiotic ingredient would not significantly increase the amount naturally present in the formula and would be considered as incidental additives in the final product.

Given the above, we believe the use of amino acids as processing aids in the manufacturing of probiotic bacteria is considered safe and suitable.

6. Please clarify any other components directly added to the ingredient, including the source of maltodextrin.

Nestlé Response:

There are no other directly added ingredients. The maltodextrin conforms to 21 CFR 184.1444 and is derived from corn.

7. On page 30 of the notice, it states that "toddler drinks refer to powder-based products for children 1-3 years of age intended to replace liquid milk in the diet." Please clarify whether the powder-based products referred to are formula-type drinks for young children (e.g., "toddler formulas"); if this category does not capture your intended uses, please provide an expanded description and examples of these products.

Nestlé Response:

In section 3.1.2, the reference to toddler drinks does refer to formula-type drinks ("toddler formulas"). Our description "toddler drinks refer to powder-based products for children 1-3 years of age intended to replace liquid milk in the diet" was intended to distinguish these products from infant formulas.

8. On page 31 of the notice, it states "…the estimated exposure estimates represent intake from all sources. If an older infant were to consume a toddler drink with Bifidobacterium longum ssp. infantis LMG 11588, it is assumed that consumption of such product would displace consumption of infant formula." We note that uses in infant formula and toddler drinks would not be substitutional given that drinks intended for toddlers would not be suitable for consumption by infants and formulas for older infants (e.g., 9-12 months of age) would still be "infant formula" and must comply with the infant formula regulations under Section 412 of the Federal Food, Drug, and Cosmetic Act. Please confirm that, the exposure assessment does not include replacement of infant formula with a formula-type drink for young children.

Nestlé Response:

We agree that toddler formulas are not intended to substitute for infant formula in the diet of infants less than 12 months of age. We apologize for the misunderstanding as it was not our intent to suggest otherwise. The statement referenced in your question was meant to clarify that if a child did consume a toddler formula under 1 year of age, it is not expected that the consumption would be in addition to infant formula. An example might be a situation where infant formula was unavailable and a health care provider recommended short term use of a toddler formula.

9. The notifier provides a dietary exposure for the subpopulation of infants (14-27 days of age) with the highest estimated energy intake per kg body weight. However, this estimate does not address the estimated dietary exposure for older infants that consume greater amounts of infant formula consumed on a per person basis. We request that you provide the mean and 90th percentile estimates of dietary exposure on a per person basis for infants 0-6 months and 7-12 months. Alternatively, if a single value is used to represent the mean for infants 0-12 months, please clearly state this for the record and include the reasoning for the use of a single value.

Nestlé Response:

The determination of dietary intake of *Bifidobacterium longum* ssp. *infantis* LMG 11588 based on energy intake of infants 14-27 days of age represents the maximum intake for infants from 0-12 months of age.

Based on the Dietary Reference Intakes (DRI), the estimated energy requirements for infants ages 1 month, 6 months, 7 months and 12 months are shown below:

Estimated Energy Requirements

Age (mo)	Median Reference Weight (kg)	EER (kcal/day)	Energy intake kcal/kg bw/day
1	4.4	472	107.3
6	7.9	645	81.6
7	8.4	668	79
12	10.3	793	77

Adapted from Table 5-16 of the DRI for Energy

In our exposure estimate, we used the 90th percentile of energy intake as 141.3kcal/kg bw/day for infant boys – which is greater than the 107 kcal/kg bw/day required for infants at one month of age as shown above and significantly more than required between 6 and 12 months.

In our exposure assessment we also used the 90th percentile formula intake of 207 mL/kg bw/day. 207 ml formula is about 7 fl oz of infant formula per kg bw/day (assumes sole source of calories/nutrition). The conversion to oz of formula and calories per day is shown below and demonstrates that using 207 mL/kg bw would be a conservative estimate for children 6-12 months of age.

Age (mo)	Reference weight (kg)	Oz formula /day	Energy intake /day
1	4.4	30.8	616
6	7.9	55.3	1106
7	8.4	58.8	1176
12	10.3	72.1	1442

A review of grams of infant formula consumed by infants (per consumer) in the 2016 Nestlé Feeding Infants and Toddlers Study, shows infants 0-3.9 months of age consumed 202.8g of formula; infants 4-5.9 months of age consumed 167.9g formula; infants 6-8.9 months consumed 155.1g of formula and infants 9-11.9 months of age consumed 121.8 g of formula. These data support that the amount of infant formula consumed between 7-12 months of age does not increase and, in fact, decreases during the 7-12 month period. This would be expected as complementary foods are added to the diet at around 6 months of age and contribute to calorie and nutrient intake during the 6-12 month period.

10. For the administrative record, please describe the in-process controls that are in place during fermentation and how the fermentation process is monitored for potential contaminants.

Nestlé Response:

The fermentation process of the starter culture and the main culture is done with an overpressure in the fermenter tanks to avoid potential microbial contamination coming from the external environment. The overpressure is automatically registered, in a continuous way, during the entire fermentation and an alarm is triggered if the overpressure falls below the limit. At the end of the fermentation, the operators print the trending of the pressure and check if the required minimum pressure has been achieved. For verification purposes, a microbiological analysis is done for every finished batch of culture powder.

09/25/2023
Nestlé Gerber Response to FDA Questions dated 9/01/2023– GRN 001107

FDA Question:

A. The notice states that an intended use of *Bifidobacterium longum* ssp. *Infantis* LMG 11588 is in non-exempt infant formula for term infants. Please identify the protein source(s) included in the intended infant formula (e.g., cow milk-based, soy-based).

Nestlé Response: The nonexempt formulas for term infants would include those made with milk-based proteins (including partially hydrolyzed whey protein, cow milk-based and goat milk-based); or soy-based protein.

B. In your response to Question 5 (see attached), you stated that, "At the proposed levels of use of for *Bifidobacterium longum* ssp. *Infantis* LMG 11588 in infant formula products, the amino acids present in the probiotic ingredient would not significantly increase the amount naturally present in the formula and would be considered as incidental additives in the final product." We request that you provide additional information to support the use of the ingredient.

1. Please state the intended use level of amino acids used as protective agents in the *Bifidobacterium longum* ssp. *Infantis* LMG 11588 ingredient and confirm that the amount added is not more than necessary to achieve the intended technical effect during processing.

Nestlé Response:
The levels of amino acids in the probiotic ingredient are added at the amount needed to accomplish the intended functional effect. Amino acids help support survival of the bacterial cells during processing. The amino acid level is self-limiting for technical reasons – higher levels of use can result in stickiness during the drying process.

The level of amino acids in the probiotic ingredient (culture powder) is (b) (4) [redacted]. This range is considered Nestlé proprietary business information and is not to be disclosed publicly.

The level of culture powder ingredient added to infant formula is about 0.012g/100 g dry infant formula powder. At [redacted], the amount contributed by the culture powder would be (b) (4) [redacted]/100 g of dry infant formula powder. This would equate to (b) (4) [redacted]) per 100 kcal.

2. Please confirm that the amino acids used as protective agents are of the L-form,[1] of a purity suitable for their intended use, and, as an ingredient added

[1] Martin CR et al. 2016. Review of infant feeding: key features of breast milk and infant formula. Nutrients. 8(5): 1-11. doi: 10.3390/nu8050279

to *Bifidobacterium longum* ssp. *Infantis* LMG 11588, do not contain one or more major allergens.

Nestlé Response:
The L-form of the amino acid(s) is used. All amino acids are suitable for their intended use and do not contain any of the major food allergens.

Lastly, as a general point, we recommend that you consider submitting a GRAS notice for the use of amino acids as cryoprotectants or for other protective effects since the intended use has not previously been the subject of a GRAS notification and is not the subject of an existing regulation.

Nestle Response: The recommendation is noted and will be considered further by the Nestlé team.

[i] The range for amino acids in the probiotic ingredient is Nestlé proprietary business information and should not be disclosed publicly.