CHR. HANSEN
Improving food & health

Susan J. Carlson, Ph.D.
Director, Division of Food Ingredients
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

Chr. Hansen A/S
Boege Allé 10-12
2970 Hoersholm
Denmark

www.chr-hansen.com
info@chr-hansen.com

August 04, 2022
CAWINN

Re: Chr. Hansen GRAS notice for *Bifidobacterium breve* DSM 33444

Dear Dr. Carlson,

In accordance with the final rule of August 17, 2016 (81 FR 159) and 21 CFR Part 170 Subpart E on the Generally Recognized as Safe (GRAS) notice, Chr. Hansen A/S is notifying the U.S. Food and Drug Administration (FDA) of our conclusion that *Bifidobacterium (B.) breve* DSM 33444 is GRAS through scientific procedures for its intended use as a microbial ingredient in conventional foods and non-exempt infant formula for term infants, and is not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetics Act*. The maximum incorporation level in conventional foods will be 5.0×10^9 colony-forming units (CFU)/serving to account for loss of viability throughout the shelf-life of the product, and 1.0×10^8 CFU/g of term, non-exempt infant formula.

Please do not hesitate to contact us should you require any clarifications regarding this GRAS notice.

Yours sincerely,

[redacted]

Winnie Ng, Ph.D., DABT
Principal Regulatory Affairs Specialist
cawinn@chr-hansen.com

cc: Katharine Urbain, Head of North America Regulatory Affairs (uskaur@chr-hansen.com)

Generally Recognized as Safe (GRAS) Conclusion for the Intended Uses of *Bifidobacterium breve* DSM 33444 in Conventional Foods and Non-Exempt Infant Formula

Prepared by Chr. Hansen A/S

August 2022

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Abbreviations

ATCC	American Type Culture Collection
BE	Bioengineering
BIOHAZ	Panel on Biological Hazards
BSL	Biosafety level
CCP	Critical Control Points
CFR	Code of Federal Regulations
CFU	Colony forming units
cGMP	Current good manufacturing practice
DNA	Deoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EFFCA	European Food and Feed Cultures Association
EFSA	European Food Safety Authority
EU	European Union
FEEDAP	The Panel on Additives and Products or Substances used in Animal Feed
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
FSSC	Food Safety System Certification
GM	Genetic modification
GRAS	Generally Recognized as Safe
GS-MS	Gas chromatography-mass spectrometry
HACCP	Hazard Analysis and Critical Control Point
IDF	International Dairy Federation
ISO	International Standardization Organization
MIC	Minimum inhibitory concentration
NCBI	National Center for Biotechnology Information
NBFDS	National Bioengineered Food Disclosure Standard
OPRP	Operational Prerequisite Program
ONT	Oxford Nanopore MiniON technology
PCR	Polymerase chain reaction
PEG	Protein encoding gene
Ph.Eur.	European Pharmacopeia
PRP	Prerequisite Program
QPS	Qualified Presumption of Safety
rDNA	Recombinant deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
tRNA	Transfer ribonucleic acid
U.S.	United States
USDA	United States Department of Agriculture
USP	United States Pharmacopeia
VFDB	Virulence Factor Database

Part 1. Signed statements and certification

1.1. Statement of intent

In accordance with the Title 21 Code of Federal Regulation (CFR) Part 170 Subpart E on the Generally Recognized as Safe (GRAS) notice, Chr. Hansen A/S has concluded, through scientific procedures, that *Bifidobacterium (B.) breve* DSM 33444 is GRAS and is not subject to the premarket approval requirements for use as a microbial ingredient in conventional food and non-exempt infant formula for term infants.

Name and Address of Organization

Chr. Hansen A/S
Boege Alle 10-12
2970 Hoersholm
Denmark

Chr. Hansen, Inc. (local office)
9015 W Maple St.
Milwaukee, WI 53214
USA

Contact Person:
Kate Urbain
Head of NA Regulatory Affairs
uskaur@chr-hansen.com
Telephone: 414-607-5819
Mobile: 414-520-3441

1.2. Name of GRAS substance

Bifidobacterium (B.) breve DSM 33444

1.3. Intended conditions of use

B. breve DSM 33444 is intended for use as a microbial ingredient in a variety of conventional foods to be consumed by populations of all ages at levels consistent with current good manufacturing practice (cGMP). *B. breve* DSM 33444 is also intended for use in non-exempt infant formula for term infants.

The level of inclusion of *B. breve* DSM 33444 will vary depending on the type of food and application under which it will be used; however, the maximum incorporation level will be 5.0×10^9 colony-forming units (CFU)/serving in conventional foods, and 1.0×10^8 CFU/g of non-exempt infant formula for term infants.

B. breve DSM 33444 is not intended for use in products regulated by the United States Department of Agriculture (USDA).

1.4. Statutory basis for conclusion of GRAS status

Pursuant to the GRAS rule [81 Fed. Reg. 159 (August 17, 2016)], Chr. Hansen has concluded that the intended use of *B. breve* DSM 33444 is GRAS through scientific procedures in accordance with 21 CFR 170.30 (b).

1.5. Premarket approval status

It is the opinion of Chr. Hansen that *B. breve* DSM 33444 is not subject to premarket approval requirements of the Federal Food, Drug, and Cosmetics Act based on our conclusion that *B. breve* DSM 33444 is GRAS under the intended conditions of use.

1.6. Availability of information

The data and information that form the basis of Chr. Hansen's conclusion that the intended use of *B. breve* DSM 33444 is GRAS are available for review and copying by FDA during customary business hours, at the location below, or will be sent to FDA upon request made to:

Chr. Hansen, Inc.
Winnie Ng
Principal Regulatory Affairs Specialist
9015 W Maple St., Milwaukee, WI 53214
cawinn@chr-hansen.com

1.7. Freedom of Information Act

It is our opinion that the information contained in this GRAS notification is not exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

1.8. Certification

To the best of our knowledge, this GRAS notification is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of *B. breve* DSM 33444 under the intended conditions of use.

1.9. FSIS statement

Not applicable. *B. breve* DSM 33444 is not intended for use in applications under the jurisdiction of the USDA.

1.10. Name, position, and signature of responsible person who signs dossier

[redacted]

August 4, 2022

Winnie Ng
Principal Regulatory Affairs Specialist

Date

[redacted]

August 4, 2022

Katharine Urbain
Head of North America Regulatory Affairs

Date

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

2.1. Identity of the GRAS substance

The subject of this GRAS notice is a strain of the bacterial species *Bifidobacterium (B.) breve* designated as DSM 33444.

2.2. Source of the GRAS organism

B. breve DSM 334444 was originally isolated from the intestinal flora of a healthy infant and belongs to the taxa *Bifidobacterium breve*.

The *B. breve* DSM 33444 that is the subject of this notice was deposited in the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) under the accession number DSM 33444.

2.3. Description of the GRAS organism

2.3.1. Bifidobacterium

Bifidobacterium are Gram-positive, non-spore forming, non-motile, rod-shaped bacteria belong to the taxonomic Actinobacteria branch of the phylum Firmicutes that are primarily strictly anaerobic (Ventura et al., 2004). Bifidobacteria may be defined by their characteristic ability as saccharolytic microorganisms to ferment glucose, galactose, and fructose, wherein the product of the metabolism of sugars is acetic and lactic acid, and differing *Bifidobacterium* species may ferment other carbohydrates and alcohols.

Bifidobacteria are a ubiquitous part of the human microbiota and are the predominant organisms in the gastrointestinal tract in infants following birth (Harmsen et al., 2000; Yang et al., 2019). Along with the gastrointestinal tract, Bifidobacteria have also been identified in the oral cavity, breast milk, vagina, and feces of humans (Haarman & Knol, 2005; Korshunov et al., 1999; Martín et al., 2009; Matsuki et al., 1999; Reuter, 2001; Russell et al., 2011; Soto et al., 2014). *Bifidobacterium* species including *B. animalis*, *B. longum*, *B. breve*, *B. bifidum*, and *B. adolescentis* have a history of use in the production of fermented foods including dairy products such as yoghurt, yoghurt and fermented milk drinks, sour milk, and other milk-based products. Industrial application of *Bifidobacterium* species in food are often combined with other lactic acid bacteria (Klein et al., 1998; Reuter, 1990, 1997; Reuter et al., 2002). Due to the long history of consumption and human exposure, Bifidobacteria associated with food are considered generally safe by the scientific community (Adams & Marteau, 1995).

2.3.2. *Bifidobacterium breve*

B. breve is a member of the phylum Actinobacteria in bacterial taxonomy. *B. breve* is a well-characterized, non-pathogenic, non-toxigenic, homogeneous species grouping. *B. breve* was originally isolated from the feces of a breast fed infant (Reuter, 1963).

The taxonomic lineage of *B. breve* DSM 33444 is detailed in Table 1.

Table 1. Taxonomic lineage of *Bifidobacterium breve* DSM 33444

Taxonomy	Taxonomic Assignment
Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteridae
Order	Bifidobacteriales
Family	Bifidobacteriaceae
Genus	*Bifidobacterium*
Species	*Bifidobacterium breve*
Strain	*Bifidobacterium breve* DSM 33444

2.3.3. Genotypic classification of *Bifidobacterium breve* DSM 33444

2.3.3.1. Species identification

Sequence analysis of the DSM 33444 strain's 16S rDNA sequence was compared to a database of 16S rDNA sequences of type strains (Ludwig et al., 2021). The 16S rDNA sequence of the DSM 33444 strain is 99.9% identical to the sequence of the type strain of *Bifidobacterium breve* (GenBank acc. No. AB006658). The DSM 33444 strain is identified as *Bifidobacterium breve*.

2.3.3.2. Genome sequencing and annotation

The genome of *B. breve* DSM 33444 was sequenced using both Illumina MiSeq technology and Oxford Nanopore MinION technology (ONT). By combining the sequence reads from both technologies in the same assembly a closed genome of high quality can be obtained. Accordingly, based on MiSeq reads and ONT reads a draft closed genome was obtained and annotated using the RASTtk pipeline (http://rast.nmpdr.org/) with default settings.

The annotated ONT/MiSeq hybrid genome sequence of the DSM 33444 strain consists of a single circular chromosome of 2.3 Mb (2,322,388 bp) with a GC content of 58.8%. The ONT/MiSeq hybrid genome sequence has an average MiSeq read coverage of 105x and a ONT read coverage of 104x. The RAST annotation of the ONT/MiSeq hybrid genome revealed 1,989 protein encoding genes (PEGs), 53 tRNAs and 9 rRNAs. This was comparable to closed *B. breve* genomes in the National Center for Biotechnology Information (NCBI) genome database (2.2-2.6 Mb in size and 1,921-2,327 PEGs).

2.3.4. Phenotypic analysis of *Bifidobacterium breve* DSM 33444

B. breve DSM 33444 is a Gram positive, non-spore forming, catalase-negative, non-motile, anaerobic bacterium. In terms of cell morphology, *B. breve* DSM 33444 are thin, irregular, and occasionally branched rods, occurring singly and in pairs, and does not contain plasmids. The carbohydrate fermentation profile of *B. breve* DSM 33444, as determined using the Rapid ID 32 A test system, is presented in Table 2 below.

Table 2. Carbohydrate fermentation (Rapid ID 32 A) of the *B. breve* DSM 33444 strain

Urease	-	Indole production	-
Arginine dehydrolase	-	Alkaline phosphatase	+
α-galactosidase	+	Arginine arylamidase	+
β-galactosidase	+	Proline arylamidase	+
β-galactosidase-6 phosphate	-	Leucyl-glycine arylamidase	-
α-glucosidase	+	Phenylalanine arylamidase	+
β-glucosidase	+	Leucine arylamidase	+
α-arabinosidase	-	Pyroglutamic acid arylamidase	-
β-glucuronidase	-	Tyrosine arylamidase	+
N-acetyl-β-glucosaminidase	+	Alanine arylamidase	-
Mannose	+	Glycine arylamidase	+
Raffinose	+	Histidine arylamidase	+
Glutamic acid decarboxylase	-	Glutamyl-glutamic acid arylamidase	-
α-fucosidase	-	Serine arylamidase	+
Nitrate reduction	-		

2.4. Genetic modification status

B. breve DSM 33444 is not genetically modified by use of recombinant DNA techniques.

In accordance with U.S. regulations, Chr. Hansen cultures and enzyme products are not subject to bioengineered (BE) labeling under the National Bioengineered Food Disclosure Standard (NBFDS), codified in 7 CFR Part 66.

Pursuant with European Union (EU) Regulation (EC) No 1829/2003 and Regulation (EC) No 1830/2003, the use of Chr. Hansen cultures including *B. breve* DSM 33444 does not trigger genetic modification (GM) labeling of the final food product.

2.5. Method of manufacture

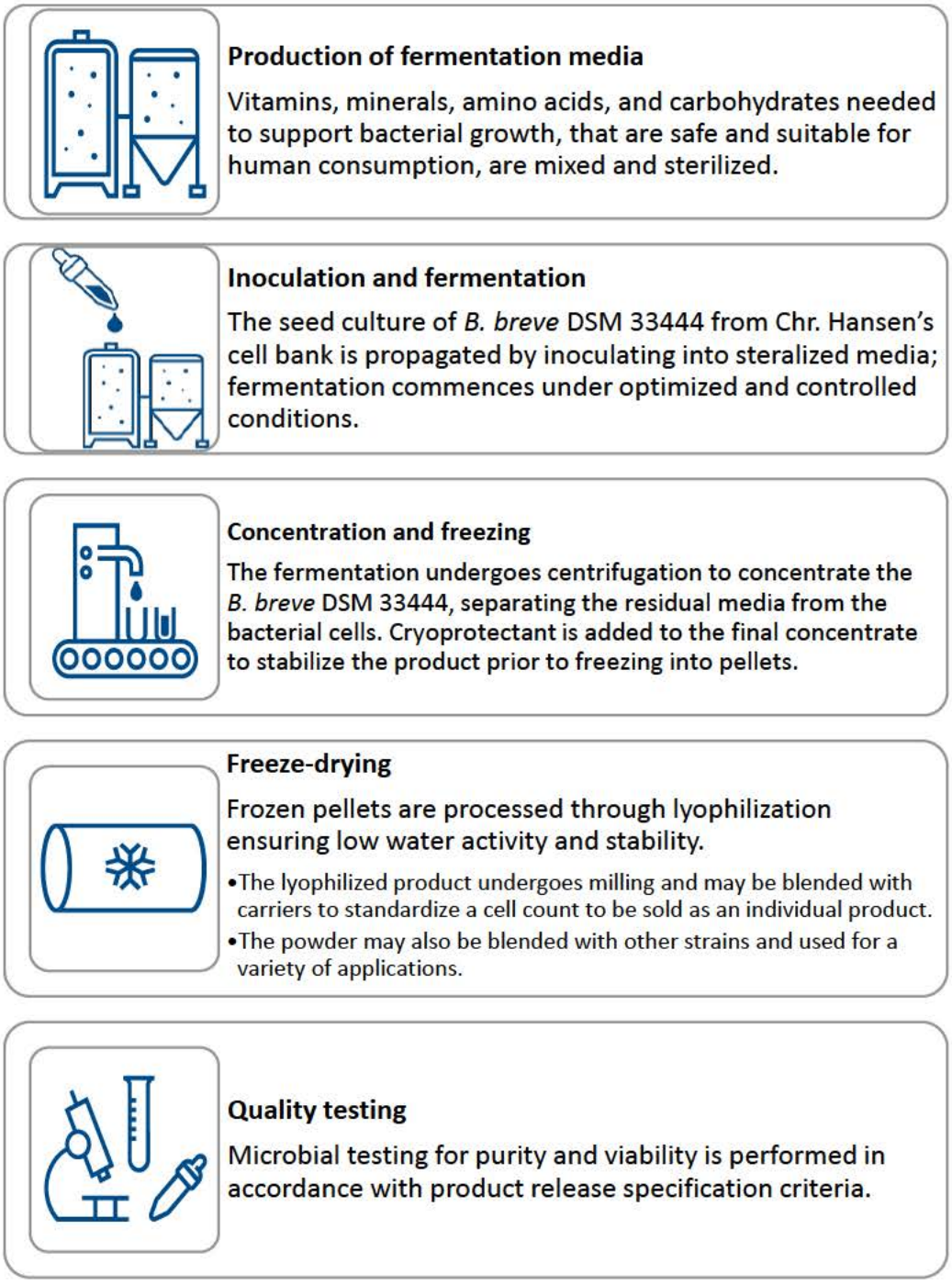
Viable *B. breve* DSM 33444 is produced by industrial batch fermentation following Chr. Hansen's global protocol for production of cultures which are in accordance with cGMP.

Pure strain of the microorganism (*B. breve* DSM 33444 seed culture) is inoculated into sterilized growth medium specifically designed to meet the nutritional needs of *B. breve*. The seed preparation is further scaled up by incubation and fermentation processes until the established fermentation end point is obtained. Strict conditions are maintained throughout the fermentation process to ensure optimal growth. These include maintaining a controlled sterile environment in a closed system and strict control of the temperature and pH. Once the fermentation enters stationary growth, it is cooled to stop the growth process. The fermentation is subjected to centrifugation for the removal of water-soluble material and to concentrate the desired *B. breve* DSM 33444. Appropriate food-safe cryoprotectants are added to improve the survival during freeze-drying. The concentrated microorganisms are then frozen into pellets. The substances used in the production process are primarily based on carbohydrates, amino acids, vitamins, and minerals that are safe. All raw materials used in the media are suitable for human consumption.

Frozen pellets are tested for quality and are freeze-dried. The resulting lyophilized pellets have a very low water activity which ensures stability of the culture. Lyophilized *B. breve* is then ground into powder. It can be tested for quality and sold as-is, or blended with other food grade microbial ingredients, carriers, or food-grade materials appropriate for their intended use. The products are packaged, labeled with necessary information, tested for quality and sold. A schematic overview of this process is outlined in Figure 1 below.

All manufacturing is done in accordance with cGMP consistent with 21 CFR Parts 110 and 117. All Chr. Hansen plants have fully implemented Hazards Analysis and Critical Control Points (HACCP) plans, standard operating procedures, and quality control programs to ensure quality of the product being produced. Each plant complies with a set of basic GMP rules, also called Pre-Requisite Program (PRP) according to Chr. Hansen's Quality, GMPs and Food Safety Principles, which are publicly available from our website www.chr-hansen.com. As part of the HACCP plan, each manufacturing process has appointed an OPRP (Operational Pre-Requisite Program) and CCPs (Critical Control Points). The OPRP and CCP's are documented and classified as specifically critical for the safety of food ingredients produced in the plant. All Chr. Hansen facilities manufacturing final products maintain FSSC 22000 certification.

Figure 1. Manufacturing overview of *B. breve* DSM 33444



2.5.1. Raw materials and processing aids

B. breve DSM 33444 is produced using standard fermentation techniques. This includes the use of fermentation and standardizing substances that are safe and suitable for use in human food. These substances have no technical function in the finished food product and are all permitted for use in this application.

2.5.2. Quality program

Chr. Hansen's extensive quality program includes a FSSC 22000 standard and hygienic monitoring program. This program serves to verify the process control of the production facility. It includes testing surfaces of process equipment and air quality to document the cleanliness of production.

2.5.3. Allergen control

Chr. Hansen controls all major food allergens as listed and established in the U.S. Food Allergen Labeling and Consumer Protection Act of 2004, in addition to control of the substances or products causing allergies or intolerances as outlined in Annex II of Regulation (EU) No 1169/2011, as amended. Chr. Hansen communicates the allergen status of its products in accordance with the U.S. and EU legislations.

Allergen control is managed via the company's cGMP and food safety programs that are FSSC 22000 certified at each of the company's production sites. In some cases, dairy or dairy components may be used during the fermentation process. In this case, dairy would be declared as a major allergen on product information sheets and communicated to customers. Allergen communication is managed via our quality management and food safety programs that are ISO 22000 certified.

2.6. Product specifications and product stability

2.6.1. Specifications and batch analyses

The final *B. breve* DSM 33444 freeze-dried ingredient is in the form of a white to light beige powder containing a total viable cell count of at least 4.8 x 10^{11} CFU/g *B. breve* DSM 33444. The quality control specifications that must be satisfied prior to the commercial release of *B. breve* DSM 33444 are outlined in Table 3 along with the methods of analysis that are all internationally recognized and/or validated.

Table 3. Product specifications for freeze-dried *B. breve* DSM 33444

Parameter	Units	Specification	Method of Analysis
Total cell count	CFU/g	≥4.8 x 10^{11}	USP 64, ISO 4833-1
Non-lactic acid bacteria	CFU/g	<500	ISO 13559:2002-M
Total aerobic microbial count	CFU/g	≤2,000	Ph.Eur. 2.6.12 (modified)
Yeast	CFU/g	<10	ISO 6611:2004
Mold	CFU/g	<10	ISO 6611:2004
Enterobacteriaceae	/10 g	Not detected	ISO 21528-1:2004
Cronobacter spp.	/10 g	Not detected	NF ISO 22964
Coagulase-positive *Staphylococcus*	/1 g	Not detected	NMKL 66:2009
Salmonella spp.	/25 g	Not detected	ISO 6579-1:2017/Amd 1:2020
Listeria spp.	/25 g	Not detected	ISO 11290-1:2017

Abbreviations: CFU, colony forming unit; ISO, International Standardization Organization; NMKL, Nordic Committee on Food Analysis Ph.Eur., European Pharmacopeia; USP, U.S. Pharmacopeia.

Analyses were conducted on 3 commercially representative batches of freeze-dried *B. breve* DSM 33444 and the results are summarized in Table 4. The analytical data demonstrate that the final *B. breve* DSM 33444 ingredient is produced consistently and conforms to the established specifications, and adequate quality control processes are in place.

Table 4. Analytical data for 3 commercially representative batches of freeze-dried *B. breve* DSM 33444

Parameter	Units	Specification	Analytical Data		
			Batch 1	Batch 2	Batch 3
Total cell count	CFU/g	≥4.8 x 10^{11}	1.4 x 10^{12}	1.6 x 10^{12}	1.6 x 10^{12}
Non-lactic acid bacteria	CFU/g	<500	<100	<100	<100
Total aerobic microbial count	CFU/g	≤2,000	<250	<250	500
Yeast	CFU/g	<10	<10	<10	<10
Mold	CFU/g	<10	<10	<10	<10
Enterobacteriaceae	/10 g	ND	ND	ND	ND
Cronobacter spp.	/10 g	ND	ND	ND	ND
Coagulase-positive *Staphylococcus*	/1 g	ND	ND	ND	ND
Salmonella spp.	/25 g	ND	ND	ND	ND
Listeria spp.	/25 g	ND	ND	ND	ND
Abbreviations: CFU, colony forming unit; ND, not detected.					

Lead was tested in three batches of *B. breve* DSM 33444 using inductively coupled plasma mass spectrometry (ICP-MS) following standardized methods for analyzing trace elements in foodstuffs. The analytical results are presented in Table 5 and demonstrate that lead is not a concern in the final *B. breve* DSM 33444 freeze-dried ingredient.

In the absence of U.S. regulatory limits for lead in foodstuffs, an internal specification of 0.05 ppm lead was established for *B. breve* DSM 33444, taking into account the typical inclusion rate in finished product applications, since the Codex Alimentarius standards for various foods sets limits according to finished food applications, where our ingredient is typically included at levels below 0.15%.

Table 5. Lead analyses for 3 batches of *B. breve* DSM 33444

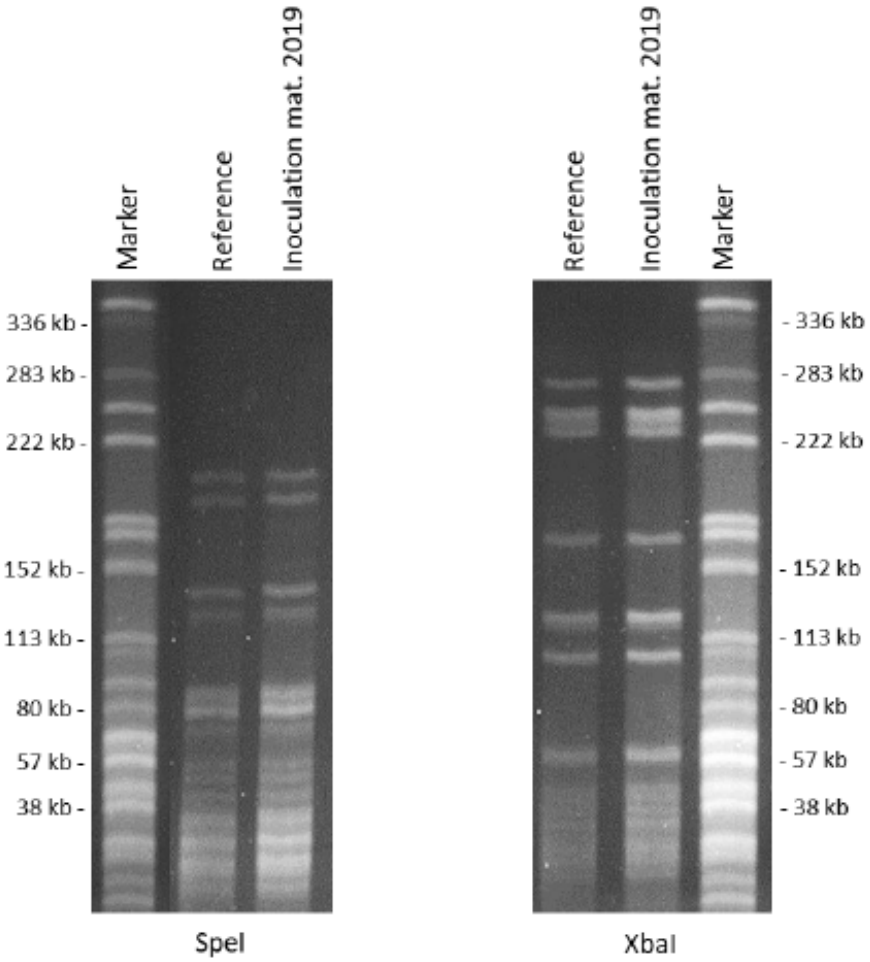
Parameter	Analytical Results (ppm)			Methods of Analysis
	Batch 1	Batch 2	Batch 3	
Lead	0.010	0.011	0.011	DIN EN ISO 15763 (2010)

2.6.2. Product stability

B breve DSM 33444 freeze-dried products have a minimum shelf life of 24 months from the date of manufacture when stored between 2-8°C in original or tightly closed foil pouch under dry conditions protected from direct sunlight.

Furthermore, the genetic stability of *B. breve* DSM 33444 has been demonstrated by DNA fingerprinting comparing the stock culture in the cell bank and the inoculation material produced in 2019 (Figure 2). The genetic stability of *B. breve* DSM 33444 shows that the strain safety analysis will hold true over time.

Figure 2. DNA fingerprint profiles of the *B. breve* DSM 33444 reference strain and the inoculation material from 2019



Part 3. Dietary exposure

3.1. Intended use

B. breve DSM 33444 is intended for use as a microbial ingredient in a variety of conventional foods to be consumed by populations of all ages at levels consistent with cGMP.

The level of inclusion of *B. breve* DSM 33444 will vary depending on the type of food and applications under which it will be used, and if it is to be blended with other microbial ingredients. Under the intended conditions of use, the maximum incorporation level will be 5.0×10^9 colony-forming units (CFU)/serving to account for loss of viability throughout the shelf-life of the product.

In addition, *B. breve* DSM 33444 is intended to be used as an ingredient in protein-based (including but not limited to soy, milk, and whey) term non-exempt infant formula at levels not to exceed 1.0×10^8 CFU/g formula product.

B. breve DSM 33444 is not intended for use in products regulated by the USDA.

3.2. Estimated dietary intake from the intended use in conventional foods

Under the intended conditions of use, it is anticipated that level of incorporation of *B. breve* DSM 33444 for conventional food applications will be up to a maximum of 5.0×10^9 CFU/serving. If it is assumed that the average consumption of a healthy individual is approximately 20 servings of all combined foods per day (Millen et al., 2006), and that all of these foods contain the strain, the maximum exposure to *B. breve* DSM 33444 as attributed to conventional foods is estimated to be in the range of 1.0×10^{11} CFU/day.

On a comparative basis, the resultant dietary exposure to *B. breve* DSM 33444 under the intended conditions of use is comparable to the dietary intake of other *B. breve* strains as referenced in a GRAS notice that received a letter of "no questions" from the U.S. FDA (GRN no. 453), where the intended use is up to 5.0×10^9 CFU/serving conventional foods (see Section 6.3.1).

Moreover, it is well recognized that the adult microbiome is very stable and only shifts with significant dietary changes or extreme weight loss (Faith et al., 2013). Considering that *Bifidobacterium* spp. are ubiquitous and *B. breve* is readily present as part of the human microbiome, any exposure to *B. breve* DSM 33444 in conventional foods under the intended conditions of use in the diet, as subject to this GRAS notice, is not anticipated to significantly alter or contribute to the overall homeostatic nature of the gut microbiota in the general population. Likewise, *B. breve* DSM 33444 may be an alternative to other *B. breve* species for the same existing uses as a microbial ingredient in conventional food (*e.g.*, GRN No. 453), and therefore the estimated daily exposure will be comparable and is not anticipated to increase the existing overall dietary intake of the species.

Indeed, the estimated dietary exposure to *B. breve* DSM 33444 under the intended use conditions in the present notice is considered extremely conservative, as it assumes that there is no loss in viability of the cells during shipping and storage. It is also unlikely that individuals would consume even half (10 servings/day) of conventional food products containing *B. breve* DSM 33444. Additionally, there is the assumption that *B. breve* DSM 33444 will be incorporated in all foods consumed on a daily basis, which includes foods explicitly excluded from the envisioned uses (*e.g.*, meat and poultry products) and foods that are not compatible with the addition of viable microbial ingredients (*e.g.*, canned foods). In reality, dietary exposure to *B. breve* DSM 33444 under the intended conditions of use will not increase the

existing exposure to the species given the aforementioned considerations and the transient nature of commensal organisms within the gastrointestinal (GI) tract.

3.3. Estimated daily exposure from the intended use in infant formula

B. breve DSM 33444 is intended for use in term, non-exempt infant formula at a maximum incorporation level of 1.0 x 10^8 CFU/g formula product. Infant formula intake data described in (Grimes et al., 2017) confirms that infants age 0-6 months consume the highest amount of formula at 834 g reconstituted formula per day. Using the Grimes daily intake data, along withs the average reconstitution rate (14.1 g powdered infant formula per 100 mL water), the maximum daily exposure to *B. breve* DSM 33444 as attributed to its use in infant formula is estimated as follows:

$$\left(\frac{1.0 \times 10^8 \text{ CFU } B.breve \text{ DSM } 33444}{1\text{g powdered formula}}\right)\left(\frac{14.1 \text{ g powdered formula}}{100 \text{ mL}}\right)\left(\frac{834 \text{ g reconstituted formula}}{1 \text{ day}}\right)$$

$$= \frac{1.2 \times 10^{10} \text{CFU } B.breve \text{ DSM } 33444}{\text{day}}$$

The estimated daily intake is in line with other microbial ingredients intended for infant formula, for example, as per GRN no. 454 and 455 where *B. breve* strain M-16V is intended for use at levels of up to 1.0 x 10^8 CFU/g powdered term infant formula, with dietary intake of up to 9.9 x 10^9 and 1.35 x 10^{10} CFU/day for one-month and six-month old infants, respectively. Considering that *B. breve* DSM 33444 may be an alternative to other *B. breve* species for the same existing uses as a microbial ingredient in infant formula, the estimated daily dietary exposures will be comparable. Likewise, in clinical trials, *B. breve* species were demonstrated to be well tolerated in infants at levels of 1.5 x 10^{10} CFU/day (see Section 6.4) (Hattori et al., 2003; Taniuchi et al., 2005; van der Aa et al., 2010, 2011, 2012).

There is no potential for cumulative exposure to *B. breve* DSM 33444, as all *Bifidobacterium* are transient in the gastrointestinal tract. Grimes *et al*. (2017) also demonstrated that as non-formula beverage intake increases, a corresponding decrease in formula occurs, such that it can be reasonably expected that the amount of *B. breve* DSM 33444 consumed will not significantly increase as the infant ages. Moreover, *B. breve* DSM 33444 will not proliferate in the foods for which it is intended for inclusion.

Part 4. Self-limiting levels of use

The intended levels of use for *B. breve* DSM 33444 are not self-limiting; however, the addition of the strain is restricted to applications that can sustain viable *B. breve* DSM 33444 at the intended use levels throughout the shelf life of the food and infant formula products.

Part 5. Experience based on common use in food

The conclusion of GRAS status for the intended uses of *B. breve* DSM 33444 is based on scientific procedures and not common use in food before 1958.

Part 6. Narrative

6.1. Approach of the safety assessment

The data and information providing the basis for our conclusion that the addition of *B. breve* DSM 33444 under the intended conditions of use is GRAS through scientific procedures are presented in the following sections. The information provided below and elsewhere in this notice is generally available in the public domain and has been properly cited. To demonstrate the safety of *B. breve* DSM 33444 under the intended conditions of use, Chr. Hansen has rigorously applied the decision tree approach to *"Determining the safety of microbial cultures for human and animal consumption"* as established by Pariza *et al.* (2015), as well as the qualified presumption of safety (QPS) approach implemented by the European Food Safety Authority (EFSA) (EFSA Scientific Committee, 2007).

As discussed in Section 2.3.3, the taxonomic identification of the organism has been definitively confirmed as *B. breve* by genomic analysis. *In silico* analyses on *B. breve* DSM 33444 demonstrates the absence of potential virulence factors and genes related to pathogenicity (see Section 6.3.2.1), as well as the absence of antibiotic resistance genes (see Section 6.3.3.1), which were further confirmed by *in vitro* assays (see Section 6.3.3.2). The strain does not exhibit undesirable metabolic activities (*e.g.*, cytotoxicity, hemolysis, or production of biogenic amines and D-lactate) (see Sections 6.3.2.1 and 6.3.4). Additionally, the *B. breve* species has been demonstrated to be well-tolerated in a number of human clinical studies (see Section 6.4).

6.2. History of safe consumption in foods

Bifidobacteria have an extensive history of use on a global scale in food products such as yoghurt, milk, infant formula, cheese, and dietary supplements (Champagne et al., 2005; Charalampopoulos et al., 2002; Mattila-Sandholm et al., 2002; Phillips et al., 2006; Vinderola et al., 2000). Mogensen et al. (2002) estimated that the average European ingests about 2.2×10^{12} LAB/year, which is equivalent to 6.0×10^{9} LAB/day. *B. breve* have been widely consumed in fermented foods for years including *B. breve* Yakult, *B. breve* SBT-2028, and *B. breve* C50. Indeed, *B. breve* strain M-16V, subject to existing GRNs 453,454, and 455, has been commercialized in Japan since 1976, while *B. breve* strain MCC1274 has been commercialized in Japan, Denmark, Italy, and Spain since 2012 as a food ingredient.

The consumption of Bifidobacteria in fermented foods and dairy products generally has low risk of infection from ingestion (Gasser, 1994; A. Ouwehand et al., 2002), and has not been associated with human clinical disease nor any specific safety concerns (EFSA Scientific Committee, 2007). A review of 54 cases of endocarditis in which LAB were isolated found none of these isolates belonging to *Bifidobacterium* (Mogensen et al., 2002). The genus *Bifidobacterium* and the *B. breve* species have been the subject of several safety assessment papers and have been found to be safe with no cause for concern regarding adverse effects or production of virulence factors or toxins (Borriello et al., 2003; Kitajima & Hirano, 2017; Meile et al., 2008; A. C. Ouwehand et al., 2004; Wong et al., 2019). Of the species that fall within the *Bifidobacterium* genus, only the *Bifidobacterium dentium* species has been associated with safety concerns related to cases of peritonsillar abscess (Civen et al., 1993) and pulmonary and subcutaneous abscesses (Slack, 1974).

Bifidobacterium breve is presently listed on the International Dairy Federation (IDF)/European Food and Feed Cultures Association (EFFCA)'s "*Inventory of microbial food cultures with safety demonstration in fermented food products"* as having a safe history of use in a variety of fermented foods such as dairy

and soy, fermented milks, and infant formula (Bourdichon et al., 2018, 2022; Mogensen et al., 2002). The IDF maintains the list using a panel of recognized experts. The source of the organisms in the IDF list may be from addition of commercially prepared starter cultures or from autochthonous organisms present on food raw materials. In either case, the organisms must be characterizing and not merely incidental components of the food microflora to be included in the IDF list.

Bifidobacterium breve is also on the Danish Veterinary and Food Administration (DVFA)'s *List of notified microbial cultures applied in food* (Danish Veterinary and Food Administration, 2016).

6.3. Safety of *B. breve* DSM 33444

6.3.1. Recognition of safety by authoritative bodies and qualified experts

The species *Bifidobacterium breve* has been evaluated and concluded by the EFSA Panel on Biological Hazards (BIOHAZ Panel) to be suitable to be granted QPS status since the initial introduction of the QPS approach in 2007 (EFSA Scientific Committee, 2007). The QPS concept was developed to provide a harmonized generic pre-evaluation to support safety risk assessments of microorganisms intentionally introduced into the food chain. Within the QPS approach the four principal considerations for evaluation of the QPS status of a microorganism include: (i) taxonomic identification, (ii) body of knowledge, (iii) safety (including virulence factors causing pathogenicity and antimicrobial resistance of valid taxonomic units), and (iv) intended use. QPS status is granted provided that the taxonomic group does not raise safety concerns or, if safety concerns exist, can be defined and excluded. The list of QPS recommended biological agents is updated regularly, wherein the most recent release in 2022 included the monitoring of any new data pertinent to the safety of species with existing QPS status (EFSA BIOHAZ Panel et al., 2021). From the updated evaluation on the *Bifidobacterium* genus, the BIOHAZ Panel concluded that the QPS status of the QPS species within this genus remained unchanged including *Bifidobacterium breve* (EFSA BIOHAZ Panel et al., 2022).

To date, there have been four GRAS notices for the *B. breve* species filed with the FDA of which all have received "no questions" letters with the exception of one GRAS notice that is pending under evaluation at the present time. The details of the GRAS notices are summarized in Table 6. Of the three GRAS notices with "no questions" from the Agency, the notified strain *B. breve* M-16V was concluded to have no safety concerns under the intended conditions of use based on the evaluation of strain-specific toxicological and clinical studies as well as clinical studies on other *B. breve* strains (see Section 6.4). While the *B. breve* strains previously notified to the FDA differ from the DSM 33444 strain as subject to this GRAS notice, it illustrates that the species *B. breve* is safe for human consumption at levels in the region of 10^{10} CFU/day in conventional foods and 9.9 x 10^{9} CFU/day for a one-month-old infant and 1.35 x 10^{10} CFU/day for a six-month-old infant.

Table 6. GRAS notices for *B. breve* filed by the FDA

GRN No.	Species/Strain	Intended Use	Use Level/ Dietary Exposure	Status
453	*Bifidobacterium breve* M-16V	As an ingredient in baked goods, breakfast cereals, fruit juices and nectars, fruit ices, vegetable juices, milk-based drinks and powders, dairy product analogs, frozen dairy desserts, processed cheese, imitation cheese, cheese spreads, butter-type products, snack foods, gelatin, pudding, fillings, meal replacements, snack bars, nut and peanut spreads, hard and soft candies, cocoa-type powder, and condiment sauces	Up to 5.0 x 10^9 CFU/serving Mean and 90th percentile dietary intake of 3.8 x 10^{10} and 6.0 x 10^{10} CFU/day, respectively	FDA has "no questions" Date of closure: September 27, 2013
454	*Bifidobacterium breve* M-16V	As an ingredient in non-exempt powdered term infant formulas (milk- or soy-based) and exempt powdered term infant formula containing partially-hydrolyzed milk or soy proteins.	Up to 1.0 x 10^8 CFU/g infant formula powder Estimated intake (both exempt and non-exempt infant formula) of 9.9 x 10^9 CFU/day for a one-month infant and 1.35 10^{10} CFU/day for a six-month infant	FDA has "no questions" Date of closure: September 27, 2013
455	*Bifidobacterium breve* M-16V	As an ingredient in exempt term powdered amino acid-based formulas	Up to 1.0 x 10^8 CFU/g infant formula powder Estimated intake of 9.9 x 10^9 CFU/day for a one-month infant and 1.35 10^{10} CFU/day for a six-month infant	FDA has "no questions" Date of closure: September 30, 2013
1002	*Bifidobacterium breve* MCC1274	As an ingredient in conventional foods including baked goods, breakfast cereals, fruit juices and nectars, fruit ices, vegetable juices, milk-based drinks and powders, yogurt, dairy product analogs, frozen dairy desserts, cheeses, condiments and spreads, nut and peanut spreads, gelatins and puddings, milk and non-milk meal replacements, soft and hard candies and snack foods, and infant and toddler foods	Up to 5.0 x 10^9 CFU/serving Estimated mean intake of 5.79 x 10^{10} CFU/day and 90th percentile 1.07 x 10^{11} CFU/day	Pending

Abbreviations: CFU, colony forming unit; FDA, Food and Drug Administration; GRAS, Generally Recognized as Safe; GRN, GRAS notice

6.3.2. Pathogenicity/Toxigenicity

Bifidobacterium breve is classified as Risk Group 1 by the German Federal Institute for Occupational Health and Safety under their Technical Rule for Biological Agents (Committee on Biological Agents, 2015). Risk Group 1 is defined as organisms that are highly unlikely to cause an infectious disease in humans; however, may in certain individual cases be a pathogen in people with reduced immunity. In the U.S., the American Type Culture Collection (ATCC) classifies the *Bifidobacterium breve* sp. as Biosafety Level (BSL) 1 which is defined as "*well-characterized agents not known to consistently cause disease in immunocompetent adult humans and present minimal potential hazard to laboratory personnel and the environment*" (Centers for Disease Control and Prevention, 2020).

6.3.2.1. *In silico* and *in vitro* analyses

To confirm the safety of *B. breve* DSM 33444, *in silico* genome screening for potential virulence factors (genes encoding for or enhancing pathogenicity, virulence, or toxigenicity) was performed as recommended by EFSA (EFSA FEEDAP Panel, 2018). The genome was screened for virulence factors against the curated Virulence Factor Database (VFDB). Furthermore, phenotypic tests for cytotoxicity and hemolysis were also conducted on the DSM 33444 strain.

I*n silico* genome screening for potential virulence factors and other genes related to pathogenicity, virulence, or toxicity in the *B. breve* DSM 33444 strain did not reveal any virulence or toxicity genes or other genes of safety concern. This was further supported by the phenotypic tests which found the *B. breve* DSM 33444 strain to be non-hemolytic when grown on blood agar plates and non-cytotoxic in a Vero cell assay. On the basis of these results, the *B. breve* DSM 33444 strain is of no safety concern with regard to pathogenicity, virulence, and toxigenicity.

6.3.2.2. Case reports

Bifidobacterium spp. including *B. breve* are generally regarded to be safe with a long history of use in food production. This is supported by the EFSA's BIOHAZ Panel during the development of the QPS list and evaluation of suitable species, where it was concluded that, "*Safety concerns are so far related mainly only to one species, B. dentium, which has been associated with dental caries*" and "*None of the bifidobacteria [sic] used for industrial purposes have been associated with human clinical disease*" (EFSA Scientific Committee, 2007).

In this respect, a report was published on a case of neonatal sepsis associated with *B. breve* BBG-01 in a female infant diagnosed with omphalocele at 13 weeks gestation who was delivered at 37 weeks (Ohishi et al., 2010). Surgery was performed to correct the omphalocele 4 hours after birth. Two days after the surgery *B. breve* BBG-01 was administered as 0.5 mL (3.3×10^8 CFU) in sterile water. On Day 10 the infant's gastric fluid became bilious, and C-reactive protein and white blood cell counts were elevated. Antibiotics were initiated, and enteral feedings were discontinued. Blood cultures taken on Day 10 grew Bifidobacterium spp., and the oral microbial therapy was discontinued. Polymerase chain reaction (PCR) analysis of the *Bifidobacterium* spp. in the blood cultures gave a positive indication of presence of *B. breve* BBG-01. A monoclonal antibody against *B. breve* BBG-01 gave a positive response as well. The patient eventually recovered without any additional complications. This case is an example where *B. breve* infection may have been a result of an underlying condition and is rare.

Additional case reports in the literature have identified *B. breve* infections in infants and children with serious underlying conditions including cancer (Avcin et al., 2015), abdominal organ abnormalities (Sato

et al., 2003), and compromised immune systems (Esaiassen et al., 2017). These infections are uncommon and opportunistic in individuals with underlying medical conditions and are not considered relevant to this current GRAS conclusion. The general safety and lack of reported adverse events have been confirmed in recent retrospective studies and reviews (Athalye-Jape et al., 2017; Kitajima & Hirano, 2017; Wong et al., 2019) which have confirmed the general absence of adverse events other than those reported herein in the case of underlying conditions.

For completeness, Chr. Hansen has also conducted a comprehensive review of the literature through June 2022 to identify publications pertinent to the safety evaluation of *B. breve* with respect to pathogenicity and toxigenicity in humans. The literature search followed the same search strategy as EFSA's QPS approach for *Bifidobacterium* (more specifically, *Bifidobacterium breve*) (EFSA BIOHAZ Panel, 2022); the details of the search criteria and identified studies are outlined in Table 7. Considering that EFSA monitors new data pertinent to the safety of species with existing QPS status, the literature search was an update to the existing information and covered publications following June 2020 to the present.

Table 7. Search strategy for *B. breve* studies related to pathogenicity and toxigenicity

Source	Outcome	Search String	Number of hits
PubMed Date filter: June 2020 to June 2022	Antimicrobial/ antibiotic/antimycotic	*Bifidobacterium breve* AND antibiotic resistan* OR antimicrobial resistan* OR antimicrobial susceptibil*	12
	Infection/bacteremia/ fungemia/sepsis	*Bifidobacterium breve* AND infection* OR abscess* OR sepsis* OR septic* OR bacteremia OR bacteraemia OR toxin*	29
	Type of disease	*Bifidobacterium breve* AND endocarditis OR abscess OR meningitis	0
	Mortality/morbidity	*Bifidobacterium breve* AND clinical* OR death* OR morbidit* OR mortalit* OR disease* OR illness*	74
	Disease risk	*Bifidobacterium breve* AND opportunistic OR virulen*	2

The search results were then screened for relevance in terms of safety concerns where *B. breve* acted as a human pathogen. Results were screened at the title and abstract level for relevance based on a select set of selection criteria as outlined in Table 8.

Table 8. Selection criteria for *B. breve* studies related to pathogenicity and toxigenicity

Screening Strategy
Inclusion criteria: • The subject of the study is *Bifidobacterium breve* • The study pertains to safety concerns of *Bifidobacterium breve* • The study was conducted in humans. • Effects must be able to be attributable to *Bifidobacterium breve.* • The study was published from June 2020 to June 2022. • The study is derived from primary research • The publication is not a review, conference proceeding, etc. • The full-text of the article is available. • The publication is in English.

Screening Strategy
Exclusion criteria: • The subject of the study is not *Bifidobacterium breve*. • The study does not assess or describe safety concerns. • The study was not conducted in humans. • Effects are not attributable to *Bifidobacterium breve*. • The study was not published from June 2020 to June 2022. • The study is not derived from primary research. • The publication is a review, conference proceeding, etc. • The full-text of the article is not available. • The publication is not in English.

On the basis of the above literature search strategy and selection criteria, only one relevant publication was identified concerning the pathogenicity of *B. breve* in infants following oral consumption. The details of the study are described as follows:

Sakurai et al. (2021) conducted a study on 298 infant patients (preterm and term, with and without congenital surgical conditions) in the neonatal intensive care unit at Miyagi Children's Hospital. Infants were administered *B. breve* BBG-01 at 1.0 x 10^9 CFU/day from the date of birth in preterm infants or from the postoperative period to infants with surgical conditions. Of this, six cases of *B. breve* bacteremia were reported (incidence rate of 2%) of which the underlying diseases in these patients included gastrointestinal perforations (2 cases), food protein-induced enterocolitis syndrome (2 cases), adhesive ileus (1 case), ileal volvulus (1 case), necrotizing enterocolitis (2 cases), cloacal exstrophy (1 case), esophageal atresia postoperative (1 case), congenital heart disease (1 case), and aspiration pneumonia following esophageal atresia repair (1 case). Clinical symptoms of bacteremia included respiratory disorders (apnea), fever, and tachycardia; however, no septic shock, death, nor other severe symptoms were observed in these cases. All patients recovered following treatment with antibiotics. The investigators concluded that "*The high incidence of B. breve bacteremia in this study may be explained by the differences in underlying diseases of patients.... ileus or intestinal mucosal damage is a risk for bacteremia due to bacterial translocation, thus infants with malformations may have an increased risk of B. breve bacteremia owing to an increased risk of ileus or intestinal mucosal damage*".

These findings are consistent with the observation that *Bifidobacterium* spp. can present as opportunistic pathogens under very rare circumstances in individuals with serious underlying conditions. Thus, the pathogenicity of *B. breve* may be considered opportunistic in nature, similar to other *Bifidobacterium* spp. that are commonly used in the food supply. Additionally, as described above, *in silico* and *in vitro* analyses have demonstrated that *B. breve* DSM 33444 does not exhibit pathogenic/virulent traits.

6.3.3. Antibiotic resistance

6.3.3.1. Genome search

The genome sequence of *B. breve* DSM 33444 was analyzed *in silico* for the presence of known antibiotic resistance genes by Blastn analysis against the ResFinder database (Zankari et al., 2012) and BlastX analysis against the NCBI Bacterial Antimicrobial Resistance Reference Gene Database. The genome of *B. breve* DSM 33444 did not contain any antibiotic resistance genes. Any resistance observed in the strain is therefore intrinsic and not due to acquired antibiotic resistance genes. This is consistent with the results of the *in vitro* analysis, where *B. breve* DSM 33444 was demonstrated to be sensitive to all antibiotics tested (see Section 6.3.3.2).

6.3.3.2. *In vitro* assay

The minimum inhibitory concentration (MIC) values of 9 antibiotics were determined for *B. breve* DSM 33444 according to the ISO 10932 | IDF 223 international standard with three biological replicates. The medium was controlled as recommended in the ISO standard by the use of *Bifidobacterium longum* ATCC 15707, which was tested in parallel and had MIC values within the ranges given in the ISO standard. The range of antibiotics tested complies with the EFSA *"Guidance on the characterization of microorganisms used as feed additives or as production organisms"* (EFSA FEEDAP Panel, 2018). The analytical results are summarised in Table 9.

Table 9. MIC Values for *B. breve* DSM 33444

Antibiotic type	Antibiotic	MIC (µg/ml)	EFSA cut-off values[a] (µg/ml)
Aminoglycoside	Gentamicin	64-128	64
	Kanamycin	512	n.r.
	Streptomycin	64-128	128
Tetracycline	Tetracycline	2	8
Macrolide	Erythromycin	0.12	1
Lincosamide	Clindamycin	≤0.03-0.06	1
Chloramphenicol	Chloramphenicol	2	4
B-lactam	Ampicillin	0.25-0.5	2
Glycopeptide	Vancomycin	0.5	2

Abbreviations: MIC, minimum inhibitory concentration; n.r., not required to be tested by EFSA.
[a] For *Bifidobacterium* group as established by EFSA FEEDAP Panel (2018).

B. breve DSM 33444 is sensitive to all of the antibiotics tested according to the EFSA guidance. The MIC value for gentamycin is one two-fold dilution above the EFSA cut-off value in one replicate, however, that is considered acceptable due to the technical variation of the phenotypic method as also recognized by EFSA in several published opinions. The low susceptibility to kanamycin is intrinsic for *Bifidobacterium* including *B. breve* as reported by several studies (Mayrhofer et al., 2011; Moubareck et al., 2005; Xiao et al., 2010). This is consistent with the results of the analysis of the genome of *B. breve* DSM 33444 where no antibiotic resistance genes were detected (as detailed in Section 6.3.3.1) and, therefore, any phenotypic resistance observed for the strain is intrinsic and not due to acquired antibiotic resistance genes.

6.3.4. Metabolic activities

6.3.4.1. Biogenic amines production

B. breve DSM 33444 was tested for biogenic amine production based on a method modified from Bover Cid et al. (2008). Detection of histamine, tyramine, cadaverine and putrescine was done by use of a gas chromatography-mass spectrometry (GS-MS) (modified from Smart et al., 2010). The method was optimized and validated for both qualitative and quantitative detection of the four biogenic amines. Positive and negative controls as well as an internal standard were included. *B. breve* DSM 33444 did not produce any of the four biogenic amine compounds tested when grown in the presence of specific amino acid precursors known to induce production.

6.3.4.2. D-/L-lactate production

B. breve DSM 33444 was tested for production of D-lactate/L-lactate by the use of an in-house method based on scientific literature (Dunlop & Neidle, 1987). The DSM 33444 strain was found to produce 100% L-lactate. *B. breve* DSM33444 produces L-lactate and is thereby characterized as a L-lactate producing strain in line with published literature (McCartney, 2003).

6.4. Human studies

The safety of *B. breve* DSM 33444 under the intended conditions of use can be supported by studies of *B. breve* following consumption in humans. Although there are no product-specific studies on the DSM 3344 strain itself, the studies of other *B. breve* strains are pivotal to support safety at the species level, while strain level safety is supported by the biosafety analyses conducted on *B. breve* DSM 33444 as previously detailed in Sections 6.3.2 to 6.3.4.

In this respect, studies supporting the safety of the *B. breve* species were extensively detailed in GRNs 453, 454, and 454 and included toxicological studies (*i.e.*, bacterial reverse mutation test, 90-day oral study in rats) and clinical studies on *B. breve* in infants, children, and adults. In general, *B. breve* was demonstrated to be safe under the intended conditions of use with no specific safety related concerns. *B. breve* was well tolerated in infants when ingested at levels of up to 1.5 x 10^{10} CFU/day (Hattori et al., 2003; Taniuchi et al., 2005; van der Aa et al., 2010, 2011, 2012) and in children when ingested at levels of up to 3.0 x 10^{9} CFU/day (Kanamori et al., 2004; Tojo et al., 1987; Wada et al., 2010). In adults, studies demonstrated the tolerability of *B. breve* at levels of up to 2.0 x 10^{10} CFU/day (Van De Pol et al., 2011; Yoshida et al., 2010) and 8.0 x 10^{11} CFU/day (Shimakawa, 2003).

In follow-up to the *B. breve* GRAS notices, Chr. Hansen conducted a comprehensive search of the literature from 2012 through to June 2022 using the PubMed database to identify randomized controlled human clinical studies relevant to the tolerability of *B. breve* as a species. A summary of the identified studies is presented in Table 10 along with the major safety-related findings.

In general, the identified studies were designed to evaluate the efficacy of *B. breve* strains; however, these studies are also pivotal to corroborate the safety of *B. breve* and demonstrated that the species is well tolerated in all identified studies. In pre-term infants, no adverse events associated with *B. breve* were reported when given at levels up to 6.7 x 10^{9} CFU/day until 36-37 weeks corrected age (Costeloe et al., 2016; Patole et al., 2014; Patole et al., 2016). Likewise, for healthy infants and those with colic, levels of *B. breve* in the region of 10^{8} to 10^{9} CFU/day were well tolerated with no adverse events attributed to the intervention (Giglione et al., 2016; Maldonado-Lobón et al., 2021; Maldonado et al., 2019). In youths, *B. breve* was well tolerated at levels of 2.0 x 10^{9} CFU/day (Solito et al., 2021). In healthy and elderly adults, *B. breve* was well tolerated at levels of 2.0 x 10^{10} CFU/day for 16 weeks (Bernier et al., 2021; Kobayashi et al., 2019; Plaza-Diaz et al., 2013; Xiao et al., 2020). The findings in the more recent studies are consistent to what has been observed in the previous *B. breve* GRAS notices.

While not specifically the DSM 33444 strain, another *B. breve* strain (Bif-195; as manufactured by Chr. Hansen) has been studied as described in Mortensen *et al.* (2019), wherein 75 healthy volunteers were administered aspirin (300 mg daily[1]) in addition to either placebo (n=31[14 male:17 female]; 31.2 ± 6.4 years of age; 23.8 ± 2.2 kg/m^2 body mass index) or *B. breve* Bif-195 (n=35 [16 male:19 female]; 30.5 ± 6.8 years of age; 24.6 ± 2.1 kg/m^2) for 8 weeks. This study was conducted as single-site, randomized,

[1] Aspirin was administered daily for the first 6 weeks of the 8-week intervention.

double-blind, placebo-controlled, parallel-group, proof-of-concept trial. The daily administered level of *B. breve* was 5 x 10^{10} CFU/day taken in the capsule form. Ingestion of *B. breve* Bif-195 appeared to decrease intestinal damage related to repeated low-dose aspirin exposure. A total of 32 adverse events were reported from 22 different participants over the course of the study; however, the events were more common in the placebo group (20 events in 37.8% of the subjects within the group) than in the *B. breve* Bif-195 group (12 events in 21.1% of the subjects within the group), and none were attributable to the administration of the *B. breve* strain. Ten adverse events were presumed to be related to aspirin intake; however, the incidence was comparable between the two intervention groups (6 in placebo and 4 in the *B. breve* group). On the basis of the study, the investigators concluded that *B. breve* Bif-195 is well-tolerated under the conditions of the trial.

Overall, the important point, for the purposes of this notice, is that the clinical evidence corroborates the safety of the *B. breve* species in general when fed to various age groups, including infants in the first days of life and adults at levels in the region of 10^{10} CFU/day. Likewise, in children, *B. breve* species were well tolerated at levels of 10^{9} CFU/day. Further, strain specific safety is supported by the biosafety analyses conducted on *B. breve* DSM 33444 (see Sections 6.3.2 to 6.3.4) and follows the Pariza *et al*. (2015) decision tree analysis approach which supports the conclusion that *B. breve* DSM 33444 is safe under its intended conditions of use.

Table 10. Summary of clinical studies conducted on *B. breve*

Reference	Study Design	Study Population	Intervention[a]	Duration of Intervention	Safety-Related Outcomes
Costeloe et al., 2016	Randomized, double-blind, placebo-controlled trial	Preterm infants (median gestation age of 28 weeks and birth weight 1,010 g) N=1,310 (744 male; 566 female)	Enteral, sachet Control: placebo infant formula Intervention: *Bifidobacterium breve* BBG-001 at 6.7 x 10^7 to 6.7 x 10^9 CFU/day	Within 48 hours of birth until 36 weeks	• One death due to toxic epidermal necrolysis (placebo group). • One report of massive pulmonary hemorrhage (intervention group); was not considered related to the intervention. • No AE associated with the interventions were reported through the duration of the study.
Patole et al., 2014; Patole et al., 2016	Randomized double blinded placebo controlled trial	Preterm infants (up to 32 weeks gestation; birth weight < 1,500 g; ready to commence or on enteral feeds for <12 h) N=153 (88 male; 65 female)	Enteral, sachet Control: placebo Intervention: *Bifidobacterium breve* M-16V at 3.0 x 10^9 CFU/day	Until corrected age 37 weeks	• No cases of positive blood culture sepsis by *B. breve* M-16V and no deaths. • No AE reported through the duration of the study.
Maldonado-Lobón et al., 2021	Multicenter, randomized, open-label, parallel, controlled trial	Infants with colic (5.6 ± 2.7 weeks old; 4,451.4 ± 1,047.8 g BW) N=150 (78 male; 72 female)	Oral, capsule (contents added to human milk, infant milk, or water) Control: simethicone drops (20 mg, 4 times daily) Intervention: 1. *Bifidobacterium breve* CECT7263 at 2.0 x 10^8 CFU/day 2. *Bifidobacterium breve* CECT7263 and *Lactobacillus fermentum* CECT5176 at 1.0 x10^8 CFU/day for each strain	28 days	• Interventions were well tolerated in all test groups and no AE reported in the study.

Reference	Study Design	Study Population	Intervention[a]	Duration of Intervention	Safety-Related Outcomes
Giglione et al., 2016	Randomized, double-blind, placebo-controlled trial	Healthy infants (within 15 days of birth; no other demographic or inclusion/exclusion criteria reported) N=60	Oral, drops Control: placebo Intervention: *Bifidobacterium breve* B632 and BR03 at 2.0 x 10^8 CFU/day (1:1 per strain).	90 days	• Number of evacuations, regurgitations, or vomits was not significantly different between groups. • No AE reported in the study.
Maldonado et al., 2019	Randomized, double blind, controlled, parallel trial	Healthy infants (1 month of age; exclusively infant formula fed) N=236 (128 male; 108 female)	Oral, infant formula Control: standard powdered infant formula Interventions: 1. Infant formula supplemented with *Bifidobacterium breve* CECT7263 of 1 × 10^9 CFU/day up to 6 months and 7.0 to 8.0 × 10^8 CFU/day between 6 and 12 months 2. Infant formula supplemented with *Lactobacillus fermentum* CECT5716 of 1 × 10^9 CFU/day up to 6 months and 7-8 × 10^8 CFU/day between 6 and 12 months	Until the age of 12 months	• Supplemented infant formula interventions were well tolerated and growth of infants consistent with standards (no differences in weight gain between groups). • No significant difference in diarrhea, upper and/or lower respiratory tract infection, conjunctivitis, otitis, urine infection, fever, or dermatitis between groups. • No AE related to consumption of any formula.
Solito et al., 2021	Cross-over, double-blind, randomized control trial	Youths with obesity and insulin resistance on diet (6 to 18 years of age; HOMA-IR >2.5 or insulin >15 µU/mL) N=101 (54 male; 47 female)	Oral, sachets Control: placebo formulation Intervention: *Bifidobacterium breve* BR03 (DSM 16604) and *Bifidobacterium breve* B632 (DSM 24706) at 2.0 x 10^9 CFU/AFU/day (1:1 mixture of 2 strains)	8 weeks	• No AE were observed through the duration of the study.

Reference	Study Design	Study Population	Intervention[a]	Duration of Intervention	Safety-Related Outcomes
Plaza-Diaz et al., 2013	Multicenter, randomized, double-blind, placebo-controlled trial	Healthy adults (average 28 years of age; BMI 23 kg/m^2) N=100 (46 male; 54 female)	Oral, capsule Control: placebo Intervention: 1. *Bifidobacterium breve* CNCM I-4035 at 9.0 x 10^9 CFU/day 2. *Lactobacillus paracasei* CNCM I-4034 at 9.0 x 10^9 CFU/day 3. *Lactobacillus rhamnosus* CNCM I-4035 at 9.0 x 10^9 CFU/day 4. Mixture of above 3 strains at 9.0 x 10^9 CFU/day	30 days	• Hematological and clinical biochemistry measures were comparable between the control and intervention groups. • No significant difference in gastrointestinal symptoms, defecation frequency, and stool consistency between groups. • No serious AE reported through the duration of the study.
Bernier et al., 2021; Xiao et al., 2020	Randomized, double-blind, placebo-controlled trial	Healthy adults with mild cognitive impairment (61.1 ± 7.2 years of age; MMSE score 24.5±1.6) N=80 (39 male; 41 female)	Oral, capsule Control: placebo Intervention: *Bifidobacterium breve* A1 (MCC1274) at 2.0 x 10^{10} CFU/day	16 weeks	• No changes in body weight, blood pressure, heart rate, and hematology and biological blood parameters between baseline and end of intervention period. • No AE were observed through the duration of the study.
Kobayashi et al., 2019	Randomized, double-blind, placebo-controlled trial	Elderly adults with subjective memory complaints (mean of 61 years of age and MMSE score of 26) N=121 (60 male; 61 female)	Oral, capsule Control: placebo Intervention: *Bifidobacterium breve* A1 at approximately > 2.0 x 10^{10} CFU/day	12 weeks	• No significant differences in vital signs, hematology and blood biochemistry parameters, nor AE between groups.

Abbreviations: AEs, adverse events; BMI, body mass index; BW, body weight; CFU, colony forming units; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; MMSE, Mini-Mental State Examination

6.5. Pariza decision tree analysis

As indicated above, in assessing the safety of *B. breve* DSM 33444 under the intended conditions of use, Chr. Hansen has consulted the "*Decision Tree for Determining the Safety of Microbial Cultures to be Consumed by Humans or Animals*" by Pariza *et al*. (2015). The decision tree is composed of thirteen questions, and their responses as they apply to *B. breve* DSM 33444 are described below:

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology?

   YES (go to 2)

2. Has the strain genome been sequenced?

   YES (go to 3)

3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity?

   YES (go to 4)

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA?

   YES (go to 5)

5. Does the strain produce antimicrobial substances (used in human or veterinary medicine)?

   NO (go to 6)

6. Has the strain been genetically modified using rDNA techniques?

   NO (go to 8a)

8a. Was the strain isolated from a food that has a history of safe consumption for which the species, to which the strain belongs, is a substantial and characterizing component?

   NO (go to 13 a). However, the DSM 33444 strain is a human commensal, and *B. breve* as a species has a history of safe use in the production of foods. Thus, it is considered appropriate to proceed to 9a.

9a: Has the species, to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been affirmed to be safe for use by an authoritative group of qualified scientific experts?

   YES (go to 10a)

10a: Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9a continue to support the conclusion that the species, to which the strain belongs, is safe for use in food?

   YES (go to 11a)

11a: Will the intended use of the strain expand exposure to the species beyond the group(s) that typically consume the species in “traditional” food(s) in which it is typically found?

NO (go to 12a)

12a: Will the intended use of the strain expand intake of the species?

NO (go to 14a)

13a. Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies?

NO (go to 14a)

14a. The strain is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.

6.6. Conclusion of GRAS status

Chr. Hansen concludes that the intended uses of *Bifidobacterium (B.) breve* DSM 33444 are GRAS based on scientific procedures

Chr. Hansen has applied the framework of the Pariza *et al*. (2015) decision tree and elements of the EFSA QPS approach to demonstrate the safety of *B. breve* DSM 33444 for use as a microbial ingredient in conventional foods and in non-exempt infant formula for term infants. The data presented in this GRAS notice fully support the conclusion that *B. breve* DSM 33444 is GRAS under the intended uses as described.

The basis of the GRAS conclusion for the intended use of *B. breve* DSM 33444 are summarized by the following pivotal considerations:

- *B. breve* has a history of safe consumption from traditional fermented foods – *Bifidobacterium breve* has QPS status and is presently included in the IDF/EFFCA's *Inventory of microbial food cultures with safety demonstration in fermented food products* (Bourdichon et al., 2018, 2022; Mogensen et al., 2002) and the DVFA's *List of notified microbial cultures applied in food* (Danish Veterinary and Food Administration, 2016).

- Chr. Hansen's manufacturing and quality control programs (cGMP, HACCP, and FSSC certification) ensure the safety and quality of the final *B. breve* DSM 33444 ingredient.

- *B. breve* DSM 33444 is not genetically modified, is not pathogenic or toxigenic, is not able to produce biogenic amines, and does not carry any transferable genes conferring antibiotic resistance.

- *B. breve* has been evaluated in a number of human clinical studies in which the species was safely consumed and well tolerated in infants, children, and adults.

Based on the above considerations, the safety of *B. breve* DSM 33444 is supported with a reasonable certainty of no harm under the intended conditions of use.

Part 7. List of supporting data and information

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From: Winnie Ng
To: Morissette, Rachel
Cc: Kate Urbain
Subject: [EXTERNAL] RE: questions for GRN 001114
Date: Friday, May 26, 2023 3:12:51 PM
Attachments: image009.png
image010.png
image011.png
image012.png
image013.png
image014.png
Chr. Hansen Response to GRN 1114 Questions_B. breve DSM 33444_2023.05.26.pdf

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Dear Dr. Morissette,

Please find attached our response to the questions for GRN 001114.

If you have any questions, please let me know.

Kindest Regards,
Winnie

Winnie Ng, Ph.D., DABT
Principal Regulatory Affairs Specialist
Human Health - Probiotics
Chr. Hansen Inc.
Mobile: +1 705 746 0491
Email: cawinn@chr-hansen.com
www.chr-hansen.com

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Wednesday, May 17, 2023 3:01 PM
To: Winnie Ng <CAWINN@chr-hansen.com>
Subject: questions for GRN 001114

Dear Dr. Ng,

Please see attached our questions for GRN 001114.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

CHR. HANSEN

Improving food & health

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist
Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration

Chr. Hansen, Inc.
9015 West Maple Street
Milwaukee, WI 53214 - 4298

www.chr-hansen.com
info@chr-hansen.com

May 26, 2023
CAWINN

Response to Questions Regarding GRN 001114

Dear Dr. Morissette,

In regard to the questions on GRN 001114 for the intended use of *Bifidobacterium breve* DSM 33444 received from the U.S. FDA on May 17, 2023, please find Chr. Hansen's responses attached.

We trust that this meets with your immediate needs and remain available for any other questions you may have.

Yours sincerely,

Winnie Ng
Principal Regulatory Affairs Specialist

E-mail: cawinn@chr-hansen.com
Phone: +1 705 746 0491

RESPONSE TO FDA QUESTIONS ON GRN 001114 FOR *BIFIDOBACTERIUM BREVE* DSM 33444 RECEIVED ON MAY 17, 2023

The following is Chr. Hansen's response to the questions on GRN 001114 for the intended use of *Bifidobacterium* (*B.*) *breve* DSM 33444 as received from the U.S. FDA on May 17, 2023.

RESPONSES:

1. **Chr. Hansen states that a literature search was performed through June 2022. Please confirm that no new information that may appear counter to your GRAS conclusion has been published since then.**

   An updated literature search was conducted to identify any publications pertinent to the safety of *B. breve* and the DSM 33444 strain published since the original GRAS notice submission. The literature search followed the same method and search strategy as the European Food Safety Authority (EFSA)'s qualified presumption of safety (QPS) approach for *Bifidobacterium* (specifically *B. breve*), and as outlined in Section 6.3.2.2 of the original GRAS notice, with the date filter ranging from June 2022 to May 2023.

   The search results were screened at the title and abstract level for relevance in terms of any safety concerns related to the pathogenicity and toxigenicity of *B. breve* in humans. On this basis, 2 new publications were identified concerning the pathogenicity of the *B. breve* species that consisted of case reports of necrotizing fasciitis; however, in both cases the patients had severe underlying conditions that were likely to have predisposed them to infection and the source of *B. breve* was unclear. In the first case report, a 43-year old female with an 8-year history of untreated and uncontrolled Type 2 diabetes mellitus (with chronic diabetic foot ulcers) presented at the hospital with swelling of the right leg, fever, and dizziness that was diagnosed as necrotizing fasciitis – *B. breve* was recovered from tissue samples and blood culture (Wakabayashi et al., 2022). In the second case report, a 42-year old male presented to the hospital with erythema, swelling and severe pain in the right inguinal region which was diagnosed as necrotizing fasciitis (Takeda et al., 2023). Blood culture and wound abscess swab confirmed the presence of *B. breve*. The patient had a history of Type 2 diabetes mellitus, dyslipidemia, obesity, cellulitis of the back, and a 2-year repeated subcutaneous abscess in the right inguinal region. In both case reports, the patients responded well to treatment which included antibiotic therapy.

   Similar to the original GRAS notice, the findings from the new case reports are consistent with the observation that *Bifidobacterium* spp. can present as opportunistic pathogens, occurring uncommonly under rare circumstances in individuals with underlying conditions that may predispose them to clinical pathologies. The pathogenicity of *B. breve* is considered opportunistic in nature and is not considered a significant concern in the general healthy population. Thus, the newly identified case reports do not counter our original GRAS conclusion for *B. breve* DSM 33444.

   In addition, an updated search was conducted to identify randomized controlled human clinical studies published since the submission of the original GRAS notice. The updated literature search was conducted using the PubMed database to identify pertinent publications from June 2022

through to May 2023. While there were no new studies specifically on the DSM 33444 strain, the search identified 3 additional studies relevant to the tolerability of *B. breve* as a species. A summary of the studies is presented in Table 1 below.

Notably, although all of the identified studies were conducted to investigate the efficacy of different strains of *B. breve*, no significant adverse events were attributable to the test articles within these studies. *B. breve* was well tolerated at levels of up to 5.0 x 10^{10} CFU/day in healthy adults when consumed over a 6-week period. Furthermore, *B. breve* was well tolerated in pregnant women at a level of 1 x 10^{9} CFU/day.

Thus, to the best of our knowledge, there are no new scientific data published since June 2022 that would counter our GRAS conclusion for *B. breve* DSM 33444 under the intended conditions of use. This is consistent with the most recent QPS update in January 2023, where EFSA concluded that the QPS status of the QPS species within the *Bifidobacterium* genus remained unchanged including *Bifidobacterium breve* (EFSA BIOHAZ Panel, 2023).

Table 1. Summary of randomized controlled clinical studies on *B. breve* published June 2022 to May 2023.

Reference	Study Population	Intervention	Duration of Intervention	Safety-Related Outcomes
Engel et al., 2022	Healthy adults (mean 35.0 ± 9.2 years of age; well-trained, ≥4-hour endurance sports per week) N=126	Oral, capsule Control: placebo Intervention: *B. breve* DSM 33360 at 5.0 x 10^{10} CFU/day	6 weeks	The test article was well-tolerated. All AEs reported were mild to moderate and none were considered related to the test-article – placebo (ligament injury, strained back); *B. breve* DSM 33360 (cold and flu symptoms, flu, injured Achilles' tendon when jogging).
Sung et al., 2022	Healthy adults (19 -60 years of age) N=100	Oral, capsule Control: placebo Intervention: *B. breve* BB-3 at 5.0 x 10^{9} CFU/day	12 weeks	No severe AEs were reported through the duration of the study. Hematology and blood biochemistry did not differ between groups. No changes in urinalysis were observed.
Moore et al., 2023	Pregnant women (mean of 33.6 ± 3.9 years of age) N=160	Oral, capsule Control: placebo Intervention: *B. breve* 702258 at 1 x 10^{9} CFU/day	From 16-weeks' gestation to 3 months postpartum	No AEs were reported through the duration of the study. No differences on mode of delivery or preterm births.

Abbreviations: AE, adverse event; CFU, colony forming unit.

References:

EFSA Panel on Biological Hazards (BIOHAZ), Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., De Cesare, A., Hilbert, F., Lindqvist, R., Nauta, M., Peixe,

L., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Cocconcelli, P. S., Escámez, P. S. F., Maradona, M. P., … Herman, L. (2023). Update of the list of qualified presumption of safety (QPS) recommended microbiological agents intentionally added to food or feed as notified to EFSA 17: suitability of taxonomic units notified to EFSA until September 2022. *EFSA Journal. European Food Safety Authority*, *21*(1), e07746. https://doi.org/10.2903/j.efsa.2023.7746

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Wakabayashi, Y., Nakayama, S., Yamamoto, A., Yoshino, Y., Ishigaki, S., Furukawa, T., & Kitazawa, T. (2022). First case of necrotizing fasciitis and bacteremia caused by Bifidobacterium breve. *Anaerobe*, *76*, 102613. https://doi.org/10.1016/j.anaerobe.2022.102613

2. **Page 26 of the notice contains an apparent typo under section 6.4 Human Studies in the second paragraph "…detailed in GRNs 453, 454, and 454…". Please clarify what the third GRN should be in this series.**

   Chr. Hansen apologies for the error. The third GRN in the series should be GRN 455 and the statement should be corrected to "…*studies supporting the safety of the B. breve species were extensively detailed in GRNs 453, 454, and* ***455***…".

3. **Please confirm that the batch analyses in the notice are non-consecutive.**

   Chr. Hansen confirms that the batch analyses in the GRAS notice for *B. breve* DSM 33444 are from non-consecutive batches.

4. **Chr. Hansen estimates the dietary exposure to *B. breve* DSM 33444 for infants 0 to 6 months of age based on the mean, per consumer estimates of infant formula intake reported in Grimes et al.,**

2017. However, the notice does not include the estimated dietary exposure to *B. breve* DSM 33444 for other infant age groups that are within the expected consumer population (e.g., infants up to 12 months of age), nor does the notice compare exposure between male and female infant consumers. Please provide dietary exposure estimates to *B. breve* DSM 33444 based on the maximum intended use level and infant formula consumption throughout infancy that represents the intended population, male and female infants from 0 to 12 months of age.

The estimated dietary exposure to *B. breve* DSM 33444 for male and female infants up to 12 months of age are summarized in Table 2 below. The estimates were calculated using the intended use of *B. breve* DSM 33444 as subject to GRN 001114 (*i.e.*, 1.0×10^8 CFU/g infant formula), the estimated caloric intake requirements of infants as outlined by the Institute of Medicine (2005), and assuming an average reconstitution rate of 14.1 g powdered infant formula per 100 mL water, wherein commercial infant formulas in the U.S. typically provide an energy content of 0.67 kcal/mL (20 kcal/fl oz) (Martinez & Ballew, 2011).

Table 2. Estimated dietary exposure to *B. breve* DSM 33444 under the intended use in infant formula.

Age (months)	Male Estimated Caloric Intake[a] (kcal/day)	Maximum Intended Use of *B. breve* DSM 33444[b] (CFU/g infant formula)	Estimated Daily Intake[c] (CFU/day)	Female Estimated Caloric Intake[a] (kcal/day)	Maximum Intended Use of *B. breve* DSM 33444[b] (CFU/g infant formula)	Estimated Daily Intake[c] (CFU/day)
1	472	1.0×10^8	9.9×10^9	438	1.0×10^8	9.2×10^9
2	567		1.2×10^{10}	500		1.1×10^{10}
3	572		1.2×10^{10}	521		1.1×10^{10}
4	548		1.2×10^{10}	508		1.1×10^{10}
5	596		1.3×10^{10}	553		1.2×10^{10}
6	645		1.4×10^{10}	593		1.2×10^{10}
7	668		1.4×10^{10}	608		1.3×10^{10}
8	710		1.5×10^{10}	643		1.4×10^{10}
9	746		1.6×10^{10}	678		1.4×10^{10}
10	793		1.7×10^{10}	717		1.5×10^{10}
11	817		1.7×10^{10}	742		1.6×10^{10}
12	844		1.8×10^{10}	768		1.6×10^{10}

Abbreviations: CFU, colony forming units.

[a] As estimated energy requirement (equivalent to total energy expenditure plus energy deposition) (IOM, 2005).

[b] Subject to the intended use in infant formula as outlined in GRN 001114.

[c] Calculated as: (caloric intake [kcal/day] x reconstitution rate [14.1 g powdered infant formula for 100 mL water] x intended use [CFU/g powdered infant formula]) / energy content (0.67 kcal/mL infant formula).

Under the intended conditions of use at a maximum incorporation level of 1.0×10^8 CFU/g infant formula, the estimated intake of *B. breve* DSM 33444 will range from 9.9×10^9 to 1.8×10^{10} CFU/day in male infants at 1 and 12 months, respectively. In female infants, the estimated daily intake will range from 9.2×10^9 to 1.6×10^{10} CFU/day at 1 and 12 months, respectively.

As indicated in the original GRAS notice, the estimated dietary exposure of the DSM 33444 strain is comparable to another *B. breve* strain (M-16V) for use in infant formula at the same intended use levels (*i.e.*, up to 1.0 x 10^8 CFU/g powdered infant formula) – this strain has received "no questions" from the FDA to their GRAS notices (GRN 454 and 455). Collectively, with the QPS status of the *B. breve* species and the extensive history of safe use in food, the dietary exposure to *B. breve* DSM 33444 under its intended conditions of use in infant formula is not expected to be a significant concern.

References:

Institute of Medicine. (2005). *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids*. National Academies Press. https://doi.org/10.17226/10490

Martinez, J. A., & Ballew, M. P. (2011). Infant formulas. *Pediatrics in Review*, *32*(5), 179–189; quiz 189. https://doi.org/10.1542/pir.32-5-179

5. **Please identify if any of the raw materials used in the fermentation process are major allergens or are derived from major allergens and discuss whether these pose a safety concern. If none of the raw materials used in the manufacturing process are major allergens or are derived from major allergens, please provide a statement of affirmation. Please note that under the Food Allergy Safety, Treatment, Education, and Research (FASTER) Act, sesame is now considered a major food allergen: (https://www.fda.gov/food/cfsan-constituent-updates/faster-act-video-food-industry-and-other-stakeholders).**

   Chr. Hansen complies with the Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) and the Food Allergy Safety, Treatment, Education, and Research (FASTER) Act.

   Chr. Hansen confirms that no major allergens nor substances derived from major allergens are used as raw materials in the fermentation media for the production of *B. breve* DSM 33444. Furthermore, sesame and substances derived thereof are not used in the manufacturing process of any of our strains. Therefore, major allergens do not pose a safety concern in the *B. breve* DSM 33444 ingredient.

6. **On page 13 of the notice, Chr. Hansen lists a specification for *Cronobacter* spp. and states that the method used is NF ISO 22964. The current version of this method is ISO 22964:2017, which corresponds to "Microbiology of the Food Chain - Horizontal Method for the Detection of Cronobacter spp." Please state whether presumptive positives are further analyzed to determine if the isolate is *C. sakazakii*.**

   Chr. Hansen confirms that the method used to test *Cronobacter* spp. is ISO 22964:2017. According to our quality control processes, if there are presumptive positives for *Cronobacter* spp. identified in the batch analyses, the entire batch is scrapped/discarded, and does not move forward in production nor commercialization – further analysis is not performed at the species level.

7. Please provide a statement that all processing aids used in the manufacture 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.

Chr. Hansen confirms that all processing aids used in the manufacture of *B. breve* DSM 33444 are used in accordance with applicable U.S. regulations and/or are GRAS for their intended uses. All processing aids are safe and suitable for human consumption under the intended conditions of use.