# Generally Recognized As Safe (GRAS) Notification for the Use of *Bifidobacterium breve* MCC1274 in Conventional Foods

**Prepared for:**

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## LIST OF ABBREVIATIONS

ADI: acceptable daily intake

ADP: adenosine diphosphate

BGLB: brilliant green bile lactose broth

*B. breve*: *Bifidobacterium breve*

CFP: carbohydrate fermentation pattern

CFR: United States Code of Federal Regulations

CFU: colony forming units

CIP: clean in place

COA: Certificate of Analysis

EDI: Estimated Daily Intake

EFFCA: European Food & Feed Cultures Association

EFSA: European Food Safety Authority

FAO/WHO: Food and Agriculture Organization of the United Nations/World Health Organisation

FCC: Food Chemical Codex

FDA: United States Food and Drug Administration

FFDCA: Federal Food, Drug, and Cosmetic Act

FNDDS: Food and Nutrition Database for Dietary Studies

FOS: fructo-oligosaccharides

FSSC: Food Safety System Certification

GI: gastrointestinal

GMO: genetically modified organism

GOS: galacto-oligosaccharides

GRAS: Generally Recognized As Safe

GRN: GRAS Notification

HACCP: Hazard Analysis and Critical Control Point

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

IDF: International Dairy Foundation

ISO: International Organization for Standardization

LLDPE: linear low-density polyethylene

LOQ: Limit of quantification

MEC: mobile examination center

MIC: minimum inhibitory concentration

MMSE: mini-mental state examination

NCBI: National Center for Biotechnology Information

ND: not detected

NEC: necrotizing enterocolitis

NHANES: National Health and Nutrition Examination Survey

NITE: National Institute of Technology (Japan)

NOAEL: no observed adverse effect level

NR: not required

PEG: polyethylene glycol

ppm: parts per million

PSU: primary sampling unit

QPS: qualified presumption of safety

RAPD PCR: random amplification of polymorphic DNA polymerase chain reaction

RO: reverse osmosis

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

WT: wild-type

# I. SIGNED STATEMENT OF THE CONCLUSION OF GENERALLY RECOGNIZED AS SAFE (GRAS) AND CERTIFICATION OF CONFORMITY TO 21 CFR §170.205-170.260

## A. SUBMISSION OF GRAS NOTICE

Morinaga Milk Industry Co., Ltd. is hereby submitting a GRAS notice in accordance with subpart E of part 170.

## B. NAME AND ADDRESS OF THE SPONSOR

Morinaga Milk Industry Co., Ltd.
33-1, Shiba 5-Chome, Minato-ku
Tokyo 108-8384
JAPAN

## C. COMMON OR USUAL NAME

*Bifidobacterium breve* MCC1274*; B. breve* B-3*; B. breve* A-1, *B breve* A1

## D. TRADE SECRET OR CONFIDENTIAL INFORMATION

This notification does not contain any trade secret or confidential information.

## E. INTENDED USE

*B. breve* MCC1274 will be used as a source of *B. breve* in general foods.

## F. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of *B. breve* MCC1274 (also known as B-3) as an ingredient in conventional foods at a maximum level of $5 \times 10^{10}$ CFU per serving at the end of shelf-life is based upon scientific procedures as described under 21 CFR §170.30(b). The intake of *B. breve* MCC1274 from the intended uses specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s). To demonstrate that *B. breve* MCC1274 is safe, and GRAS, under the intended conditions of use, the safety of the intake of *B. breve* MCC1274 has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

The proposed use of *B. breve* MCC1274 as an ingredient in foods has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

- Bifidobacteria are naturally occurring bacteria that contribute to the composition of the gut microflora of humans. *Bifidobacterium breve* species have been detected in the feces of infants and adults.
- *B. breve* MCC1274 is a non-motile, non-spore forming, rod-shaped, anaerobic, Gram-positive bacterium. It was isolated from the feces of an infant in 2009. The bacterium has been deposited with the National Institute of Technology and Evaluation (NITE, Japan) and is designated FERM BP-11175.
- *B. breve* MCC1274 has been commercially available since 2012 and has since been sold in a variety of markets including Japan, Denmark, Italy, and Spain.
- The original stock culture of *B. breve* MCC1274 has been maintained at −80°C since it was obtained by Morinaga Milk Industry, and no selective pressures have been applied.
- *B. breve* MCC1274 cultures are used to produce the final B-3-EX formulation freeze-dried powder mixed with cornstarch carrier to be used in conventional foods only.
- Finished products made with *B. breve* MCC1274 consistently comply with established, food-grade product specifications. Specifications are in place to control anaerobic plate count (MCC1274 count), moisture, microbial contamination, and heavy metals.
- Fifteen GRAS Notices (GRNs) on *Bifidobacterium* species have received "no questions" letters from the FDA. This includes GRN 453, 454, and 455, which allows for the use of a strain of *B. breve* M-16V for use in general foods at levels up to 5 x $10^9$ CFU/serving as well as powdered, milk- or soy-based, non-exempt term infant formula and exempt, powdered, amino acid-based infant formula at levels providing 1 x $10^9$ CFU/g.
- *B. breve* has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) for use in the member countries of the European Union.
- *B. breve* MCC1274 has been tested for parameters outlined in the Food and Agriculture Organization of the United Nations/World Health Organization's (FAO/WHO) guidelines. Results from these tests provide evidence that *B. breve* MCC1274 is safe for use in foods, namely:

- *B. breve* MCC1274 is not atypically resistant to conventional antibiotics.
- *B. breve* MCC1274 produces L-lactic acid but does not produce D-lactic acid.
- *B. breve* MCC1274 is shown to deconjugate bile salts, but no secondary bile acids are produced.
- An *in vitro* study indicates *B. breve* MCC1274 does not produce biogenic amines.
- An *in vitro* study indicates that *B. breve* MCC1274 does not produce ammonia.
- The use of 3 different methods indicates that *B. breve* MCC1274 does not degrade mucins.
- Testing has confirmed the absence of plasmids in *B. breve* MCC1274.
- Genomic analysis of *B. breve* MCC1274 did not reveal the presence of known toxin or virulence genes.
- *B. breve* MCC1274 was not observed to have hemolytic activity.
- *B. breve* MCC1274 does not induce platelet aggregation.

- The safety of *B. breve* MCC1274 is supported by a published acute toxicology study and a pivotal published 90-day repeated dose toxicology study, both in rats. In the single dose oral toxicity test using $8.4 \times 10^{11}$ CFU/kg of *B. breve* MCC1274, there were no deaths or MCC1274 related adverse findings. The no observed adverse effect level (NOAEL) from the 90-day study was determined to be at least $1.3 \times 10^{10}$ CFU/kg bw/day (Arai et al., 2018).
- Four published studies of *B. breve* MCC1274 alone in adults support the safety of *B. breve* MCC1274. No adverse events were reported in any study. These studies support the safe use of *B. breve* MCC1274 in adults at doses up to $5.0 \times 10^{10}$ CFU/day for 12 weeks.
- Additionally, an acute toxicity study and subchronic toxicity study on *B. breve* M-16V corroborate the safety of *B. breve* MCC1274 (Abe et al., 2009). *B. breve* M-16V was not acutely toxic at 3,000 mg/kg ($6.9 \times 10^{11}$ CFU/kg) and has a NOAEL of 1000 mg/kg/day ($2.3 \times 10^{11}$ CFU/kg bw/day) based on results of the 90-day subchronic toxicity study which were used in support the GRAS designation of *B. breve* M-16V in conventional foods and exempt and non-exempt infant formula (GRN 453,454, and 455).
- Since the GRAS notifications of *B. breve* M-16V in 2013, nine additional clinical studies of other *B. breve* strains in infants and children are used to corroborate the

safety of *B. breve* MCC1274. These studies administered doses of 1 x $10^7$ to 1.5 x $10^{10}$ CFU/day to term and preterm infants and children up to 17 years of age. The study duration ranged from 30 days to 12 months. There were no study reported adverse events related to *B. breve* ingestion.

- Since the GRAS notifications of *B. breve* M-16V in 2013, three additional clinical studies of other *B. breve* strains in adults are used to corroborate the safety of *B. breve* MCC1274. These studies administered doses of 3 x $10^8$-8.0 x $10^{11}$ CFU/day for durations from 2 weeks to 12 months. There were no study reported adverse events related to *B. breve* ingestion.
- *B. breve* MCC1274 will be added to select general foods at levels sufficient to provide 5 x $10^{10}$ CFU/serving at the end of shelf life. This will result in a mean estimated daily intake (EDI) for consumers age 2+ of 5.79 x $10^{10}$ CFU/day (8.64 x $10^8$ CFU/kg bw/day) and a 90$^{th}$ percentile intake of 1.07 x $10^{11}$ CFU/day (1.60 x $10^9$ CFU/kg bw/day).

Determination of the GRAS status of *B. breve* MCC1274 under the intended conditions of use has been made through the deliberations of Roger Clemens, DrPH, CNS, FACN, FASN, FIFT, A. Wallace Hayes, PhD, DABT, FATS, ERT, CNS, and Thomas E. Sox, PhD, JD. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of *B. breve* MCC1274 and the potential human exposure to *B. breve* MCC1274 resulting from its intended use as an ingredient in foods and have concluded:

> *There is no evidence in the available information on B. breve* MCC1274 *that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when B. breve* MCC1274 *is used at levels that might reasonably be expected from the proposed applications. B. breve* MCC1274 *is GRAS for use in foods as proposed by Morinaga Milk Industry Co, Ltd.*

Therefore, *B. breve* MCC1274 is safe and GRAS at the proposed levels of addition to conventional foods. *B. breve* MCC1274 is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

## G. PREMARKET APPROVAL

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of intended use.

## H. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Claire L. Kruger, PhD, DABT, Managing Partner, Spherix Consulting Group, Inc., at 751 Rockville Pike, Unit 30-B, Rockville, MD 20852. Telephone: 301-775-9476; Email: ckruger@spherixgroup.com, or be sent to FDA upon request.

## I. FREEDOM OF INFORMATION ACT (FOIA)

Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA.

## J. INFORMATION INCLUDED IN THE GRAS NOTIFICATION

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to Morinaga Milk Industry Co., Ltd. and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

April 29, 2021

Claire L. Kruger, PhD, DABT
Managing Partner, Spherix Consulting Group, Inc.
Signature of Authorized Representative of
Morinaga Milk Industry Co., Ltd.

Date

# II. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

## A. COMMON OR USUAL NAME

*Bifidobacterium breve* MCC1274*; B. breve* B-3*; B. breve* A-1, *B. breve* A1

## B. TRADE NAME

B-3-EX

## C. DESCRIPTION OF *BIFIDOBACTERIUM BREVE* MCC1274

### 1. Taxonomy and Origin

*B. breve* MCC1274 (also known as *B. breve* B-3) is a non-motile, non-spore forming, rod-shaped, anaerobic, Gram-positive bacterium (Figure 1). It was isolated from the feces of an infant in 2009.

The full taxonomic classification of *B. breve* is as follows:

**Kingdom:** Bacteria
**Subkingdom:** Posibacteria
**Phylum:** Actinobacteria
**Subclass:** Actinobacteridae
**Order:** Bifidobacteriales
**Family:** Bifidobacteriaceae
**Genus:** Bifidobacterium
**Species:** *Bifidobacterium breve*

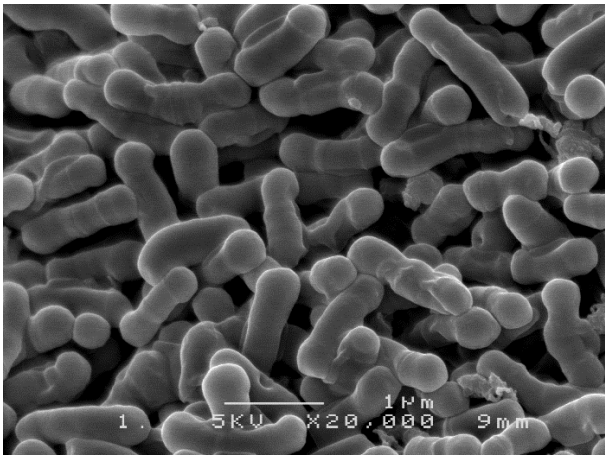



**Figure 1. Scanning Election Microscope Image of *B. breve* MCC1274**

The bacterium has been deposited with the National Institute of Technology and Evaluation (NITE) in Japan and is designated FERM BP-11175.

## 2. Phenotypic Identification

To confirm that *B. breve* MCC1274 is phenotypically similar to other strains of *B. breve*, Morinaga Milk compared the carbohydrate fermentation pattern (CFP) of *B. breve* MCC1274 to that of *B. breve* ATCC 15700$^T$ (type strain) using the method developed by Boyd et al. (2005). The CFPs are qualitatively similar (Table 1), indicating that *B. breve* MCC1274 is phenotypically similar to *B. breve* ATCC 15700$^T$.

**Table 1. Carbohydrate Fermentation Patterns of *B. breve***

| Carbohydrate | *B. breve* | |
|---|---|---|
| | **MCC1274** | **ATCC 15700$^T$** |
| Glycerol | – | – |
| Erythritol | – | – |
| D-Arabinose | ± | (+) |
| L-Arabinose | – | – |
| Ribose | + | + |
| D-Xylose | – | – |
| L-Xylose | – | – |
| Adonitol | – | – |
| βMethyl-xyloside | – | – |
| Galactose | + | + |
| D-Glucose | + | + |
| D-Fructose | + | + |
| D-Mannose | + | + |
| L-Sorbose | – | – |
| Rhamnose | – | – |
| Dulcitol | – | – |
| Inositol | – | – |
| Mannitol | + | + |
| Sorbitol | + | + |
| αMethyl-D-mannoside | ± | – |
| αMethyl-D-glucoside | + | + |
| N Acetyl glucosamine | + | (+) |
| Amygdalin | + | + |
| Arbutin | + | + |
| Esculin | + | + |
| Salicin | + | + |
| Cellobiose | + | + |
| Maltose | + | + |

**Table 1. Carbohydrate Fermentation Patterns of *B. breve***

| Carbohydrate | *B. breve* | |
|---|---|---|
| | **MCC1274** | **ATCC 15700$^T$** |
| Lactose | + | + |
| Melibiose | + | + |
| Sucrose | + | + |
| Trehalose | + | – |
| Inulin | – | – |
| Melezitose | + | – |
| Raffinose | + | + |
| Starch | + | +S |
| Glycogen | – | + |
| Xylitol | + | +S |
| Gentiobiose | + | + |
| D-Turanose | + | + |
| D-Lyxose | – | – |
| D-Tagatose | – | – |
| D-Fucose | – | – |
| L-Fucose | + | + |
| D-Arabitol | – | – |
| L-Arabitol | – | – |
| Gluconate | – | – |
| 2 keto-gluconate | – | – |
| 5 keto-gluconate | ± | ±S |

+ positive ; - negative ; (+) weakly positive;
± very weakly positive ; S delayed reaction

### 3. Genotypic Identification

Morinaga Milk has sequenced the entire genome of *B. breve* MCC1274. BLASTN analysis (Ver. 2.6.1+) of the *B. breve* MCC1274 16S rDNA sequence with the 16S rDNA of the type strain *B. breve* ATCC 15700 (Accession number AB006658) obtained from the National Center for Biotechnology Information (NCBI) website revealed that there is 99.7% homology between the two strains with four mismatches, confirming that *B. breve* MCC1274 is a member of *B. breve*.

## D. PRODUCTION PROCESS

The production of *B. breve* MCC1274 consists of a culturing process and a non-culturing process (Figure 2), which are typical for the production of microbial ingredients used in food. The culturing process is a series of sequential expansions of working stocks, derived from the original stocks of *B. breve* MCC1274, yielding a manufacturing culture. The manufacturing culture provides the material for the non-culturing process where *B. breve* MCC1274 is refined, resuspended, and prepared for distribution.

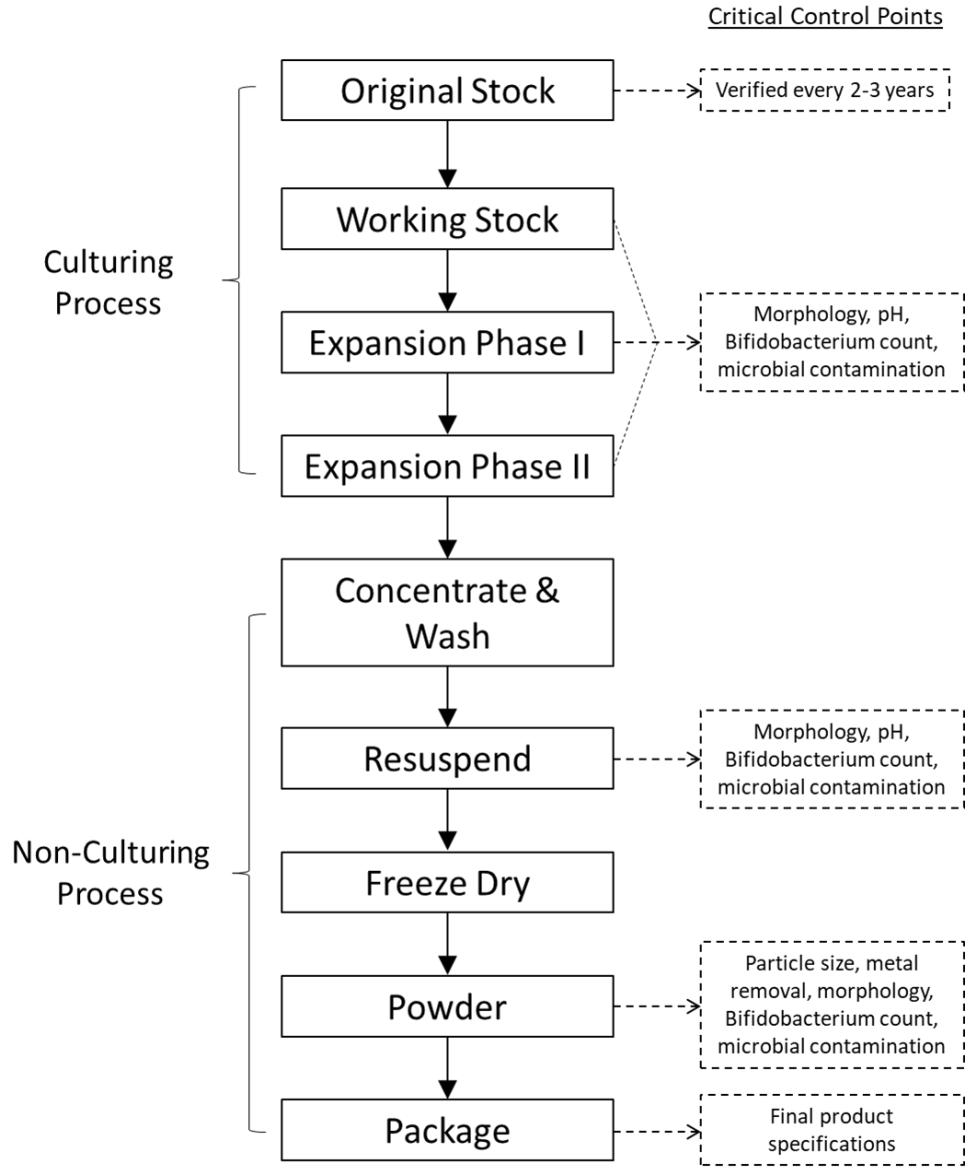



**Figure 2. Production process for *B. breve* MCC1274**

This production process results in the final B-3-EX formulation of a freeze-dried powder mixed with a cornstarch carrier. B-3-EX is used only in conventional foods.

## 1. Regulatory Compliance

*B. breve* MCC1274 is manufactured in Japan by Morinaga Milk Industry Co., Ltd., in FDA-registered facilities. Products are manufactured under food grade conditions and do not contain genetically modified organisms (GMOs) or ingredients derived from GMO-derived products. Morinaga Milk Industry Co., Ltd. operates under a Hazard Analysis Critical Control Point (HACCP) management system. Their facilities have been audited by a third party and determined to be compliant with the Food Safety System Certification (FSSC) 22000 and International Organization for Standardization (ISO) 22000:2005 standards.

The manufacturing process utilizes well-water, filtered through a reverse osmosis (RO) membrane, which is regularly tested and complies with the quality standards set forth in the Japan Water Supply Act (MHLW, 2003). All food contact surfaces used in manufacturing *B. breve* MCC1274 are either stainless steel, aluminum, or otherwise suitable for use in the production of food ingredients. Media ingredients are nutritional substances necessary for fermentation, do not contain major food allergens nor are they derived from major food allergens, and are safe and suitable for human consumption. *B. breve* MCC1274 is thoroughly washed during the non-culturing process to minimize carry-over of the fermentation medium to the finished ingredient. Resuspension medium is not washed from the final product; all components comply with their respective conditions of use and specifications stipulated within 21 CFR and/or Food Chemicals Codex (FCC). Carbohydrate carriers that are directly added to *B. breve* MCC1274 to generate the final ingredient comply with the respective Food Chemicals Codex (FCC) specifications. Packaging materials comply with 21 CFR (Table 2).

**Table 2. Compliance with US Regulations**

| Role in Production | Processing Aid | Compliance |
|---|---|---|
| Carbohydrate carrier | Cornstarch (unmodified) | 21 CFR §182.70; FCC 11 3S |
| Packaging | LLDPE/aluminum foil bag | 21 CFR §177.1520 |

CFR: United States Code of Federal Regulations; FCC: Food Chemicals Codex; LLDPE: linear low-density polyethylene

## 2. Quality Control

Morinaga Milk Industry routinely evaluates the quality of the *B. breve* MCC1274 product during the production process to ensure that the finished product is free of contaminants and the genotype and phenotype of the *B. breve* MCC1274 in the finished product are consistent with that of the original stock. The timing and parameters measured during the culturing and non-culturing processes are provided in Table 3. Although *B. breve* MCC1274 is produced in the same facility and using the same line/equipment as other microbial ingredient strains, a clean-in-place (CIP) procedure is used, and manual cleaning is performed after each production run to prevent cross-contamination between the various strains. Additionally, the production line is sterilized with steam or by dry heating before every production run. All cleaning is performed under approved SOPs and operators document the completion of each cleaning step.

**Table 3. Quality Control Parameters Monitored During the Production of *Bifidobacterium breve* MCC1274**

| Parameter* | Culturing Process | | | Non-Culturing Process | | |
|---|---|---|---|---|---|---|
| | Seed Culture | Expansion Phase 1 | Expansion Phase 2 | Resuspension | Powdering | Finished Product |
| Culture pH | X | X | X | X | | |
| Cell morphology | X | X | X | X | X | |
| Foreign body | X | X | X | X | X | X |
| Anaerobic CFU | | X | X | X | X | X |
| Aerobic CFU | | X | X | X | X | X |
| Mold | | X | X | X | X | X |
| Yeast | | X | X | X | X | X |
| Coliforms (including *E. coli*) | | | | X | X | X |
| *Staphylococcus aureus* | | | | X | X | X |
| *Salmonella* | | | | | | X |
| Moisture | | | | | | X |
| Heavy Metals | | | | | | X |
| Appearance | | | | | | X |
| Odor and Taste | | | | | | X |
| RAPD PCR | | | | | | X |

"X" denotes that the parameter is measured.
CFU: colony forming units; RAPD PCR: random amplification of polymorphic DNA polymerase chain reaction.
*Methods are validated and the same as those described in the product specifications.
Heavy Metals tested: arsenic, lead, mercury, cadmium

## E. FINISHED PRODUCT SPECIFICATIONS AND OTHER QUALITY ATTRIBUTES

### 1. Analytical Methods

All testing is performed using compendial and/or internal methods that have been validated.

### 2. Product Specifications

B-3-EX is a white to light brown powder consisting of freeze-dried active *B. breve* MCC1274 and a carbohydrate filler. To ensure a consistent food-grade product, each lot of B-3-EX is evaluated against an established set of product specifications using validated methods. RAPD PCR fingerprinting, which is performed annually, is used to verify that the *B. breve* MCC1274 PCR profile in the final products in the represented lots is identical to the original stocks. Additional product specifications are in place for anaerobic plate count (which includes *B. breve* MCC1274), microbial contamination, and heavy metals.

Data from three lots are shown in Table 4 demonstrating control of the production process and compliance with the product specifications.

**Table 4. Final Product Specifications and Lot Data for B-3-EX for Use in General Foods**

| Parameter | Specification | Method | Lot Number | | |
|---|---|---|---|---|---|
| | | | 2019.06.12 | 2019.07.29 | 2019.08.29 |
| Anaerobic plate count* | > $1.0 \times 10^{11}$ CFU/g | Reinforced Clostridial Agar | $1.4 \times 10^{11}$ | $1.8 \times 10^{11}$ | $1.6 \times 10^{11}$ |
| *B. breve* MCC1274[a] | Banding pattern | RAPD PCR | Confirmed | Confirmed | Confirmed |
| Appearance | White to slightly brown powder | Visual | White to slightly brown powder | White to slightly brown powder | White to slightly brown powder |
| Foreign Body | Negative | Visual | Negative | Negative | Negative |
| Odor and Taste | No abnormal odor and taste | Sensory Evaluation | No abnormal odor and taste | No abnormal odor and taste | No abnormal odor and taste |
| Moisture | < 6g/100 g | Gravimetric method at 105°C for 4h | 1.8 | 1.8 | 2.2 |
| **Microbial Contamination** | | | | | |
| Aerobic plate count | < 300 CFU/g | ISO 4833-1 | < 300 | < 300 | < 300 |
| Molds | < 30 CFU/g | ISO 21527-2 | < 30 | < 30 | < 30 |
| Yeast | < 30 CFU/g | ISO 21527-2 | < 30 | < 30 | < 30 |
| Coliform bacteria | Negative/1 g | BGLB Broth | Negative | Negative | Negative |
| *Staphylococcus aureus* | Negative/0.01 g | ISO 6888-1 | Negative | Negative | Negative |
| *Salmonella* | Negative/25 g | ISO 6579 | Negative | Negative | Negative |
| **Heavy Metals** | | | | | |
| Arsenic | < 1 ppm | ICP-MS | < 1 | < 1 | < 1 |
| Lead | < 0.5 ppm | ICP-MS | < 0.5 | < 0.5 | < 0.5 |
| Mercury | < 1 ppm | ICP-MS | < 1 | < 1 | < 1 |
| Cadmium | < 1 ppm | ICP-MS | < 1 | < 1 | < 1 |

**B. breve* MCC1274 is included in anaerobic plate count. Differential selective medium is used occasionally to distinguish *B. breve* MCC1274 from other anaerobes.
[a]RAPD PCR identification is performed annually.
CFU = colony forming units; ppm = parts per million; ICP-MS = inductively coupled plasma mass spectrometry.
Lot numbers indicate the date of production.
Limits of quantitation: heavy metals = 0.04 ppm.

## F. STABILITY OF *BIFIDOBACTERIUM BREVE* MCC1274

The stability of powdered B-3-EX was examined following storage in aluminum bags. The storage temperature was maintained at less than 10°C, and humidity was not monitored.

For MCC1274, data on three non-consecutive lots demonstrate that the anaerobic plate count (representing *B. breve*) continues to meet specifications through 36 months (Table 5). Measurements of moisture and microbial contamination are ongoing, but data on three lots demonstrate that these parameters continue to meet specifications at timepoints up to 45 months (Table 6).

Overall, these data support a shelf life of B-3-EX of up to 36 months when stored at less than 10°C.

**Table 5. Stability Analysis of B-3-EX less than 10°C**

| Parameter | Specification | Lot Number | Time (Months) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 3 | 6 | 12 | 18 | 24 | 36 |
| Anaerobic CFU/g | >$1.0x10^{11}$ | 2015.12.17 | $1.8x10^{11}$ | $2.0x10^{11}$ | $1.6x10^{11}$ | $1.6x10^{11}$ | $1.7x10^{11}$ | $1.4x10^{11}$ | $1.2x10^{11}$ |
| | | 2016.03.01 | $2.1x10^{11}$ | $1.7x10^{11}$ | $2.1x10^{11}$ | $1.9x10^{11}$ | $1.8x10^{11}$ | $1.8x10^{11}$ | $1.5x10^{11}$ |
| | | 2016.10.26 | $2.1x10^{11}$ | $1.9x10^{11}$ | $2.4x10^{11}$ | $1.7x10^{11}$ | $1.9x10^{11}$ | $1.9x10^{11}$ | $1.6x10^{11}$ |
| CFU: colony forming units. | | | | | | | | | |

**Table 6. Moisture and Microbial Contamination Stability Data for B-3-EX less than 10°C**

| Parameter | Method | Specification | Lot Number | | |
|---|---|---|---|---|---|
| | | | 2015.12.17 | 2016.03.01 | 2016.10.26 |
| | | Timepoint: | 45 months | 43 months | 35 months |
| Moisture | Gravimetric 105°C, 4 hours | < 6 g/100 g | 1.5 | 1.8 | 2.3 |
| Total aerobic bacteria | ISO 4833-1 | < 300 CFU/g | <300 | <300 | <300 |
| Yeast | ISO 21527-2 | < 30 CFU/g | <30 | <30 | <30 |
| Mold | ISO 21527-2 | < 30 CFU/g | <30 | <30 | <30 |
| Coliform bacteria | BGLB broth | Negative/1 g | Negative | Negative | Negative |
| *Staphylococcus aureus* | ISO 6888-1 | Negative/0.01 g | Negative | Negative | Negative |
| *Salmonella* spp. | ISO 6579 | Negative/25 g | Negative | Negative | Negative |
| CFU: colony forming units. | | | | | |

# III. DIETARY EXPOSURE

## A. INTENDED EFFECT

B-3-EX containing *B. breve* MCC1274 will be used as a source of *B. breve* in general foods.

## B. HISTORY OF USE

Bifidobacteria are a natural component of the normal human gut microflora. Bifidobacteria comprise up to 25% of the cultivatable fecal bacteria in adults and 80% in infants (Picard et al. 2005). *Bifidobacterium breve* MCC1274 was isolated from the feces of an infant in 2009.

Bifidobacteria have been consumed in fermented foods for decades and currently used commercial strains include *Bifidobacterium animalis* ssp. lactis strain Bf-6, *Bifidobacterium lactis* Bb-12, *Bifidobacterium lactis* DR10 (HN019), *Bifidobacterium longum* BB536, *Bifidobacterium breve* Yakult, *Bifidobacterium breve* SBT-2928, and *Bifidobacterium breve* C50. In the United States *B. animalis* ssp. lactis Bf-6 has been approved for use in conventional foods (GRN 377), *B. lactis* Bb-12 has been approved for use in formulas for infants four months of age and older (GRN 49), *B. longum* BB536 has been approved for use in selected foods (GRN 268), *B. breve* M-16V has been approved for use in selected foods and infant formulas (GRN 453, 454, 455), and *Bifidobacterium longum* subsp. *infantis* R0033 has been approved for use in infant formulas (GRN 758). Other microbial ingredients, such as *Lactobacillus reuteri* DSM 17938, *Lactobacillus fermentum* CECT5716, and *Bacillus coagulans* GBI-30, 6086, have been approved for use in term infant formulas (GRN 410, 531, and 660). Furthermore, there is no evidence showing that the consumption of viable bifidobacteria in fermented foods is unsafe.

Morinaga Milk Industry is the sole proprietor of *B. breve* MCC1274 and introduced the bacterium into the Japanese market in 2012. *B. breve* MCC1274 is also used in Japan in general foods. It is also used as a food ingredient in the several countries of the European Union such as Denmark, Italy, and Spain.

## C. INTENDED USE

Morinaga Milk Industry Co., Ltd., intends to add *B. breve* MCC1274 to selected food products (Table 7) to contain up to $5 \times 10^{10}$ CFU/serving at the end of shelf life.

**Table 7. Proposed Conventional Food Categories for the Addition of *B. breve* MCC1274**

| Food Category | Specific Food | CFU/serving |
|---|---|---|
| Breads/baked goods | • Bars; includes meal replacement, high protein, snack bars<br>• Biscuits<br>• Breads/roll (yeast), including bagels, croissants, English muffins, pizza crust<br>• Breakfast pastries; includes Danish<br>• Cakes, includes coffee cakes<br>• Cobblers, turnovers, strudels, crisps<br>• Cookie bars<br>• Crackers<br>• Doughnuts<br>• Pies<br>• Quick breads; includes breads, muffins, popovers, cornbread | $5 \times 10^{10}$ |
| Cereals | • Breakfast cereals, cooked; includes grits, oatmeal, cream of wheat, and wheat cereal<br>• Breakfast cereals, ready-to-eat | $5 \times 10^{10}$ |
| Fruits | • Juices and nectars, including citrus, non-citrus, vegetable and blends, frozen fruit, frozen juice bars, ices | $5 \times 10^{10}$ |
| Dairy products/dairy-based foods and dairy substitutes | • Skim milk<br>• Cheese spreads<br>• Cheese, imitation<br>• Cheese, processed<br>• Cream substitutes<br>• Cream, heavy<br>• Fermented milk (flavored, heat treated), including buttermilk, kefir, and flavored milk beverage mixes<br>• Frozen desserts, including ice cream, ice milk, frozen yogurt, frozen novelties, and imitation milk<br>• Meal replacements, liquids and dry mixes<br>• Milk shakes<br>• Milk (plain and flavored), including cocoa, chocolate milk, fruit milks, coffee drinks (fluid/dry)<br>• Puddings and custards<br>• Smoothies<br>• Whipped toppings<br>• Yogurt<br>• Butter and dried milk products<br>• Milk powder for pregnant women, plain and flavored<br>• Milk powder for adult people, plain and flavored<br>• Milk powder for elderly people, plain and flavored | $5 \times 10^{10}$ |
| Miscellaneous | • Candies, including hard candies, mints, chocolate and all other types of confections (i.e., chewing gum), cocoa powder, condiment sauces, (i.e., catsup, BBQ, taco, steak, cocktail, Worcestershire, teriyaki, cheese-based, hollandaise, tartar, béarnaise)<br>• Gelatin desserts, plain or with fruit gravies<br>• Peanut and other nut butters/spreads<br>• Snack foods, including chips, popcorn mixtures<br>• Weaning foods, including meals, desserts, fruits, cereal, vegetables, snacks, juices | $5 \times 10^{10}$ |

## D. ESTIMATED DAILY INTAKE

### 1. Assessment of *B. breve* MCC1274 Use in General Foods

The estimated daily intake (EDI) of *B. breve* MCC1274 in general foods is calculated based on the food uses and maximum use levels listed in Table 7, in conjunction with food consumption data included in the National Center for Health Statistics' (NCHS) 2015-2016 National Health and Nutrition Examination Surveys (NHANES) (CDC, 2018a; USDA, 2018). Food codes representative of each use were chosen from the Food and Nutrition Database for Dietary Studies (FNDDS) for the corresponding biennial NHANES survey. Calculations from NHANES for the mean and 90th percentile intakes were performed for representative food uses of *B. breve* MCC1274.

#### *a. Food Consumption Survey Data*

##### i. Survey Description

The most recent NHANES data for the years 2015-2016 are available for public use. NHANES are conducted as a continuous, annual survey, and are released in 2-year cycles. In each cycle, approximately 10,000 people across the U.S. completed the health examination component of the survey. Any combination of consecutive years of data collection is a nationally representative sample of the U.S. population. It is well established that the length of a dietary survey affects the estimated consumption of individual users and that short-term surveys, such as the typical 1-day dietary survey, overestimate consumption over longer time periods (Hayes et al., 2014). Because two 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) are available from the NHANES 2015-2016 survey, these data were used to generate estimates for the current intake analysis.

The NHANES provide the most appropriate data for evaluating food-use and food-consumption patterns in the United States, containing 2 years of data on individuals selected via stratified multistage probability sample of civilian non-institutionalized population of the U.S. NHANES survey data were collected from individuals and households via 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) throughout all 4 seasons of the year. Day 1 data were collected in-person in the Mobile Examination Center (MEC), and Day 2 data were collected by telephone in the following 3 to 10 days, on different days of the week, to achieve the desired degree of statistical independence. The data were collected by first selecting Primary Sampling Units (PSUs), which were counties throughout the U.S. Small counties were combined to attain a minimum population size. These PSUs were segmented and households were chosen within each segment. One or more participants within a household were interviewed.

Fifteen PSUs are visited each year. For example, in the 2009-2010 NHANES, there were 13,272 persons selected; of these 10,253 were considered respondents to the MEC examination and data collection. 9754 of the MEC respondents provided complete dietary intakes for Day 1 and of those providing the Day 1 data, 8,405 provided complete dietary intakes for Day 2. The release data do not necessarily include all the questions asked in a section. Data items may have been removed due to confidentiality, quality, or other considerations. For this reason, it is possible that a dataset does not completely match all the questions asked in a questionnaire section. Each data file has been edited to include only those sample persons eligible for that particular section or component, so the numbers vary.

In addition to collecting information on the types and quantities of foods being consumed, the NHANES surveys collect socioeconomic, physiological, and demographic information from individual participants in the survey, such as sex, age, height and weight, and other variables useful in characterizing consumption. The inclusion of this information allows for further assessment of food intake based on consumption by specific population groups of interest within the total population.

Sample weights are incorporated with NHANES surveys to compensate for the potential under-representation of intakes from specific population groups as a result of sample variability due to survey design, differential non-response rates, or other factors, such as deficiencies in the sampling frame (CDC, 2018b; USDA, 2012).

ii. Statistical Methods

Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer in Octave and used to generate estimates for the intake of *B. breve* MCC1274 by the U.S. population. Estimates for the daily intake of *B. breve* MCC1274 represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES data; these average amounts comprised the distribution from which mean and percentile intake estimates were produced. Mean and percentile estimates were generated incorporating sample weights in order to provide representative intakes for the entire U.S. population. “All-user” intake refers to the estimated intake of *B. breve* MCC1274 by those individuals consuming food products containing *B. breve* MCC1274. Individuals were considered users if they consumed 1 or more food products containing *B. breve* MCC1274 on either Day 1 or Day 2 of the survey.

*b. Food Usage*

The estimated "all-user" total intakes of *B. breve* MCC1274 from the proposed food uses listed in NHANES in the U.S. by population group is described in Table 8. The mean intake by all *B. breve* MCC1274 consumers age 2+ from the selected food uses was estimated to be $5.79 \times 10^{10}$ CFU/person/day or $8.64 \times 10^{8}$ CFU/kg bw/day. The heavy consumer ($90^{th}$ percentile) intake was estimated to be $1.07 \times 10^{11}$ CFU/person/day or $1.60 \times 10^{9}$ CFU/kg bw/day.

**Table 8. Estimated "All-user" Daily Intake of *B. breve* MCC1274 in Targeted Foods by Population Group (2015-2016 NHANES Data)**

| Population Group | N users | N population | % Users | Mean mass (kg) | Mean EDI (CFU) | 90th % EDI (CFU) | Mean EDI (CFU/kg) | 90th % EDI (CFU/kg) |
|---|---|---|---|---|---|---|---|---|
| ages 0-1 | 223 | 293 | 76.11 | 11.22 | 3.69E+10 | 6.19E+10 | 3.29E+09 | 5.52E+09 |
| ages 1-2 | 223 | 291 | 76.63 | 13.59 | 3.98E+10 | 6.89E+10 | 2.93E+09 | 5.07E+09 |
| ages 2-5 | 665 | 915 | 72.68 | 16.92 | 4.17E+10 | 7.23E+10 | 2.45E+09 | 4.27E+09 |
| ages 6-12 | 1186 | 1505 | 78.80 | 36.59 | 5.10E+10 | 9.14E+10 | 1.39E+09 | 2.50E+09 |
| ages 13-19 | 994 | 1143 | 86.96 | 67.35 | 6.39E+10 | 1.13E+11 | 9.49E+08 | 1.68E+09 |
| ages 20 and up | 4880 | 5748 | 84.90 | 79.95 | 6.03E+10 | 1.15E+11 | 7.55E+08 | 1.43E+09 |
| ages 2 and up | 7725 | 9311 | 82.97 | 66.96 | 5.79E+10 | 1.07E+11 | 8.64E+08 | 1.60E+09 |

CFU: colony forming units; N = number; EDI = estimated daily intake.

# IV. SELF-LIMITING LEVELS OF USE

This part does not apply.

# V. COMMON USE IN FOOD BEFORE 1958

This part does not apply.

# VI. NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The general recognition of safety of *B. breve* MCC1274 under the specified conditions of use in general foods is based on the established safety of Bifidobacteria and *Bifdobacterium breve*, as well as published and unpublished studies of *B. breve* MCC1274. Bifidobacteria are ubiquitous, generally recognized to be non-pathogenic to humans, lack invasive properties, and have been the subject of numerous GRNs. *B. breve* strains have been granted Qualified Presumption of Safety (QPS) status by EFSA (EFSA BIOHAZ Panel, 2019). In the United States, *Bifidobacterium breve* strain M-16V (Morinaga Milk Industry) is GRAS for use in general foods at levels up to $5 \times 10^9$ CFU/serving as well as powdered, milk- or soy-based, non-exempt term infant formula and exempt, powdered, amino acid-based infant formula at levels providing $1 \times 10^9$ CFU/g (GRN 453, 454, and 455). *In vitro* studies of *B. breve* MCC1274, including antibiotic resistance, bile salt conjugation, biogenic amine production, ammonia production, mucin degradation, absence of plasmids, genomic analysis for toxins and pathogenic markers, and measurement of hemolytic potential provide supportive evidence that *B. breve* MCC1274 does not pose safety risks.

The pivotal studies that directly support the safety of *B. breve* MCC1274 include a published acute oral toxicity study and a published 90-day repeated oral dose toxicity study, both conducted in rats (Arai et al., 2018). No adverse test article-related effects were observed in either study and the NOAEL from the 90-day study was determined to be at least $1.3 \times 10^{11}$ CFU/kg bw/day.

The safety of *B. breve* MCC1274 is also corroborated by five published clinical studies in adults. Additionally, other *B. breve* strains have been tested in one published study in rats and nine clinical studies in infants and children, and three published clinical studies in adults since the GRAS notifications in 2013. No test article-related adverse effects were reported in any study.

Thus, based on the weight of the evidence, Morinaga concludes there is reasonable certainty that the use of *B. breve* MCC1274 in conventional foods is expected to be safe under its intended uses and is therefore GRAS.

## A. REVIEW OF BIFIDOBACTERIA

The safety of Bifidobacteria is reviewed in GRN 268, pg. 42-46, which is incorporated by reference. *Bifidobacterium* spp. lack invasive properties, *i.e.*, they do not pass the epithelial boundary of the intestine (Zhou et al., 2000a; 2000b). *Bifidobacterium* spp. have been used in a variety of food products and are regularly consumed by humans on a daily basis. Bifidobacteria are components of the normal flora of the human gastrointestinal tract (Ahrné et al., 1998; Germond et al., 2002; Picard et al., 2005; Reuter, 2001; Yang et al., 2019). The lack of pathogenicity has also been demonstrated across all age groups and opportunistic infections resulting from Bifidobacterium ingestion have not been reported in immunocompromised

individuals(Borriello et al., 2003). Although there are a limited number of case reports documenting opportunistic infections associated with Bifidobacteria species reported in GRN 453, pg. 54-55, no new reports have been published that would change the conclusion that the ingestion of Bifidobacterium is safe.

As of August 18, 2020, 15 GRAS Notices have been submitted for various *Bifidobacterium* species and all have either received "no questions" letters or are pending review by the FDA (GRNs 49, 268, 377, 445, 453, 454, 455, 758, 813, 814, 855, 856, 872, 875, and 877).

## B. REVIEW OF *BIFIDOBACTERIA BREVE*

*Bifidobacterium breve* has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA BIOHAZ Panel, 2019). A strain belonging to a species listed on QPS and meeting the established criteria can freely be added to foods in the European Union. Additionally, the International Dairy Federation (IDF) in collaboration with the European Food and Feed Cultures Association (EFFCA) has included *Bifidobacterium breve* on its list of microorganisms with a documented history of safe use in food (IDF, 2018).

Recently, Yang et al. (2019) showed that *B. breve* is a major component of the cultivable infant gastrointestinal microbiota. Although some significant differences were seen between delivery (cesarian section versus vaginal) and feeding methods (breast fed, milk-powder fed, and mixed fed), *B. breve* was a major gastrointestinal component in all tested groups (~8-15% of total).

In the United States, *Bifidobacterium breve* strain M-16V (Morinaga Milk Industry) is the subject of GRNs 453, 454, and 455 for use in general foods at levels up to $5 \times 10^9$ CFU/serving as well as powdered, milk- or soy-based, non-exempt term infant formula and exempt, powdered, amino acid-based infant formula at levels providing $1 \times 10^9$ CFU/g, which received "no questions" letters from the FDA. The safety of *B. breve* M-16V provides corroborative evidence for the safety of *B. breve* MCC1274.

## C. *IN VITRO* SAFETY STUDIES OF *BIFIDOBACTERIA BREVE MCC1274*

### 1. Antibiotic Resistance

Morinaga Milk Industry evaluated the antibiotic resistance of *B. breve* MCC1274 and the type strain of *B. breve* (ATCC $15700^T$) to several antibiotics using a modified ISO/IDF method (ISO 10932/IDF 223). The resultant minimum inhibitory concentrations (MICs) along with their EFSA breaking points are shown in Table 9. The susceptibility of *B. breve* MCC1274 for each antibiotic was below the EFSA breaking points and no atypical characteristics were observed for the antibiotic resistance of strain MCC1274.

| **Table 9. Antibiotic Resistance of *B. breve* MCC1274** | | | |
|---|---|---|---|
| **Antibiotic** | **EFSA MIC for *Bifidobacterium* (mg/L)** | ***B. breve* strains** | |
| | | **MCC1274** | **ATCC 15700$^T$** |
| Ampicillin | 2 | 1 | 0.125 |
| Vancomycin | 2 | 0.5 | 0.5 |
| Gentamicin | 64 | 64 | 128 |
| Streptomycin | 128 | 64 | 64 |
| Erythromycin | 1 | 0.125 | < 0.02 |
| Clindamycin | 1 | 0.13 | < 0.03 |
| Tetracycline | 8 | < 0.13 | < 0.13 |
| Chloramphenicol | 4 | 0.5 | 0.25 |
| NR = Not Required | | | |

## 2. Metabolic Activity

### *a. Lactic Acid Production*

Lactic acid isomer production by *B. breve* MCC1274 was compared to that of *L. delbrueckii* subsp. *bulgaricus* MCC526 (a known D-lactic acid producer), *L. acidophilus* MCC605 (produces both D- and L-lactic acid), and *L. rhamnosus* JCM1136 (produces only L-lactic acid). The results of lactic acid production in Figure 3 are consistent with what is known about the above strains and show that *B. breve* MCC1274 produces only L-lactic acid. This is similar to other *Bifidobacterium* strains (GRN 453, 454, 455, and 877). Thus, there is no concern of increased D-lactic acid in the gut which may cause acidosis in infants by *B. breve* MCC1274.

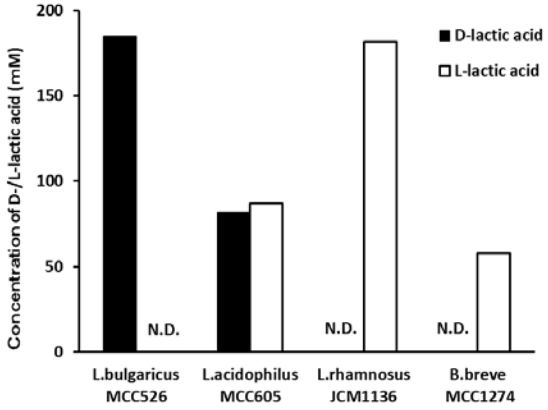



**Figure 3. D- and L-lactic Acid Production by *Bifidobacterium breve* MCC1274**
D- and L-lactic acid production by *B. breve* MCC1274, *L. delbrueckii* subsp. *bulgaricus* MCC526, *L. acidophilus* MCC605, and *L. rhamnosus* JCM1136 was determined using enzymatic assays involving D- and L-lactate dehydrogenase.

*b. Bile Salt Conjugation*

Although many *Bifidobacterium* strains are capable of deconjugating bile acids (Ridlon et al., 2006), the production of secondary bile acids by *B. breve* strains has not been reported. To confirm this finding, *B. breve* MCC1274 was incubated with broth containing 0.1 mM of various bile acids (taurocholic acid sodium salt hydrate (T-CA), glycocholic acid hydrate (G-CA), taurochenodeoxycholic acid sodium salt (T-CDCA), and sodium glycochenodeoxycholate (G-CDCA)) anaerobically at 37°C overnight. *B. breve* MCC1274 converted all tested conjugated bile acids into the corresponding unconjugated forms to some extent (Table 10). No secondary bile acids were detected after incubation. Thus, although *B. breve* MCC1274 can deconjugate bile salts, it does not produce secondary bile acids.

**Table 10. Bile Salt Deconjugation Activity of *B. breve* MCC1274**

| Bile Acid Substrate | Product After Culturing | Residual Substrate (µM) | Product Concentration (µM) | Ratio of Product to Total Bile Acid[1] |
|---|---|---|---|---|
| Taurocholic acid (T-CA) | Cholic acid (CA) | 15.2 ± 1.2 | 60.2 ± 3.1 | 79.8 ± 2.1% |
| Glycocholic acid (G-CA) | CA | ND | 81.2 ± 1.7 | 100% |
| Taurochenodeoxy cholic acid (T-CDCA) | Chenodeoxy cholic acid (CDCA) | 70.0 ± 3.4 | 27.6 ± 0.1 | 28.3 ± 1.0% |
| Glycochenodeoxy cholic acid (G-CDCA) | CDCA | ND | 85.6 ± 2.9 | 100% |
| CA | Deoxycholic acid (DCA) | 85.0 ± 1.4 | ND | 0% |
| CDCA | Lithocholic acid (LCA) | 76.8 ± 1.9 | ND | 0% |

Data are shown as mean ± SD, n=3.
ND = not detected.
[1]Ratio of product in total bile acid was calculated by the following equation: Product concentration / (Residual concentration of bile acid + Product concentration).

*c. Biogenic Amine Production*

The ability of *B. breve* MCC1274 to produce biogenic amines was examined. *Enterococcus faecalis* ATCC 19433 and *Clostridium perfringens* ATCC 13124 were used as positive controls for tyramine and histamine production, respectively. Strains were cultured overnight in the appropriate medium at 37°C under aerobic or anaerobic conditions, as required. The culture supernatant was collected for analysis, and the bacteria were pelleted by centrifugation and resuspended in test buffer containing no additives or supplemented with tyrosine or histidine. The pH of the buffer was tested before and after a 6 hour incubation with the bacteria. As expected, incubation of *E. faecalis* ATCC 19433 and *C. perfringens* ATCC 13124 with tyrosine and histidine, respectively, resulted in significant increases in the buffer pH (Figure 4). Incubation with *B. breve* MCC1274 did not cause changes in buffer pH following incubation with either tyrosine or histidine, indicating no biogenic amine production.

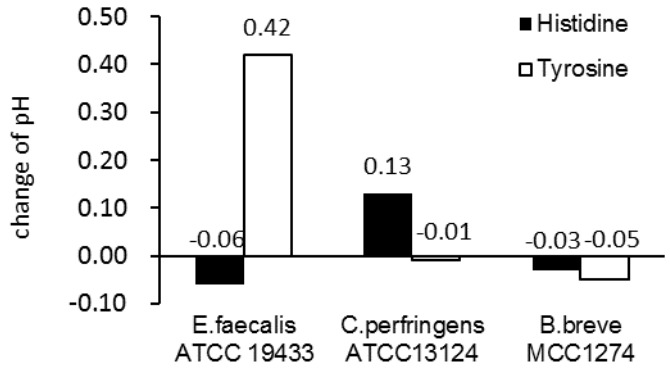



**Figure 4. Change in pH of histidine- or Tyrosine-Containing Test Buffer Incubated with *E. faecalis, C. perfringens,* or *B. breve* MCC1274 for 6 Hours at 37˚C**

Following the incubation, unsupplemented test buffer was also analyzed for tyrosine and histidine content by HPLC-MS. As expected, buffer from *E. faecalis* and *C. perfringens* contained significant amounts of tyramine and histamine, respectively (Figure 5). However, buffer from *B. breve* MCC1274 cultures contained trace or undetectable amounts of the amines. Therefore, *B. breve* MCC1274 does not produce biogenic amines.

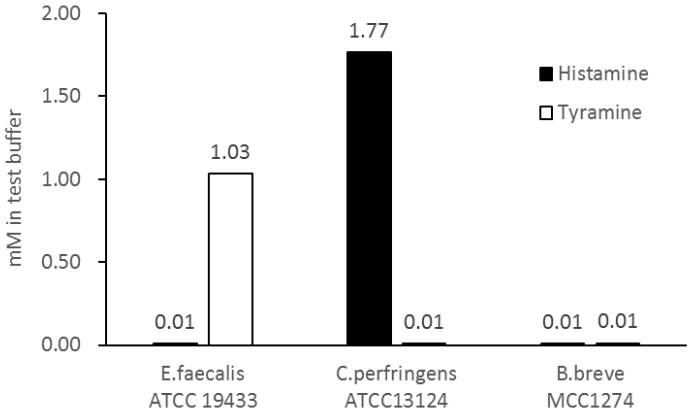



**Figure 5. Histamine and Tyramine Content in Buffer Incubated with *E. faecalis, C. perfringens,* or *B. breve* MCC1274 for 6 Hours at 37˚C.**

*d. Ammonia Production*

The ability of *B. breve* MCC1274 to produce ammonia was examined. *Enterococcus faecium* JCM5804 and *Lactobacillus rhamnosus* JCM1136 were used as positive and negative controls, respectively. Bacteria were cultured at 37°C overnight under anaerobic conditions as precultures. An aliquot of each preculture was inoculated into Gifu Anaerobic Medium (GAM broth), then incubated anaerobically at 37˚C for 48 hours. The culture medium was collected and adjusted to pH 7 with sodium hydroxide. Samples were diluted appropriately, and ammonia concentration was determined using an enzymatic photometric assay kit (Roche Diagnostics GmbH, Germany). Uncultured GAM broth was used for the baseline value. As expected, medium from *E. faecium* JCM5804 contained ammonia whereas the medium from *L. rhamnosus* JCM1136 contained 45 times less ammonia (Table 11). The ammonia content of medium from *B. breve* MCC1274 was below the baseline level. Thus, *B. breve* MCC1274 does not produce ammonia *in vitro* and is not expected to increase blood ammonia levels *in vivo*.

**Table 11. Ammonia Concentration in GAM Broth Following 48 Hour Culture**

| Bacteria | Ammonia (mM) |
|---|---|
| *E. faecium* JCM5804 | 6.42 |
| *L. rhamnosus* JCM1136 | 0.14 |
| *B. breve* MCC1274 | -1.49 |

*e. Mucin Degradation*

*B. breve* MCC1274 was also evaluated for mucin degradation, using three different methods: 1) *B. breve* MCC1274 growth in liquid medium providing mucin as a carbon source; 2) sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] analysis of mucin residues obtained from the growth medium containing mucin; and 3) degradation of mucins during *B. breve* MCC1274 growth on a Petri dish (Abe et al. 2010). None of the methods indicated that *B. breve* MCC1274 possesses mucin degradation activity.

**3. Presence of Plasmids**

DNA was extracted from *B. breve* MCC1274 using a QIAGEN Plasmid kit according to the manufacturer's instructions. Extracted DNA was analyzed by gel electrophoresis on a 1.0% agarose gel together with wide-range DNA Ladder (50-10,000 bp, Takara Bio Inc.) (Figure 6). No discrete bands, which would be indicative of plasmids were detected between 500 and 10,000 bp, confirming that *B. breve* MCC1274 does not harbor plasmids. To corroborate the gel electrophoresis results, whole cell genome sequencing of *B. breve* MCC1274 did not reveal any non-chromosomal DNA indicative of plasmids.

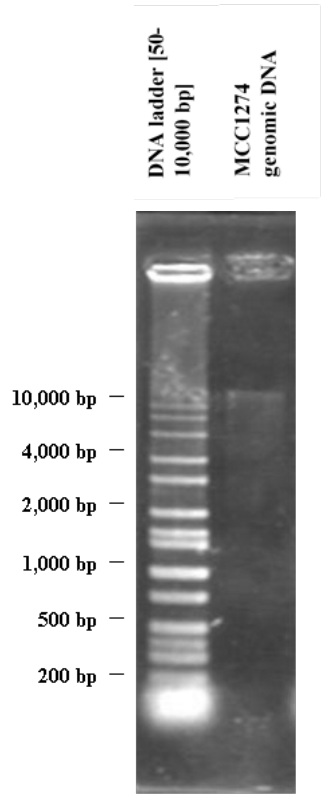



**Figure 6. Evaluation of *B. breve* MCC1274 for the Presence of Plasmids by Gel Electrophoresis**
*Plasmids, which are not present, migrate as discrete bands between 500 bp and 10,000 bp.*

### 4. Genomic Analysis for Toxins and Pathogenic Markers

To confirm the absence of toxin-producing genes and pathogenic markers, the genomic sequence of *B. breve* MCC1274 was determined using a PacBio RS II sequencer. The entire sequence was assembled using SMRT Analysis ver.2.3 with a program of RS_HGAP_Assembly.3. Open Reading Frame prediction was performed using PRODIGAL prediction software version 2.6. Automatic annotation was performed on the basis of BLASTP v2.2.26 analysis using the non-redundant protein database curated by the National Centre for Biotechnology (e-value $< 10^{-5}$). Where necessary, manual editing was performed using Artemis v.15, which was employed for output visualization. No known toxin or virulence genes were identified (Chen et al., 2011).

### 5. Hemolytic Potential

To evaluate the hemolytic potential of MCC1274, *B. breve* MCC1274, *Listeria ivanovii* subsp. *ivanovii* ATCC 19119$^{T}$ (positive control) and *Bifidobacterium breve* M-16V (negative control) were plated on horse blood-supplemented agar and incubated at 37°C for up to 72 hours (24

hr for *Listeria*). Compared to *L. ivanovii* subsp. *ivanovii* ATCC 19119$^{T}$, which is known to cause hemolysis, no hemolysis was detected for *B. breve* MCC1274 and *B. breve* M-16V (Figure 7). Thus, *B. breve* MCC1274 does not induce hemolysis *in vitro*.

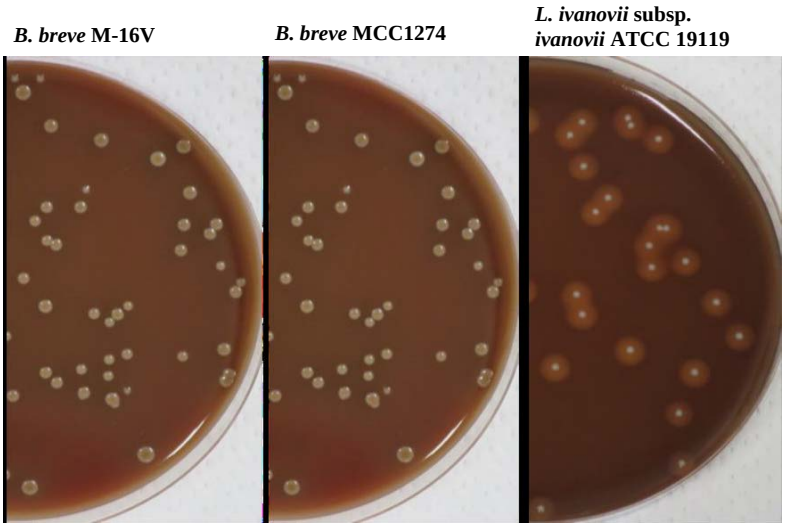



**Figure 7. Hemolysis Induced by *B. breve* M-16V, *B. breve* MCC1274, and *L. ivanovii* subsp. *ivanovii* ATCC 19119$^{T}$ Plated on Blood Agar**
*Absence or presence of zone of hemolysis in blood agar by strain colonies.*

### 6. Platelet Aggregation

The potential for *B. breve* MCC1274 to induce platelet aggregation was evaluated by Sanquin Research (Amsterdam, Netherlands). Blood was collected from informed, consenting donors and processed to isolate the platelets. All platelet samples were verified for proper aggregation prior to use by initial testing with adenosine diphosphate (ADP) as a stimulus. Medium alone (negative control) or bacterial suspensions of *B. breve* MCC1274, *S. aureus* (positive control), and *B. breve* B1 (negative control) were incubated with platelets with gentle mixing. Aggregation was measured after 1 and 3 hours of incubation using light transmission platelet aggregometry. *S. aureus* at a concentration of 0.05-0.35 x $10^{9}$/mL induced aggregation, while medium alone or incubation with *B. breve* B1 did not, confirming the validity of the assay. *B. breve* MCC1274 at concentrations of 0.06 x $10^{9}$/mL and 0.24 x $10^{9}$/mL did not induce aggregation. Thus, *B. breve* MCC1274 does not induce platelet aggregation.

## D. TOXICOLOGY STUDIES

The toxicity of *B. breve* MCC1274 has been evaluated in published acute and subchronic oral toxicity studies (Arai et al., 2018). *B. breve* MCC1274 is not acutely toxic at levels up to 6000 mg/kg (8.4 × $10^{11}$ CFU/kg) and has a no observed adverse effect level (NOAEL) of at least 1000 mg/kg (1.3 x $10^{11}$ CFU/kg) or 1.3 x $10^{10}$ CFU/kg bw/day, based on the results from a 90-day

oral toxicity study. Additional studies in mice models have also been published and, although the studies were designed to evaluate the beneficial effects of ingesting *B. breve* MCC1274 ($10^8$-$10^9$ CFU/day), they reported that the ingestion of *B. breve* MCC1274 was well-tolerated (Kondo et al, 2010).

Additionally, an acute toxicity study and a subchronic toxicity study on *B. breve* M-16V corroborate the safety of *B. breve* MCC1274 (Abe et al., 2009). *B. breve* M-16V was not acutely toxic at 3,000 mg/kg (6.9 x $10^{11}$ CFU/kg) and has a NOAEL of 1000 mg/kg/day (2.3 x $10^{11}$ CFU/kg bw/day) based on results of the 90-day subchronic toxicity study.

**1. Acute Toxicity**

In a single dose oral toxicity study, 5 male and 5 female Crj:CD (SD)IGS(SPF) rats received a single oral dose of *B. breve* MCC1274 at 6000 mg/kg (8.4 x $10^{11}$ CFU/kg suspended in saline) using a metal gastric tube (Arai et al., 2018). All animals were housed individually in a climate-controlled room (20-24°C, 40-70% humidity) with a 12-hour light/dark cycle and pelleted feed and water were available *ad libitum*. Clinical signs and body weight were monitored continuously over the following 14 days and at the end of the study period, animals were euthanized and necropsied.

No deaths or clinical signs of toxicity were reported in any animal during the study. At necropsy, no abnormalities were reported in any animal. The $LD_{50}$ for *B. breve* MCC1274 was determined to be higher than the tested dose of 6000 mg/kg (8.4 x $10^{11}$ CFU/kg).

**2. Subchronic Toxicity**

*B. breve* MCC1274 was evaluated in a 90-day repeated dose oral toxicity study conducted in compliance with Japanese guidelines: Guidelines for Designation of Food Additives and Revision of Standards for Use of Food Additives (1996). The results of this study were published by Arai et al. (2018).

Crj:CD(SD)IGS rats (10/sex/group; Charles River Japan Inc. (Tokyo, Japan)) were administered a cornstarch suspension in saline (control) or 1000 mg/kg (1.3 x $10^{11}$ CFU/kg bw) *B. breve* MCC1274 suspended in saline via gavage for 90 days. All animals were housed individually in bracket-type metallic wire-mesh cages in a room with temperature controlled at 21–24°C and humidity at 25–58% with a 12 hour light/dark cycle, allowed free access to a pelleted feed and water and acclimatized for 8 days before the study.

Over the course of the 90-day treatment period, all animals were observed for general conditions such as external appearance, nutritional condition, posture, behavior, and abnormalities

in excreta three times a day. Body weight and feed consumption were measured continuously. Ophthalmological examinations were conducted before and at the end of the experiments. Urinalysis was performed using urine collected during week 13 (day 85 to day 87). At the end of the test period, all animals were subjected to laparotomy under ether anesthesia. Blood and serum were collected for measurement of hematological, biochemical, and coagulation parameters. All animals were killed by exsanguination and subjected to necropsy, organ weight measurements, and histopathological examination.

No mortalities or abnormalities in clinical signs were observed in either group. Animals receiving *B. breve* MCC1274 showed no difference in body weight, water, or feed consumption compared to the control group. No abnormalities in ophthalmological examinations, urinalysis, blood chemistries, or gross pathology were detected. No hematological parameter exhibited any significant difference between the test group and controls outside historical control ranges. Histopathological examination and organ weights showed no abnormalities in any organ or differences between the test group and control.

Due to the lack of observed toxicity, the NOAEL for *B. breve* MCC1274 was determined to be at least 1000 mg/kg or $1.3 \times 10^{11}$ CFU/kg bw/day under the tested conditions.

### 3. Corroborative Studies of *B. breve*

While no additional toxicology studies of *B. breve* MCC1274 were found in the published literature, there are 2 published animal toxicology studies of *B. breve* M-16V that corroborate the safety of *B. breve* MCC1274 (Abe et al., 2009).

A single oral dose toxicity rat test was conducted on *B. breve* M-16V, where a powder containing $2.3 \times 10^{11}$ CFU/g was suspended in saline and administered by gavage to 2 groups of 10 male and 10 female three week old rats at 3,000 mg/kg ($6.9 \times 10^{11}$ CFU/kg body weight) or 6,000 mg/kg ($1.4 \times 10^{12}$ CFU/kg body weight) and compared to a control group in support of GRNs 454 and 455. (Abe et al., 2009). Observations of general signs revealed no deaths or abnormalities related to treatment. For males, body weight was significantly lower in the 6,000 mg/kg group than in the 3,000 mg/kg group and the control group on days 8 and 10. By days 12 and 14, there were no differences among the different groups. For females, the changes in body weight in both high and low dose groups were equivalent to those in the control group.

A 90-day oral repeat dose toxicity study using 5-week old rats was conducted on *B. breve* M-16V in support of GRNs 453, 454, and 455 (Abe et al., 2009). Rats were randomized into one test and one control group of 10 male and 10 female rats/group. *B. breve* M-16V powder containing $2.3 \times 10^{11}$ CFU/g suspended in saline was administered by gavage at a dose of 1,000

mg/kg/day (i.e., 2.3 x $10^{11}$ CFU/kg bw/day). No significant treatment-related changes in clinical signs, anthropometric measurements, hematology, clinical chemistries, organ weights, or histopathological examinations were observed between test article and control groups. In conclusion, the no observed adverse effect level (NOAEL) from administration of *B. breve* M-16V to rats is 1000 mg/kg/day or 2.3 x $10^{11}$ CFU/kg bw/day for 90 days. Overall, these studies provide corroborative evidence for the safety of *B. breve* MCC1274.

## E. CLINICAL STUDIES

Pivotal clinical studies assessing the safety of *B. breve* MCC1274 have been conducted in adults, while corroborative studies of other strains of *B. breve* have been conducted in adults, children, and infants. The following discussion provides the safety-related parameters reported from each study.

### 1. Studies of *B. breve* MCC1274 in Adults

There are five published clinical studies of *B. breve* MCC1274 in adults (Minami et al., 2015; Minami et al., 2018; Kobayashi et al., 2019; Okubo et al., 2019; Xiao et al., 2020). These studies are summarized in Table 12.

Minami et al. (2015) conducted a randomized, double-blind, placebo-controlled, parallel group comparative study with *B. breve* MCC1274 in adults ages 40-69 years who had obesity tendencies (BMI 24 to 30 kg/$m^2$ and treated for diabetes). Subjects were administered placebo or a microbial mixture capsule containing 5 x $10^{10}$ CFU/day *B. breve* MCC1274 (n=24/group) for 12 weeks. Blood analysis showed significant positive inter-group differences of γ-glutamyltranspeptidase (γ-GTP) (P = 0.011) and high-sensitivity C-reactive protein (hCRP) (P = 0.039) in the test article compared to control at week 12. There were no drop-outs due to the tolerability of the test article, and no serious adverse effects were reported.

Minami et al. (2018) conducted a randomized, double-blind, placebo-controlled study with *B. breve* MCC1274 in pre-obese adults ages 20-64 years (BMI 25-30 kg/$m^2$). Subjects were administered placebo or a microbial mixture capsule totaling 2 x $10^{10}$ CFU/day *B. breve* MCC1274 (n=40/group) for 12 weeks. All participants completed the study and no contraventions with respect to compliance were reported. There were no observed significant differences in blood parameters, biochemical, and urine tests between the groups. No serious adverse events were reported, and mild adverse events were considered unrelated to test article ingestion.

In a randomized, double-blind, placebo-controlled study, Kobayashi el al. (2019) examined *B. breve* MCC1274 supplementation effects on older adults ages 50-80 years with subjective memory complaints. Participants were randomly allocated to a placebo group or microbial mixture group that consumed capsules totaling 2 x $10^{10}$ CFU/day *B. breve* MCC1274 (n=61/group) for 12 weeks and then completed a battery of cognitive tests that were compared to baseline. There were no adverse events related to the test article during the study period. All blood parameter changes were within historical normal ranges, and no significant differences were observed between groups after the study period.

Okubo et al., (2019) examined the effect of ingestion of *B. breve* MCC1274 in schizophrenic adults in an open-label, single-arm study. Subjects ingested 1 x $10^{11}$ CFU *B. breve* MCC1274/day (n= 30) for 4 weeks and were observed for a total of 8 weeks. One patient declined to continue participation in the middle of the baseline assessment. There were no adverse events related to the ingestion of *B. breve* MCC1274.

In another randomized, double-blind, placebo-controlled study, Xiao et al. (2020) examined *B. breve* MCC1274 supplementation on older adults ages 50-79 years suffering from mild cognitive impairment (MCI; mini-mental state evaluation MMSE 22 or more). Subjects received placebo or *B. breve* MCC1274 capsules (n=40/group) totaling 2 x $10^{10}$ CFU/day for 12 weeks. There were no significant hematological or blood parameter changes between the two groups. Vital signs such as blood pressure and heart rate were unchanged due to intake. No study related adverse events were reported.

Collectively, there were no safety related concerns due to the intake of *B. breve* MCC1274 in any of the cited studies. These studies report the same use of *B. breve* MCC1274 at doses up to 1 x $10^{11}$ CFU/day for 4 weeks and doses up to 5 x $10^{11}$ CFU/day for 12 weeks are safe for use in adults.

**Table 12. Clinical Studies of *B. breve* MCC1274 in Adults**

| Reference | Study Design and Population | Groups (Numbers of Subjects) | Duration | Safety Parameters |
|---|---|---|---|---|
| Minami et al., 2015 | Randomized, double-blind, placebo-controlled, parallel group comparative study with *B. breve* MCC1274 in adults ages 40-69 years who had obesity tendencies (BMI 24 to 30 $kg/m^2$ and treated for diabetes) | Control: maize starch gelatin capsule, n=28<br><br>Group 1 (microbial mixture): 3 gelatin capsules containing in total $5 \times 10^{10}$ CFU *B. breve* MCC1274/day, n=24 | 12 weeks | • Blood analysis showed significant positive inter-group differences of γ-glutamyltranspeptidase (γ-GTP) (P = 0.011) and high-sensitivity C-reactive protein (hCRP) (P = 0.039) in the test article group compared to control at week 12.<br>• There were no drop-outs due to the tolerability of the test article.<br>• No serious adverse effects were observed. |
| Minami et al., 2018 | Randomized, double-blind, placebo-controlled study with *B. breve* MCC1274 in pre-obese adults ages 20-64 years (BMI 25-30 $kg/m^2$) | Control: corn starch capsule, n=40<br><br>Group 1 (microbial mixture): 2 capsules containing in total $2 \times 10^{10}$ CFU *B. breve* MCC1274/day, n=40 | 12 weeks | • All participants completed the study and no contraventions with respect to compliance were reported.<br>• There were no observed significant differences in blood parameters, biochemical, and urine tests between the groups.<br>• No serious adverse events were reported, and mild adverse events were considered unrelated to test article ingestion. |
| Kobayashi et al., 2019 | Randomized, double-blind, placebo-controlled study examining *B. breve* MCC1274 supplementation effects on older adults ages 50-80 years with subjective memory complaints | Control: maize starch gelatin capsule, n=60<br><br>Group 1 (microbial mixture): 2 gelatin capsules containing in total $2 \times 10^{10}$ CFU *B. breve* MCC1274/day, n=61 | 12 weeks | • There were no adverse events related to the test article during the study period.<br>• All blood parameter changes were within historical normal ranges, and no significant differences were observed between groups after the study period. |
| Okubo et al., 2019 | Open-label, single-arm study of *B. breve* MCC1274 ingestion in schizophrenic adults | Microbial mixture: 2 sachets totaling $1 \times 10^{11}$ CFU *B. breve* MCC1274/day, n=30 | 4 weeks | • There were no adverse events related to the test article during the study period. |
| Xiao et al., 2020 | Randomized, double-blind, placebo-controlled study examining *B. breve* MCC1274 supplementation in older adults ages 50-79 years suffering from mild cognitive impairment (MCI; MMSE 22 or more) | Control: maize starch gelatin capsule, n=40<br><br>Group 1 (microbial mixture): 2 gelatin capsules containing in total $2 \times 10^{10}$ CFU *B. breve* MCC1274/day, n=40 | 12 weeks | • There were no significant hematological or blood parameter changes between the two groups.<br>• Vital signs such as blood pressure and heart rate were unchanged due to intake.<br>• No study related adverse events were reported. |

## 2. Corroborative Studies of *B. breve*

### *a. Infant and Children Studies*

Numerous studies of other *B. breve* strains by themselves or in combination with other microbial mixtures have been conducted in infants, children, and adults. Studies conducted prior to the GRAS determination of *B. breve* M-16V in infant formula and general foods are summarized in GRN 453, 454, and 455, and are incorporated by reference (see GRN 453, pg. 45-81). In summary, twenty studies in infants with doses of 2 x $10^8$ CFU/day to 1.5 x $10^{10}$ CFU/day at durations up to 6 months showed that the ingestion of *B. breve* strains at levels up to 1.5 x $10^{10}$ CFU/day by infants is safe and well-tolerated; four studies in children with doses ranging from 1 x $10^9$ CFU/day to 9 x $10^9$ CFU/day for durations up to 55 months showed that the ingestion of *B. breve* at levels up to 3 x $10^9$ CFU/day by children is safe and well-tolerated in children.

Since the GRAS notifications of *B. breve* M-16V in 2013, nine additional clinical studies conducted in infants and children with other *B. breve* strains are summarized below and corroborate the safety of *B. breve* MCC1274 (Table 13).

In a randomized, placebo-controlled, double-blind, multicenter study on healthy, full-term (≥37 weeks) formula-fed infants, Abrahmse-Berkeveld et al. (2016) evaluated the safety of formula supplemented with short-chain galacto-oligosaccharides (GOS) and long-chain fructo-oligosaccharides (FOS) and *B. breve* M-16V. Infants were randomized and received either standard whey protein-based, isoenergetic control formula (Nutricia Research, n=111) or control formula supplemented with 9:1 ratio of short-chain GOS and long-chain FOS (0.8 g/100 mL) plus 1.3 x $10^9$ CFU/100 ml *B. breve* M-16V (Morinaga Milk Industry, n=100) for 13 weeks. It was noted that growth parameters were similar between the groups and stayed close to the WHO growth standards. The test formula was well tolerated as the number of adverse events and the percentage of children experiencing one serious adverse event were similar between the groups. Additionally, no significant difference in blood parameters measured was seen between the two groups.

Aloisio et al. (2018) utilized a randomized, placebo-controlled, double-blind study to analyze the gut colonization of *B. breve* in breast-fed and bottle-fed healthy newborns (< 15 days post-birth). Infants were divided between breast-fed and bottle-fed and then either received a daily dose of 5 drops of oily placebo suspension (breast-fed: n=59; bottle-fed: n=14) or 5 drops of 1 x $10^8$ CFU 1:1: mixture of *B. breve* BR03 (DSM 16604) and *B. breve* B632 (DSM 24706) (breast-fed: n=71; bottle-fed: n=11) for 90 days. No adverse events after microbial administration were reported in any group. *Bifidobacteria breve* counts increased significantly in all administered newborns ($p < 0.02$). The authors found that a significant reduction in

incidence of daily vomiting ($p<0.03$), daily regurgitations ($p<0.03$), and an improvement in stool consistency ($p<0.0001$) was observed in both microbial mixture groups compared to their respective control groups. Additionally, a significant reduction in fecal *B. fragilis* ($p<0.03$) in the bottle-fed group receiving microbial mixture supplementation was seen.

A randomized, placebo-controlled, double-blind study in newborn, healthy infants (>36 weeks) examined the safety of formula supplemented with several *Bifidobacterium* strains for 12 months (Bazanella et al., 2017). Infants were randomized to groups that received whey-based, isoenergetic control formula (n=49) or control formula formulated with a total of 1 x $10^7$ CFU/g of *B. bifidum, B. breve, B. longum,* and *B. longum* subspecies *infantis* (n=48). The trial also included a breast-fed reference group (n=9). Parameters such as size, weight, antibiotic treatment, age of weaning, and start of solid food intake were similar between groups. There were no adverse events reported in association with microbial intake. Long-term colonization of *Bifidobacterium* strains was not detected.

A randomized, placebo-controlled, double-blind study of microbial mixture-supplemented formula was conducted in a population of very low birth weight, pre-term infants (750-1499 g) in Brazil (Braga et al, 2011). Infants were given 3 mL of human milk from a milk bank administered 1x/day enterally (n=121) or 3 mL of human milk supplemented with 3.5 x $10^7$-3.5 x $10^9$ CFU total of *L. casei* and *B. breve*, administered 1x/day enterally (n=122) for 30 days. No adverse events were reported. Four confirmed cases of NEC were observed in the control group with none occurring in the microbial mixture-supplemented formula group.

Chua et al. (2017) utilized a randomized, placebo-controlled, double-blind study of symbiotic formula in Cesarean-delivered infants to examine the effect and gut colonization of microbial-supplemented formula plus GOS/FOS. Infants were randomized to a cow's milk-based, non-hydrolyzed formula group (n=50), control formula with 0.8 g/100mL short chain (sc) GOS/long chain (lc) FOS (n=51) group, or control formula with 0.8 g/100mL scGOS/lcFOS and 7.5 x $10^8$ CFU/100mL *B. breve* M-16V group (n=52) for 16 weeks. The authors concluded that all formulas were well-tolerated and groups had a similar number of adverse events and growth profiles. Post-hoc analysis revealed a lower percentage of adverse event-related skin disorders in the GOS/FOS/*B. breve* group compared to controls (20% vs 42%, $p = 0.017$). GOS/FOS/*B. breve* supplementation resulted in a higher bifidobacteria proportion from day 3/5 ($p < 0.0001$) until week 8 ($p < 0.05$), a reduction of *Enterobacteriaceae* from day 3/5 ($p = 0.002$) till week 12 ($p = 0.016$) compared to controls. This was accompanied by a lower fecal pH and higher acetate.

In a randomized, placebo-controlled, double-blind, multicenter study of pre-term infants (born between 23-30 weeks), Costeloe et al. (2016) examined the effect of daily formula

supplemented with *B. breve* BBG-001 for 36 weeks. Infants were randomized to one of two groups and received either 3 mL of basic formula (Neocate®, Nutricia Ltd.) with maize starch, administered enterally (n=661) or 3 mL control formula with 1.6 x $10^8$ to 1.6x$10^9$ CFU *B. breve* BBG-001, administered enterally (n=654), once daily. No serious adverse events related to the test article were observed and the microbial was well-tolerated.

Del Giudice et al. (2017) examined a *Bifidobacterium* mixture in children (4-17 years of age) with season allergic rhinitis and intermittent asthma in a randomized, placebo-controlled, double-blinded study. Children were divided into two groups receiving a placebo sachet or oral supplementation sachet containing *B. longum* BB536 (3 x $10^9$ CFU), *B. infantis* M-63 (1 x $10^9$ CFU), and *B. breve* M-16V (1 x $10^9$ CFU) (n=20/group) to be diluted in water or milk for 8 weeks. All children completed the study and ingestion of the *Bifidobacterium* mixture was well-tolerated. No serious adverse events related to the test article were reported.

Maldonado et al. (2019) used a randomized, placebo-controlled, double-blind, parallel study to test the safety of microbial mixture-supplemented formula in healthy, formula-fed infants. Infants were randomized to receive either standard powdered formula consistent with European Union (EU) regulations (n=77), standard formula supplemented with 1 x $10^7$ CFU/g *L. fermentum* CECT5716 (n=83), or standard formula supplemented with 1 x $10^7$ CFU/g *B. breve* CECT7263 (n=76) for 12 months. It was noted that anthropometric measurements were similar among all groups and no significant difference was observed in formula intake among groups. The microbial mixture-supplemented formulas were well-tolerated as no adverse effects associated with microbial supplementation were reported.

In a randomized, controlled, unblinded study, Russo et al. (2017) assessed a microbial mixture on children (ages 4-12 years) with functional constipation. Children were randomized into two groups, one receiving polyethylene glycol (PEG; 3.6 g; n=28) doses from 0.4 g/kg/day to 0.8 g/kg/day and the other receiving PEG plus a microbial mixture of *B. breve* M-16V, *B. infantis* M-63, and *B. longum* BB536 (Tribif® sachets 3 g; dose not provided; n=27) for 8 weeks. No significant adverse events associated with the test article besides diarrhea were reported, but that occurred in both groups. There were no differences in anthropometric measurements and the test article was well tolerated with equal dropouts due to taste in both groups.

In these studies, *B. breve* was administered at doses of 1 x $10^7$-1.5 x $10^{10}$ CFU/day to pre-term, term infants, and children up to 17 years of age. The duration of *B. breve* administration ranged from 30 days to 12 months. No serious adverse events related to *B. breve* ingestion were reported. Overall, these studies provide corroborative evidence for the safe use of *B. breve* MCC1274 at dose of up to 1.5 x $10^{10}$ CFU/day in infants and children.

**Table 13. Clinical Studies of *B. breve* in Infants and Children**

| Reference | Study Design and Population | Groups (Numbers of Subjects) | Duration | Safety Parameters |
|---|---|---|---|---|
| Abrahmse-Berkeveld et al. 2016 | Randomized, placebo-controlled, double-blind, multicenter study on healthy, full term (≥37 weeks) formula-fed infants | Control: standard whey protein-based, isoenergetic control formula (Nutricia Research), n=111<br><br>Group 1: control formula supplemented with 9:1 ratio of short-chain GOS and long-chain FOS (0.8 g/100 mL) plus $1.3x10^9$ CFU/100 ml *B. breve* M-16V (Morinaga Milk Industry), n=100 | 13 weeks | • Growth parameters were similar and stayed close to the WHO growth standards<br>• The number of adverse events and the percentage of children experiencing one serious adverse event were similar between groups<br>• There was no significant difference between groups in blood parameters measured |
| Aloisio et al., 2018 | Randomized, placebo-controlled, double-blind study on healthy newborns (< 15 days post-birth) | **Breast-fed:**<br>Control: Daily dose of 5 drops of placebo oily suspension, excipients-only, n=59<br><br>Group 1 (microbial mixture): Daily dose of 5 drops of $1x10^8$ CFU 1:1: mixture of *B. breve* BR03 (DSM 16604) and *B. breve* B632 (DSM 24706), n=71<br><br>**Bottle-fed:**<br>Control: Daily dose of 5 drops of placebo oily suspension, excipients-only, n=14<br><br>Group 2 (microbial mixture): Daily dose of 5 drops of $1x10^8$ CFU 1:1: mixture of *B. breve* BR03 (DSM 16604) and *B. breve* B632 (DSM 24706), n=11 | 90 days | • No adverse events after microbial mixture administration were reported<br>• *B. breve* counts increased significantly in all administered newborns ($p < 0.02$)<br>• A significant reduction in incidence of daily vomiting ($p<0.03$), daily regurgitations ($p<0.03$), and an improvement in stool consistency ($p<0.0001$) was observed in both microbial mixture groups compared to their respective control groups.<br>• A significant reduction in fecal *B. fragilis* ($p<0.03$) in the bottle-fed group receiving microbial mixture supplementation was seen. |

**Table 13. Clinical Studies of *B. breve* in Infants and Children**

| Reference | Study Design and Population | Groups (Numbers of Subjects) | Duration | Safety Parameters |
|---|---|---|---|---|
| Bazanella et al., 2017 | Randomized, placebo-controlled, double-blind study in newborn, healthy infants (>36 weeks) | Control: whey-based isoenergetic control formula, n=49<br><br>Group 1 (microbial mixture): control formula supplemented with $1x10^7$ CFU/g of *B. bifidum, B. breve, B. longum, B. longum* subspecies *infantis*, n=48<br><br>Breast-fed reference group, n=9 | 12 months | • Size, weight, antibiotic treatment, age of weaning, and start of solid food intake were similar between groups<br>• No adverse events were reported in association with microbial mixture intake<br>• Long-term colonization of *Bifidobacterium* strains was not detected |
| Braga et al., 2011 | Randomized, placebo-controlled, double-blind study in very low birth rate, pre-term infants (750-1499 g) in Brazil | Control (Placebo): 3 mL of human milk from milk bank, administered 1x/day enterally, n=121<br><br>Group 1 (microbial mixture): 3 mL of human milk from milk bank supplemented with $3.5x10^7$-$3.5x10^9$ CFU total of *L. casei* and *B. breve*, administered 1x/day enterally, n=122 | 30 days | • Adverse events were not monitored and none were reported<br>• Four confirmed cases of NEC were observed in the control group with none occurring in the microbial mixture-supplemented formula group |
| Chua et al., 2017 | Randomized, placebo-controlled, double-blind study of symbiotic formula in Cesarean-delivered infants | Control: cow's milk-based nonhydrolyzed formula, n=50<br><br>Group 1: cow's milk-based nonhydrolyzed formula with 0.8g/100mL scGOS/lcFOS, n=51<br><br>Group 2: cow's milk-based nonhydrolyzed formula with 0.8g/100mL scGOS/lcFOS and $7.5x10^8$ CFU/100mL *B. breve* M-16V, n=52 | 16 weeks | • All formulas were well-tolerated and groups had similar number of adverse events and growth profiles<br>• Post-hoc analysis revealed lower percentage of adverse event-related skin disorders in group 2 compared to controls (20% vs 42%, $p = 0.017$)<br>• Group 2 had a higher bifidobacteria proportion from day 3/5 ($p < 0.0001$) until week 8 ($p < 0.05$), a reduction of Enterobacteriaceae from day 3/5 ($p = 0.002$) till week 12 ($p = 0.016$) compared to controls. This was accompanied with a lower fecal pH and higher acetate. |

**Table 13. Clinical Studies of *B. breve* in Infants and Children**

| Reference | Study Design and Population | Groups (Numbers of Subjects) | Duration | Safety Parameters |
|---|---|---|---|---|
| Costeloe et al., 2016 | Randomized, placebo-controlled, double-blind, multicenter study of pre-term infants (born between 23-30 weeks | Group 1 (placebo): 3 mL of basic formula (Neocate®, Nutricia Ltd.) with maize starch, administered 1x/day, enterally, n=661<br><br>Group 2 (microbial): 3 mL of basic formula (Neocate®, Nutricia Ltd.) with 1.6 x $10^8$ to 1.6 x $10^9$ CFU *B. breve* BBG-001, administered 1x/day enterally, n=654 | 36 weeks | • Administration of the microbial was well tolerated, and no serious adverse events related to test article were observed |
| Del Giudice et al., 2017 | Randomized, placebo-controlled, double-blinded study examining a *Bifidobacterium* mixture in children (4-17 years of age) with season allergic rhinitis and intermittent asthma | Control: Placebo sachet, n=20<br><br>Group 1: Oral supplementation sachet containing *B. longum* BB536 (3 x $10^9$ CFU), *B. infantis* M-63 (1 x $10^9$ CFU), and *B. breve* M-16V (1 x $10^9$ CFU), n=20 | 8 weeks | • All children completed the study and ingestion of *Bifidobacterium* mixture was well tolerated.<br>• No serious adverse events related to the test article were reported. |
| Maldonado et al., 2019 | Randomized, placebo-controlled, double-blind, parallel study in healthy, formula fed infants | Control: standard powdered formula consistent with EU nutritional regulations, n=77<br><br>Group 1: standard formula supplemented with 1x$10^7$ CFU/g *L. fermentum* CECT5716, n=83<br><br>Group 2: standard formula supplemented with 1x$10^7$ CFU/g *B. breve* CECT7263, n=76 | 12 months | EC |
| Russo et al. 2017 | Randomized, controlled, unblinded study, assessing a microbial mixture on children (ages 4-12 years) with functional constipation | Control: polyethylene glycol (PEG; 3.6 g) doses from 0.4 g/kg/day to 0.8 g/kg/day, n=28<br><br>Group 1: PEG plus a microbial mixture of *B. breve* M-16V, *B. infantis* M-63, and *B. longum* BB536 (Tribif® sachets 3 g; dose not provided), n=27 | 8 weeks | • No significant adverse events associated with the test article besides diarrhea were reported, but that occurred in both groups.<br>• There were no differences in anthropometric measurements and the test article was well tolerated with equal dropouts due to taste in both groups. |

3′-GL, 3′galactosyllactoses; CFU, colony forming unit; EU, European Union; lcFOS, long-chain fructo-oligosaccharides; NEC, necrotizing enterocolitis; scGOS, short-chain galacto-oligosaccharides; WHO, World Health Organization

*b. Adult Studies*

All studies conducted in adults are summarized in GRN 453, pg. 82-109 and are incorporated by reference. In summary, twenty-one studies of *B. breve* alone or in combination with other microbials were examined with doses of 3 x $10^8$ CFU/day to 8 x $10^{11}$ CFU/day at durations from 2 weeks to 1 year. Results show that *B. breve* was safe and well-tolerated at doses up to 8 x $10^{11}$ CFU/day.

Since the GRAS notifications of *B. breve* M-16V in 2013, three additional clinical studies of other *B. breve* strains in adults are summarized below that corroborate the safety of *B. breve* MCC1274 (Table 14).

Jager et al. (2016) utilized a randomized, double-blind, placebo-controlled, crossover to determine the effect of prior ingestion of microbial strains *B. breve* BR03 and *S. thermophilus* FP4 in health resistance-training men. Fifteen total participants were randomized to receive either placebo or a microbial mixture consisting of 5 x $10^9$ active fluorescent units (AFU) of *S. thermophilus* FP4 (DSMZ 18616) and 5 x $10^9$ AFU *B. breve* BR03 (DSMZ 16604) per day for 21 days. There was a 21-day washout period, then the participants switched regiments. No serious adverse events were reported.

In a randomized, double-blind, placebo-controlled study, Inoue et al. (2018) assessed whether a combination of *Bifidobacterium* spp. supplementation and moderate resistance training had an effect on health-related parameters in healthy elderly subjects (ages 66-78 years). For 12 weeks, subjects were assigned to either a placebo group (n=19) or a microbial mixture group (n=20) consisting of a sachet of *B. longum* BB536, *B. infantis* M-63, *B. breve* M-16V, and *B. breve* MCC1274 (approximately 1.25×$10^{10}$ CFU each). No adverse effects were reported during the study period and only one subject dropped out in the microbial mixture group because they could not adhere to the study protocol. There were no significant differences in biochemical analysis between the two groups. The microbial mixture group showed a significant increase in the defecation frequency ($P$=0.023) after 12 weeks.

Madempudi et al. (2019) assessed UB0316, a multi-strain microbial mixture formulation in patients with type 2 diabetes mellitus using a randomized, double-blind, placebo-controlled study. Subjects were randomized into two groups, one receiving a maltodextrin placebo (n=39) and the other receiving microbial UB0316 consisting of 3 x $10^{10}$ CFU (*L. salivarius* UBLS22, *L. casei* UBLC42, *L. plantarum* UBLP40, *L. acidophilus* UBLA34, *B. breve* UBBr01, and *B. coagulans* Unique IS2) and 100 mg of FOS (n=40) twice a day for 12 weeks. No study dropouts were due to tolerability of the test article. Hematology, vital signs, and physical examinations

remained normal and within normal range throughout the trial period. Only mild adverse events such as flatulence and constipation occurred which were associated with the test article. No serious adverse events were reported.

In these studies, *B. breve* was administered at doses of $3 \times 10^8$ to $8.0 \times 10^{11}$ CFU/day to adults for durations ranging from 2 weeks to 12 months. There were no reported serious adverse events related to *B. breve* ingestion. Overall, these studies corroborate the safe use of *B. breve* MCC1274 at doses of up to $8 \times 10^{11}$ CFU/day in adults.

**Table 14. Clinical Studies of *B. breve* in Adults**

| Reference | Study Design and Population | Groups (Numbers of Subjects) | Duration | Safety Parameters |
|---|---|---|---|---|
| Inoue et al. 2018 | Randomized, placebo-controlled, double-blind, assessing whether a combination of *Bifidobacterium* spp. supplementation and moderate resistance training had an effect on health-related parameters in healthy elderly subjects (ages 66-78 years) | Control: placebo, n=19<br><br>Group 1 (microbial mixture): Microbial mixture group consisting of a sachet of *B. longum* BB536, *B. infantis* M-63, *B. breve* M-16V and *B. breve* MCC1274 (approximately $1.25\times10^{10}$ CFU each), n=20 | 12 weeks | • No adverse effects were reported during the study period and only 1 subject dropped out in the microbial mixture group because they could not adhere to the study protocol.<br>• There were no significant differences in biochemical analysis between the two groups.<br>• The microbial mixture group showed a significant increase in the defecation frequency (P=0.023) after 12 weeks. |
| Jager et al., 2016 | Randomized, double-blind, placebo-controlled, crossover to determine the effect of prior ingestion of microbial strains *B. breve* BR03 and *S. thermophilus* FP4 in health resistance-training men | Control: Placebo<br><br>Group 1 (microbial mixture): Microbial mixture consisting of $5 \times 10^9$ active fluorescent units (AFU) *S. thermophilus* FP4 (DSMZ 18616) and $5 \times 10^9$ AFU *B. breve* BR03 (DSMZ 16604) per day<br><br>n=15 total subjects randomly assigned then switched after 21 day washout period | 12 weeks | • No serious adverse events were reported. |
| Madempudi et al., 2019 | Randomized, double-blind, placebo-controlled study assessing UB0316, a multi-strain microbial formulation in patients with type 2 diabetes mellitus | Control: maltodextrin placebo, n=39<br><br>Group 1 (microbial mixture): Microbial UB0316 consisting of $3 \times 10^{10}$ CFU (*L. salivarius* UBLS22, *L. casei* UBLC42, *L. plantarum* UBLP40, *L. acidophilus* UBLA34, *B. breve* UBBr01, and *B. coagulans* Unique IS2) and 100 mg of FOS twice a day, n=40 | 12 weeks | • No study dropouts were due to tolerability of test article.<br>• Hematology, vital signs, and physical examinations remained normal and within normal range throughout the trial period.<br>• Only mild adverse events such as flatulence and constipation occurred which were associated with the test article. No serious adverse events were reported. |

## F. ALLERGENICITY

No published reports of allergic reactions resulting from the ingestion of *B. breve* generally or *B. breve* MCC1274 specifically were found.

## G. REGULATORY APPROVALS ACROSS THE WORLD

### 1. *B. breve* MCC1274

*B. breve* MCC1274 is used in Japan in general foods. It is also used as a food ingredient in several countries of the European Union such as Denmark, Italy, and Spain.

### 2. *B. breve*

In the United States, *Bifidobacterium breve* strain M-16V (Morinaga Milk Industry) is GRAS for use in general foods at levels up to $5 \times 10^9$ CFU/serving as well as powdered, milk- or soy-based, non-exempt term infant formula and exempt, powdered, amino acid-based infant formula at levels providing $1 \times 10^9$ CFU/g (GRN 453, 454, and 455).

*Bifidobacterium breve* has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA BIOHAZ Panel, 2019). A strain belonging to a species listed on QPS and meeting the established criteria can freely be added to foods in Europe. Additionally, the International Dairy Federation (IDF) in collaboration with the European Food and Feed Cultures Association (EFFCA) has included *Bifidobacterium breve* on its list of microorganisms with a documented history of safe use in food (IDF, 2018).

In Canada, *Bifidobacterium breve* is listed in a monograph of microbial mixtures that may be used to support Product License Applications (Health Canada, 2019).

# VII. SUPPORTING DATA AND INFORMATION

## A. REFERENCES

All information included in the following list of references is generally available.

Abe, F., Yaeshima, T. and Iwatsuki, K. (2009) Safety Evaluation of Two Probiotic Bifidobacterial Strains, *Bifidobacterium breve* M-16V and *Bifidobacterium infantis* M-63, by Oral Toxicity Tests Using Rats. *Bioscience and Microflora*. 28: 7-15.

Abe, F., Muto, M., Yaeshima, T., Iwatsuki, K., Aihara, H., Ohashi, Y. and Fujisawa, T. (2010) Safety evaluation of probiotic bifidobacteria by analysis of mucin degradation activity and translocation ability. *Anaerobe*. 16: 131-136.

Abrahamse-Berkeveld, M., Alles, M., Franke-Beckmann, E., Helm, K., Knecht, R., Köllges, R., Bufe, A. (2016). Infant formula containing galacto-and fructo-oligosaccharides and Bifidobacterium breve M-16V supports adequate growth and tolerance in healthy infants in a randomised, controlled, double-blind, prospective, multicentre study. *Journal of Nutritional Science, 5,* e42–e42. https://doi.org/10.1017/jns.2016.35

Ahrné S, Nobaek S, Jeppsson B, Adlerberth I, Wold AE, Molin G. (1998) The normal Lactobacillus flora of healthy human rectal and oral mucosa. *J Appl Microbiol*. 85(1):88-94.

Aloisio, I., Prodam, F., Giglione, E., Bozzi Cionci, N., Solito, A., Bellone, S., … Di Gioia, D. (2018). Three-Month Feeding Integration With Bifidobacterium Strains Prevents Gastrointestinal Symptoms in Healthy Newborns . *Frontiers in Nutrition* , Vol. 5, p. 39. Retrieved from https://www.frontiersin.org/article/10.3389/fnut.2018.00039

Arai, S., Minami, J., Muto, M., Iwabuchi, N., Yamauchi, K., Xiao, J., & Abe, F. (2018). Safety evaluation of Bifidobacterium breve MCC1274 via oral toxicity tests in rats . *Toxicology Research and Application, 2*.

Bazanella, M., Maier, T. V, Clavel, T., Lagkouvardos, I., Lucio, M., Maldonado-Gòmez, M. X., … Haller, D. (2017). Randomized controlled trial on the impact of early-life intervention with bifidobacteria on the healthy infant fecal microbiota and metabolome. *The American Journal of Clinical Nutrition*, *106*(5), 1274–1286. https://doi.org/10.3945/ajcn.117.157529

Borriello, S.P., Hammes, W.P., Holzapfel, W., Marteau, P., Schrezenmeir, J., Vaara, M., Valtonen, V. (2003) Safety of probiotics that contain lactobacilli or bifidobacteria. *Clin Infect Dis*. 15;36(6):775-80.

Boyd, M.A., Antonio, M.A., and Hillier, S.L. (2005) Comparison of API 50 CH strips to whole-chromosomal DNA probes for identification of *Lactobacillus* species. *J Clin Microbiol.* 43: 5309–5311.

Braga, T. D., Da Silva, G. A. P., De Lira, P. I. C., & De Carvalho Lima, M. (2011). Efficacy of Bifidobacterium breve and Lactobacillus casei oral supplementation on necrotizing enterocolitis in very-low-birth-weight preterm infants: A double-blind, randomized, controlled trial. *American Journal of Clinical Nutrition*, *93*(1), 81–86. https://doi.org/10.3945/ajcn.2010.29799

CDC 2018a. National Health and Nutrition Examination Survey (NHANES): Analytic Guidelines, 2011-2014 and 2015-2016. National Center for Health Statistics, Centers for Disease Control and Prevention; Hyattsville, Maryland. Available from: https://wwwn.cdc.gov/nchs/data/nhanes/analyticguidelines/11-16-analytic-guidelines.pdf

CDC 2018b. NHANES 2015-2016 Dietary Data. Available from: https://wwwn.cdc.gov/nchs/nhanes/search/datapage.aspx?Component=Dietary&CycleBeginYear=2015.

Chen, L., Xiong, Z., Sun, L., Yang, J., & Jin, Q. (2012). VFDB 2012 update: Toward the genetic diversity and molecular evolution of bacterial virulence factors. *Nucleic Acids Research*, *40*(D1), 641–645. https://doi.org/10.1093/nar/gkr989

Chua, M. C., Ben-Amor, K., Lay, C., Neo, A. G. E., Chiang, W. C., Rao, R., … Chongsrisawat, V. (2017). Effect of Synbiotic on the Gut Microbiota of Cesarean Delivered Infants: A Randomized, Double-blind, Multicenter Study. *Journal of Pediatric Gastroenterology and Nutrition*, *65*(1), 102–106. https://doi.org/10.1097/MPG.0000000000001623

Costeloe, K., Hardy, P., Juszczak, E., Wilks, M., Millar, M. R., & Group, P. in P. I. S. C. (2016). Bifidobacterium breve BBG-001 in very preterm infants: a randomised controlled phase 3 trial. *Lancet (London, England)*, *387*(10019), 649–660.

Del Giudice, M. M., Indolfi, C., Capasso, M., Maiello, N., Decimo, F. and Ciprandi, G. (2017) *Bifidobacterium* mixture (*B. longum* BB536, *B. infantis* M-63, *B. breve* M-16V) treatment in children with seasonal allergic rhinitis and intermittent asthma. *Italian journal of pediatrics*. 43: 25-25.

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), Koutsoumanis, K, Allende, A, Alvarez-Ordóñez, A, Bolton, D, Bover-Cid, S, Chemaly, M, Davies, R, De Cesare, A, Hilbert, F, Lindqvist, R, Nauta, M, Peixe, L, Ru, G, Simmons, M, Skandamis, P, Suffredini, E, Cocconcelli, PS, Fernández Escámez, PS, Maradona, MP, Querol, A, Suarez, JE, Sundh, I, Vlak, J, Barizzone, F, Correia, S and Herman, L. (2019) Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 10: Suitability of taxonomic units notified to EFSA until March 2019. *EFSA Journal*. 17(7):5753, 79 pp.

Germond, J.E., Mamin, O., Mollet, B. (2002) Species specific identification of nine human Bifidobacterium spp. in feces. *Syst Appl Microbiol*. 25(4):536-43.

GRN 49 (2002). *Bifidobacterium lactis* strain Bb12 and *Streptococcus thermophilus* strain Th4. Nestle USA. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=grasnotices&id=49.

GRN 268 (2009). *Bifidobacterium longum* strain BB536. Morinaga Milk Industry Co., Ltd. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=grasnotices&id=268

GRN 377 (2011). *Bifidobacterium animalis* subsp. *lactis* strain Bf-6. Cargill Inc. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=grasnotices&id=377.

GRN 410 (2011). *Lactobacillus reuteri* strain DSM 17938. Nestle Nutrition, US. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=grasnotices&id=410.

GRN 445 (2012). *Bifidobacterium animalis* subsp. *lactis* strains HN019, Bi-07, Bl-04 and B420. Danisco USA, Inc. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=grasnotices&id=445.

GRN 453 (2013). *Bifidobacterium breve* M-16V. Morinaga Milk Industry Co., Ltd. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=grasnotices&id=453.

GRN 454 (2013). *Bifidobacterium breve* M-16V. Morinaga Milk Industry Co., Ltd. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=grasnotices&id=454.

GRN 455 (2013). *Bifidobacterium breve* M-16V. Danone Trading B.V. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=grasnotices&id=455.

GRN 531. (2015). *Lactobacillus fermentum* CECT5716. Biosearch Life, S.A. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=531.

GRN 660 (2015). *Bacillus coagulans* GBI-30, 6086 spores. Ganeden Biotech, Inc. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=660.

GRN 758 (2018). *Lactobacillus helveticus* R0052, *Bifidobacterium longum* subsp. *infantis* R0033, *and Bifidobacterium bifidum* R0071. Lallemand Health Solutions. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=grasnotices&id=758.

GRN 813 (2019). *Bifidobacterium longum*. BORI. BIFICO CO., LTD. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=813.

GRN 814 (2019). *Bifidobacterium bifidum* BGN4. BORI. BIFICO CO., LTD. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=814.

GRN 855 (2019). *Bifidobacterium animalis* subsp. *lactis* strain R0421. Lallemand Health Solutions. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=855.

GRN 856 (2019). *Bifidobacterium animalis* subsp. *lactis* strain BB-12. Chr. Hansen Inc. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=856.

GRN 872 (2019). *Bifidobacterium animalis* subsp. *lactis* UABla-12. UAS Laboratories, LLC. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=872.

GRN 875 (Pending). *Bifidobacterium animalis* subsp. *lactis* AD011. BIFICO CO., LTD. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=875.

GRN 877 (2019). *Bifidobacterium longum* BB536. Morinaga Milk Industry Co., Ltd. https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=877.

Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives (1996), https://www.mhlw.go.jp/english/topics/foodsafety/foodadditives/dl/tenkabutu-shiteikijunkaiseishishin-english.pdf.

Hayes, A.W., and Kruger, C. (2014). Hayes' Principles and Methods of Toxicology. CRC Press. Taylor and Francis Group. p.631.

Health Canada. (2019) Nautral Health Product: Probiotics Monograph. March 25, 2019.

IDF. (2018). Inventory of microbial food cultures with safety demonstration in fermented food products. *Bulletin of the IDF*. No. 495/2018. https://store.fil-idf.org/product/bulletin-idf-n-495-2018-inventory-microbial-food-cultures-safety-demonstration-fermented-food-products/.

Inoue, T., Kobayashi, Y., Mori, N., Sakagawa, M., Xiao, J.-Z., Moritani, T., Sakane, N. and Nagai, N. (2018) Effect of combined bifidobacteria supplementation and resistance training on cognitive function, body composition and bowel habits of healthy elderly subjects. *Beneficial Microbes*. 9: 843-853.

ISO/IDF, 2010, https://www.iso.org/obp/ui/#iso:std:iso:10932:ed-1:v1:en.

Jäger, R., Purpura, M., Stone, J. D., Turner, S. M., Anzalone, A. J., Eimerbrink, M. J., … Oliver, J. M. (2016). Probiotic Streptococcus thermophilus FP4 and Bifidobacterium breve BR03 supplementation attenuates performance and range-of-motion decrements following muscle damaging exercise. *Nutrients*, *8*(10), 1–11.

Kobayashi, Y., Kuhara, T., Oki, M., & Xiao, J. Z. (2019). Effects of bifidobacterium breve a1 on the cognitive function of older adults with memory complaints: A randomised, double-blind, placebo-controlled trial. *Beneficial Microbes, 10*(5), 511–520.

Kondo, S., Xiao, J.Z., Satoh, T., et al. Antiobesity effects of Bifidobacterium breve strain B-3 supplementation in a mouse model with high-fat diet-induced obesity. Biosci Biotechnol Biochem. 2010;74(8):1656-1661. doi:10.1271/bbb.100267.

Madempudi, R. S., Ahire, J. J., Neelamraju, J., Tripathi, A., & Nanal, S. (2019). Efficacy of UB0316, a multi-strain probiotic formulation in patients with type 2 diabetes mellitus: A double blind, randomized, placebo controlled study. *PLoS ONE*, *14*(11), 1–16.

Maldonado, J., Gil-Campos, M., Maldonado-Lobón, J. A., Benavides, M. R., Flores-Rojas, K., Jaldo, R., … Olivares, M. (2019). Evaluation of the safety, tolerance and efficacy of 1-year consumption of infant formula supplemented with Lactobacillus fermentum CECT5716 Lc40 or Bifidobacterium breve CECT7263: a randomized controlled trial. *BMC Pediatrics*, *19*(1), 361.

Minami, J. I., Kondo, S., Yanagisawa, N., Odamaki, T., Xiao, J. Z., Abe, F., … Shimoda, T. (2015). Oral administration of Bifidobacterium breve B-3 modifies metabolic functions in adults with obese tendencies in a randomised controlled trial. *Journal of Nutritional Science*, *4*, 1–7.

Minami, J., Iwabuchi, N., Tanaka, M., Yamauchi, K., Xiao, J. zhong, Abe, F., & Sakane, N. (2018). Effects of Bifidobacterium breve B-3 on body fat reductions in pre-obese adults: A randomized, double-blind, placebo-controlled trial. *Bioscience of Microbiota, Food and Health*, *37*(3), 67–75.

Ministry of Health, Labor, and Welfare (MHLW). (2003). Water Supply in Japan. https://www.mhlw.go.jp/english/policy/health/water_supply/menu.html

Okubo, R., Koga, M., Katsumata, N., Odamaki, T., Matsuyama, S., Oka, M., … Matsuoka, Y. J. (2019). Effect of bifidobacterium breve A-1 on anxiety and depressive symptoms in schizophrenia: A proof-of-concept study. *Journal of Affective Disorders*, *245*(November 2018), 377–385.

Picard, C., Fioramonti, J., Francois, A., Robinson, T., Neant, F., Matuchansky, C. (2005) Review article: bifidobacteria as probiotic agents -- physiological effects and clinical benefits. *Aliment Pharmacol Ther*. 22(6):495-512.

Reuter, G. (2001) The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. *Curr Issues Intest Microbiol*. 2(2):43-53.

Ridlon, J. M., Kang, D.-J. and Hylemon, P. B. (2006) Bile salt biotransformations by human intestinal bacteria. *Journal of Lipid Research*. 47: 241-259.

Russo, M., Giugliano, F.P., Quitadamo, P., Mancusi, V., Miele, E., Staiano, A. (2017) Efficacy of a mixture of probiotic agents as complementary therapy for chronic functional constipation in childhood. *Ital J Pediatr*. 43(1):24.

USDA 2012. What We Eat In America (WWEIA), NHANES: overview. Available from: http://www.ars.usda.gov/Services/docs.htm?docid=13793#release. Retrieved 29 January 2018.

USDA 2018. U.S. Department of Agriculture, Agricultural Research Service. 2018. USDA Food and Nutrient Database for Dietary Studies 2015-2016. Food Surveys Research Group Home Page, http://www.ars.usda.gov/nea/bhnrc/fsrg. Available at https://www.ars.usda.gov/northeast-area/beltsville-md-bhnrc/beltsville-human-nutrition-research-center/food-surveys-research-group/docs/fndds-download-databases/.

Xiao, J., Katsumata, N., Bernier, F., Ohno, K., Yamauchi, Y., Odamaki, T., … Kaneko, T. (2020). Probiotic Bifidobacterium breve in Improving Cognitive Functions of Older Adults with Suspected Mild Cognitive Impairment: A Randomized, Double-Blind, Placebo-Controlled Trial. *Journal of Alzheimer's Disease*, 1–9.

Yang, B., Chen, Y., Stanton, C., et al. Bifidobacterium and Lactobacillus Composition at Species Level and Gut Microbiota Diversity in Infants before 6 Weeks. Int J Mol Sci. 2019;20(13):3306. Published 2019 Jul 5. doi:10.3390/ijms20133306

Zhou, J.S., Shu, Q., Rutherfurd, K.J., Prasad, J., Birtles, M.J., Gopal, P.K., Gill, H.S. 2000a. Safety assessment of potential probiotic lactic acid bacterial strains *Lactobacillus rhamnosus* HN001, *Lb. acidophilus* HN017, AND *Bifidobacterium lactis* HN019 in BALB/c mice. *Intl J Food Microbiol.* 56:87-96.

Zhou, J.S., Shu, Q., Rutherfurd, K.J., Prasad, J., Gopal, P.K., Gill, HS. 2000b. Acute oral toxicity and bacterial translocation studies on potentially probiotic strains of lactic acid bacteria. *Food Chem Toxicol.* 38: 153-161.

## B. EXPERT PANEL STATEMENT

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized As Safe (GRAS) status of *B. breve* MCC1274 as an ingredient in conventional foods. *B. breve* MCC1274 has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), as described under 21 CFR §170.30(b).

This GRAS determination for the use of *B. breve* MCC1274 as an ingredient in conventional foods at a maximum level of $5 \times 10^{10}$ CFU per serving at the end of shelf-life is based upon scientific procedures as described under 21 CFR §170.30(b). The intake of *B. breve* MCC1274 from the intended uses specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s). To demonstrate that *B. breve* MCC1274 is safe, and GRAS, under the intended conditions of use, the safety of the intake of *B. breve* MCC1274 has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food, and is based on generally available and accepted information.

The proposed use of *B. breve* MCC1274 as an ingredient in foods has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

- Bifidobacteria are naturally occurring bacteria that contribute to the composition of the gut microflora of humans. *Bifidobacterium breve* species have been detected in feces from infants and adults.
- *B. breve* MCC1274 is a non-motile, non-spore forming, rod-shaped, anaerobic, Gram-positive bacterium. It was isolated from the feces of an infant in 2009. The bacterium has been deposited with the International Patent Organism Depositary of the National Institute of Advanced Industrial Science and Technology and is designated FERM BP-11175.
- *B. breve* MCC1274 was first commercially available in 2012 and has since been sold in a variety of markets including Japan, Denmark, Italy, and Spain.
- The original stock culture of *B. breve* MCC1274 has been maintained at −80°C since it was obtained by Morinaga Milk Industry, and no intentional selective pressures have been applied.

- *B. breve* MCC1274 cultures are used to produce the final B-3-EX formulation freeze-dried powder mixed with cornstarch carrier to be used in conventional foods only.
- Finished product made with *B. breve* MCC1274 consistently comply with established, food-grade product specifications. Specifications are in place to control anaerobic plate count (MCC1274 count), moisture, microbial contamination, and heavy metals.
- Fifteen GRAS Notices (GRNs) on *Bifidobacterium* species have received "no questions" letters from the FDA. This includes GRN 453, 454, and 455, which allows for the use of a strain of *B. breve* M-16V for use in general foods at levels up to 5 x $10^9$ CFU/serving as well as powdered, milk- or soy-based, non-exempt term infant formula and exempt, powdered, amino acid-based infant formula at levels providing 1 x $10^9$ CFU/g.
- *B. breve* has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) for use in the member countries of the European Union.
- *B. breve* MCC1274 has been tested for parameters outlined in the Food and Agriculture Organization of the United Nations/World Health Organization's (FAO/WHO) guidelines for the evaluation of microbes. Results from these tests provide evidence that *B. breve* MCC1274 is safe for use in foods, namely:
  - *B. breve* MCC1274 is not atypically resistant to conventional antibiotics.
  - *B. breve* MCC1274 produces L-lactic acid but does not produce D-lactic acid.
  - *B. breve* MCC1274 deconjugates bile salts, but no secondary bile acids are produced.
  - An *in vitro* study shows that *B. breve* MCC1274 does not produce biogenic amines.
  - An *in vitro* study indicates that *B. breve* MCC1274 does not produce ammonia.
  - The use of 3 different methods indicates that *B. breve* MCC1274 does not degrade mucins.
  - Testing has confirmed the absence of plasmids in *B. breve* MCC1274.
  - Genomic analysis of *B. breve* MCC1274 did not reveal the presence of known toxin or virulence genes.
  - *B. breve* MCC1274 was not observed to have hemolytic activity.

- *B. breve* MCC1274 does not induce platelet aggregation.

- The safety of *B. breve* MCC1274 is supported by a published acute toxicology study and a pivotal published 90-day repeated dose toxicology study, both in rats. In the single dose oral toxicity test using $8.4 \times 10^{11}$ CFU/kg of *B. breve* MCC1274, there were no deaths or MCC1274 related adverse findings. The no observed adverse effect level (NOAEL) from the 90-day study was determined to be at least $1.3 \times 10^9$ CFU/kg bw/day (Arai et al., 2018).
- Four published studies of *B. breve* MCC1274 alone in adults support the safety of *B. breve* MCC1274. No adverse events were reported in any study. These studies support the safe use of *B. breve* MCC1274 in adults at doses up to $5.0 \times 10^{10}$ CFU/day for 12 weeks.
- An acute toxicity study and subchronic toxicity study on *B. breve* M-16V corroborates the safety of *B. breve* MCC1274 (Abe et al., 2009). *B. breve* M-16V was not acutely toxic at 3,000 mg/kg ($6.9 \times 10^{11}$ CFU/kg) and has a NOAEL of 1000 mg/kg/day ($2.3 \times 10^{11}$ CFU/kg bw/day) based on results of the 90-day subchronic toxicity study which were used in support the GRAS designation of *B. breve* M-16V in conventional foods and exempt and non-exempt infant formula (GRN 453,454, and 455).
- Since the GRAS notifications of *B. breve* M-16V in 2013, nine additional clinical studies of other *B. breve* strains in infants and children are used to corroborate the safety of *B. breve* MCC1274. These studies administered doses of $1 \times 10^7$-$1.5 \times 10^{10}$ CFU/day to term and preterm infants and children up to 17 years of age. The study durations ranged from 30 days to 12 months. There were no study reported adverse events related to *B. breve* ingestion.
- Since the GRAS notifications of *B. breve* M-16V in 2013, three additional clinical studies of other *B. breve* strains in adults corroborate the safety of *B. breve* MCC1274. These studies administered doses of $3 \times 10^8$-$8.0 \times 10^{11}$ CFU/day for durations from 2 weeks to 12 months. There were no study reported adverse events related to *B. breve* ingestion.

- *B. breve* MCC1274 will be added to select general foods at levels sufficient to provide $5 \times 10^{10}$ CFU/serving at the end of shelf life. This will result in a mean estimated daily intake (EDI) for consumers age 2 and above of $5.79 \times 10^{10}$ CFU/day ($8.64 \times 10^8$ CFU/kg bw/day) and a 90$^{th}$ percentile intake of $1.07 \times 10^{11}$ CFU/day ($1.60 \times 10^9$ CFU/kg bw/day).

Determination of the GRAS status of *B. breve* MCC1274 under the intended conditions of use has been made through the deliberations of Roger Clemens, DrPH, CNS, FACN, FASN, FIFT, A. Wallace Hayes, PhD, DABT, FATS, ERT, CNS, and Thomas E. Sox, PhD, JD. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of *B. breve* MCC1274 and the potential human exposure to *B. breve* MCC1274 resulting from its intended use as an ingredient in foods and non-exempt infant formula and have concluded:

> *There is no evidence in the available information on B. breve* MCC1274 *that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when B. breve* MCC1274 *is used at levels that might reasonably be expected from the proposed applications. B. breve* MCC1274 *is GRAS for use in foods and non-exempt infant formula as proposed by Morinaga Milk Industry Co, Ltd.*

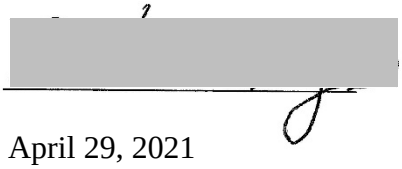
Therefore, *B. breve* MCC1274 is GRAS at the proposed levels of use. It is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

Roger Clemens, DrPH, CNS, FACN, FIFT
GRAS Expert Panel Member
School of Pharmacy
University of Southern California

Signature: 

Date: April 29, 2021

A. Wallace Hayes PhD, DATS, FACT, FACN
GRAS Expert Panel Member
University of South Florida
College of Public Health

Signature: 

Date: April 29, 2021

Thomas E. Sox, PhD, JD
GRAS Expert Panel Member
Principal, Pondview Consulting LLC

Signature: 

Date: April 29, 2021

Spherix Consulting Group

January 14, 2022

Katie Overbey, Ph.D., M.S.
Regulatory Review Scientist
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
5001 Campus Drive, HFS-225
College Park, MD 20740

RE: Questions Regarding GRN 001002

Dear Dr. Overbey:

Below are our responses to your requests for additional information regarding GRN 001002 as stated in your email on December 10, 2021. Please note that there is an error in Notice. The subject of GRN 001002, *B. breve* MCC1274, was isolated from the feces of an infant in 2002, not 2009. Your requests for additional information are in italicized text and our responses are below in plain text:

1. *Under Part D (Production Process), you provide general information about the manufacture of Bifidobacterium breve (B. breve) strain MCC1274 along with a flowchart outlining the production process. In addition, under Part D (1), you note that the resuspension media is not washed from the final product and that the carbohydrate carriers that are directly added to B. breve MCC1274 to generate the final ingredient comply with respective Food Chemical Codex (FCC) specifications. We note that in Table 2 you list unmodified cornstarch as a carbohydrate carrier and cite 21 CFR 182.70 as the appropriate regulation for this use. However, 21 CFR 182.70 is not applicable to the use of cornstarch as a carbohydrate carrier. Please provide a narrative of the manufacturing process that coincides with the provided flowchart, including the identification of the major components of the resuspension medium. In addition, please confirm that all materials used in the manufacturing process are used in accordance with U.S. regulations and that the ingredient is manufactured in accordance with good manufacturing practice.*

   Yes, we erroneously cited 21 CFR 182.70 to substantiate the use of cornstarch as a carbohydrate carrier. As cited in GRN 952, the use of cornstarch as an ingredient in food is substantiated by SCOGS report 115.

   Regarding a narrative of the manufacturing process, original stocks of *B. breve* MCC1274 were established by aliquoting a mixture *B. breve* MCC1274 in sterilized medium and storing them at -80°C at Morinaga Milk Industry. Working stocks were then

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established by expanding an original stock in sterilized medium and freezing aliquots in cryogenic vials at -80°C at the production facilities.

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. The *B. breve* MCC1274 resuspension medium mixture is then freeze-dried, crushed to a powder, mixed with cornstarch as a carrier, sifted to remove large particles, and passed through a metal detector, which monitors for and removes contaminating metal particles. The finished product is qualified for release by testing for compliance with the product specifications, packed into air-tight linear low-density polyethylene (LLDPE)-lined aluminum bags, and stored at less than 10°C.

The critical control points during the expansion phases and resuspension of the *B. breve* MCC1274 biomass in the resuspension medium include checks for *B. breve* MCC1274 morphology and cell count, culture pH, and microbial contamination. *B. breve* MCC1274 morphology and cell count, particle size and the presence of contaminating metal particles and other microbes are also evaluated in the freeze-dried powdered product prior to testing for compliance with the product specifications. Importantly, the production of *B. breve* MCC1274 occurs according to good manufacturing practices and all materials used in the manufacturing process are used in accordance with US regulations.

2. *In Table 4, you provide the specifications for heavy metals and the corresponding results from the analyses of three batches. You also state that the limit of quantification (LOQ) for heavy metals is 0.04 mg/kg. However, the results from the batch analyses are reported as either <1 mg/kg for arsenic, cadmium, and mercury, and <0.5 mg/kg for lead. Please provide the results of the heavy metal analyses as actual measured values. We also note that the specification limits for heavy metals should reflect the results of the batch analyses and be as low as possible.*

The measured values for the arsenic, lead, mercury, and cadmium are provided in Table 1. The limits of detection for arsenic, lead, mercury, and cadmium are 0.04 ppm. Arsenic levels ranged from 0.05 to 0.06 ppm whereas lead, mercury, and cadmium were not detected.

| Table 1. Heavy Metal Residues in B-3-EX | | | | | |
|---|---|---|---|---|---|
| **Parameter** | **Specification** | **Method** | **Lot Number** | | |
| | | | **2019.06.12** | **2019.07.29** | **2019.08.29** |
| Arsenic | < 1 ppm | ICP-MS | 0.05 | 0.06 | 0.06 |
| Lead | < 0.5 ppm | ICP-MS | < 0.04* | < 0.04 | < 0.04 |
| Mercury | < 1 ppm | ICP-MS | < 0.04* | < 0.04 | < 0.04 |
| Cadmium | < 1 ppm | ICP-MS | < 0.04* | < 0.04 | < 0.04 |
| *Limit of quantitation. | | | | | |

3. *In Table 7, you list the intended food categories and state the intended use level to be 5 x $10^{10}$ CFU/serving. However, you do not provide serving sizes for the intended food categories. Please confirm that the serving sizes for the food categories listed in Table 7 correspond to the reference amounts customarily consumed per eating occasion listed in 21 CFR 101.12.*

The serving sizes used to calculate the Estimated Daily Intake (EDI) of *B. breve* MCC1274 correspond to the reference amounts customarily consumed (RACC) per eating occasion as specified in 21 CFR 101.12. The food categories and their corresponding RACCs are shown below in a revised version of Table 7. For the RACCs that are listed in imperial measurement units in 21 CFR 101.12 (e.g. tablespoons and cups), a conversion factor of 15 g/1 tablespoon was assumed for the exposure estimates. Any RACC that was provided in milliliters was converted to grams, assuming 1 mL is equal to 1 g.

The EDIs provided in the GRAS Notice were calculated using only the RACCs for ages 4 years and above for all food categories and did not use the RACCs specific to foods intended for infants and young children 1 through 3 years of age, as described in 21 CFR 101.12 (a)(2), for the "weaning foods" described in Table 7 in the GRAS Notice. The EDIs have now been recalculated using the RACCs for infants and young children 1 through 3 years of age for the weaning products and the RACCs for ages 4 years and above for all of the other food products (Table 7). The mean and 90$^{th}$ percentile EDIs for all users ages 2 and up are now 5.68 x $10^{10}$ CFU/day and 1.05 x $10^{11}$ CFU/day (Table 2), respectively, and are comparable to the EDIs provided in the GRAS Notice. Importantly, the new EDIs do not change Morinaga's conclusion regarding the GRAS status of the use of *B. breve* MCC1274 in conventional food products.

**Table 7. Proposed Conventional Food Categories for the Addition of *B. breve* MCC1274**

| Food Category | Specific Food | CFU/serving | RACCs* Used |
|---|---|---|---|
| Breads/baked goods | Bars; includes meal replacement, high protein, snack bars[1] | $5 \times 10^{10}$ | 40 g |
| | Biscuits, croissants, English Muffins, pizza crusts | $5 \times 10^{10}$ | 55 g |
| | Bagels | $5 \times 10^{10}$ | 110 g |
| | Breads/roll (yeast) | $5 \times 10^{10}$ | 50 g |
| | Breakfast pastries; includes Danish | $5 \times 10^{10}$ | 55 g |
| | coffee cakes | $5 \times 10^{10}$ | 55 g |
| | Cakes, heavyweight (as defined in 21 CFR 101.12) | $5 \times 10^{10}$ | 125 g |
| | Cakes, mediumweight (as defined in 21 CFR 101.12) | $5 \times 10^{10}$ | 80 g |
| | Cakes, lightweight (as defined in 21 CFR 101.12) | $5 \times 10^{10}$ | 55 g |
| | Cobblers, turnovers, strudels, crisps | $5 \times 10^{10}$ | 125 g |
| | Cookie bars | $5 \times 10^{10}$ | 30 g |
| | Crackers that are usually used as snacks | $5 \times 10^{10}$ | 30 g |
| | Crackers that are not usually used as snacks (as defined in 21 CFR 101.12) | $5 \times 10^{10}$ | 15 g |
| | Doughnuts | $5 \times 10^{10}$ | 55 g |
| | Pies | $5 \times 10^{10}$ | 125 g |
| | Quick breads; includes breads, muffins, popovers, cornbread | $5 \times 10^{10}$ | 55 g |
| Cereals | Breakfast cereals, cooked; includes grits, oatmeal, cream of wheat, and wheat cereal | $5 \times 10^{10}$ | 40 g |
| | Breakfast cereals, ready-to-eat, weighing less than 20 g per cup, e.g., plain puffed cereal grains | $5 \times 10^{10}$ | 15 g |
| | Breakfast cereals, ready-to-eat, weighing 20 g or more but less than 43 g per cup; high fiber cereals containing 28 g or more of fiber per 100 g | $5 \times 10^{10}$ | 40 g |
| | Breakfast cereals, ready-to-eat, weighing 43 g or more pre cup; biscuit type | $5 \times 10^{10}$ | 60 g |
| Fruits | Juices and nectars, including citrus, non-citrus, vegetable and blends, frozen fruit, frozen juice bars, ices | $5 \times 10^{10}$ | 240 mL (g)[2] |
| Dairy products/dairy-based foods and dairy substitutes | Skim milk | $5 \times 10^{10}$ | 240 mL (g)[2] |
| | Cheese spreads | $5 \times 10^{10}$ | 30 g |
| | Cheese, imitation | $5 \times 10^{10}$ | 30 g |
| | Cheese, processed | $5 \times 10^{10}$ | 30 g |
| | Cream substitutes | $5 \times 10^{10}$ | 15 g |
| | Cream, heavy | $5 \times 10^{10}$ | 15 g |
| | Fermented milk (flavored, heat treated), including buttermilk, kefir, and flavored milk beverage mixes | $5 \times 10^{10}$ | 240 mL (g)[2] |
| | Frozen desserts, including ice cream, ice milk, frozen yogurt, frozen novelties, and imitation milk | $5 \times 10^{10}$ | 2/3 c (160g)[3] |
| | Meal replacements, liquids and dry mixes | $5 \times 10^{10}$ | 240 mL (g)[2] |
| | Milk shakes | $5 \times 10^{10}$ | 240 mL (g)[2] |
| | Milk (plain and flavored), including cocoa, chocolate milk, fruit milks, coffee drinks (fluid/dry) (coffee 360) | $5 \times 10^{10}$ | 240 mL (g)[2] |
| | Puddings and custards | $5 \times 10^{10}$ | ½ c (120 g)[3] |
| | Smoothies[4] | $5 \times 10^{10}$ | 240 mL (g)[2] |
| | Whipped toppings | $5 \times 10^{10}$ | 30 g |
| | Yogurt | $5 \times 10^{10}$ | 170 g |
| | Butter and dried milk products[5] | $5 \times 10^{10}$ | 15 g |
| | Milk powder for pregnant women, plain and flavored | $5 \times 10^{10}$ | 240 g |

**Table 7. Proposed Conventional Food Categories for the Addition of *B. breve* MCC1274**

| Food Category | Specific Food | CFU/serving | RACCs* Used |
|---|---|---|---|
| | Milk powder for adult people, plain and flavored | $5 \times 10^{10}$ | 240 g |
| | Milk powder for elderly people, plain and flavored | $5 \times 10^{10}$ | 240 g |
| Miscellaneous | Hard candies[6] | $5 \times 10^{10}$ | 15 g |
| | All other candies | $5 \times 10^{10}$ | 30 g |
| | Chewing gum | $5 \times 10^{10}$ | 3 g |
| | Cocoa powder | $5 \times 10^{10}$ | 1 Tbsp (15 g)[3] |
| | All sauces for dipping | $5 \times 10^{10}$ | 30 g |
| | Catsup, steak sauce, soy sauce, vinegar, teriyaki marinades | $5 \times 10^{10}$ | 15 g |
| | Minor condiments: horseradish, horseradish, hot sauces, mustards, Worcestershire sauce | $5 \times 10^{10}$ | 5 g |
| | Gelatin desserts, plain or with fruit gravies | $5 \times 10^{10}$ | 1/2 c (120 g)[3] |
| | Peanut and other nut butters/spreads | $5 \times 10^{10}$ | 2 Tbsp (30 g)[3] |
| | Snack foods, including chips, popcorn mixtures | $5 \times 10^{10}$ | 30 g |
| Weaning foods | Cereals, dry instant | $5 \times 10^{10}$ | 15 g |
| | Cereals, prepared, ready-to-serve | $5 \times 10^{10}$ | 110 g |
| | Other cereal and grain products, dry ready-to-eat, e.g., ready-to-eat cereals, cookies, teething biscuits, and toasts | $5 \times 10^{10}$ | 7 g |
| | Dinners, desserts, fruits, vegetables or soups, ready-to-serve, junior type | $5 \times 10^{10}$ | 110 g |
| | Dinners, desserts, fruits, vegetables or soups, ready-to-serve, strained type | $5 \times 10^{10}$ | 110 g |
| | Dinners, stews or soups for young children, ready-to-serve | $5 \times 10^{10}$ | 170 g |
| | Fruits for young children, ready-to-serve | $5 \times 10^{10}$ | 125 g |
| | Vegetables for young children, ready-to-serve | $5 \times 10^{10}$ | 70 g |
| | Juices all varieties | $5 \times 10^{10}$ | 120 mL (g)[2] |

*RACC: Reference amounts customarily consumed, as described in 21 CFR 101.12.

[1]"Grain-based bars with or without filling or coating, e.g., breakfast bars, granola bars, rice cereal bars" was chosen as a surrogate category to estimate the RACC for "Bars, includes meal replacement, high protein, snack bars".

[2]When RACC is described in mL, the RACC was converted to grams, assuming 1 mL is equal to 1 gram.

[3]When the RACC is provided in imperial measurements (cups and tablespoons), the value was converted to metric grams assuming 1 tablespoon is equal to 14.79 mL, or approximately 15 g (As described in the National Institute of Standards and Technology (NIST) Guide for the use of the International System of Units https://www.nist.gov/pml/special-publication-811).

[4]"Shakes or shake substitutes, e.g. dairy shake mixes, fruit frost mixes" was chosen as a surrogate category to estimate the RACC for "Smoothies".

[5]"Butter, margarine, oil, shortening" was chosen as a surrogate category to estimate the RACC for "butter and dried milk products".

[6]"Hard candies, others" was chosen as a surrogate category to estimate the RACC for "Hard candies." This RACC is for all hard candies except breath mints, or roll-type, mini-size hard candies.

**Table 2. Estimated "All-user" Daily Intake (EDI) of *B. breve* MCC1274 in Targeted Foods by Population Group (2015-2016 NHANES Data), Calculated with Corrected RACCs**

| Population Group | N users | N population | % Users | Mean mass (kg) | Mean EDI (CFU) | 90th % EDI (CFU) | Mean EDI (CFU/kg) | 90th % EDI (CFU/kg) |
|---|---|---|---|---|---|---|---|---|
| ages 0-1 | 446 | 689 | 64.73 | 9.22 | 3.50E+10 | 6.03E+10 | 3.80E+09 | 6.53E+09 |
| ages 1-2 | 368 | 487 | 75.56 | 14.49 | 3.97E+10 | 6.85E+10 | 2.74E+09 | 4.73E+09 |
| ages 3-5 | 467 | 643 | 72.63 | 21.50 | 4.28E+10 | 7.17E+10 | 1.99E+08 | 3.33E+09 |
| ages 6-12 | 1170 | 1473 | 79.43 | 40.94 | 5.23E+10 | 9.41E+10 | 1.28E+09 | 2.30E+09 |
| ages 13-19 | 840 | 960 | 87.50 | 69.01 | 6.26E+10 | 1.09E+11 | 9.08E+08 | 1.57E+09 |
| ages 20 and up | 4814 | 5665 | 84.98 | 79.97 | 5.93E+10 | 1.11E+11 | 7.41E+08 | 1.38E+09 |
| ages 2 and up | 7291 | 9020 | 80.83 | 66.96 | 5.68E+10 | 1.05E+11 | 8.48E+08 | 1.57E+09 |

4. *Please confirm that the subject of this notice, B. breve MCC1274, is not intended for use in infant formula or any products under the jurisdiction of the United States Department of Agriculture.*

   The subject of this Notice is not intended for use in infant formula or any products under the jurisdiction of the United States Department of Agriculture.

5. *You provide a specification for Staphylococcus aureus, listed as negative by test in 0.01 grams. The method referenced is ISO 6888-1. We note that this method is based on the analysis of a 0.1 mL test portion. Please clarify that the analytical method used to detect Staphylococcus aureus has been validated for the stated sample size.*

   Morinaga uses ISO 6888-1 for the quantitation of *S. aureus* as it is intended to be used and has set a specification based on the dilutions of the product and volumes specified in ISO 6887-1 and ISO 6888-1. Specifically, Section 8 of ISO 6888-1 states, "Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned: follow the procedures specified in the ISO 6887 series." According to section 3.6 of ISO 6887-1, an initial suspension is "obtained after a weighed or measured quantity of the product under examination (or of a test sample prepared from the product) has been mixed with, normally, a nine-fold quantity of diluent, allowing large particles, if present, to settle." Section 9.2 of ISO 6888-1 then states, "Transfer, by means of a sterile pipette (6.7), 0,1 ml of the test sample if liquid, or 0,1 ml of the initial suspension ($10^{-1}$ dilution) in the case of other products, to a Baird-Parker agar (BPA) plate (see B.2)." Therefore, the 0.1 ml of the initial suspension that is added to the BPA plate represents 0.01 grams of the product and the method used and specification established by Morinaga are fit-for use and comply with ISO 6888-1.

*6. Please confirm that B. breve strain MCC1274 is produced in compliance with current good manufacturing practices (cGMP).*

Per our response to Question 1, *B. breve* MCC1274 is produced in compliance with good manufacturing practices.

Should you need any additional information, please feel free to contact me at 240-367-6089 or dconze@spherixgroup.com.

Sincerely,

Dietrich B. Conze, Ph.D.
Managing Partner

GRN 1002
5/23/2022 Amendment

Just following up to FDA's question regarding GRN 1002. Below are FDA's question in italicized text and our response in plain text.

*1. As the notified substance is intended for use in infant foods, please provide a specification for Cronobacter sakazakii, including a level, sample size, and analytical method. Please also provide batch analysis data from at least three non-consecutive batches demonstrating that the Cronobacter sakazakii specification can be met.*

Setting a specification for *C. sakazakii* for the subject of GRN 001002 and providing batch data showing that the specification can be met is not justified. *C. sakazakii* infections in infants less than 12 months old are often linked to the ingestion of powdered infant formula and the intended uses for the subject of GRN 001002 do not include infant formula. Additionally, the Dietary Guidelines for Americans and the American Academy of Pediatrics recommend that children be introduced to foods other than breast milk or infant formula when they are approximately 6 months old (https://www.cdc.gov/nutrition/infantandtoddlernutrition/foods-and-drinks/when-to-introduce-solid-foods.html). Consistent with this guidance, the weaning foods that will contain the subject of GRN 001002 are intended to be consumed by infants once they have been weaned from breast milk and/or infant formula.

## Overbey, Katie

**From:** Dietrich Conze <dconze@spherixgroup.com>
**Sent:** Thursday, June 9, 2022 9:27 AM
**To:** Overbey, Katie
**Cc:** Claire Kruger; Kathy Brailer
**Subject:** Re: [EXTERNAL] Additional FDA Follow-up Question - GRN 1002

**Follow Up Flag:** Follow up
**Flag Status:** Flagged



Thanks Katie.
Dietz

On Jun 8, 2022, at 11:17 AM, Overbey, Katie <Katie.Overbey@fda.hhs.gov> wrote:

Hello Dietz,
Thank you for your response, this will be sufficient to remove these uses from GRN 1002. I will follow-up if we have any additional questions.

Thank you,
Katie

---

**From:** Dietrich Conze <dconze@spherixgroup.com>
**Sent:** Monday, June 6, 2022 8:38 AM
**To:** Overbey, Katie <Katie.Overbey@fda.hhs.gov>
**Cc:** Claire Kruger <ckruger@spherixgroup.com>; Kathy Brailer <kbrailer@spherixgroup.com>
**Subject:** [EXTERNAL] Re: Additional FDA Follow-up Question - GRN 1002



Hi Katie,
FDA's question is in italicized text and our response is in plain text below.

*Cronobacter sakazakii has been isolated from foods intended for very young children and can cause infection in infant and toddler populations. Because Morinaga lists an intended use of B. breve MCC1274 as an ingredient in infant and toddler foods there remains a potential risk to these vulnerable populations if C. sakazakii is not controlled for during the production of B. breve MCC1274 or if foods formulated with this ingredient are not treated with an inactivation step (ie. autoclaving) before consumption by infants or toddlers. We note the following publications that discuss the prevalence and potential concerns of C. sakazakii presence in infant and toddler foods:*

*Chen, Q., Zhu, Y., Qin, Z., Qiu, Y., & Zhao, L. (2018). Cronobacter spp., foodborne pathogens threatening neonates and infants. Frontiers of Agricultural Science and Engineering, 5(3), 330-339.*
*Forsythe, S. J. (2015). New insights into the emergent bacterial pathogen Cronobacter. In Food Safety*

*(pp. 265-308). Academic Press.*

*Given that the intended uses include very young children, does Morinaga plan to control for the presence of C. sakazakii? If not, please provide a discussion why this is not necessary from a safety perspective.*

Morinaga is not going to control for the presence of *C. sakazakii* in the subject of GRN 1002. Therefore, Morinaga is going to remove "Weaning foods, including meals, desserts, fruits, cereal, vegetables, snacks, juices", which are intended for infants and toddlers, from the intended uses of *B. breve* MCC1274 to mitigate the potential risk to infants and toddlers. Please remove "Weaning foods, including meals, desserts, fruits, cereal, vegetables, snacks, juices" from the intended uses.

Regards.
Dietz

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On Jun 1, 2022, at 2:28 PM, Overbey, Katie <Katie.Overbey@fda.hhs.gov> wrote:

Hello Dr. Kruger,
Thank you for sending your response to the FDA's most recent question about GRN 1002. We still need further clarification on the issue of Cronobacter sakazakii; please see our follow-up question below.

• Cronobacter sakazakii has been isolated from foods intended for very young children and can cause infection in infant and toddler populations. Because Morinaga lists an intended use of B. breve MCC1274 as an ingredient in infant and toddler foods there remains a potential risk to these vulnerable populations if C. sakazakii is not controlled for during the production of B. breve MCC1274 or if foods formulated with this ingredient are not treated with an inactivation step (ie. autoclaving) before consumption by infants or toddlers. We note the following publications that discuss the prevalence and potential concerns of C. sakazakii presence in infant and toddler foods:

• Chen, Q., Zhu, Y., Qin, Z., Qiu, Y., & Zhao, L. (2018). Cronobacter spp., foodborne pathogens threatening neonates and infants. Frontiers of Agricultural Science and Engineering, 5(3), 330-339.
• Forsythe, S. J. (2015). New insights into the emergent bacterial pathogen Cronobacter. In Food Safety (pp. 265-308). Academic Press.

Given that the intended uses include very young children, does Morinaga plan to control

for the presence of C. sakazakii? If not, please provide a discussion why this is not necessary from a safety perspective.

Please format your response such that each answer immediately follows the stated question. Please ensure that your responses do not contain confidential business information and please do not submit a revised version of the GRAS notice. We respectfully request a response to these questions within 10 business days. If you are unable to complete the response within that time frame, please contact me to discuss further options.

Thank you in advance for your attention to our comments.

Katie

Katie Overbey, Ph.D., M.S (she/her/hers)
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