CHR. HANSEN
Improving food & health

Division of Biotechnology and GRAS Notice Review
Center for Food Safety & Applied Nutrition (HFS-255)
U.S. Food & Drug Administration
5100 Campus Drive, College Park, MD 20740

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

Telephone: +1 (414) 607 5700
www.chr-hansen.com
info@chr-hansen.com

May 14, 2021

USEMGR

Reference: Chr. Hansen GRAS Notification for
Lactobacillus rhamnosus, LGG®

To Whom it May Concern,

In accordance with the Federal Register [81 Fed. Reg. 159 (17 August 2016)] issuance on Generally Recognized as Safe (GRAS) notifications (21 CFR Part 170), Chr. Hansen is pleased to submit a notice that we have concluded, through scientific procedures, that the organism *Lactobacillus rhamnosus* LGG®, is generally recognized as safe and is not subject to the pre-market approval requirements for use as an ingredient in milk and dairy products such as yogurt and other fermented milk products; dairy alternatives (plant-based (oat, soy, almond, coconut, pea, etc.) fermented milk and yogurt products); beverages such as juice and protein shakes; shelf-stable products such as bars (granola, protein, meal replacement bars), confectionery (gummy candy, hard candy, soft chew candy, chewing gum, coatings), breakfast cereals (RTE and hot), and non-exempt infant formula (including cow-milk, soy, and protein hydrolysate based formulas).

Yours sincerely,

Emily Gregoire
Probiotics Regulatory Affairs Manager – North America

usemgr@chr-hansen.com
Phone: 414-607-5877

Generally Recognized as Safe (GRAS) Determination for *Lactobacillus rhamnosus*, LGG®

Prepared by Chr. Hansen, Inc.

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Attachments

Allergen management in Chr. Hansen - HH statement

Part 1. Signed Statements and Certification

Name and Address of Notifier

Chr. Hansen, Inc.
9015 W Maple St.
Milwaukee, WI 53214

Name of Notified Substance

The bacterium *Lactobacillus rhamnosus,* LGG® DSM 33156. The strain is also known as *Lactobacillus casei* subspecies *rhamnosus* GG or LGG®.

Intended Conditions of Use

L. rhamnosus, LGG® is intended to be used as a microbial ingredient in conventional foods and non-exempt infant formula at levels consistent with current good manufacturing practices (cGMPs). It is intended to be consumed by the general population as well as term infants. Intended applications include but are not limited to the following: milk and dairy products such as yogurt and other fermented milk products; dairy alternatives (plant-based (oat, soy, almond, coconut, pea, etc.) fermented milk and yogurt products); beverages such as juice and protein shakes; shelf-stable products such as bars (granola, protein, meal replacement bars), confectionery (gummy candy, hard candy, soft chew candy, chewing gum, coatings), breakfast cereals (RTE and hot), and non-exempt infant formula (including cow-milk, soy, and protein hydrolysate based formulas). The maximum intended level of use is 10^{11} cfu/ serving of conventional food and 10^{8} cfu/g in infant formula.

Basis for GRAS Determination

Lactobacillus rhamnosus, LGG® has been determined to be GRAS through scientific procedures in accordance with 21 C.F.R. § 170.30(a) and (b).

Premarket Approval Status

Lactobacillus rhamnosus, LGG® is not subject to premarket approval requirements of the Federal Food, Drug, and Cosmetics Act based on a conclusion that the notified substance is GRAS under the conditions of intended use.

Availability of Information

The information and data that serve as the basis for the GRAS determination will be sent to FDA upon request, or will be available for review and copying at reasonable times at Chr. Hansen's office in the USA at the following address:

Chr. Hansen, Inc.
9015 W. Maple St
Milwaukee, WI 53214
Telephone: (414) 607-5700
Fax: (414) 607-5959

Freedom of Information Act Statement

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

Certification

To the best of our knowledge, this GRAS notification is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of *Lactobacillus rhamnosus, LGG®*.

FSIS Statement

Lactobacillus rhamnosus, LGG® is not intended for use in applications under the jurisdiction of the United States Department of Agriculture (USDA)

Name, Position, and Signature of Notifier

May 12, 2020

_____ _____

Emily Gregoire Date

Senior Regulatory Specialist
North America
Chr. Hansen, Inc.

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

2.1 Identity of the Organism

2.1.1 Source and Description of GRAS Organism

Lactobacillus rhamnosus, LGG® (commercially known as LGG®) was isolated from a healthy human by two US researchers, Prof. Barry Goldin and Prof. Sherwood Gorbach in 1985. After extensive research by Valio R&D and the global scientific community, the first LGG® products were launched in Finland under Valio Gefilus® brand in 1990. Since 1994, Chr. Hansen produced LGG® for Valio as a contract manufacturing organization (CMO) until the company acquired the strain from Valio in 2016. LGG® was most recently deposited into in the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) under the accession number DSM 33156.

A comprehensive genomic analysis was performed to assess the differences of LGG® at three manufacturing stages over the history, including LGG produced by Valio (1990-1994), LGG as CMO material for the two companies (1994-2016) and LGG as current production strain at Chr. Hansen (2016-now). The analysis comprised state-of-the-art sequencing technologies (HiSeq, MiSeq and Oxford nanopore technology long-read sequencing) and a robust variant calling pipeline intersecting outputs from multiple widely-used genomics tools. The results showed no differences of LGG® from the three manufacturing stages, indicating that Valio or Chr. Hansen produced LGG® remained stable at least for the last 25 years. The conclusion was consolidated by replicating the analyzed sample using different sequencing methods, and by repeating the analysis using different tools (Stage, et al., 2020).

2.1.2 Genome Sequencing and Annotation

To obtain a high-quality genome sequence of the LGG® *Lactobacillus rhamnosus*, the strain was genome sequenced using the Illumine HiSeq Technology and Oxford Nanopore Technology. Output from the HiSeq sequencing (5,786,431 raw reads and coverage 514x) was assessed and trimmed as previously described (Agersø et al., 2018) and the ONT (452,095 raw reads and coverage 162x) was filtered and down sampled to obtain high ONT quality consisting of the top 6% of ONT reads.

Combing reads from both sequencing technologies lead to a circular genome of 3.01 Mbp with a GC content of 46.7%. No plasmid was observed in the genome assembly and plasmid profiling verifies the lack of plasmids.

The Oxford Nanopore Technology (ONT)/HiSeq combined genome sequence of the LGG® *Lactobacillus rhamnosus* strain was subjected to annotation using RAST (Aziz, R.K. *et al*., 2008). RAST (Rapid Annotation using Subsystem Technology) is an annotation tool for bacterial and archaeal genomes and provides a high-quality annotation. The RAST annotation of the combined genome sequence for the LGG® *Lactobacillus rhamnosus* strain contained 2968 coding sequences (CDS) and 74 RNAs.

The genome size, GC content and number of CDSs in the LGG® *Lactobacillus rhamnosus* strain was comparable to *Lactobacillus rhamnosus* in the NCBI genome database.

Search Against Antibiotic Resistance Gene Databases

To identify genes with high identity to previously published antibiotic resistance genes, the annotated genome for *L. rhamnosus* strain LGG® was analyzed against a curated database of antibiotic resistance genes. The database focus on acquired antibiotic resistance genes from the scientific literature and covers both Gram-positive and Gram-negative bacteria including pathogenic species. The analysis did not detect any antibiotic resistance gene in line with the strain being sensitive to relevant antibiotics the strain was tested. One exception was for chloramphenicol where the minimal inhibitory concentration was one, two-fold over the EFSA cutoff value, but this is considered acceptable due to the technical variation of the phenotypic method as also recognized by EFSA in several published opinions.

Search Against the Virulence Factor Database and Phenotypic Test

The annotated genome of *L. rhamnosus* strain LGG® was analyzed against a published database containing virulence factors and other genes related to pathogenicity and toxicity from 30 different pathogens including Gram-positive pathogens such as *Enterococcus*, *Staphylococcus*, *Streptococcus* and *Listeria*. Most of the hits were associated with stress regulation (Clp), heat shock proteins, biosynthesis, capsule formation, transport systems or secretion systems. None of the hits were assessed to be virulence factors and all hits could be regarded as niche factors (Hill et al. 2012), since they are also found in commensal bacteria.

In general, most hits had low coverage and identity to the target sequences in the virulence factor database and the annotated CDSs in *L. rhamnosus* strain LGG® were found in all 22 *L. rhamnosus* genomes present in the NCBI NR database. The screening revealed 35 unique hits. Of these hits 26 were to genes which could be categorized either as 'niche factors' or housekeeping genes in bacteria (Clp genes (5), transporters (15), capsule genes (5), or genes involved in heat shock (1)). Moreover, of these niche factors only eight of the hits had more that 50% coverage and more than 50% identity to the target genes (Clp genes (2), capsule genes (5), or genes involved in heat shock (1)). The term 'niche factors' refer to adhesion factors, capsule genes, stress regulators and genes involved in cell division, all genes also found in commensal bacteria. The term was suggested by Hill (5). These genes were not further assessed.

The remaining nine hits were further assessed. All nine PEGs were present in all the 22 *L. rhamnosus* genomes in the NR NCBI database with high coverage (100%) and identity (96-100%) and based on the RAST annotation they were all genes coding for proteins which are involved in 'house-keeping' functions within the cell (eg. UDP- glucose-4-epimerase, D-alanine-polyligase sub unit, phosphoglucosamine mutase, Bactoprenol glucosyl transferase, lipoate-protein ligase A, hydrolases and a manganese ABC transporter).

One hit was annotated as a fibronectin/fibrinogen-binding protein and was found in all 22 *L. rhamnosus* present in the NCBI NR database with 98.8-100% identity. Fibronectin/fibrinogen-binding proteins are involved in adhesion to extracellular matrix or to host cell surfaces and is not itself a virulence factor. In *L. rhamnosus* strain LGG®, this could be regarded as a beneficial feature rather than a safety concern.

Overall, the *in-silico* genome screening for potential virulence factors and other genes related to pathogenicity, virulence or toxicity in *Lactobacillus rhamnosus* (LGG®) did not reveal any virulence or toxicity genes or other genes of safety concern. This was further supported by the phenotypic tests which showed the strain LGG® to be non-hemolytic and to not cause cytotoxic activity in a Vero cell assay.

Conclusion

Lactobacillus rhamnosus (LGG®) is of no safety concern with regard to pathogenicity, virulence and toxigenicity. Moreover, as no virulence genes were detected horizontal transfer of virulence to other bacteria is not considered a safety concern.

2.1.3 Phenotypic Properties

Carbohydrate Fermentation Profile

The carbohydrate fermentation profile of *L. rhamnosus, LGG®* using API 50 CHL medium, is shown in Table 1.

Control	-	Esculine	+
Glycerol	-	Salicine	+
Erythritol	-	Cellobiose	+
D-Arabinose	+	Maltose	-
L-Arabinose	-	Lactose	-
Ribose	+	Melibiose	-
D-Xylose	-	Saccharose	-
L-Xylose	-	Trehalose	+
Adonitol	-	Inuline	-
β-Methyl-xyloside	-	Melezitose	+
Galactose	+	D-Raffinose	-
D-Glucose	+	Amidon	-
D-Fructose	+	Glycogen	-
D-Mannose	+	Xylitol	-
L-Sorbose	-	β-Gentiobiose	+
Rhamnose	-	D-Turanose	-
Dulcitol	+	D-Lyxose	-
Inositol	+	D-Tagatose	+
Mannitol	+	D-Fucose	-
Sorbitol	+	L-Fucose	+
α-Methyl-D-mannoside	-	D-Arabitol	-
α-Methyl-D-glucoside	-	L-Arabitol	-
N-acetyl glucosamine	+	Gluconate	+
Amygdaline	+	2-keto-gluconate	-
Arbutine	+	5-keto-gluconate	-

Table 1: Carbohydrate Fermentation Profile of Lactobacillus rhamnosus LGG®

Antibiotic Resistance

Minimum inhibitory concentrations (MICs) of 9 antibiotics were determined for *L. rhamnosus, LGG®* according to the ISO 10932 | IDF 223 international standard (Table 2). These MICs were compared with the cut-off values established for *Lactobacillus rhamnosus* by the European Food Safety Authority (EFSA Journal, 2018).

	Antibiotic	**MIC in µg/ml**	**EFSA cut-off values in µg/ml[a]**
Aminoglycoside	Gentamicin	2	16
	Kanamycin	32-64	64
	Streptomycin	8	32
Tetracycline	Tetracycline	1-2	8
Macrolide	Erythromycin	0.12-0.25	1
Lincosamide	Clindamycin	1	4
Chloramphenicol	Chloramphenicol	8	4
β-lactam	Ampicillin	1	4
Glycopeptide	Vancomycin	>128	n.r.

Table 2: MIC Values for Lactobacillus rhamnosus, LGG®

n.r.: not required to be tested by EFSA. a: EFSA cut-off values for *Lactobacillus rhamnosus* as listed in 'Guidance on microorganisms used as feed additives or as production organisms', EFSA Journal 2018, 16(3):5206

The *L. rhamnosus,* LGG® strain is sensitive to most of the antibiotics tested with MIC values that are less than or equal to EFSA 2018 cut-off values for *Lactobacillus rhamnosus*. The MIC values for chloramphenicol is one two-fold dilution above the EFSA cut-off value, however, that is considered acceptable due to the technical variation of the phenotypic method as also recognized by EFSA in several published opinions.

The resistance to vancomycin is intrinsic to many *Lactobacillus* species, including *L. rhamnosus* (Billot-Klein et al. 1994; Klare et al. 2007; Kirtzalidou et al. 2011; Solieri et al. 2014).

Production of Biogenic Amines

The strain *L. rhamnosus*, LGG® was tested for production of histamine, tyramine, cadaverine and putrescin using an in-house procedure based on published methods and no production of the four biogenic amines were detected.

Production of L-lactate

L. rhamnosus, LGG® was tested for production of L- and D-lactate. The ratio between L- and D-Lactic acid was detected, and it was found that over 95% of the lactate produced was the L-enantiomer.

Inhibitory Activities

L. rhamnosus, LGG® does not produce antimicrobials relevant for use in humans and animals. The inhibitory effect of compounds produced by this strain have been investigated in several scientific papers and evaluated to be a positive trait as the inhibitory compounds were able to inhibit human pathogenic bacteria such as *Salmonella enterica* Serovar Typhimurium and *Listeria monocytogenes* (Oliveira et al. 2017; Marianelli et al. 2010). The inhibitory effect against *Salmonella enterica* Serovar Typhimurium has been found to depend on pH, lactic acid and a non-lactic acid molecule leading to full inhibitory effect at low pH (Marianelli et al. 2010).

Another scientific publication found a putative prebacteriocin belonging to Enterocin A (a class II bacteriocin) in *L. rhamnosus* strains including L. rhamnosus, LGG® (Oliveira et al. 2017). The gene was also found in all 22 *L. rhamnosus* genomes present in the NCBI NR database. Bacteriocins are compounds commonly found in *Lactobacillus* strains, but it is mainly their inhibitory effect against pathogenic bacteria that has been studied. Umu *et al.* (2016) investigated the potential of class II bacteriocins to modify the gut microbiota of mice and found that the main structure of the gut bacterial composition was largely unaffected and lower taxonomic groups were only transiently affected.

Laursen *et al.*, 2017, BMC Microbiol 17:175 investigated the effects of a 6 month placebo-controlled intervention with *Bifidobacterium* (BB-12®) and *L. rhamnosus* (LGG®) on gut microbiota composition and diversity in more than 200 Danish infants and concluded that consumption of the strains during early life did not change gut microbiota community structure or diversity, despite active proliferation of the strains.

It can be concluded that the inhibitory effect of *Lactobacillus rhamnosus, LGG®* on pathogens (*Salmonella* Typhimurium and *Listeria monocytogenes*) is caused by lactic acid and potentially bacteriocin class II compounds commonly found in lactic acid bacteria. The production of these inhibitory compounds does not affect the main commensal bacterial groups in the gut.

2.2 Method of Manufacture

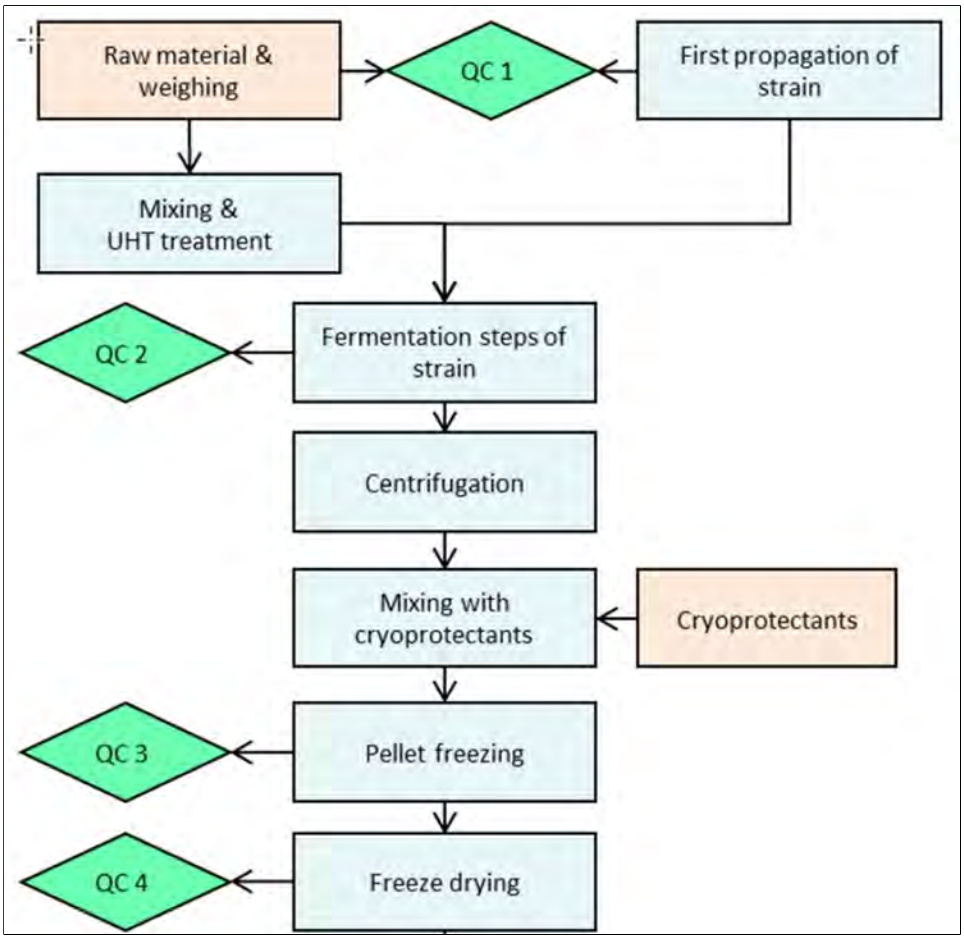
2.2.1 Cell Banking System

L. rhamnosus, LGG® cultures are maintained in the Chr. Hansen Culture Collection which is operated according to written procedures. The storage conditions employed have proven to ensure both genetic and physiological stability. The strain identification and DNA fingerprint serve as reference for the Cell Banking System. The Cell Banking System consists of a Master Cell Bank (MCB) and a Working Cell Bank (WCB). Each MCB and WCB vial is labeled with an internal culture collection number and a batch number. In order to reduce the risk of genetic drift and microbial contamination, as few propagations as possible are done when using the Working Cell Bank materials. The WCB is used as starting material for the production process.

2.2.2 Manufacturing Process

L. rhamnosus, LGG® is manufactured in compliance with FDA's current Good Manufacturing Practice (21 CFR Parts 110 and 117) and Food Safety System Certification 22000. A general outline of the manufacturing process for *L. rhamnosus, LGG®* is illustrated in the flow chart in Figure 1.

Figure 1: Flow Diagram of Lactobacillus rhamnosus, LGG® Manufacturing Process



The individual production steps are as follows:

1. *Production of media for fermentation*. The media ingredients used in the manufacturing process are primarily carbohydrates, amino acids, vitamins and minerals that are safe and suitable for human consumption.

2. *Allergens in fermentation media and raw materials*. Chr. Hansen produces LGG® products in several different forms. Due to this, there may be different formulations for different products. Milk allergen is present in both the fermentation media and finished product ingredients for some forms of LGG®. Additionally, Chr. Hansen produces dairy-free products which contain no allergens in either the fermentation media or finished product ingredients. For

any products containing allergens in the finished product, Chr. Hansen declares the allergen's presence per (section 403(w)) of the FD&C Act.

3. *Inoculation and fermentation*. From Chr. Hansen's Culture Collection, *L. rhamnosus, LGG®* working cell bank (inoculation culture) is propagated throughout different production steps. This includes the first propagation from a small vial followed by a number of fermentation processes using the above-mentioned media for fermentation. Upon completion of the fermentation processes the bacterial cells are harvested and proceed to the concentration step.

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

5. *Freezing into pellets.* The bacterial cell suspension mixture is frozen into pellets.

6. *Freeze-drying.* The frozen pellets are lyophilized resulting in very low water activity and ensuring stability of the culture. The freeze-dried granules may be ground to a powder and blended with excipients to a standardized cell count and sold as an individual product. The powder may also be blended with other strains and excipients to produce new products.

2.3 Analytical Program and Product Specifications

Production batches of *L. rhamnosus, LGG®* are thoroughly tested throughout the production process as described below by identification, viability and Quality Program:

1. *Strain characterization.* The strain is characterized by colony and cell morphology. The strain is identified according to the current recognized and accepted taxonomy by appropriate molecular testing techniques. During strain characterization, other valuable characteristics are studied such as temperature tolerance, antibiotic resistance profile, bile sensitivity, immunology and salt tolerance. Genotypically, the strain is characterized by DNA fingerprinting and plasmid content.

2. *Identification of the strain.* An unambiguous identification test is used to confirm the identity of the strain used by Chr. Hansen before fermentation. The method used is a DNA fingerprinting by pulse-field gel electrophoresis (PFGE).

3. *Viability (Total Cell Count (CFU)).* Viability of the strain is measured as colony forming units per gram (CFU/g) of individual lyophilized bulk product, blended and finished products.

4. *Microbial purity.* The microbial purity of the product is determined in accordance with the product release specification criteria (Table 3).

5. *Quality Program.* Chr. Hansen's extensive Quality Program includes a FSSC 22000 standard and hygienic monitoring program. The Quality Program serves to verify the control of the production facility and includes testing surfaces of process equipment and air quality to document the cleanliness of production as well as analyzing total aerobic microbial count, and coliform bacteria.

6. *Allergen Control.* Chr. Hansen controls all allergens listed in EU Labeling Regulation 1169/2011 and the US Food Allergen Labeling and Consumer Protection Act of 2004. Chr. Hansen also communicates the allergen status of our products in accordance with these two regulations. Allergen control is managed via our GMP and HACCP programs that are FSSC 22000 certified at all of our production sites. Allergen communication is managed via our Quality Management and HACCP programs that are ISO 22000 certified in our head office, R&D, and Support functions. See attached statement regarding our allergen management program.

7. *Release of the product.* All finished products are tested and released according to a product release specification (Table 3) to guarantee the identity, total count, and purity of the microorganisms.

Table 3: Release Specifications for Lactobacillus rhamnosus, LGG®

Criterion	Specification
Viable cell count	≥ $5x10^{11}$ cfu/g
Non-lactic cell count	< 500 cfu/g
Enterococci	< 100 cfu/g
Enterobacteriaceae	< 10 cfu/g
Staphylococcus (coagulase +)	< 10 cfu/g
Salmonella	absent
Listeria	absent
Molds and Yeast	< 10 cfu/g

2.4 Stability

An in-house, two-year stability study was conducted for *L. rhamnosus, LGG®* concentrate. The study included analysis of long-term storage stability at -20°C and 5°C as well as at accelerated storage condition (25°C/ 60% Relative Humidity) to simulate short term shipping and handling conditions. Two commercial batches (3277415 and 3255756) of *L. rhamnosus,* LGG® concentrate, packed in aluminum foil pouches, were tested. The test parameters included total cell count (Table 4) and water activity (Table 5).

Table 4: Total Cell Count Results of Lactobacillus rhamnosus LGG® Stability Study

Months	CFU/gram		
	´-20°C	5°C	25°C/60%RH
0	3.5E+10	3.5E+10	3.5E+10
3	NA	4.0E+10	3.0E+10
6	3.0E+10	3.4E+10	3.1E+10
12	3.6E+10	3.4E+10	NA
24	3.2E+10	NA	NA
25	NA	3.2E+10	NA

Table 5: Water Activity Results of Lactobacillus rhamnosus LGG®

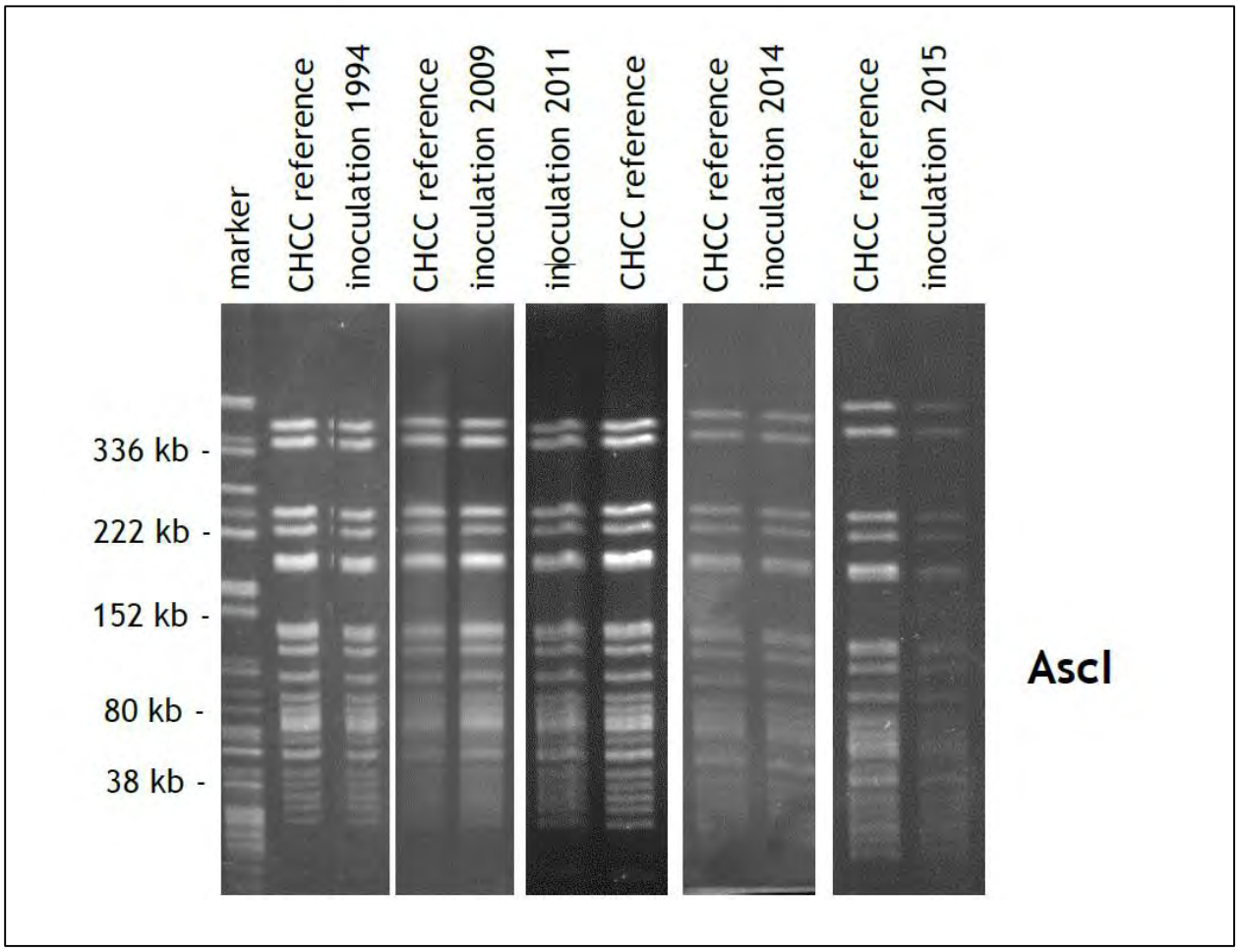
Months	Water activity		
	-20°C	5°C	25°C/60%RH
0	0.05	0.05	0.05
3	NA	0.05	0.05
6	0.06	0.06	0.06
12	0.06	0.06	NA
24	0.06	NA	NA
25	NA	0.07	NA

The data obtained demonstrates that *L. rhamnosus, LGG®* is stable for up to 24 months of storage at 5°C and -20°C. For all tested storage conditions, the water activity remained constant throughout the study. The study conclusion: stability trials conducted at accelerated storage condition (25°C/60%RH) indicated that *L. rhamnosus, LGG®* can be handled and shipped at room temperature.

The genomic stability of *L. rhamnosus, LGG®* following manufacturing process and exposure to various conditions of environmental storage in infant formula powder was described in GRN 231 (Mead Johnson, 2007). Using pulse-field gel electrophoresis (PFGE) method, a comparison of *L. rhamnosus, LGG®* cultures before and after manufacturing and storage showed that the genome of *L. rhamnosus, LGG®* is stable under normal conditions of processing and storage in infant formula products.

The genomic stability of *L. rhamnosus, LGG®* during long-term storage in Chr. Hansen Culture Collection (CHCC) was demonstrated by comparing the DNA fingerprints of reference stock material from 1994 and inoculation materials produced in 1994, 2009, 2011, 2014, and 2015. The DNA fingerprints (obtained with PFGE) showed identical patterns (Figure 2), further demonstrating genome stability and the value of highly controlled storage and production.

Figure 2: Fingerprints Profiles of Lactobacillus rhamnosus LGG® Reference Stock and Inoculation Materials



Part 3. Dietary Exposure

L. rhamnosus, LGG® is intended to be used as a microbial ingredient in conventional foods and non-exempt infant formula at levels consistent with current good manufacturing practices (cGMPs). It is intended to be consumed by the general population as well as term infants. Intended applications include but are not limited to the following: milk and dairy products such as yogurt and other fermented milk products; dairy alternatives (plant-based (oat, soy, almond, coconut, pea, etc.) fermented milk and yogurt products); beverages such as juice and protein shakes; shelf-stable products such as bars (granola, protein, meal replacement bars), confectionery (gummy candy, hard candy, soft chew candy, chewing gum, coatings), breakfast cereals (RTE and hot), and non-exempt infant formula (including cow-milk, soy, and protein hydrolysate based formulas). The maximum intended level of use is 10^8 cfu/ serving for conventional foods, and 10^8 cfu/g for infant formula.

In several products, *L. rhamnosus, LGG®* is expected to be present at concentration of 10^8 to 10^{10} cfu/serving at the time of consumption. The maximum ingestion of *L. rhamnosus, LGG®* through conventional foods is likely to be less than 10^{11} cfu/day based on the assumption that the average consumption of a healthy individual is approximately 20 servings of all combined food per day. A consumer would have to consume 100 servings of foods supplemented with *L. rhamnosus, LGG®* per day to ingest 10^{11} cfu of this strain. This concentration is well within the levels ($2x10^{12}$ cfu/day and $5.6x10^{11}$ cfu/day) that have been tested to be safe in numerous clinical trials involving children and adults (Lawrence et al. 2005).

Powdered term infant formulas will contain 10^8 cfu B *L. rhamnosus, LGG®*/g to produce an intended target intake level of 10^9 - 10^{10} cfu *L. rhamnosus, LGG®*/day. Infant formulas in the US market typically provide 0.67 kcal/ml (20 kcal/fl oz) (Martinez and Ballew, 2011). Assuming that these formulas are the sole source of nutrition, reconstituted at 14.1 g/100 ml with a caloric density of 0.67 kcal/ml, and the caloric requirements of one month-old and a six month-old infant are 472 kcal/day and 645 kcal/day (Institute of Medicine (US) Panel on Macronutrients and Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 2005), the addition of 10^8 cfu *L. rhamnosus, LGG®*/g infant formula will result in intakes of 9.9×10^9 and 1.35×10^{10} cfu *L. rhamnosus, LGG®*/day. These levels are consistent with intake levels reported in other GRAS notifications where *B. lactis* Bb12, *Streptococcus thermophilus* Th4, *L. reuteri* DSM 17938, *B. breve* M-16V are used in infant formulas (GRN 49; GRN 410; GRN 454, 455).

Part 4. Self-limiting Levels of Use

Lactobacillus rhamnosus, LGG® does not have any self-limiting use levels under the conditions of use described in this GRAS notification, other than it is restricted to applications that can sustain living *Lactobacillus rhamnosus, LGG®* for the intended level throughout the shelf life of the product.

Part 5. Experience Based on Common Use in Food Before 1958

The basis for this GRAS conclusion for *Lactobacillus rhamnosus, LGG®* is based on scientific procedures and not based on common use in food before 1958.

Part 6. Narrative

6.1 History of Safe Use and Recognition of Safety by Regulatory Authorities

The first commercial products with *L. rhamnosus, LGG®* were launched in Finland in 1990. Since then, *L. rhamnosus, LGG®* has been incorporated in a variety of product applications, including yogurt, fermented milk, pasteurized (uncultured) milk, semi hard cheese, and a few milk-free products such as juice drink and food supplements in the form of capsules, tables, and sachets (Saxelin & Kajander, 2009).

The species *Lactobacillus rhamnosus* has been evaluated by the European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ) and found to be suited for the Qualified Presumption of Safety (QPS) status since 2007. The QPS concept was developed in 2007 to provide a harmonized generic pre-evaluation to support safety risk assessments of microorganisms intentionally introduced into the food chain. The identity, body of knowledge, safety concerns and antimicrobial resistance of valid taxonomic units were assessed. The QPS status is given if the taxonomic group does not raise safety concerns or, if safety concerns exist, can be defined and excluded. The list of QPS recommended biological agents is updated annually, with the latest version being released in January 2018. *Lactobacillus rhamnosus* has remained valid up to and including the latest 2018 list (EFSA BIOHAZ Panel, 2018).

The Codex Alimentarius standard for infant formula (Codex Stan 71-1981, Revision 2007) (FAO/WHO 1981) and follow-up formula (Codex Stan 156-1987) (FAO/WHO 1987) allow the addition of L(+) lactic acid producing cultures in infant formula products.

Based on the strong safety and scientific profile of *L. rhamnosus, LGG®*, this bacterium has been incorporated in infant formulas in Europe since 2003 by Mead Johnson Nutrition. Through the end of 2005, this represented an estimated 8.5 million days of feeding with no adverse events being reported that could be attributed to the presence of *L. rhamnosus, LGG®*. In 2008, the U.S Food and Drug Administration responded with a no comments letter to a GRAS notice submitted by Mead Johnson & Company, that *L. rhamnosus, LGG®* is GRAS (GRN 231) as an ingredient in infant formula powder intended for consumption by term infants from the time of birth (Mead Johnson, 2007).

The safety of *L. rhamnosus, LGG®* was further evaluated using the decision tree of Pariza et. al. (2015). Based on the outcome of the decision tree for determining safety of microbial cultures for consumption by human and animals (Appendix 3), including strain characterization, genome sequencing, screening for undesirable attributes and metabolites, and experimental evidence of safety by appropriately designed safety evaluations studies. Chr. Hansen concluded that *L.*

rhamnosus, LGG® is non-pathogenic, non-toxigenic and is safe for use as a microbial ingredient in the foods and beverages listed in this notification.

6.2 Specific Safety Concerns

The following paragraph regarding safety of *L. rhamnosus, LGG®* and surveillance studies is repeated from GRN 231:

> "The safety of LGG® is supported by surveillance studies that evaluated potential increases in clinical infections with increased consumption. Such studies showed that during a nine-year period, despite a notable increase in LGG® consumption (-10-fold) in Finland, the number of infections involving *Lactobacillus* species reported to Helsinki health authorities remained at a constant background level of 10-20 cases per year (Salminen et al*.,* 2002, Saxelin et al., 1996a). Saxelin et al *(*1996a) found that over the 1989 - 1992 period, "the results did not provide evidence that any particular species or subspecies of *Lactobacillus* was the cause of the infections; no infections caused by isolates similar to [LGG®] were observed." Salminen et al. (2002) identified 11 out of a total of 48 isolates to be identical to LGG® over the 1994-2000 period but concluded that "[t]he results indicate that increased use of LGG® has not led to an increase in *Lactobacillus* bacteremia."

Cases of infection by lactic acid bacteria are extremely rare and the majority of these cases have occurred in patients with compromised immune status and/or mucosal barrier function due to underlying conditions such as heart disease, diabetes, or therapeutic treatment (*e.g*., dental surgery). Seven case reports where the use of *L. rhamnosus, LGG®* is implicated as potential source of infection were presented in GRN 231, which is incorporated by reference. Since the preparation of GRN 231 in 2008, there have been six documented cases of adverse events associated with *L. rhamnosus, LGG®* consumption.

A case reported by Vahabnezhad et al. (2013) involved a 17-year-old man with severe ulcerative colitis. Initially his symptoms were attributed to *Clostridium difficile* colitis. His symptoms persisted despite treatment of vancomycin and documented clearance of *C. difficile*. He was refractory to intravenous ethyl prednisolone but appeared to respond well to infliximab. After his initial hospitalization and diagnosis, he was managed as an outpatient with mesalamine and prednisone. After consumption of *L. rhamnosus, LGG®,* He developed high fevers and initial blood culture was positive for *Lactobacillus.* He was treated empirically with intravenous piperacillin/tazobactam and gentamicin for 5 days and defervesce by day 8 of his illness.

Using 16S rRNA sequence analysis, the isolates from the blood culture and the product containing *L. rhamnosus* LGG® were identified as *L. rhamnosus* with a 99.78% match for both strains. The phenotypic relatedness of the two *L*. *rhamnosus* isolates was determined by evaluating the profile of each strain's susceptibility and resistance to a panel

of antibiotics. Of the 13 drugs tested on the panel, all were either the same or within 1 serial dilution, indicating a high probability that these 2 strains are identical. The authors stated that "…disruption to the intestinal mucosal barrier may serve as a predisposing factor to the invasion of gastro intestinal flora such as *Lactobacillus* into the bloodstream…. making him more susceptible to translocation of the strain into the bloodstream. In addition, the immunosuppressive effects from systemic corticosteroids and a tumor necrosis factor-α antagonist such as infliximab may have also predisposed our patient to higher risk of infection, as there is a clear risk of adverse infectious outcomes associated with these medications" (Vahabnezhad et al. 2013).

A second case of *L. rhamnosus, LGG®* bacteremia associated with a patient with severe active ulcerative colitis (UC) was reported by Meini at al. (2015). The patient was a 64-year-old female affected by UC for 31 years, admitted to the hospital due to exacerbation of the disease, with fever and diarrhea that persisted for 2 months. During hospital admission, she was treated with methylprednisolone, mesalazine, and different antibiotic regimens. The patient consumed *L. rhamnosus, LGG®*, once daily at an amount of 6×10^9 cfu. The fever initially subsided, but after 13 days relapsed. The blood cultures yielded *L. rhamnosus* (confirmed by pulsed-field gel electrophoresis) along with *Candida albicans*. After administration of a new regimen of antibiotics, the fever was resolved with no more positive blood cultures. However, due to worsening of the abdominal condition, the patient underwent surgical colectomy.

Ishihara et al. (2014) reported on a case of an oral infection in a 31-year-old man diagnosed with acute monoblastic leukemia. He received induction of chemotherapy administered daily by intravenous infusion. He developed fever and extensive oral plaques and ulcers on his palate and bottom lip. Clindamycin was administered due to isolation of Gram-positive bacteria from the oral plaques. Repetitive blood cultures during his persistent fever were all negative. The patient consumed a relatively large number of dairy products on a daily basis, some of which contained *L. rhamnosus, LGG®*. Subsequent pulsed-field gel electrophoresis (PFGE) and 16S rRNA sequence analysis show that the strain isolated from the patient as identical to *L. rhamnosus, LGG®*. The oral lesions and high fever improved after his neutrophil count recovered.

In summary, all documented cases of adverse events following *L. rhamnosus, LGG®* consumption developed in subjects who had some type of underlying disease or health condition (*e.g.,* severe ulcerative colitis, acute leukemia, neonates with intrauterine growth restriction, chromosomal disorder, and extreme prematurity). Four of the six infections involved hospitalized patients who consumed *L. rhamnosus, LGG®*. The identity of *L. rhamnosus, LGG®* consumed and the clinical isolates were obtained through molecular

methods in only four of the six cases. The following summary regarding systemic infections attributable to *L. rhamnosus, LGG®* is repeated from GRN 231:

> These results establish that LGG® has the potential, in rare instances, to be an opportunistic pathogen in severely compromised subjects. Nevertheless, the extensive clinical studies involving the use of LGG® in healthy subjects and those with less severe medical conditions – and the usual absence of adverse effects of LGG® in these populations – go far towards establishing that LGG® is generally recognized as safe in these populations.

6.2.1 Gene Transfer Capability

Information regarding the identification, characterization, and conjugation experiments that showed *L. rhamnosus, LGG®* does not contain plasmids and is unable to transfer its chromosomal vancomycin resistance genes to other bacteria is contained in GRN 231 (Mead Johnson, 2007).

Since the preparation of GRN 231, the complete genome sequence of *L. rhamnosus, LGG®* has been published (Kankainen et al. 2009), and screening for antimicrobial resistance genes via genome sequencing and *in silico* analysis has been evaluated. No analog to any known vancomycin resistance gene was found, suggesting that *L. rhamnosus, LGG®* resistance to vancomycin is an inherent factor due to the structure of their cell wall. This is supported by the scientific literature as resistance to vancomycin is intrinsic to many *Lactobacillus* species, including *L. rhamnosus* (Billot-Klein et al. 1994).

6.3 Inconsistent Information

Chr. Hansen A/S is not aware of information that appears to be inconsistent with the determination of safety or general recognition of safety for the proposed intended uses of *L. rhamnosus, LGG®*.

6.4 Recognition of Safety by an Authoritative Group of Qualified Experts

The species *Lactobacillus rhamnosus* has been evaluated by the European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ) and found to be suited for the Qualified Presumption of Safety (QPS) status since 2007. *Lactobacillus rhamnosus* has remained valid up to and including the latest 2018 list (EFSA BIOHAZ Panel, 2018).

6.5 Common Knowledge Elements of GRAS Conclusion

All references used to establish this GRAS status conclusion have been published in the scientific literature, thus generally available. The intended use of *L. rhamnosus, LGG®* has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b). This safety was shown by establishing the identity and characteristics of the strain, demonstrating its freedom from pathogenic or other risk factors, and concluding that the expected exposure to *L. rhamnosus, LGG®* is without significant risk of harm. Finally, because this safety assessment satisfies the common knowledge requirement of a GRAS determination, this intended use can be considered GRAS.

6.6 Conclusion

The history of safe use of *Lactobacillus rhamnosus, LGG®* is strongly supported by a large body of published research. This strain has been incorporated in a variety of conventional food products and has been consumed in the United States and internationally by general population. All the available evidence demonstrates that there is no reason to suspect harm to healthy individuals consuming foods supplemented with *L. rhamnosus, LGG®*. We concluded that the intended use of *L. rhamnosus, LGG®* to be added as an ingredient of conventional foods consistent with current good manufacturing practice, can be considered GRAS. The basis of this conclusion are scientific procedures set forth under the U.S. Food & Drug Administration Final Rule, 81 FR 54959 and the data and information presented in this notice.

6.7 Pariza Decision Tree Evaluation

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

   YES (go to 2)

2. Has the strain genome been sequenced?

   YES (go to 3)

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

   YES (go to 4)

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

   YES (go to 5)

5. Does the strain produce antimicrobial substances?

NO (go to 6)

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

NO (go to 8a)

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

NO (isolated from a healthy human gut) (go to 13a)

13a. For strains to be used in human food: Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies?

NO (go to 14a)

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

Part 7. List of Supporting Data and Information

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From:	Arie Carpenter
To:	Morissette, Rachel
Cc:	Kate Urbain; Anna Gehrmann
Subject:	[EXTERNAL] RE: questions for GRN 001013
Date:	Friday, October 29, 2021 9:09:30 AM
Attachments:	image001.png image002.png image003.png image004.png image005.png image006.png 29OCT21 FDA response GRN 1013.pdf Allergen_Management_EN.pdf

Hi Rachel,

Please see the attached answers regarding your questions dated October 19, 2021 on GRN 1013.

Please let me know if you have any further questions.

Thanks so much,

Arie Carpenter
Principal Regulatory Affairs Specialist, Food Cultures and Enzymes
Cell: 414-544-2317 Desk: 414-777-7526
usarbr@chr-hansen.com | www.chr-hansen.com

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Tuesday, October 19, 2021 1:30 PM
To: Kate Urbain <USKAUR@chr-hansen.com>
Subject: questions for GRN 001013

Dear Kate,

Please see attached our questions for GRN 001013. Let me know if you have any questions.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

Division of Food Ingredients

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

Statement

November 20, 2018

Valid two years from date of issue

To whom it may concern

Allergen Management in Chr. Hansen

Food safety has the highest priority in Chr. Hansen; as such allergen management is one of our core programs to secure the safety of our products.

We *control* all allergens listed in EU Labeling Regulation 1169/2011 and the US Food Allergen Labeling and Consumer Protection Act of 2004. Chr. Hansen also *communicates* the allergen status of our products in accordance with these two regulations.

Allergen *control* is managed via our Good Manufacturing Practice (GMP) and HACCP programs that are FSSC 22000 certified at all our production sites. The programs include (but are not limited to):

- Segregation of all food allergens during storage and handling
- Risk assessment and control of all processes where allergens are handled
- Cross contamination control via validated/verified allergen cleaning programs
- Full traceability on all raw materials, rework and finished products

Allergen *communication* is managed via our Quality Management and HACCP programs that are ISO 22000 certified in our head office, R&D, and Support functions. The programs include (but are not limited to):

- Declaration of allergens, and confirmation of allergen management from all suppliers
- Allergen risk assessment of all raw materials and finished products
- Allergen profiles on all finished products
- Product Allergen Information sheets on all finished products

More information about Chr. Hansen's 'Quality, GMP and Food Safety principles' is available at our global homepage www.chr-hansen.com. Please refer to our site on policies and positions and open the subfolder on 'Quality & Product Safety'.

Chr. Hansen A/S -10-12 Bøge Allé - DK-2970 Hørsholm, Denmark - Phone: +45 45 74 74 74 - Fax: +45 45 74 88 88 www.chr-hansen.com

Statement

Allergens and other sensitizing substances, for example on the LEDA and ALBA lists

Chr. Hansen only control the allergens listed in the EU Labeling Regulation 1169/2011 and the US Food Allergen Labeling and Consumer Protection Act of 2004. Cross contamination from other allergens or sensitizing substances mentioned in for example the LEDA and ALBA lists is covered by our standard GMP, but with no specific cleaning programs for these allergens or substances. We can inform upon request if other allergens or sensitizing substances mentioned in for example the LEDA and ALBA lists have been used as ingredients in our finished products.

If you have any further questions, please contact your local sales representative.

Yours sincerely
Global Business Support

Chr. Hansen A/S - Food Cultures & Enzymes
Chr. Hansen Natural Colors A/S

Electronically generated, therefore not signed

Chr. Hansen A/S -10-12 Bøge Allé - DK-2970 Hørsholm, Denmark - Phone: +45 45 74 74 74 - Fax: +45 45 74 88 88 www.chr-hansen.com

Rachel Morissette, Ph.D.

Regulatory Review Scientist
FDA Center for Food Safety and
Applied Nutrition
Office of Food Additive Safety
Division of Food Ingredients

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

Telephone: +1 (414) 607 5700
www.chr-hansen.com
info@chr-hansen.com

October 29, 2021
usarbr

Chr. Hansen response to FDA's questions regarding GRN 001013

Dear Dr. Morissette,

We are happy to provide you with answers to your questions regarding GRN 001013 on *L. rhamnosus* DSM 33156. Please see the responses below.

Yours sincerely,

Arie Carpenter
Principle Regulatory Affairs Specialist

usarbr@chr-hansen.com
Mobile: 414-544-2317

1. **Please provide a statement that Chr. Hansen does not intend to use *L. rhamnosus* DSM 33156 in juices for which a standard of identity may preclude its use.**

   L. rhamnosus DSM 33156 is not intended for use in juices for which a standard of identity may preclude its use.

2. **On p. 19 of the notice, Chr. Hansen states "The maximum ingestion of *L. rhamnosus*,LGG® through conventional foods is likely to be less than 10^{11} cfu/day based on the assumption that the average consumption of a healthy individual is approximately 20 servings of all combined food per day." Please provide a citation for this statementand state whether this is a maximum consumption estimate.**

   Estimated dietary exposure described in the notice for the intended use in conventional foods is based on assumptions that an average, healthy individual consumes approximately 20 servings of food/day and that approximately 10 of those servings would contain 1 x 10^{10} cfu/serving of the notified substance.

   By calculating intake assuming that half of all servings of food per day (10 servings) contain *L. rhamnosus* DSM 33156, we are using a "worst-case-scenario" approach, as it is highly unlikely that half of the conventional food consumed in a day would contain *L. rhamnosus*, so 10^{11} cfu/day is a maximum consumption estimate.

   This is based on the following reference:

   Millen, A. E., Midthune, D., Thompson, F. E., Kipnis, V., & Subar, A. F. (2006). The National Cancer Institute Diet History Questionnaire: Validation of Pyramid. American Journal of Epidemiology, 279-288.

3. **A GRAS conclusion is based on the safety of the microorganism and does not consider any suggested benefits. On p. 8 of the notice, Chr. Hansen states "In *L. rhamnosus* strain LGG®, this could be regarded as a beneficial feature rather than a safety concern." Please provide a statement either removing this sentence or restate the sentence without discussing any potential benefits.**

   Please remove this last sentence from the dossier so that the paragraph reads as follows:

   "One hit was annotated as a fibronectin/fibrinogen-binding protein and was found in all 22 *L. rhamnosus* present in the NCBI NR database with 98.8-100% identity. Fibronectin/fibrinogen-binding proteins are involved in adhesion to extracellular matrix or to host cell surfaces and is not itself a virulence factor."

4. **Please clarify if *L. rhamnosus* DSM 33156 is manufactured with milk in the fermentation media that is not in the final product. If this is the case, please statehow the allergen (milk) is removed from the final product.**

Chr. Hansen produces *L. rhamnosus* products for use in a variety of conventional foods as well as infant formula. Due to this, there are different formulations for different products. For some formulations, milk allergen is present in both the fermentation media and finished product ingredients. We have dairy-free products as well which contain no allergens in either the fermentation media or finished product ingredients. Please see the attached statement regarding our allergen management program (Allergen_Management_EN).

5. **For the microbial specifications listed in Table 3 on p. 16 of the notice, please provide the following:**

To provide a bit of clarity on all parts of question 5, because *L. rhamnosus* products are used in a variety of consumer good applications, the testing parameters vary depending on the intended use. As an example, release criteria for use in infant formula is more strict than use in conventional foods. Below you will find examples of specifications for a *L. rhamnosus* for use in an infant application (Table 1) as well as for use in a conventional food application (Table 3).

Methods used for cell count are based on well-established principles for counting of microorganisms and the principle of analyses are similar to the enumeration methods described in USP 64 and ISO 4833-1.

Testing methods used are based on internationally recognized standard methods and modified when appropriate for optimal recovery of the target organism.

Table 1 Product Release Specifications for the Purity of Bacterial Cultures. Example of specifications intended for an infant application

Microorganism/Parameter	Criteria (CFU/g unless otherwise stated)	Frequency of Testing	Method
Cell count	>=1.7 x 10^{10}	Every Batch	Based on USP 64 and ISO 4833-1
Enterobacteriaceae	Absent/10X10g	Every Batch	ISO 21528
Bacillus cereus	<100	Every Batch	ISO 7932
Cronobacter spp	Absent/10X10g	Every Batch	ISO 22964
S. aureus*	<10	Every Batch	Ph.Eur. 2.6.13
Total Yeasts and Moulds Count	<=100	Every Batch	Ph.Eur. 2.6.12
Total Aerobic Microbial Count	<=2000	Every Batch	Ph.Eur. 2.6.12
Salmonella spp	Absent/10X10g	Every Batch	ISO 6579
Listeria monocytogenes	Absent/10X25g	Every Batch	ISO 11290-1

*** Not detected in 0.1g**

Table 2 Product test results for 5 non-consecutive batches destined for infant applications

Microorganism	Criteria (CFU/g unless otherwise stated)	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
Cell count	>1.7 x 10^{10}	2.8 x 10^{10}	2.6 x 10^{10}	3.3 x 10^{10}	2.9 x 10^{10}	3.2 x 10^{10}
Enterobacteriaceae	Absent /10X10g	Absent /10X10g	Absent /10X10g	Absent /10X10g	Absent /10X10g	Absent /10X10g
Bacillus cereus	<100 cfu/g	<100	<100	<100	<100	<100
Cronobacter spp	Absent /10X10g	Absent /10X10g	Absent /10X10g	Absent /10X10g	Absent /10X10g	Absent /10X10g
S. aureus	<10 cfu/g	<10	<10	<10	<10	<10
Total Yeasts and Moulds Count	<=100 cfu/g	<100	<100	<100	<100	<100
Total Aerobic Microbial Count	<=2000 cfu/g	<2000	<2000	<2000	<2000	<2000
Salmonella spp	Absent /10X10g	Absent/ 10X10g	Absent/ 10X10g	Absent/ 10X10g	Absent/ 10X10g	Absent/ 10X10g
Listeria monocytogenes	Absent /10X25g	Absent/ 10X25g	Absent/ 10X25g	Absent/ 10X25g	Absent/ 10X25g	Absent/ 10X25g

Table 3 Product Release Specifications for the Purity of Bacterial Cultures. Example of specifications intended for a conventional food application

Microorganism	Criteria (CFU/g unless otherwise stated)	Frequency of Testing	Method
Total cell count	>=5.0 x 10^{10}	Every Batch	ISO 4833-1
Enterobacteriaceae	<1	Every Batch	ISO 21528-2
Non lactic acid bacteria	<500	Every Batch	ISO 13559 | IDF 153
Coagulase-positive Staphylococci	<1	Every Batch	ISO 6888-1 & NMKL no 66
Yeasts and moulds	<1	Every Batch	ISO 96611 | IDF 94
Listeria monocytogenes	Absent in 25g	Per Monitoring Program	ISO 11290/ ISO 16140*
Salmonella spp	Absent in 25g	Per Monitoring Program	ISO 6579 ISO 16140*

*** VIDAS Listeria and VIDAS Salmonella are alternative methods to ISO 11290-1 and ISO 6579, respectively. The VIDAS methods are widely known and accepted in the food industry and have been validation according to ISO 16140 whereas BACGene methods are AOAC and AFNOR certified.**

Table 4 Product test results for 5 non-consecutive batches of product destined for a conventional food application

Microorganism	Criteria (CFU/g unless otherwise stated)	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
Total cell count	>=5.0 x 10^{10}	>=5.0 x 10^{10}	>=5.0 x 10^{10}	>=5.0 x 10^{10}	>=5.0 x 10^{10}	>=5.0 x 10^{10}
Enterobacteriaceae	<1	<1	<1	<1	<1	<1
Non lactic acid bacteria	<500	<500	<500	<500	<500	<500
Coagulase-positive Staphylococci	<1	<1	<1	<1	<1	<1
Yeasts and moulds	<1	<1	<1	<1	<1	<1
Listeria monocytogenes	Absent in 25g	Absent in 25g	Absent in 25g	Absent in 25g	Absent in 25g	Absent in 25g
Salmonella spp	Absent in 25g	Absent in 25g	Absent in 25g	Absent in 25g	Absent in 25g	Absent in 25g

a. **All sample sizes used for monitoring.**

Please see tables above

i. **Please note that we recommend that *Salmonella* testing be performed on sample sizes no larger than 25 g to prevent the possibility of false negatives, unless the method used is validated forlarger samples. If analysis is performed on a sample size larger than25 g, please discuss the method and how it was validated**.

100 g total of sample is used when testing *Salmonella spp.* in infant applications. That 100 g sample is split into 10 x 10g samples tested separately.

25 g of sample is used when testing for *Salmonella spp.* in conventional food applications.

b. **The analytical methods used to analyze batches for conformance with the stated specifications**.

Please see Tables 1 and 3 above.

c. **A statement indicating that the analytical methods have been validated for that particular purpose.**

All methods for have either gone through a validation or verification when applied to the food culture in question.

d. **A specification for *Cronobacter sakazakii*. Please note that we recommend that *C. sakazakii* testing be performed on sample sizes no larger than 10 g to prevent the possibility of false negatives, unless the method used is validatedfor larger samples. If analysis is performed on a sample size larger than 10 g, please discuss the method and how it was validated.**

100 g total of sample is used when testing *Cronobacter spp.* in infant applications. That 100 g sample is split into 10 x 10g samples tested separately.

e. **Data from the analyses of at least three non-consecutive batches to demonstrate that *L. rhamnosus* DSM 33156 can be manufactured to meetall specifications.**

Please see Tables 2 and 4 above.

From:	Arie Carpenter
To:	Morissette, Rachel
Cc:	Kate Urbain
Subject:	[EXTERNAL] RE: follow-up questions for GRN 1013
Date:	Tuesday, November 23, 2021 2:55:24 PM
Attachments:	image001.png image002.png image003.png image004.png image005.png image006.png 18NOV21 FDA response GRN 1013.pdf

Hi Dr. Morissette,

Thanks for the opportunity to address these questions. You can find the answers in the attached document.

If you have any further questions surrounding *L. rhamnosus* DSM 33156, or I can clarify any information, please let me know.

Thanks so much and have a great Thanksgiving!

Arie Carpenter
Principal Regulatory Affairs Specialist, Food Cultures and Enzymes
Cell: 414-544-2317 Desk: 414-777-7526
usarbr@chr-hansen.com | www.chr-hansen.com

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Thursday, November 18, 2021 9:16 AM
To: Arie Carpenter <USARBR@chr-hansen.com>
Subject: follow-up questions for GRN 1013

Hi Arie,

We have a few follow-up questions for GRN 1013.

1. Please provide phenotypic information for *L. rhamnosus* DSM 33156, including gram stain, colony morphology, and motility.
2. Please provide a specification for lead with at least three non-consecutive batch analyses or else please provide a rationale for why this heavy metal was excluded as part of the manufacturing.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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

FDA U.S. FOOD & DRUG ADMINISTRATION

I GOT MY COVID-19 VACCINATION

Rachel Morissette, Ph.D.

Regulatory Review Scientist
FDA Center for Food Safety and
Applied Nutrition
Office of Food Additive Safety
Division of Food Ingredients

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

Telephone: +1 (414) 607 5700
www.chr-hansen.com
info@chr-hansen.com

November 23, 2021
usarbr

Chr. Hansen response to FDA's questions regarding GRN 001013

Dear Dr. Morissette,

We are happy to provide you with answers to your questions regarding GRN 001013 on *L. rhamnosus* DSM 33156. Please see the responses below.

Yours sincerely,

Arie Carpenter
Principle Regulatory Affairs Specialist

usarbr@chr-hansen.com
Mobile: 414-544-2317

CHR HANSEN

Improving food & health

1. **Please provide phenotypic information for *L. rhamnosus* DSM 33156, including gram stain, colony morphology, and motility.**

L. rhamnosus DSM 33156 is a thermophilic, Gram-positive, catalase-negative, rod appearing in singles or pairs. It is facultative anaerobic, heterofermentative, non-motile, non-spore forming. Colonies form round, convex, smooth, shiny, soft, white colonies. It is able to ferment many carbohydrates such as glucose, galactose, and ribose as determined by use of Api50 CHL as shown below:

Control	-	Esculine	+
Glycerol	-	Salicine	+
Erythritol	-	Cellobiose	+
D-Arabinose	+	Maltose	-
L-Arabinose	-	Lactose	-
Ribose	+	Melibiose	-
D-Xylose	-	Saccharose	-
L-Xylose	-	Trehalose	+
Adonitol	-	Inuline	-
β-Methyl-xyloside	-	Melezitose	+
Galactose	+	D-Raffinose	-
D-Glucose	+	Amidon	-
D-Fructose	+	Glycogen	-
D-Mannose	+	Xylitol	-
L-Sorbose	-	β-Gentiobiose	+
Rhamnose	-	D-Turanose	-
Dulcitol	+	D-Lyxose	-
Inositol	+	D-Tagatose	+
Mannitol	+	D-Fucose	-
Sorbitol	+	L-Fucose	+
α-Methyl-D-mannoside	-	D-Arabitol	-
α-Methyl-D-glucoside	-	L-Arabitol	-
N-acetyl glucosamine	+	Gluconate	+
Amygdaline	+	2-keto-gluconate	-
Arbutine	+	5-keto-gluconate	-

2. **Please provide a specification for lead with at least three non-consecutive batch analyses or else please provide a rationale for why this heavy metal was excluded as part of the manufacturing.**

We set our spec at 0.05 ppm of lead taking into account the typical inclusion rate of our product in finished food applications since CODEX sets limits according to finished food applications. Our product is typically included at levels below 0.15%.

Three non-consecutive batch results are as follows:

Batch	Result	Specification
Batch 1	<0.05 mg/kg	<=0.05 mg/kg
Batch 2	<0.05 mg/kg	<=0.05 mg/kg
Batch 3	<0.05 mg/kg	<=0.05 mg/kg

From: Arie Carpenter
To: Morissette, Rachel
Cc: Kate Urbain
Subject: RE: [EXTERNAL] RE: one more question for GRN 1013
Date: Thursday, December 2, 2021 2:16:47 PM
Attachments: image001.png
image002.png
image003.png
image004.png
image005.png
image006.png
image008.png
image009.png
image010.png
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image012.png
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image018.png
image019.png
image020.png
image021.png

Hi Rachel,

Chr. Hansen confirms that the lead analysis was performed at an accredited laboratory. The analysis is an application of inductively coupled plasma mass spectrometry (ICP-MS) with Methods References ISO 13805m:2014 and ISO 17294-2:2016. These are standardized methods for analyzing trace elements in foodstuff, and the technology behind the methods are the same as in Pharmacopeias, such as USP or Ph.Eur. The test is suitable for foodstuffs by applying the method reference ISO 13805m:2014 for sample preparation of foodstuff samples for ICP-MS.

Please let me know if you had additional questions.

Thanks!

Arie Carpenter
Principal Regulatory Affairs Specialist, Food Cultures and Enzymes
Cell: 414-544-2317 Desk: 414-777-7526
usarbr@chr-hansen.com | www.chr-hansen.com

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Tuesday, November 30, 2021 10:35 AM
To: Arie Carpenter <USARBR@chr-hansen.com>

Cc: Kate Urbain <USKAUR@chr-hansen.com>
Subject: RE: [EXTERNAL] RE: one more question for GRN 1013

Great, thank you!

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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

FDA U.S. FOOD & DRUG ADMINISTRATION

I GOT MY COVID-19 VACCINATION

From: Arie Carpenter <USARBR@chr-hansen.com>
Sent: Tuesday, November 30, 2021 11:31 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Kate Urbain <USKAUR@chr-hansen.com>
Subject: [EXTERNAL] RE: one more question for GRN 1013

Hey Rachel,

Thanks so much and yes, I got your email.

Our heavy metal testing is performed by an external ISO 17025 certified lab in Europe.

I am working on due diligence and just waiting for confirmation from that lab.

As soon as I get it, I will respond to you.

Thanks so much for your patience!

Arie

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Tuesday, November 30, 2021 9:32 AM
To: Arie Carpenter <USARBR@chr-hansen.com>
Cc: Kate Urbain <USKAUR@chr-hansen.com>
Subject: RE: one more question for GRN 1013

Dear Arie,

I just wanted to follow-up that you received my email from last week.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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

From: Morissette, Rachel
Sent: Wednesday, November 24, 2021 9:17 AM
To: Arie Carpenter <USARBR@chr-hansen.com>
Cc: Kate Urbain <USKAUR@chr-hansen.com>
Subject: one more question for GRN 1013

Hi Arie,

One more question.

Please cite the analytical method used for the lead batch analyses and provide a statement that the method has been validated its purpose.

Thank you.

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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

From: Arie Carpenter <USARBR@chr-hansen.com>
Sent: Tuesday, November 23, 2021 2:55 PM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Kate Urbain <USKAUR@chr-hansen.com>
Subject: [EXTERNAL] RE: follow-up questions for GRN 1013

Hi Dr. Morissette,

Thanks for the opportunity to address these questions. You can find the answers in the attached document.

If you have any further questions surrounding *L. rhamnosus* DSM 33156, or I can clarify any information, please let me know.

Thanks so much and have a great Thanksgiving!

Arie Carpenter
Principal Regulatory Affairs Specialist, Food Cultures and Enzymes
Cell: 414-544-2317 Desk: 414-777-7526
usarbr@chr-hansen.com | www.chr-hansen.com

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Thursday, November 18, 2021 9:16 AM
To: Arie Carpenter <USARBR@chr-hansen.com>
Subject: follow-up questions for GRN 1013

Hi Arie,

We have a few follow-up questions for GRN 1013.

1. Please provide phenotypic information for *L. rhamnosus* DSM 33156, including gram stain, colony morphology, and motility.
2. Please provide a specification for lead with at least three non-consecutive batch analyses or else please provide a rationale for why this heavy metal was excluded as part of the manufacturing.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

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