CHR. HANSEN
Improving food & health

1189

January 31, 2019

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

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

Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Office of Food Additive Safety:

In accordance with the guidance issued by the Food and Drug Administration (FDA) under 21 81 Fed. Reg. 54960 (Aug. 17, 2016), Chr. Hansen hereby provides notice of a claim that the use of *Lactobacillus rhamnosus* LGG® in conventional foods as described in the enclosed notification is Generally Recognized As Safe (GRAS) based on scientific procedures.

The enclosed triplicate copies of the submission each include form FDA 3667, a comprehensive GRAS assessment, and appendix documents in support of the assessment, for your consideration.

Please feel free to contact me directly to discuss any aspects of this submission.

Sincerely,

(b) (6)

Sarah F. Kraak-Ripple
Regulatory Affairs Manager
Human Health – North America
Chr. Hansen, Inc.

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

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

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000845	2/13/2019
ESTIMATED DAILY INTAKE	**INTENDED USE FOR INTERNET**
NAME FOR INTERNET	
KEYWORDS	

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

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

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

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

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

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

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Sarah F. Kraak-Ripple	Regulatory Affairs Manager

Organization *(if applicable)*: Chr. Hansen, Inc.

Mailing Address *(number and street)*: 9015 West Maple Street

City	State or Province	Zip Code/Postal Code	Country
Milwaukee	Wisconsin	53214	United States of America

Telephone Number	Fax Number	E-Mail Address
414-614-9348	414-607-5959	ussakr@chr-hansen.com

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

Name of Contact Person	Position or Title

Organization *(if applicable)*:

Mailing Address *(number and street)*:

City	State or Province	Zip Code/Postal Code	Country

Telephone Number	Fax Number	E-Mail Address

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

Lactobacillus rhamnosus LGG®

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

☐ Electronic Submission Gateway

☐ Electronic files on physical media

☒ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes 3

Total number of pages

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

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

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

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

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

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

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

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

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

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

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

☐ No

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

☐ Yes, a redacted copy of the complete submission

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

☐ No

SECTION D – INTENDED USE

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

L. rhamnosus LGG is intended to be used as an ingredient in conventional foods at levels consistent with current good manufacturing practices. It is intended to be consumed by the general population. Intended applications included but are not limited to; 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). The intended level of use is between 10^8 to 10^{10} cfu/serving with an initial load of up to 10^{11} cfu/serving.

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

(Check one)

☐ Yes ☒ No

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

(Check one)

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

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

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

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

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

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

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

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

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

Other Information

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

☒ Yes ☐ No

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

☒ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Sarah F. Kraak-Ripple

(name of notifier)

has concluded that the intended use(s) of Lactobacillus rhamnosus LGG®

(name of notified substance)

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

2. Sarah F. Kraak-Ripple *(name of notifier)* agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

9015 West Maple Street, Milwaukee, WI 53214

(address of notifier or other location)

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

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
(b) (6)	Sarah F. Kraak-Ripple, Regulatory Affairs Manager, HHNA	01/31/2019

SECTION G – LIST OF ATTACHMENTS

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

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
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Add Continuation Page

OMB Statement: Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, PRAStaff@fda.hhs.gov. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

Generally Recognized as Safe (GRAS) Determination for the Intended Use of *Lactobacillus rhamnosus* LGG®

Prepared by
Chr. Hansen A/S
Denmark

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Appendices

Part 1. Signed Statements and Certification

1.1 Name and Address of Notifier

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

1.2 Name of Notified Substance

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

LGG was isolated by Drs. Gorbach and Goldin of Tufts University in 1985, patented, and deposited to American Type Culture Collection (ATCC, number 53103). In 1987, Valio, Ltd. (Valio) of Helsinki, Finland was granted an exclusive license to manufacture, market, and distribute LGG. After manufacturing LGG for a number of years for Valio for various intended uses including for application in infant formula (as specified in additional correspondence by Mead Johnson Nutrition to FDA regarding GRN 231 on August 1, 2007), Chr. Hansen A/S acquired LGG from Valio in 2016. This GRAS notification is for LGG® manufactured by Chr. Hansen A/S under the trademark *Lactobacillus rhamnosus* LGG®.

1.3 Intended Conditions of Use

L. rhamnosus LGG® is intended to be used as an ingredient in conventional foods at levels consistent with current good manufacturing practices (cGMPs). It is intended to be consumed by the general population. 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).

The intended level of use in each category is between 10^8 to 10^{10} cfu/ serving throughout the shelf life of the products. The initial addition level of *L. rhamnosus* LGG® in the products may be as high as 10^{11} cfu/ serving to allow for loss of viability over time.

1.4 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).

1.5 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 condition of intended use.

1.6 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

1.7 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.

1.8 Certification

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

1.9 FSIS Statement

Not applicable

1.10 Name, Position, and Signature of Notifier

(b) (6)

1/31/19

Sarah F. Kraak-Ripple
Regulatory Affairs Manager
Human Health – North America
Chr. Hansen, Inc.

Date

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® was isolated from a fecal sample from a healthy human adult by Drs. Gorbach and Goldin of Tufts University in 1985, and deposited to American Type Culture Collection (ATCC, number 53103). The strain has been used worldwide since 1990 as an ingredient in food and dietary supplements.

Analysis of the strain's 16S rDNA sequence revealed that the strain is identical to the sequence of the type strain of *Lactobacillus rhamnosus* (GenBank acc. No. D16552). The colony morphology is round with smooth and shiny surface, convex elevation. The appearance on MRS medium after anaerobic incubation for 3 days at 37°C is white and soft consistency. Microscopically, the strain is Gram-positive non-motile single rods or pairs. The strain shows negative catalase reaction.

2.1.2 Genome Sequencing and Annotation

In 2009, the complete 'closed genome' of *L. rhamnosus*, LGG® was sequenced, annotated, and published by Kankainen et al. (2009). The genome sequences have been deposited in the European Molecular Biology Laboratory (EMBL) database under accession number FM179322 (GenBank accession number NC013198). The complete 'closed genome' sequence of *L. rhamnosus*, LGG® consists of a single circular chromosome of 3.01 million base pairs (Mbp) with a GC content of 46.7% and with no plasmids.

The strain LGG® was also sequenced in-house at Chr. Hansen by purifying total DNA, Illumina and assembly by use of published methods (Agersø et al., 2018, Appl. Environ. Microbiol. 84: ahead of print). The obtained 'draft genome' consisted of 71 contigs with a total contig length of 2.92 Mbp and a GC content of 46.7%.

For the assessment the 'closed genome' was used and the results were verified by use of the 'draft genome'. Both genomes were subjected to annotation using published methods. The LGG® 'closed genome' and the 'draft genome' contained 2,978 coding sequences (CDS) and 72 RNAs and 2,766 CDSs and 53 RNAs, respectively. The number of CDSs on both genomes were comparable to *L. 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 focused 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 for and the one, two-fold over the EFSA cutoff value for chloramphenicol 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 18 *L. rhamnosus* genomes present in the NCBI NR database. Two hits differed by being present in only six of the 17 *L. rhamnosus* genomes. The two CDSs were located adjacent to each other and were both annotated as UDP-N-acetylglucosamine 2-epimerase, an enzyme that converts UDP-N-acetyl-D-glucosamine to UDP-N-acetyl-D-mannosamine. This epimerase is also involved in the synthesis of the capsule precursor UDP-ManNAcA. The fact that the gene is not present in all *L. rhamnosus* strains may explain why some *L. rhamnosus* strains are able to form polysaccharides and aggregate whereas others are unable to perform this function (Polak et al., 2014). The genes in *L. rhamnosus* LGG® were only half the size of the epimerase in the other *L. rhamnosus* genomes so it may not be functional in *L. rhamnosus* LGG®. In any case, the gene is a housekeeping gene of no safety concern.

One hit was annotated as a fibronectin/fibrinogen-binding protein and was found in all 17 *L. rhamnosus* present in the NCBI NR database with 98-100% identity. Fibronectin/fibrinogen-binding proteins are involved in adhesion to extracellular matrix or to host cell surfaces and are not themselves a virulence factor. In *L. rhamnosus* strain LGG®, this could be regarded as a probiotic feature rather than a safety issue.

In addition to *in silico* genome screening, phenotypic tests for cytotoxicity and hemolysis were also performed. Results of these phenotypic tests showed that *L. rhamnosus* LGG® did not cause cytotoxic activity in a Vero cell assay and the strain is non-hemolytic.

In conclusion, the *in-silico* genome screening for potential virulence factors and other genes related to pathogenicity, virulence or toxicity in *L. rhamnosus*, LGG® did not reveal any virulence or toxicity genes or other genes of 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.

Table 1. Carbohydrate Fermentation Profile of *Lactobacillus rhamnosus* LGG®

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	-

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).

Table 2: MIC Values for *Lactobacillus rhamnosus* LGG®

	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.

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; Fayol-Messaoudi et al. 2005).

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 17 *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. Umo 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.

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 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

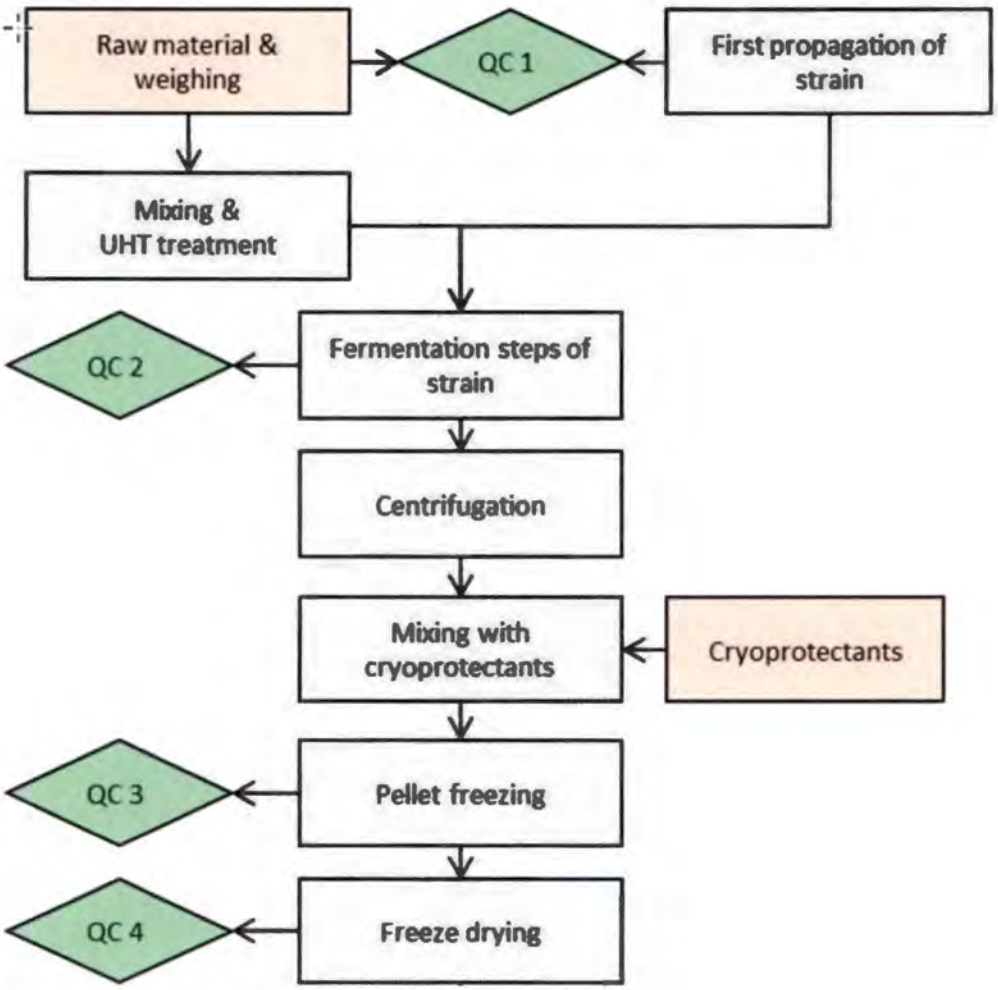
2.2.1 Cell Banking System

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. *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.

3. *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.

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

5. *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 before it is filled into the appropriate product forms such as capsules, tablets or sticks.

2.3 Analytical Program and Product Specifications

L. rhamnosus LGG® Product Description and Customer Specification sheet is attached in Appendix 1.

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.

7. *Release of the product.* All products are tested and released according to a product release specification (Table 3) to guarantee the identity, total count, and purity of the microorganisms. Certificates of Analysis of three non-consecutive batches of *L. rhamnosus* LGG® are in the Appendix 2.

Table 3: Release Specifications for *Lactobacillus rhamnosus* LGG®

Criterion	Specification	Method
Description	Fine powder	
Color	White to light beige	
Odor	Representative	
Taste	Representative	
Viable Cell Count	≥ $5x10^{11}$ CFU/g	ISO 7889/IDF117
Microbiological		
Non-Lactic Cell Count	< 500 CFU/g	ISO 13559/IDF 153:2002
Enterococci	<100 CFU/g	NMKL no 68. 2011
Enterobacteriaceae	<10 CFU/g	SOP-03912
Staphylococcus (coagulase +)	<10 CFU/g	SOP-04746
Salmonella	absent	AOAC 2004,03
Listeria	absent	AOAC 2004.06.2008
Molds and Yeast	<10 CFU/g	SOP-02839

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® Stability Study

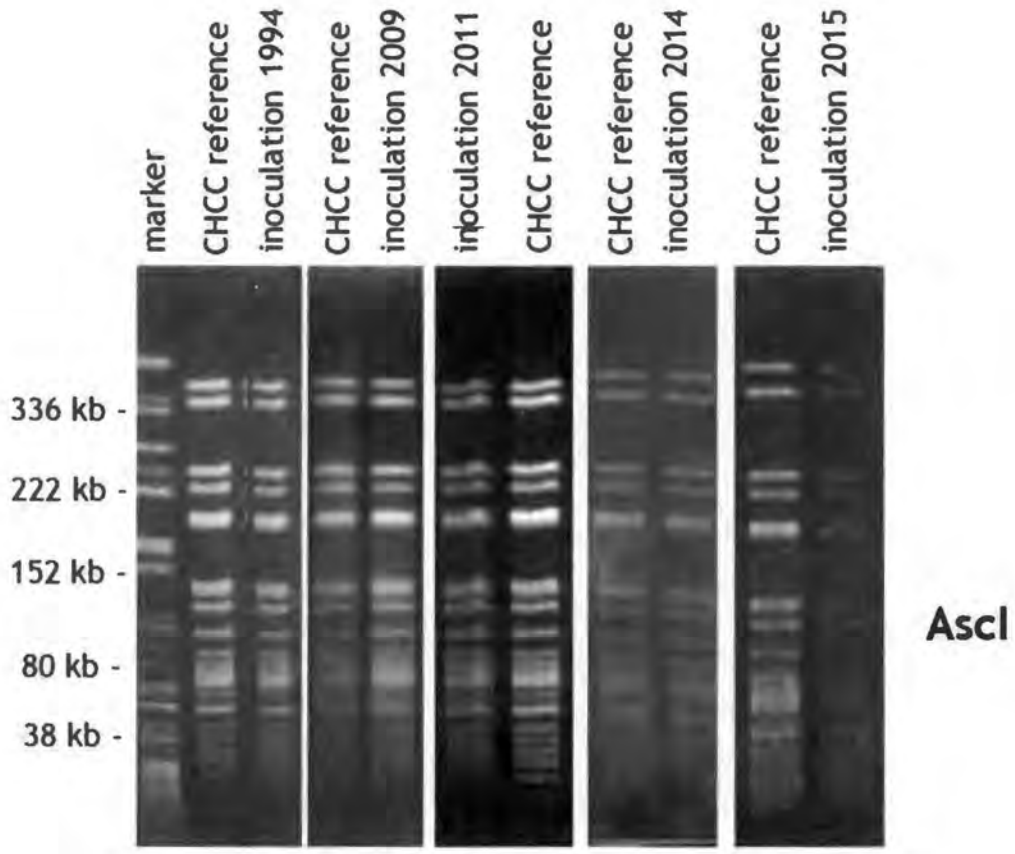
Months	Water activity		
	-20°C	5°C	25°C/60%RH
0	0.05	0.05	0.05
3	NA	0.05	0.05
6	0.06	0.06	0.06
12	0.06	0.06	NA
24	0.06	NA	NA
25	NA	0.07	NA

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

Lactobacillus rhamnosus LGG® is intended to be added as an ingredient in a variety of conventional foods with current good manufacturing practice (cGMP). It is intended to be consumed by the general population. Intended applications include but are not limited to the following: milk and dairy products, such as yogurt and other fermented milk products; dairy alternatives (fermented oat milk, fermented soy milk, fermented almond milk, fermented coconut milk); beverages such as juice and protein shakes; shelf-stable products such as bars (granola bars, protein bars, meal replacement bars); confectionery (gummy candy, hard candy, soft chew candy, chewing gum, coatings); breakfast cereals (RTE and hot).

The intended level of use in each category is between 10^8 to 10^{10} cfu/ serving throughout the shelf life of the products. The initial addition level of *L. rhamnosus,* LGG® in the products may be as high as 10^{11} cfu/ serving to allow for loss of viability over time.

The number of viable *L. rhamnosus* LGG® in conventional foods and in supplement forms will decline over the shelf-life since LGG will not proliferate in the products to which it is added. 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 (Basu et al. 2009; Lawrence et al. 2005).

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 probiotic 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 231as 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 microorganism in the foods and beverages listed in this notification.

6.2 Clinical Studies Evaluating Safety and Benefit of *L. rhamnosus*, LGG® Since 2008

In GRN 231 (Mead Johnson, 2007), safety of *L. rhamnosus* LGG® was discussed. *In vitro* studies, toxicity and dosing studies in animals, and human studies involving healthy adults, young children, term and preterm infants, as well as immunocompromised populations were presented. In the following sections, relevant studies in humans published since the preparation of GRN 231 in 2008 are discussed.

6.2.1 Studies in Infants

The studies discussed in this section are summarized in Table 6.

A randomized, prospective, double-blind, placebo-controlled study by Scalabrin et al. (2009) enrolled 289 healthy term infants in order to evaluate the effect of an extensively hydrolyzed or partially hydrolyzed formula supplemented with *L. rhamnosus*, LGG® ($1x10^8$ cfu/ gram of formula powder) on growth and tolerance from 14 to 120 days of age. The secondary objectives were to analyze the incidence of allergies, allergic sensitizations, infections, and antibody response to routine vaccinations in a subset of infants who received study formula up to 150 days of age. In addition, fatty acids (FAs) were analyzed to determine the effect of *L. rhamnosus* LGG® on plasma and red blood cells FAs profiles.

A total of 210 infants completed the study. In the total study population, 25 participants discontinued because of formula intolerance; the most common symptoms were fussiness (n=14), vomiting (n=10), and gas (n=10). The most common reason for discontinuation unrelated to study formula was parental decision (n=17). Incidence of adverse events was similar between groups; the most common were upper respiratory infection (27%), nasal congestion (21%), gas (17%), otitis media (16%), and diaper rash (15%). The only significant difference in adverse events was excessive crying: 4% in the partially hydrolyzed formula (PHF)+LGG group versus none in the extensively hydrolyzed formula (EHF) and EHF+LGG (P=0.036). Post hoc analysis demonstrated no significant group differences in incidence of adverse events categorized as infection-related or allergy-related. Serious adverse events were unrelated to study formula as assessed by study physician, except for one infant in the PHF+LGG group considered intolerant to study formula and one infant in the EHF+LGG group with gastroesophageal reflux, whose relationship with study formula was undetermined. The authors concluded that in this study all formulas supplemented with *L. rhamnosus* LGG® supported normal growth and were well tolerated. The plasma and red blood cell FAs composition was unaffected by *L. rhamnosus* LGG®. Conclusions regarding the influence of *L. rhamnosus* LGG® supplementation on tolerance to allergens and response to vaccinations could not be made.

Rougé et al. (2009) enrolled 94 premature infants with birthweight <1500 g and gestational age < 32 week to evaluate the efficacy of probiotics on digestive tolerance of enteral feeding in a randomized, double-blind, placebo-controlled trial. The infants were randomized to receive either placebo (n = 49) or *L. rhamnosus* LGG® in combination with *Bifidobacterium longum* BB536 (n = 45) at a concentration of 4 x10^8 cfu of each for 14 days. The primary endpoint was the percentage of infants receiving >50% of their nutrition needs enterally on the 14th day of life.

No statistically significant difference was observed regarding the primary endpoint, however in infants who weighed >1000 g, probiotic supplementation was associated with a shortening in the time to reach full enteral feeding. There was no significant effect on the composition of intestinal microbiota, except for colonization by the probiotic strains. No unexpected adverse events were observed during the course of the study and no difference in the incidence of sepsis between the probiotic and placebo groups, including the subgroup that weighed <1000 g. *L. rhamnosus* LGG® and BB536 were not detected in any blood culture in the relatively small population studied.

In a randomized, blinded, placebo-controlled trial that included 90 premature infants, Underwood et al., (2009) compared the effect of prebiotics and probiotics on weight gain, stool microbiota, and stool short chain fatty acid (SCFA) content of premature infants. The subjects were randomized into 3 groups: placebo (n = 29), *L. rhamnosus* LGG® (10x10^8 cfu) and prebiotic fructooligosaccharide (n = 30), or multispecies probiotics (*L. acidophilus, B. longum, B. bifidum, and B. infantis* at 10x10^8 cfu each) and prebiotic fructooligosaccharide (n = 31). All products were given by mouth or gavage tube for 28 days or until hospital discharge, whichever came first.

There were no significant differences in weight gain and stool SCFA between the probiotics and placebo groups. The multispecies probiotics caused significant increase in the stool content of bifidobacteria compared to placebo. The authors noted: "Our study showed no differences between groups in necrotizing enterocolitis (NEC), documented infections, or adverse outcomes but it was not powered to detect such differences".

To determine the benefits of *L. rhamnosus* LGG® in an extensively hydrolyzed casein formula (EHCF) in improving hematochezia and fecal calprotectin, Baldassarre et al. (2010) conducted a prospective, randomized, double-blind, placebo-controlled study involving 62 term infants age 1 to 10 months. The infants were divided into 2 groups: Group A (n = 30, consists of infants with recurring cow's milk allergic colitis fed a casein-based routine formula or breast milk), and Group B (n = 32, healthy infants either formula or breast-fed as comparisons). The randomization was applied to the formula-fed infants in group A. Infants received 1 of 2 commercially available EHCF, one containing *L. rhamnosus* LGG® (1.46x10^7 cfu/ 100 ml formula) and the other, not. After randomization, 12 patients in group A received EHCF with LGG® and 14 EHCF without LGG®.

At the time of enrollment, fecal calprotectin in those with hematochezia was significantly higher than in comparisons. At 4 weeks of intervention, fecal calprotectin decreased to 50% of baseline but was still significantly higher than in comparisons.

Fecal calprotectin mean decrease was significantly larger among EHCF with *L. rhamnosus* LGG® compared with EHCF without LGG®. At 4 weeks, none of the EHCF with *L. rhamnosus*, LGG® had blood in stools, and 5/14 on EHCF without *L. rhamnosus* LGG® did (P = .002). The authors concluded that "EHCF with LGG® resulted in significant improvement of hematochezia and fecal calprotectin compared with the EHCF alone." There was no discussion of any adverse effects of the treatment.

The safety and tolerability of long-lasting administration of *L. rhamnosus* LGG® to immunocompromised hosts such as very low birth weight (VLBW) preterm infants were reported by Manzoni et al. (2011) in a retrospective study over 6 years. The method utilized was clinical charts review of VLBW infants admitted over the years 2003–2008 in two large tertiary NICUs in Italy. As a standard-of-care in both NICUs all VLBW infants older than three days who had started oral feeding with either breast milk (own mother's or donors') or preterm formula were eligible for receiving probiotic supplementation. *L. rhamnosus* LGG® (3×10^9 cfu/ day) was the probiotic used in both NICUs throughout the study period of 4 to 6 weeks courses (depending on the subject). Infants were given the probiotic in a single, 1ml oral dose, diluted in the feeds, beginning on their 4th day-of-life.

During the study period, 889 VLBW infants were admitted to the two facilities, 811 of them survived until discharge. Complete data were obtained and analyzed for 743 of these 811 infants. The mean birth weight was 1056 g and the mean gestational age was 29.5 weeks. A total of 17,108 *L. rhamnosus*, LGG® doses were administered (mean 23.1/infant), 5350 clinical and surveillance cultures were performed and none of these cultures ever grew LGG® or other species of *Lactobacilli*. Among the infants given *L. rhamnosus* LGG®, 142 of 743 (19.1%) experienced at least one episode of late-onset sepsis (LOS), with 75 of them having more than one. No sepsis episode was microbiologically or clinically attributable to LGG. Fourteen cases of NEC occurred (incidence rate=1.9%), and among them 5 episodes were severe (>2b stage) NEC cases. The authors noted that the low incidence rates of NEC may be partially related to routine use of *L. rhamnosus* LGG® since birth in all VLBW infants. They concluded that routine supplementation of probiotic *L. rhamnosus* LGG® in a large 6-year VLBW Italian infant cohort proved microbiologically safe and clinically well tolerated.

Interaction of *L. rhamnosus* LGG® with skin and gut microbiota in infants with atopic dermatitis (AD) was investigated by Nermes et al. (2011). Thirty-nine infants with AD were randomized to receive extensively hydrolyzed casein formula supplemented with LGG® ($3.4\text{x}10^9$ cfu/ day; n=19) or placebo (n=20) for a 3-month period. Blood and fecal samples were collected at entry of study, and at 1 and 3 months afterwards.

The proportions of IgA- and IgM-secreting cells decreased significantly in the treated group. The proportions of $CD19^+CD27^+$ B cells increased in the probiotic-treated infants but not in the untreated. There were no significant differences in bifidobacterial species composition of the gut between the study groups. On the skin, the bacterial counts of *Bifidobacterium* genus vs. *Clostridium coccoides* in the treated and untreated infants were similar. There was no adverse event reported in the study. The authors concluded

that, "Specific probiotics may enhance gut barrier function and aid in the development of immune responses".

Al-Hosni et al. (2012) investigated the effects of supplementing *L. rhamnosus*, LGG® and *Bifidobacterium infantis* to extremely low-birth-weight (ELBW) infants on improvement of growth and feeding tolerance. One hundred and one infants with birth weight between 501 to 1000 g and ≤ 14 days of age were enrolled in the prospective, randomized, blind controlled trial. The probiotic group (n = 50) received $5x10^8$ cfu each of *L. rhamnosus* LGG® and *B. infantis* daily through enteral feeding until discharge from the neonatal intensive care units or until 34 weeks postmenstrual age (PMA). The control group (n = 31) received unsupplemented milk. Infant weight at the time of birth, feeding initiation, 28 days after feeding initiation and at 34 weeks postmenstrual age or discharge was recorded.

Although probiotic-supplemented feedings improved growth velocity in ELBW infants, there was no improvement in the percentage of infants with growth delay at 34 weeks post menstrual age. Mortality or NEC was not different between probiotic supplemented and control groups. There were no probiotic-related adverse events, and sepsis related to the organisms supplemented was not reported in any of the infants studied.

The effect of *L. rhamnosus* LGG® supplemented enteral feeding on the preterm infant microflora was investigated by Chrzanowska-Liszewska et al., (2012) in a randomized, double-blind, placebo-controlled trial. The study compared the stool of bottle fed preterm, randomized to receive *L. rhamnosus* LGG® $6x10^9$ cfu/ day (n = 21) or placebo (n = 26) with formula feeding for the duration of 8 weeks. Stool samples were collected from each enrolled infant on days 7, 21, 42. The primary end point of the study was to evaluate if supplementation of formula based enteral nutrition with *L. rhamnosus* LGG® significantly increases the amount of stool bifidogenic flora and decreases pathogenic gut colonization.

In the *L. rhamnosus* LGG® fed group, the number of lactobacillus in the stool was significantly higher on both study day 7 and study day 21, but not on day 42. There was a higher rate of positive samples for *Enterobacteriaceae*, *Enterococcus* spp., and staphylococci in the *L. rhamnosus* LGG® fed group. There was no significant difference in weight gain, mean hospital stay, or average use of antibiotics. There was no discussion of any adverse effects of *L. rhamnosus* LGG®. The authors concluded that, "probiotics may not alternate the pathological colonization of preterm infants. Further larger studies are needed, which will be able to look at the specific CFU of certain microorganisms".

In a randomized, double-blind, placebo-controlled study, Pärtty et al. (2013) evaluated the impact of prebiotic and probiotic intervention on preterm infants' well-being, crying, growth, and microbiological programming. Ninety-four premature infants, gestational age between 32 and 36 weeks and birth weight >1500 g, were enrolled and randomized to receive prebiotics (mixture of galactooligosaccharide and polydextrose 1:1, n = 31), probiotics (LGG®, n = 31), or placebo (n = 32) during the first 2 months of life. Follow-up consultations were conducted at the ages of 1, 2, 4, 6, and 12 months.

During all study visits, parents reported the infant's behavior patterns, infection and other diseases, and medication use. Adverse events were asked from the parents during all visits and fecal samples were collected at the age of 1 month.

A total of 27 of 94 infants (29%) infants were classified as excessive criers, significantly less frequently in the prebiotic and the probiotic groups than in the placebo group (19% vs 19% vs 47%, respectively; P = 0.02). The placebo group had a higher percentage of *Clostridium histolyticum* group bacteria in their stools than did the probiotic group (13.9% vs 8.9%, respectively; P = 0.05). The number of *Bifidobacterium infantis* by qPCR was found to be decreased among excessive criers compared with contented infants ($1.3x10^7$ vs $2.5x10^8$, respectively; P = 0.035). There were no other statistically significant differences in gut microbiota composition between these two groups. There were no adverse events related to either supplementation. The authors concluded that early prebiotic and probiotic supplementation may alleviate symptoms associated with crying and fussing in preterm infants.

Based on the same enrollment, levels and randomization, Luoto et al. (2014) reported the effect of probiotics and prebiotics in reducing the risk of viral respiratory tract infections (RTI) in preterm infants. They found significant lower incidence of RTIs in infants receiving prebiotics or probiotics compared with those receiving placebo. In addition, the incidence of rhinovirus-induced episodes, which comprised 80% of all RTI episodes, was found to be significantly lower in the prebiotic and probiotic groups compared with the placebo group. No differences emerged among the study groups in rhinovirus RNA load during infections, duration of rhinovirus RNA shedding, duration or severity of rhinovirus infection, or occurrence of rhinovirus RNA in asymptomatic infants. The authors noted: "The absence of adverse effects in this study cohort represents safety documentation for the use of these prebiotics and probiotics in this sensitive infant population".

In a prospective cohort study, Janvier et al. (2014) compared the incidence of necrotizing enterocolitis (NEC) and death in the NICU before and after routine administration of probiotics to very preterm infants. Two hundred ninety-four infants of <32 weeks' gestation received probiotics, and 317 infants formed the comparison group. The probiotics given were a mixture of *L. rhamnosus* LGG®, *B. breve*, *B. bifidum*, *B. infantis*, and *B. longum* at a concentration of $2x10^9$ cfu per day, starting with the first feed until the infant reaches 34 weeks.

Introduction of probiotics was associated with a reduction in NEC (from 9.8% to 5.4%, P < 0.02), a nonsignificant decrease in death (9.8% to 6.8%), and a significant reduction in the combined outcome of death or NEC (from 17% to 10.5%, P < 0.05). After adjustment for gestational age, intrauterine growth restriction, and sex, the improvements remained significant. There was no effect of probiotics on health care–associated infection.

Van Niekerk et al. (2014) compared the effect of administration of *L. rhamnosus* LGG® plus *B. infantis* on feeding tolerance and growth outcomes of HIV-exposed (but uninfected) versus HIV non-exposed very low birth weight preterm infants in a

randomized, double-blind, placebo-controlled trial. A total of 184 premature infants (74 HIV-exposed and 110 HIV non-exposed) with birth weight between ≥500 g and ≤1250 g were enrolled. The HIV-exposed group was randomized into 37 infants in the study and control group, respectively. The HIV non-exposed group was randomized into 54 study infants and 56 control infants. The study group received breast milk plus daily supplement of *L. rhamnosus* LGG® and *B. infantis* ($0.35x10^9$ cfu each), the control group received breast milk plus a placebo consisting of medium-chain triacylglycerol (MCT) oil for 28 days.

The use of probiotic supplementation did not affect growth outcomes or feeding tolerance in HIV-exposed and non-exposed VLBW infants. There was significant difference in head circumference and length in the HIV-exposed group. There were no differences in the incidence of any signs of feeding intolerance and abdominal distension between the groups.

6.2.2 Studies in Children

The studies discussed in this section are summarized in Table 7.

Evaluation of the effective dose of *L. rhamnosus,* LGG® in controlling acute watery diarrhea in children was evaluated by Basu et al. (2009). The randomized, blinded, controlled trial involved 588 children over 1 year of age with acute watery diarrhea. The subjects were randomized into 3 groups: group A (n=185), the control group, received only oral rehydration solution (ORS); group B (n=188) received ORS + LGG® containing 10^{10} cfu; and group C (n=186) received ORS + LGG® containing 10^{12} cfu (n = 186) twice daily for a minimum period of 7 days or until diarrhea stopped along with correction of dehydration.

All children were given nutritional supplementation for their age during the hospital stay, breastfed children continued to receive breastfeeds. At the earliest sign of any complication such as electrolyte imbalance, septicemia, and renal failure, the children were withdrawn from the study and treated accordingly. Patients were discharged when the diarrhea had stopped, and oral intake was adequate (as in pre-diarrhea state). Follow-up was done weekly for 4 weeks.

The initial daily frequency of diarrhea was similar in the 3 groups; significant reduction in the daily frequency was observed from fourth day onwards in groups B and C compared with group A. The average duration of diarrhea, intravenous fluid requirement and hospital stay were significantly less in both the intervention groups compared with the controls. No difference was observed in the frequency and duration of vomiting between the 3 groups. The number of patients who developed complications was small and equal in both the interventions and control groups. There was no complication documented from probiotic use during the hospital stay and during the follow-up visits. The authors concluded that both doses of *L. rhamnosus* LGG® were equally effective in decreasing the frequency and duration of diarrhea and reduction of hospital stay in patients of acute watery diarrhea.

In a randomized controlled trial involving 141 children with recurrent abdominal pain, Francavilla et al. (2010) evaluated the efficacy of *L. rhamnosus* LGG® in relieving symptoms of irritable bowel syndrome. Children received *L. rhamnosus* LGG® or placebo for 8 weeks; the primary outcome was to reduce overall pain at the end of intervention period. At entry and at end of the trial, children underwent a double-sugar intestinal permeability test.

Lactobacillus rhamnosus LGG® significantly reduced the frequency and severity of abdominal pain in children with IBS. Compared with baseline, LGG®, but not the placebo, caused a significant reduction of both frequency and severity of abdominal pain. These differences still were significant at the end of follow up. At entry, 59% of the children had abnormal results from the intestinal permeability test; LGG®, but not the placebo, determined a significant decrease in the number of patients with abnormal results from the intestinal permeability testing.

Hojsak et al. (2010a) investigated the role of *L. rhamnosus,* LGG® in preventing nosocomial gastrointestinal and respiratory tract infections at a pediatric hospital by conducting a randomized, double-blind, placebo-controlled trial of 742 hospitalized children. The patients were randomly allocated to received *L. rhamnosus* LGG® at a dose of 10^9 cfu in 100 ml of fermented milk (n = 376) or placebo that was the same fermented milk without the *L. rhamnosus* LGG® (n = 366) during their hospitalization. A pediatric resident entered all data regarding product consumption and infections or adverse effects into the patient's study chart.

The risk for gastrointestinal infections and risk for respiratory tract infections were significantly reduced in the *L. rhamnosus* LGG® group compared with the placebo group. Children in the *L. rhamnosus* LGG® group had a reduced risk for vomiting episodes, diarrheal episodes, and episodes of gastrointestinal infections that lasted more than 2 days compared with the placebo group. None of the patients had a bacterial infection. In 5 patients, rotavirus (2 patients: both in the placebo group) or norovirus (3 patients: 2 in the placebo group and 1 in the *L. rhamnosus* LGG® group) was isolated. All patients were treated symptomatically, and none required antibiotic treatment. The *L. rhamnosus* LGG® group showed lower risk for episodes of respiratory tract infection that lasted more than 3 days. All patients had upper respiratory tract infections, and only 1 patient in the placebo group also had a diagnosis of pneumonia. A bacterial cause was determined and treated with antibiotics in only 5 patients with upper respiratory tract infections (4 were from the placebo group). There was no significant difference regarding the duration of hospitalization between the 2 groups. No adverse effects were noted during study, and both products were well tolerated.

The role of *L. rhamnosus* LGG® in the prevention of gastrointestinal and respiratory tract infections in healthy children who attend day care centers was investigated by Hojsak et al. (2010b). A total of 281 children with average age between 52 – 54 months old were randomized into placebo (n = 142) or LGG® (n = 139) group. During the 3-month intervention period, *L. rhamnosus* LGG® ($1x10^9$ cfu/ day) was administered in 100 ml of fermented milk product and the placebo group received the same fermented milk product without *L. rhamnosus* LGG®. Under the supervision of the

parents or day care educators, children received either the *L. rhamnosus* LGG® preparation or placebo once daily. The children were not allowed to consume other products containing probiotics or prebiotics and parents were asked to record details of all infections that the child experienced during the study period.

Children in the *L. rhamnosus* LGG® group had a significantly reduced risk of upper respiratory tract infections (RTI), RTI lasting longer than 3 days, and a significantly lower number of days with respiratory symptoms. There was no difference in the number of children with vomiting or diarrheal episodes between the groups, and no difference in the risk of episodes of gastrointestinal infections lasting longer than 2 days. None of the children developed a bacterial infection (all stool samples were negative). The rate of absence from daycare centers due to infections was lower in the *L. rhamnosus* LGG® group compared to the placebo group. No side effects or adverse effects were noted during the study. The authors concluded that *L. rhamnosus* LGG® can be recommended as a valid measure for decreasing the risk of upper respiratory tract infections in children attending day care centers.

Ritchie et al. (2010) assessed the efficacy of *L. rhamnosus* LGG® as a probiotic therapy for acute rotavirus infectious diarrhea in Australian Aboriginal children. Seventy Aboriginal children between the ages of 4 months and 2 years admitted to the hospital with a clinical diagnosis of acute diarrheal disease were randomized to receive either placebo (n = 32) or LGG® (n = 38) at a dose of 1 capsule 3 times per day for 3 days. The *L. rhamnosus*, LGG® dose per capsule was $\geq 5x10^9$ cfu. Placebo capsule and contents were identical except the capsule contained no *L. rhamnosus* LGG®. The powder from each capsule was reconstituted in 5 mL of sterile NaCl 0.9% and given via a nasogastric tube.

Both groups showed mean improvement in the sucrose breath test after 4 days; although the *L. rhamnosus* LGG® group did not change the duration of diarrhea, total diarrhea stools, or diarrhea score compared with the placebo. There was a significant ($P<0.05$) difference in diarrhea frequency on day 2 between *L. rhamnosus* LGG® and placebo groups. The author concluded that *L. rhamnosus* LGG® did not appear to enhance short-term recovery following acute diarrheal illness in this setting. There was no adverse effect attributable to *L. rhamnosus* LGG® reported in the study.

Szachta et al. (2011) evaluated the efficacy of *L. rhamnosus* LGG® supplementation in eliminating the gastrointestinal (GI) carrier state of vancomycin-resistant enterococci (VRE) in colonized children. A total of 61 children (age 0-18 years old) diagnosed with GI carrier state of VRE completed the study in a randomized, single blind, placebo-controlled design. The treatment group (n=32) received $3x10^9$ cfu of *L. rhamnosus* LGG®/ day for 21 consecutive days. Rectal swabs for VRE and Lactobacillus spp. were collected at baseline, during supplementation at weekly intervals and 1 month after supplementation.

A significant difference in the number of children colonized with VRE between the groups was observed at 3 weeks ($P=0.002$). The VRE carrier state was lost by 20 of 32 participants in the treatment group and 7 of 29 in the control group. Increased GI

counts of *Lactobacillus spp.* was observed in children receiving *L. rhamnosus* LGG®. The authors concluded that *L. rhamnosus*, LGG® supplementation temporarily eliminates the VRE carrier state and increases gastrointestinal counts of *Lactobacillus* spp. in children versus placebo.

In a randomized, double-blind, placebo-controlled study conducted by Kumpu et al. (2012) the efficacy of *L. rhamnosus*, LGG® to reduce occurrence of respiratory illness in children attending day care was evaluated. A total of 523 children aged 2-6 years were randomized to receive either normal milk (n = 262) or the same milk with *L. rhamnosus* LGG® (n = 261, average daily dose of $1x10^8$ cfu) with three daily meals for 28 weeks. For the days off from day care (weekends, holidays and sick leaves), parents were advised to serve the milk to the child at home. Fecal samples were collected at baseline and at the end of the intervention period. Fecal recovery of *L. rhamnosus* LGG® was measured from all subjects who provided both baseline and end-of-study fecal samples (n = 119 in the LGG® group; n = 98 in placebo group).

In the *L. rhamnosus* LGG® group, LGG® fecal recovery was below the detection limit before the intervention and above the detection limit at the end of the intervention in 68% (81/119) of the subjects from whom both samples were analyzed. In the placebo group, 48% (47/98) of subjects had *L. rhamnosus* LGG® below the detection limit in both samples. These subjects (n = 128) were considered completed cases in terms of recovery of *L. rhamnosus* LGG® in fecal samples. A significant reduction in days with at least one respiratory symptom was found in a completed cases subgroup analysis based on recovery of LGG in fecal samples. There was no significant reduction in the occurrence of respiratory or gastrointestinal illness observed in the total population. Of 22 reported adverse events during the intervention (LGG® = 8, placebo = 14), 15 events were related to gastrointestinal problems such as nausea or abdominal pain (LGG® = 5, placebo = 10) and seven to skin problems such as rash (LGG® = 3, placebo = 4). None of the reported adverse events were serious.

Muraro et al. (2012) evaluated the hypo allergenicity of an extensively hydrolyzed casein formula (EHF) supplemented with *L. rhamnosus* LGG® in a randomized, double-blind, placebo-controlled crossover trial. Following a 7-day period of cow's milk protein elimination, 33 children ≤ 14 years old with documented cow's milk allergy were randomized to receive EHF or EHF supplemented with LGG® ($1x10^8$ cfu/ g powder). The EHF and EHF+LGG formulas were administered in an initial 5 - 10 ml aliquot followed by gradually increasing volumes over a maximum period of 120 min to provide a cumulative volume of 150ml. If the double-blind, placebo-controlled food challenge (DBPCFC) was negative, an open challenge (OC) with 150 – 250 ml of the EHF+LGG followed. To assess long-term tolerance and reveal any false-negative results to the challenges, all participants with negative responses to both the DBPCFC and OC consumed a minimum of 240 ml of EHF+LGG formula/day during a 7-day home feeding period.

For all participants with confirmed cow's milk allergy, the DBPCFC and open challenge were classified as negative. No serious adverse events were reported during the double-blind placebo-controlled food challenge, open challenge or the 7-day home

feeding period. The authors concluded: The extensively hydrolyzed casein formula supplemented with *L. rhamnosus* LGG® is hypoallergenic and can be recommended for infants and children allergic to cow's milk who require an alternative to formulae containing intact cow's milk protein.

Sindhu et al. (2014) reported the effect of *L. rhamnosus* LGG® on intestinal function, immune response, and clinical outcome in Indian children with cryptosporidial or rotavirus diarrhea. In the randomized, double-blind, placebo-controlled trial 124 children aged 6 months to 5 years, testing positive for rotavirus (n = 82) or *Cryptosporidium* species (n = 42) in stool were enrolled. The *L. rhamnosus* LGG® group consisted of 45 children with rotavirus diarrhea and 20 children with cryptosporidial diarrhea. None of the enrolled children received a rotavirus vaccine.

At study initiation, the baseline and clinical parameters were comparable between children receiving *L. rhamnosus* LGG® at $1x10^{10}$ CFU/daily and placebo. Supplementation with *L. rhamnosus* LGG® for 4 weeks after acute infection improved intestinal permeability in children with rotavirus and cryptosporidial diarrhea, reduced the number of subsequent diarrheal episodes, and increased IgG response in children with rotavirus diarrhea. Five children experienced serious adverse events requiring hospitalization; these were for lower respiratory infections, vulval abscess, and measles. Four were in the probiotic group, but no events were considered related to the intervention. All of the children recovered.

6.2.3 Studies in Adults and Elderly

The studies discussed in this section are summarized in Table 8.

Lawrence et al. (2005) reported results from a pilot trial of adjunctive *L. rhamnosus* LGG® for prevention of recurrent *Clostridium difficile*-associated disease (RCDAD). Fifteen adults with mean age of 77.9 years old were enrolled in the study. Eight participants received *L. rhamnosus* LGG® capsules ($5.6x10^{11}$ cfu/ day) adjunctively with anti-*C. difficile* antibiotics for the duration of antibiotic therapy and for an additional 21 days. The primary outcome was subsequent RCDAD within 60 days of completing anti-*C. difficile* antibiotic therapy.

Evidence for efficacious treatments of RCDAD is sparse. Three (37.5 %) cases of RCDAD were observed in the LGG arm and one (14.3 %) in the placebo arm. The median duration of the anti-*C. difficile* antibiotic regimens was 18.0 days and similar between arms. There were no *Lactobacillus* infections, LGG-related serious adverse events or intolerances leading to study discontinuation. Mild to moderate adverse effects attributed to LGG® included bloating (25 % incidence) and excessive flatulence (37.5 % incidence).

Morrow et al., (2010) investigated whether administration of *L. rhamnosus* LGG® can reduce the incidence of ventilator-associated pneumonia (VAP) in mechanically ventilated patients. A prospective, randomized, double-blind, placebo-controlled trial

was conducted involving 146 patients at high risk of developing VAP. Patients were randomly assigned to receive LGG (10^9 cfu, n = 68) or an inert inulin-based placebo (n = 70) administrated to the oropharynx and through nasogastric tube twice a day in addition to routine care. Patients continued to receive active intervention or placebo until extubation, tracheostomy placement, or demise.

Patients treated with *L. rhamnosus* LGG® were significantly less likely to develop microbiologically confirmed VAP compared with patients treated with placebo. Patients treated with *L. rhamnosus* LGG® had significantly less *Clostridium difficile*–associated diarrhea, but the duration of diarrhea per episode was not different compare to placebo group. *Lactobacillus rhamnosus* LGG® patients had fewer days of antibiotics prescribed for VAP and for *C. difficile*–associated diarrhea. There were no adverse events attributable to *L. rhamnosus* LGG® administration, and no cases of *Lactobacillus* bacteremia or pneumonia. There was no evidence of *Lactobacillus* infection in autopsy of three patients treated with *L. rhamnosus* LGG® who died while participating in the study. The authors concluded that *L. rhamnosus* LGG® is safe and efficacious in preventing VAP in a select, high-risk ICU population.

Davidson et al. (2011) conducted a randomized, double-blind, placebo-controlled pilot study to evaluate the efficacy of *L. rhamnosus* LGG® as an immune adjuvant for live-attenuated influenza vaccine (LAIV) in healthy adults. Forty-two healthy adults who received LAIV were randomized to receive LGG ($1x10^{10}$ cfu) or placebo, twice daily for 28 days. Patients were assessed for adverse events at day 14, day 38 and day 56 study visits.

Nineteen and twenty subjects from *L. rhamnosus* LGG® and placebo groups respectively, were included in the final analysis. There was no difference in seroconversion rates between *L. rhamnosus* LGG® and placebo groups from baseline for the H1N1 and B vaccine strains. There was a significant increase in seroprotection in the *L. rhamnosus* LGG® group vs placebo for the H3N2 vaccine strain on day 28, however at day 56 the rates of seroconversion were not statistically significant. Seventeen subjects in the placebo group and 14 subjects in the *L. rhamnosus* LGG® group reported at least one adverse event at any time during the study, all were rated as mild.

The impact of *L. rhamnosus* LGG® and *Bifidobacterium lactis* supplemented dietary counseling during pregnancy on colostrum adiponectin was evaluated by Luoto et al. (2012) in a prospective, randomized, placebo-controlled study. Altogether 256 pregnant women were randomized into three study groups: dietary intervention with probiotics (diet/probiotics, n = 85, $1x10^{10}$ cfu of each LGG® and *Bifidobacterium lactis* per day) or with placebo (diet/placebo, n = 86) and a control group (control/placebo, n = 85). The intervention group received dietary counseling provided by a nutritionist, the focus being the amount and the type of dietary fat. Intervention lasted from the first trimester of pregnancy to the end of exclusive breast feeding, but no longer than to the infant age of 6 months. Breast milk samples were collected after birth (colostrum) for adiponectin concentration analysis (n=181).

No significant difference among the study groups was detected in the colostrum adiponectin concentration. The effect of dietary intervention on the adiponectin concentration was analyzed further and the dietary intervention groups were combined. The adiponectin concentration was significantly higher in the combined dietary intervention group compared to the control group. There was no discussion of any adverse effects on the probiotic dietary intervention.

Suchánek et al. (2013) evaluated the efficacy of probiotics to prevent premature birth and changes in the serum levels of IL-6, C-reactive protein, and ferritin. Thirty symptomatic pregnant women (uterine contraction and/ or progress vaginal score) with intact membrane were admitted to the study after the 28th but before the 34th week of pregnancy with a singleton pregnancy and without a previous history of preterm delivery. Fifteen enrolled women received probiotics (*L. rhamnosus* LGG® and *B. animalis* ssp. *lactis*, BB-12) in capsule containing $5.4x10^6$ cfu each day for 4 weeks.

Statistically significant reduced serum levels of all three biomarkers (IL-6, C-reactive protein, and ferritin) were observed in the probiotics group. None of the women gave birth prematurely or experienced adverse effects. The authors noted that, "Although the use of probiotics appears to treat vaginal infections in pregnancy, there are currently insufficient data from trials to demonstrate any impact of oral probiotics on preterm birth."

Smith et al. (2013) recruited apparently healthy college students and assessed the effect of *L. rhamnosus* LGG® and *Bifidobacterium animalis* ssp. *lactis* BB-12 on health-related quality of life (HRQL) during upper respiratory tract infections. Subjects (n = 231) were randomized to receive placebo (n = 117) or probiotic-containing powder (n = 114, $1x10^9$ cfu of each *L. rhamnosus* LGG® and BB-12 per day in a small foil stick) for 12 weeks.

Upper respiratory tract infection duration was 33% (2d) longer in the placebo group compared to the probiotics group and the severity scores were 34% (30 points) higher for the placebo group compared to the probiotics group. Significantly fewer days of illness and significantly lower severity scores indicate a higher HRQL in the probiotics group compared to the placebo group. The number of missed work days did not differ significantly between groups. The number of missed school days was significantly higher for the placebo group. A total of forty-three adverse events were reported during the study period; diarrhea or vomiting was the most commonly reported and increased flatulence and bloating were the second most common adverse events. There were no significant differences between groups for adverse events, and no serious adverse events were reported.

A randomized, double-blind, placebo-controlled study of *L. rhamnosus* LGG® was undertaken by Kumpu et al. (2013) to investigate its recovery in tonsil tissue after oral administration. Sixty-one healthy adults ages 18 – 30 years underwent tonsillectomy were randomized to receive *L. rhamnosus* LGG® in single strain format (n = 20), LGG® as part of multispecies probiotics (n = 20), or placebo (n = 21). The dose of *L. rhamnosus* LGG® as a single strain was $2x10^{10}$ cfu/ capsule. The probiotic multispecies

combination consisted of four bacterial strains: LGG® ($5x10^9$ cfu), *L. rhamnosus* LC705 ($5x10^9$ cfu), *Propionibacterium freudenreichii* subsp. *shermanii* JS ($3x10^9$ cfu) and *Bifidobacterium animalis* subsp. *lactis* BB12 ($1x10^9$ cfu). Subjects were asked to consume the study products for 3 weeks prior to the scheduled tonsillectomy by mixing the content of capsule in yogurt. The last serving of yogurt was to be consumed on the day preceding the operation.

Lactobacillus rhamnosus LGG® can be recovered from tonsil tissue after oral administration as a single- strain probiotic or as a part of a multispecies probiotic combination. Individual variation exists in the ability of *L. rhamnosus* LGG® to adhere to tonsil tissue. In all subjects with positive recovery of *L. rhamnosus* LGG® in the tonsil tissue, LGG® was also recovered in the fecal sample. Venous blood cultures drawn after the tonsillectomy were negative for bacterial growth. There was no significant difference between the groups in respiratory symptoms or in gastrointestinal symptoms; however, a statistically significant difference was found in the total number of subjects having a temperature $\geq 37{\cdot}5^0$C post operatively in the probiotic groups.

The safety and tolerability of *L. rhamnosus* LGG® in patients with cirrhosis was evaluated by Bajaj et al. (2014) in a randomized (Phase I) clinical trial. Thirty-seven cirrhotic patients with minimal hepatic encephalopathy (MHE) were randomized into *L. rhamnosus* LGG® (n = 18, $5x10^{10}$ cfu/ day) or placebo (n = 19). A total of thirty patients (14 LGG® and 16 placebo) completed the study without any differences in serious adverse events. Twenty five percent of patients in placebo group had serious adverse events compared to none in the probiotic group during the study period. In the *L. rhamnosus* LGG® group, self-limited diarrhea was more frequent, however endotoxemia and TNF-α decreased, and microbiomes changed with changes in metabolite/microbiome correlations pertaining to amino acid, vitamin and secondary bile acid metabolism. The authors concluded that *L. rhamnosus* LGG® is safe and well-tolerated in cirrhosis and is associated with reduction of endotoxemia and dysbiosis.

Pedersen et al. (2014) investigated the effects of *L. rhamnosus* LGG® and a low fermentable oligosaccharides, disaccharides, monosaccharide and polyols (FODMAP) diet in irritable bowel syndrome (IBS) management. One hundred twenty-three patients (median age 37 years, range: 18-74 years, 90 females) were included in a 6-week randomized unblinded controlled trial and allocated to one of three groups: FODMAP diet (LFD, n = 42), LGG® (n = 41, $1.2x10^{10}$ cfu/ day in capsules) and a non-intervention control group (ND, n = 40). All three groups were asked to register their symptoms weekly using the IBS-severity scoring system and IBS specific quality of life questionnaires on the web-application and could continue their regular IBS medication.

A significant reduction in IBS severity score from baseline to week 6 was observed between LFD vs LGG® vs ND. Adjusted changes of IBS severity score for baseline covariates showed statistically significant reduction of IBS severity score in LFD group compared to ND, but not in LGG® compared to ND. IBS quality of life was not altered significantly in any of the three groups. The authors concluded that both LFD and *L. rhamnosus* LGG® are efficacious in patients with IBS. There was no discussion of any adverse effects on the treatment.

The safety and tolerability of *L. rhamnosus* LGG® in healthy elderly was studied in a phase I open label clinical trial (Hibberd et al. 2014). Fifteen healthy elderly, aged 66–80 years received LGG capsules ($1x10^{10}$ cfu), twice daily for 28 days and were followed through day 56. Subjects completed a daily diary, a telephone call on study days 3, 7 and 14 and study visits at baseline, day 28 and day 56 to determine whether adverse events had occurred. Assessments included prompted and open-ended questions.

The stability of *L. rhamnosus* LGG® administered was tested and showed no difference between levels of *L. rhamnosus* LGG® in capsules cultured at baseline compared with capsules cultured at day 28. There were no serious adverse events reported during the trial or follow-up period. The 15 study volunteers reported a total of 47 adverse events ranging from 1–7 per volunteer, 39 (83%) of which were rated as mild and 40% of which were considered related to consuming *L. rhamnosus* LGG®. Thirty-one (70%) of the events were expected, prompted symptoms while 16 were unexpected events. The most common adverse events were gastrointestinal (bloating, gas, and nausea), 27 rated as mild and 3 rated as moderate. In the exploratory analysis, the pro-inflammatory cytokine IL-8 decreased during *L. rhamnosus* LGG® consumption, returning towards baseline one month after discontinuing *L. rhamnosus* LGG® while there was no difference in other pro- or anti-inflammatory plasma cytokines. The authors concluded that "*Lactobacillus rhamnosus* GG ATCC 53103 is safe and well tolerated in healthy adults aged 65 years and older."

Doron et al. (2015) used a randomized, double-blind, placebo-controlled trial to examine the safety and efficacy of *L. rhamnosus* LGG® for the reduction or elimination of intestinal colonization by vancomycin-resistant enterococci (VRE). Eleven adults > 18 years of age with VRE history, of whom 5 received LGG ($2x10^{10}$ cfu/ day) and 6 received a placebo for 14 days, were analyzed. Quantitative stool cultures for *L. rhamnosus* LGG® and VRE were collected at baseline and on days 7, 14, 21, 28, and 56. Day 14 stool samples from some subjects were analyzed by quantitative PCR for *L. rhamnosus* LGG®. Patients were closely monitored for adverse events.

Adverse events were common in both groups, some of them serious, but all these events were consistent with and attributable to the subjects' extensive comorbidities, and no *L. rhamnosus* LGG® related adverse events such as *Lactobacillus* infection were reported. There were no differences in VRE colony counts at any time points between groups. No decline in colony counts was seen over time in subjects who received *L. rhamnosus* LGG®. The strain was detected by PCR in all samples tested from subjects who received it but was only isolated in culture from 2 of 5 subjects in the *L. rhamnosus* LGG® group. The authors concluded: "We demonstrated that LGG could be administered safely to patients with comorbidities and is recoverable in some patients' stool cultures".

In a randomized, double-blind, controlled trial, 62 healthy university students with salivary mutans streptococci counts $\geq 10^3$ cfu/ ml used lozenges containing a combination of *L. rhamnosus* LGG® and *Bifidobacterium animalis* subsp. *lactis* BB-12 (test group, n = 29) or lozenges without added probiotics (control group, n = 31) for 4 weeks (Toiviainen et al. 2015). The aim of the study was to evaluate the effects of orally administered *L. rhamnosus* LGG® and BB-12 on the number of salivary mutans

streptococci (MS), amount of plaque, gingival inflammation and the oral microbiota in healthy young adults. The probiotic lozenge decreased both plaque index and gingival index ($p<0.05$) while no changes were observed in the control group. There were no probiotic-induced changes found in the microbial compositions of saliva in either group. Gastrointestinal problems were reported by two subjects which did not appear to relate to the consumption of the lozenge. The authors concluded that the probiotic lozenge improved the periodontal status without affecting the oral microbiota.

Solano-Aguilar et al. (2016) reported the gene expression of whole blood cells from 15 elderly volunteers who received *L. rhamnosus* LGG® who participated in the phase I open label study conducted by Hibberd et al. (2014). Data analysis for biological interpretation of differentially expressed genes revealed down-regulation of overlapping genes involved with cellular movement, cell to cell signaling interactions, immune cell trafficking and inflammatory response. The authors concluded that "these data provide evidence for LGG-induced transcriptional modulation in healthy elderly volunteers because pre-treatment transcription levels were restored at 28 days after LGG treatment was stopped."

Jäsberg et al. (2018) evaluated the levels of matrix metalloproteinases (MMPs) on their inhibitors and tissue inhibitors of metalloproteinases (TIMPs) in the saliva samples of 62 healthy adults participating in a randomized controlled trial to study the effect of *L. rhamnosus* LGG® and *Bifidobacterium animalis* subsp. *lactis* BB-12® on oral microbiota. The clinical trial was previously reported by Toiviainen et al. (2015). MMPs are a family of enzymes that are involved in physiological processes such as tissue remodeling, wound healing, and inflammation and innate immunity. MMPs activity is controlled by changes in expression and synthesis of MMPs and their major inhibitors, TIMPs.

In the probiotic group ($n = 29$), salivary MMP-9 levels increased, and TIMP-1 levels decreased significantly during the intervention. The ratio of MMP-9/TIMP-1 differed significantly from the baseline level. These changes were not observed in the control group ($n=31$). In the whole data, salivary MMP-9 and gingival index are correlated. Intergroup differences or correlations with other clinical parameters were not found. Probiotic consumption did not affect the saliva flow rate. The authors noted that this result may be an indication of a positive immunomodulatory effect of probiotics in the oral environment.

6.2.4 Meta-Analysis Report

Dugoua et al. (2009) conducted a systematic review and meta-analysis of randomized controlled trials (RCT) of *Lactobacillus, Bifidobacterium,* and *Saccharomyces spp.* which evaluated the safety of probiotics in pregnancy. Of the eight RCTs that were included for meta-analysis, the probiotic intervention was of *Lactobacillus spp.* alone or in combination with *Bifidobacterium spp.* There were no RCTs on *Saccharomyces spp.* during pregnancy. Five of these studies included *L. rhamnosus* LGG®, 4 administered *L. rhamnosus* LGG® as singe species probiotic, 1 as multi species probiotics. The participants in all studies examined included 1505 patients. The primary pregnancy outcomes were Caesarean section rate, birth weight, and

gestational age. One study reported the incidence of malformations; no malformations were reported in the probiotic group, while three cases with malformations were reported in the placebo group. The odds ratio (OR) of Caesarean section rate was 0.88 (95% CI 0.65–1.19). There was no significant increase in birth weight of 45 g or increase in gestational age of 0.4 weeks associated with taking probiotics during pregnancy. According to the authors, the best basis for determining if a probiotic intervention is a risk factor for low birth weight or preterm delivery would have been the incidences of low birth weight infants and preterm deliveries in the probiotic and placebo groups. None of these data were available in the manuscripts extracted for meta-analysis.

Lactobacillus rhamnosus LGG® and *L. reuteri* appear to play a role in decreasing the incidence of atopic disease in infants. LGG® appears to affect the mother–infant bifidobacteria transfer at birth and bifidobacteria development later in life. *Lactobacillus rhamnosus* LGG® and *B. lactis* may affect fatty acid transfer in the placenta, leading to higher placental concentrations of linoleic and dihomo-c-linolenic acids. Administration of *L. rhamnosus* LGG®, *L. rhamnosus*, *B. breve*, and *P. freudenreichii* spp. *Shermanii* to pregnant mothers did not affect antibody responses to diphtheria, tetanus, or *Haemophilus influenzae* type b vaccination.

Shen et al. (2009) evaluated the efficacy and adverse events of Lactobacilli compared with placebo in maintenance therapy of Crohn disease. Six randomized controlled trials with a total of 359 participants met the inclusion criteria. The length of the follow up of these trials ranged from 3 to 24 months; two studies were conducted in children and the remaining were conducted in adults. In two of the studies, *Lactobacillus johnsonii* (LA1) was used, and *L. rhamnosus* LGG® was used in the other four trials.

The authors reported that administration of Lactobacilli as maintenance therapy for Crohn disease is inefficacious in reducing the incidence or relapse. Five trials gave information about adverse events; no appreciable difference was found between interventions. Few serious adverse events were observed in trials and in some trials adverse events were neither severe nor were they considered trial related. Mild nausea, diarrhea, vomiting, bloating, ankle-swelling and edema could be observed in both interventions.

Szajewska et al. (2011) reported a systematic review on the efficacy of administering *L. rhamnosus* LGG® for the prevention of healthcare-associated diarrhea. Three RCTs involving 1092 children (age up to 18 years, male and female and of any ethnic group, being admitted to the hospital for any reason) were included. Studies with participants at high risk of developing infections such as intensive care unit patients and very low birth weight preterm infants were excluded from analysis. The daily dose of *L. rhamnosus* LGG® ranged from $1x10^9$ to $1x10^{10}$ cfu and the form of administration was fermented milk supplemented with LGG®, or LGG® in capsules or sachets. In all included studies, *L. rhamnosus* LGG® administration lasted for the duration of the hospital stay.

The administration of *L. rhamnosus* LGG® compared with placebo to hospitalized children reduced the overall incidence of healthcare-associated diarrhea, including

rotavirus gastroenteritis. There was no significant difference between the *L. rhamnosus* LGG® and control groups in the incidence of asymptomatic rotavirus infection, duration of hospitalization, or duration of diarrhea. *Lactobacillus rhamnosus* LGG® was well tolerated, and no harms were reported in any of the trials.

With regard to *L. rhamnosus* LGG® supplementation for preventing respiratory infections in children, Liu et al. (2013) reviewed 4 RCTs involving 1805 participants (age 0 month to 18 years). The intervention was *L. rhamnosus* LGG®, or *L. rhamnosus* LGG® together with other probiotics at any form or dose compared with placebo or with no additional intervention. The primary outcome measure was the incidence of respiratory infections using the original investigator's definition, including the overall respiratory infections, the upper and lower respiratory infections and acute otitis media. The secondary outcome measures were the incidence of antibiotic treatments and occurrence of adverse effects.

Compared with the placebo group, the *L. rhamnosus* LGG® group had a significantly reduced risk of acute otitis media and a reduced risk of upper respiratory infections. There was no difference in reduced risk of the overall respiratory infections or lower respiratory infections. There was a statistically significant difference in reduction of antibiotic treatments in the *L. rhamnosus* LGG® group compared to placebo group. Three infants receiving placebo experienced vomiting, flatulence and increased fussiness. In the trial involving newborn infants, the symptoms included abdominal discomfort, vomiting, crying, and difficulty in swallowing the products. There was no difference in adverse events reported between the *L. rhamnosus* LGG® and the placebo group, and the *L. rhamnosus* LGG® was well tolerated.

TABLE 6: *Lactobacillus rhamnosus*, LGG® CLINICAL STUDIES IN INFANTS					
Reference	**Study Design & Objective**	**Description of Subjects**	**Daily Dose and Delivery**	**Duration**	**Results**
Al-Hosni, et. al. (2012)	Prospective, randomized, blind controlled study to evaluate the effects of LGG (Culturelle) and *Bifidobacterium infantis* (Align) in extremely low-birth- weight (ELBW) premature infants on improved growth and feeding tolerance.	101 premature infants ≤ 1000 grams, less than or equal to 14 days of age. LGG + *B. infantis*: 50	$5x10^8$ cfu each of LGG and *B. infantis*, enteral feeding.	Approximately 6 weeks (34 week post menstrual age minus 26 weeks of average gestational age minus 2 weeks of age at the time of feeding).	Although probiotic-supplemented feedings improve growth velocity in ELBW infants, there was no improvement in the percentage of infants with growth delay at 34 weeks post menstrual age. Mortality or NEC was not different between probiotic supplemented and control groups. There were no probiotic-related adverse events or sepsis related to the organisms supplemented reported in any of the infants studied.
Baldassarre , et. al. (2010)	Prospective, randomized, double- blind, placebo-controlled study for formula-fed infants to determine the benefits of LGG in an extensively hydrolyzed casein formula (EHCF) in improving hematochezia and fecal calprotectin over EHCF alone.	62 infants 1-10 months of age (30 with presumptive diagnosis of CMAC; 30 healthy infants). LGG: 12 after randomization	$1.46x10^7$ cfu LGG/ 100 ml formula, bottle fed.	4 weeks	Fecal calprotectin is elevated in infants with hematochezia and possible allergic colitis. EHCF + LGG resulted in significant improvement of hematochezia and fecal calprotectin compared with the EHCF alone. There was no discussion of any adverse effects of the treatment in the publication.
Chrzanowska-Liszewska, et. al. (2012)	Randomized, double-blind, placebo-controlled trial to determine if oral supplementation with bifidogenic flora can prevent abnormal colonization of premature GIT.	47 premature infants, birth weight >1000 g, gestational age <32weeks, absence of any disease other than those linked to prematurity. LGG: 21	$6x10^9$ cfu LGG powder/ day, mixed with formula.	8 weeks	A preterm infant formula with an addition of LGG leads to a rapid growth of LGG in the gut of bottle fed infants but does not: a) decrease the number of pathogenic organisms; b) increase weight gain during enteral feeding; or c) decrease length of hospital stay. There was no discussion of any adverse effects of the treatment. The authors concluded that "probiotics may not alternate the pathological colonization of preterm infants. Further larger studies are needed, which will be able to look at the specific CFU of certain microorganisms".

TABLE 6: *Lactobacillus rhamnosus*, LGG® CLINICAL STUDIES IN INFANTS					
Reference	**Study Design & Objective**	**Description of Subjects**	**Daily Dose and Delivery**	**Duration**	**Results**
Janvier, et. al. (2014)	Prospective cohort study to determine whether routine probiotic administration to very preterm infants would reduce the incidence of necrotizing enterocolitis (NEC).	294 preterm infants <32 weeks' gestation received probiotics; 317 infants as comparison	$2x10^9$ cfu per day of LGG, *Bifidobacterium breve, B. bifidum, B. infantis, B. longum.*	Starting with the first feed until the infant reaches 34 weeks	Probiotics was associated with a reduction in NEC (from 9.8% to 5.4%, P < .02), a nonsignificant decrease in death (9.8% to 6.8%), and a significant reduction in the combined outcome of death or NEC (from 17% to 10.5%, P < .05). There was no effect of probiotics on health care–associated infection.
Luoto, et. al. (2014)	Randomized, double-blind, placebo-controlled trial to evaluate the effect of probiotic and prebiotic in reducing the risk of viral respiratory tract infections in preterm infants.	94 premature infants, gestational age between 32 and 36 weeks, birth weight >1500 g. LGG:31 Prebiotic: 31	$1x10^9$ cfu LGG/ day for day 1- 30 days; $2x10^9$ cfu/ day for 31 to 60 days, mixed with breast milk or formula. Prebiotic: a mixture of polydextrose and galacto-oligosaccharides in a 1:1 ratio.	60 days, follow up to 12 months of age.	A significantly lower incidence of RTIs was detected in infants receiving prebiotics or probiotics compared with those receiving placebo. The incidence of rhinovirus-induced episodes, which comprised 80% of all RTI episodes, was found to be significantly lower in the prebiotic and probiotic groups compared with the placebo group. No differences emerged among the study groups in rhinovirus RNA load during infections, duration of rhinovirus RNA shedding, duration or severity of rhinovirus infection, or occurrence of rhinovirus RNA in asymptomatic infants. The authors noted: "The absence of adverse effects in this study cohort represents safety documentation for the use of these prebiotics and probiotics in this sensitive infant population".
Manzoni, et. al. (2011)	Clinical charts review, retrospective study of very low-birth-weight (VLBW) infants admitted to two Italian NICUs in the years 2003–2008. The primary objective was to assess safety and tolerability of the probiotic LGG.	743 VLBW infants; mean birth weight 1056 g; mean gestational age 29.5 weeks.	Standard protocol of LGG administration: $3x10^9$ cfu/day. LGG freeze- dried powder was reconstituted in 1 ml sterile water and diluted in feeds.	4 to 6-weeks courses.	A total of 17,108 LGG doses were administered. No adverse effects or intolerances attributable to LGG occurred. Overall, 5350 clinical and surveillance cultures from 13 different sites/devices were performed. None ever grew LGG, or other Lactobacilli. No clinical sepsis episode was attributable to LGG. Full enteral feeding was achieved at 19.2 mean days-of-life; 73% of infants were exclusively/partially breastfed. Fourteen NEC cases occurred, with 5 being > 2b stage. The authors concluded: Routine supplementation of probiotic LGG in a large, 6-year VLBW infants Italian cohort proved microbiologically safe and clinically well tolerated.

TABLE 6: *Lactobacillus rhamnosus*, LGG® CLINICAL STUDIES IN INFANTS					
Reference	**Study Design & Objective**	**Description of Subjects**	**Daily Dose and Delivery**	**Duration**	**Results**
Nermes, et. al. (2011)	Randomized, double-blind, placebo-controlled trial to investigate the interaction of LGG with skin and gut microbiota in infants with atopic dermatitis (AD).	39 infants with AD LGG:19	$3.4x10^9$ cfu of LGG/ day in extensively hydrolyzed casein formula	3 months	The proportions of IgA- and IgM-secreting cells decreased significantly in the treated group. The proportions of CD191CD271 B cells increased in the probiotic-treated infants but not in the untreated. There were no significant differences in bifidobacterial species composition of the gut between the study groups. On the skin, the bacterial counts of *Bifidobacterium* genus vs. *Clostridium coccoides* in the treated and untreated infants were similar. There was no adverse event reported in the study.
Pärtty, et. al. (2013)	Randomized, double-blind, placebo-controlled trial to evaluate the impact of early prebiotic and probiotic intervention on preterm infants' well-being, crying, growth, and microbiological programming.	94 premature infants, gestational age between 32 and 36 weeks, birth weight >1500 g. LGG:31 Prebiotic: 31	$1x10^9$ cfu LGG/ day for day 1-30 days; $2x10^9$ cfu/ day for 31 to 60 days, mixed with breast milk or formula. Prebiotic: a mixture of polydextrose and galacto-oligosaccharides in a 1:1 ratio.	60 days, follow up to 12 months of age.	A total of 27 of 94 (29%) infants were classified as excessive criers, significantly less frequently in the prebiotic and the probiotic groups than in the placebo group. The placebo group had a higher percentage of *Clostridium histolyticum* group bacteria in their stools than did the probiotic group. There were no adverse events related to either supplementation.

TABLE 6: *Lactobacillus rhamnosus*, LGG® CLINICAL STUDIES IN INFANTS					
Reference	**Study Design & Objective**	**Description of Subjects**	**Daily Dose and Delivery**	**Duration**	**Results**
Rouge, et. al. (2009)	Randomized, double-blind, placebo-controlled trial to evaluate the efficacy of probiotics on the digestive tolerance to enteral feeding in preterm infants born with a very low or extremely low birth weight. The primary outcome chosen was the percentage of infants receiving >50% of their overall nutritional needs enterally on the 14th day of life.	94 premature infants, gestational age <32 wk, birth weight <1500 g, postnatal age ≤ 2 weeks, start enteral feeding. LGG: 45	4×10^8 cfu of each LGG and *B. longum* BB536 daily (lyophilized cells in capsules, mixed with 1 ml sterile water immediately before administration in enteral feeding)	14 days	No deleterious effects were observed, and no difference in the incidence of sepsis between the probiotic and placebo groups, even in the subgroup that weighed <1000 g. BB536-LGG were not detected in any blood culture in the relatively small population studied. Probiotic mixture failed to accelerate weaning from parenteral nutrition and had no significant effect on the composition of intestinal microbiota (except for colonization by the probiotic strains) or on the excretion of fecal calprotectin. In infants who weighed >1000 g, probiotic supplementation was associated with a shortening in the time to reach full enteral feeding. No colonization by probiotic strains was detected in infants who weighed ≤ 1000 g, presumably because of more frequent suspensions of enteral feeding, more courses of antibiotic treatment, or both.
Scalabrin, et. al. (2009)	Prospective, randomized, blinded, placebo-controlled trial to evaluate the effect of extensively (EH) and partially hydrolyzed (PH) formulas supplemented with LGG on growth and tolerance in healthy, term infants.	210 healthy infants, 38 to 42 weeks gestational age, birth weight ≥ 2500 g, solely formula-fed at 24 hours prior to randomization. LGG: 63 in EH + 77 in PH groups.	1×10^8 cfu of LGG/ gram formula powder.	From 14 through 120 days of age	Formula intake, overall incidence of gas and fussiness, and a low incidence of diarrhea and constipation were similar in all groups. Rates of discontinuation and adverse events were similar between groups; serious adverse event rates were low and generally deemed unrelated to study products by the study physician. The authors concluded: "The EH and PH formulas supplemented with LGG support normal growth in healthy, term infants and are well tolerated and safe".

TABLE 6: *Lactobacillus rhamnosus*, LGG® CLINICAL STUDIES IN INFANTS					
Reference	**Study Design & Objective**	**Description of Subjects**	**Daily Dose and Delivery**	**Duration**	**Results**
Underwood, et.al. (2009)	Randomized, blinded, placebo-controlled trial to compare the effect of prebiotic and probiotic products on weight gain, stool microbiota, and stool short chain fatty acid (SCFA) content of premature infants.	90 premature infants, birth weight 750-2000 g, gestational age at birth less than 35weeks, age less than 7 days. LGG: 30	$10x10^8$ cfu/ day of LGG (CUL) or each of *Lactobacillus acidophilus*, *Bifidobacterium. longum*, *B. bifidum*, and *B. infantis* (PBP), by mouth or gavage tube. Prebiotic: fructo-oligosaccharides	Up to 28 days	There were no significant differences in weight gain and stool SCFA between the probiotics and placebo groups. PBP cause significant increase in the stool content of bifidobacteria compared to placebo. The authors noted: "Our study showed no differences between groups in NEC, documented infections, or adverse outcomes but it was not powered to detect such differences".
Van Niekerk, et. al. (2014)	Randomized, double-blind, placebo-controlled trial was to compare the effect of probiotics administration on feeding tolerance and growth outcomes of HIV-exposed (but uninfected) versus HIV non-exposed preterm infants.	184 premature infants from HIV-positive or HIV-negative mothers, birth weight ≥500 g and ≤1250 g. LGG + *B. infantis*: 54 (HIV-exposed); 37 (HIV-unexposed)	$0.35x10^9$ cfu/ day of each LGG and *Bifidobacterium infantis* mixed in breast milk, administered via orogastric tube.	28 days	The use of probiotic supplementation did not affect growth outcomes or feeding tolerance in HIV-exposed and non-exposed VLBW infants. There were no differences in the incidence of any signs of feeding intolerance and abdominal distension between the groups.

TABLE 7: *Lactobacillus rhamnosus*, LGG® CLINICAL STUDIES IN CHILDREN					
Reference	**Study Design & Objective**	**Description of Subjects**	**Daily Dose and Delivery**	**Duration**	**Results**
Basu, et. al. (2009)	Randomized, blinded, controlled trial to evaluate the effective dose of LGG as probiotic in acute watery diarrhea (AWD) in Indian children.	588 children > 1 years of age with acute watery diarrhea. LGG: 374	$2x10^{10}$ and $2x10^{12}$ cfu of LGG/ day, combined with oral rehydration solution.	7 days or until diarrhea stopped	No adverse effect of LGG was documented both during the hospital stay and during the follow-up period even in malnourished children. Both the doses of LGG were equally effective to decrease the frequency and duration of diarrhea and reduction in hospital stay in patients of AWD.
Francavilla, et. al. (2010)	Randomized, controlled trial to determine whether LGG relieves symptoms in children with recurrent abdominal pain.	141 children with irritable bowel syndrome or functional pain.		8 weeks	Compared with baseline, LGG, but not placebo, caused a significant reduction of both frequency and severity of abdominal pain. LGG also determined a significant decrease in the number of patients with abnormal results from the intestinal permeability testing.
Hojsak, et. al. (2010a)	Randomized, double-blind, placebo-controlled trial to investigate the role of LGG in preventing nosocomial gastrointestinal and respiratory tract infections at a pediatric hospital.	742 hospitalized children age > 1 year. LGG: 376	10^9 cfu of LGG/ day in 100 ml fermented milk	For the duration of the hospitalization	There was a significantly reduced risk for gastrointestinal infections in the LGG group compared with placebo group. No adverse effects were noted during study and the products were well tolerated.

TABLE 7: *Lactobacillus rhamnosus*, LGG® CLINICAL STUDIES IN CHILDREN					
Reference	**Study Design & Objective**	**Description of Subjects**	**Daily Dose and Delivery**	**Duration**	**Results**
Hojsak, et. al. (2010b)	Randomized, double-blind, placebo-controlled trial to investigate the role of LGG in the prevention of gastrointestinal and respiratory tract infections in children who attend day care centers.	281 healthy children (average age between 52 -54 months old) attending 4 day care centers. LGG: 139	$1x10^9$ cfu of LGG in 100 ml fermented milk/ day	3 months	Children in the LGG group had a significantly reduced risk of upper respiratory tract infections (RTI), RTI lasting longer than 3 days, and a significantly lower number of days with respiratory symptoms. There was no significant difference between the risk of gastrointestinal infections. No side effects or adverse effects were noted during the study. The authors concluded: "...we can recommend treatment with LGG as a valid measure for the prevention of upper respiratory tract infections in children who attend day care centers".
Kumpu, et. al. (2012)	Randomized, double-blind, placebo-controlled trial to determine whether long-term daily consumption of milk containing probiotic LGG decreases respiratory illness in children.	523 healthy children aged 2-6 years attending 60 day care centers. LGG:261	$1x10^8$ cfu of LGG/ day in meals	28 weeks	Consumption of LGG reduced the occurrence of respiratory illness in children attending day care centers in the completed cases subgroup, but not in the total population. There were 22 adverse events reported by parents during the intervention, none of the events were serious or related specifically to LGG group.
Muraro, et. al. (2012)	Randomized, double-blind, placebo-controlled crossover trial to evaluate the hypoallergenicity of an extensively hydrolysed (EH) casein formula supplemented with LGG.	33 children 14 years or younger, with documented cow's milk allergy	$1x10^8$ cfu of LGG/ g formula powder. Consumption: 240 ml/ day, no information of the amount of powder to make 1 ml of formula.	Double-blind placebo-controlled food challenge, open challenge and 7-day home feeding period	No serious adverse events were reported during the double-blind placebo-controlled food challenge (DBPCFC), open challenge or the 7-day home feeding period. For all participants with confirmed cow's milk allergy, the DBPCFC and open challenge were classified as negative. The authors concluded: The EH casein formula supplemented with LGG is hypo-allergenic and can be recommended for infants and children allergic to cow's milk who require an alternative to formulae containing intact cow's milk protein.

TABLE 7: *Lactobacillus rhamnosus*, LGG® CLINICAL STUDIES IN CHILDREN					
Reference	**Study Design & Objective**	**Description of Subjects**	**Daily Dose and Delivery**	**Duration**	**Results**
Ritchie, et. al. (2010)	Randomized double-blind placebo-controlled study to assess the efficacy of probiotics in Australian Aboriginal children admitted to hospital with rotavirus infectious diarrhea	70 children between the ages of 4 months and 2 years admitted with acute diarrhea LGG: 38	Greater than $15x10^9$ cfu of LGG/ day, LGG powder in capsule was reconstituted in 5 ml sterile NaCl 0.9% and given via nasogastric tube.	3 days	Probiotics did not change the duration of diarrhea, total diarrhea stools, or diarrhea score compared with placebo. The author concluded: LGG did not appear to enhance short-term recovery following acute diarrhea illness in this setting. No adverse effect attributable to LGG in the present study.
Sindhu, et. al. (2014)	Randomized, double-blind, placebo-controlled trial to evaluate the effect of the LGG on intestinal function, immune response, and clinical outcomes in Indian children with cryptosporidial or rotavirus diarrhea.	124 children aged 6 months to 5 years, testing positive for rotavirus or *Cryptosporidium* species in stool. LGG: 45 and 20	$1x10^{10}$ cfu of LGG/ day; LGG in capsule was added to milk.	4 weeks	Fewer children with rotavirus diarrhea on LGG had repeated diarrheal episodes and impaired intestinal function. Significant increase in IgG levels post intervention was observed in children with rotavirus diarrhea receiving LGG. Among children with cryptosporidial diarrhea, those receiving LGG showed significant improvement in intestinal permeability. Five children experienced serious adverse events requiring hospitalization; these were for lower respiratory infections, vulval abscess, and measles. Four were in the probiotic group, but no events were considered related to the intervention. All the children recovered.
Szachta, et. al. (2011)	Randomized, single-blind, placebo controlled study to evaluate the efficacy of LGG in eliminating the GI carrier state of vancomycin-resistant enterococci (VRE).	61 children (0 – 18 years) diagnosed with GI carrier state of VRE. LGG: 32	$3x10^9$ cfu/ day	21 days	The VRE carrier state was lost by 20 of 32 participants in the treatment group and 7 of 29 in the control group. LGG supplementation temporarily eliminates the VRE carrier state and increases gastrointestinal counts of *Lactobacillus* spp. in children versus placebo.

TABLE 8: *Lactobacillus rhamnosus*, LGG® CLINICAL STUDIES IN ADULTS AND ELDERLY					
Reference	**Study Design & Objective**	**Description of Subjects**	**Daily Dose and Delivery**	**Duration**	**Results**
Bajaj, et. al. (2014)	Randomized clinical trial (Phase I) study to evaluate the safety and tolerability of LGG in cirrhotic patients.	30 cirrhotic patients with average age of 56 – 58 years old. LGG: 14	$5x10^{10}$ cfu/ day, mixed in yogurt.	4 weeks	No difference was observed in serious adverse events between LGG and placebo groups. Self-limiting diarrhea was more frequent in LGG group, endotoxemia and TNF-α decreased, microbiome changed with changes in metabolite/microbiome correlations pertaining to amino acid, vitamin and secondary bile acid metabolism. The authors concluded that LGG is safe and well-tolerated in cirrhosis and is associated with reduction of endotexemia and dysbiosis.
Davidson, et. al. (2011)	Randomized, double-blind, placebo-controlled trial to evaluate the effect of LGG as an immune adjuvant for live-attenuated influenza vaccine in healthy adults.	42 healthy adults between the ages of 18 – 49 years. LGG: 19	$2x10^{10}$ cfu of LGG capsules/ day.	28 days	There was a significant increase in seroprotection in the LGG group vs placebo for the H3N2 vaccine strain on day 28. However, at day 56 the rates of seroconversion were not statistically significant. 17 subjects in the placebo group and 14 subjects in the LGG group reported at least one adverse event at any time during the study. All were rated as mild.
Doron, et. al. (2015)	Randomized, double-blind, placebo-controlled trial to examine the safety and efficacy of LGG for the reduction or elimination of intestinal colonization by vancomycin-resistant enterococci (VRE).	11 adults > 18 years of age with VRE history. LGG: 5	$2x10^{10}$ cfu of LGG capsules/ day.	14 days	Adverse events were common in both groups, some of them serious, but all of these events were consistent with and attributable to the subjects' extensive comorbidities, and no LGG- related adverse events such as *Lactobacillus* infection were reported. No differences in VRE colony counts were seen at any time points between groups. LGG administration did not affect VRE colonization in this study. The authors concluded: "We demonstrated that LGG could be administered safely to patients with comorbidities and is recoverable in some patients' stool cultures".

TABLE 8: *Lactobacillus rhamnosus*, LGG® CLINICAL STUDIES IN ADULTS AND ELDERLY					
Reference	**Study Design & Objective**	**Description of Subjects**	**Daily Dose and Delivery**	**Duration**	**Results**
Hibberd, et. al. (2014)	Open label clinical trial to assess safety and tolerability of LGG in elderly population.	15 healthy adults age 66-80 years. LGG: 15	$2x10^{10}$ cfu of LGG capsules/ day.	28 days	There were no serious adverse events; the most common adverse events were gastrointestinal (bloating, gas, and nausea). In the exploratory analysis, the pro-inflammatory cytokine interleukin 8 decreased during LGG consumption, returning towards baseline one month after discontinuing LGG. The authors concluded: LGG is safe and well tolerated in healthy adults aged 65 years and older.
Jäsberg, et. al. (2018)	Evaluation of salivary samples originated from a randomized controlled trial to study the effect of LGG and *Bifidobacterium animalis* subsp. *lactis* BB-12 on levels of matrix metalloproteinases (MMPs) or their inhibitors (TIMPs) in healthy adults.	62 healthy students with average age of 24 years. LGG+BB-12: 29	$2x10^{9}$ cfu of LGG and BB-12 in lozenges.	4 weeks	The use of probiotics related to increased salivary MMP-9 and decreased of TIMP-1 levels. The authors noted that this result may be an indication of a positive immunomodulatory effect of probiotics in the oral environment.
Kumpu. Et. al. (2013)	Randomized, double-blind, placebo-controlled trial to determine whether consumption of LGG would lead to the recovery of LGG in tonsil tissue.	61 healthy adults aged 18-30 years underwent tonsillectomy. LGG as a single strain: 20 LGG as part of multispecies group: 20	$2x10^{10}$ cfu of LGG as a single strain/ capsule; or $5x10^{9}$ cfu of LGG as a part of multispecies combination/ capsule daily	3 weeks	LGG can be recovered from tonsil tissue after oral administration as a single-strain probiotic or as a part of a multi-species probiotic combination. In all subjects with positive recovery of LGG in the tonsil tissue, LGG was also recovered in the fecal sample. Venous blood cultures drawn after the tonsillectomy were negative for bacterial growth. There was no significant difference between the groups in respiratory symptoms nor in gastrointestinal symptoms. The number of subjects having a temperature $\geq 37.5^{\circ}C$ post-operatively in the probiotic groups was significantly higher from placebo group.

TABLE 8: *Lactobacillus rhamnosus*, LGG® CLINICAL STUDIES IN ADULTS AND ELDERLY					
Reference	**Study Design & Objective**	**Description of Subjects**	**Daily Dose and Delivery**	**Duration**	**Results**
Lawrence, et. al. (2005)	Pilot trial of adjunctive LGG for prevention of recurrent *Clostridium difficile*-associated disease (RCDAD).	15 adults with mean age of 77.9 years. LGG: 8	$5.6x10^{11}$ cfu/ day in capsules.	During antibiotic therapy + 21 days	Evidence for efficacious treatments of RCDAD is sparse. There were no *Lactobacillus* infections, LGG-related serious adverse events or intolerances leading to study discontinuation.
Luoto, et. al. (2012)	Prospective, randomized, placebo-controlled study to determine the impact of probiotic supplementation on colostrum adiponectin concentration during pregnancy.	256 pregnant women. LGG + *Bifidobacterium lactis* BB-12: 85	$1x10^{10}$ cfu of each LGG and *Bifidobacterium lactis* BB-12 per day in capsule.	From the first trimester of pregnancy to the end of exclusive breast feeding, but no longer than to the infant age of 6 months.	No significant difference among the study groups was detected in the colostrum adiponectin concentration. The effect of dietary intervention on the adiponectin concentration was analyzed further and the dietary intervention groups were combined. The adiponectin concentration was significantly higher in the combined dietary intervention group compared to the control group. There was no discussion of any adverse effects on the treatment.
Morrow, et. al. (2010)	Prospective, randomized, blinded, placebo-controlled trial to determine whether oropharyngeal and gastric administration of LGG can reduce the incidence of ventilator-associated pneumonia (VAP).	146 mechanically ventilated patients at high risk of developing VAP; range of age 19 - 91 years. LGG: 68	$2x10^{9}$ cfu of LGG per day; administrated as a slurry to the oropharynx and through nasogastric tube.	Patients continued to receive active intervention or placebo until extubation, tracheostomy placement, or death.	LGG-treated group were significantly less likely to develop VAP compared with placebo group. Patients treated with LGG had significantly less *Clostridium difficile*–associated diarrhea, but the duration of diarrhea per episode was not different compare to placebo group. The LGG® group had fewer days of antibiotics prescribed for VAP and for *C. difficile*–associated diarrhea. No adverse events related to probiotic administration were identified. The authors concluded that LGG is safe and efficacious in preventing VAP in a select, high-risk ICU population.

TABLE 8: *Lactobacillus rhamnosus*, LGG® CLINICAL STUDIES IN ADULTS AND ELDERLY					
Reference	**Study Design & Objective**	**Description of Subjects**	**Daily Dose and Delivery**	**Duration**	**Results**
Pedersen, et. al. (2014)	Randomized, un-blinded controlled trial to investigate the effect of low fermentable, oligo-saccharides, disaccha-rides, monosaccharides and polyols (FODMAP) diet (LFD) and LGG in IBS patients.	123 patients age 18-74 years. LGG: 41	$1.2x10^{10}$ cfu of LGG/ day in capsules	6 weeks	There was a significant reduction of IBS severity score from baseline to week 6 between LFD vs LGG vs ND. IBS quality of life was not altered significantly in any of the three groups. The authors concluded that both LFD and LGG are efficacious in patients with IBS. There was no discussion of any adverse effects on the treatment.
Smith, et. al. (2013)	Prospective, randomized, double-blind, placebo-controlled trial to assess the effect of LGG and *Bifidobacterium animalis* ssp. *lactis* BB-12 on health-related quality of life (HRQL) in college students affected by upper respiratory infections (URI).	231 apparently healthy college students. LGG+BB-12: 101	$1x10^{9}$ cfu of each LGG and BB-12 per day in a small foil stick.	12 weeks	Probiotic group showed significantly shorter duration of URI indicating higher HRQL during infection and missed significantly fewer school days compared to placebo group. Number of missed work days was not different between groups. There were no significant differences between groups for adverse events, and no serious adverse events were reported.
Solano-Aguilar. et. al. (2016)	Open label study to examine the gene expression of whole blood cells from elderly subjects fed LGG.	11 elderly patients, age 65 – 80 years. LGG: 11	$2x10^{10}$ cfu of LGG/ day in capsules	28 days	Data analysis for biological interpretation of differentially expressed genes revealed down-regulation of overlapping genes involved with cellular movement, cell to cell signaling interactions, immune cell trafficking and inflammatory response. The authors concluded that "these data provide evidence for LGG-induced transcriptional modulation in healthy elderly volunteers because pre-treatment transcription levels were restored at 28 days after LGG treatment was stopped."

TABLE 8: *Lactobacillus rhamnosus*, LGG® CLINICAL STUDIES IN ADULTS AND ELDERLY					
Reference	**Study Design & Objective**	**Description of Subjects**	**Daily Dose and Delivery**	**Duration**	**Results**
Suchánek, et. al. (2013)	Study to evaluate the efficacy of probiotics to prevent premature birth.	30 symptomatic pregnant women. LGG+BB-12: 15	$5.4x10^6$ cfu/ day in capsules	4 weeks	Statistically significant reduced serum levels of all three biomarkers (IL-6, C-reactive protein, and ferritin) were observed in the probiotics group. None of the women gave birth prematurely or experienced adverse effects.
Toiviainen, et. al. (2015)	Randomized, double-blind, controlled trial to evaluate the effects of orally administered LGG and *Bifidobacterium animalis* subsp. *lactis* BB-12 on plaque accumulation, gingival health and the oral microbiota in healthy subjects.	62 healthy university students with salivary mutans streptococci counts $\geq 10^3$ cfu/ ml. LGG+BB-12: 29	$2x10^9$ cfu of each LGG and BB-12 in lozenges	4 weeks	The probiotic lozenge decreased both plaque index and gingival index while no changes were observed in the control group. No probiotic-induced changes were found in the microbial compositions of saliva in either group. Gastrointestinal problems were reported by two subjects which did not appear to relate to the consumption of the lozenge.

6.3 Specific Safety Considerations

According to a 2002 report jointly released by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations (http://www.fda.gov/ohrms/dockets/dockets/95s0316/95s-0316-rpt0282-tab-03-ref-19-joint-faowho-vol219.pdf), "probiotics may theoretically be responsible for four types of side effects: 1) Systemic infections; 2) Deleterious metabolic activities; 3) Excessive immune stimulation in susceptible individuals; and 4) Gene transfer" (Doron and Snydman 2015). All these topics in relation to *L. rhamnosus* LGG® were discussed in GRN 231, which was incorporated by reference. Updated publications regarding these theoretical types of effects are as follows.

6.3.1 Systemic Infections

A large number of clinical studies in healthy and compromised populations of newborn, preterm infants, children, adults, pregnant woman, and elderly, demonstrated the safety of *L. rhamnosus* LGG®. The strain is the most documented probiotic strain with over 760 scientific publications and evaluated in more than 260 clinical trials (as of May 2017), in dosages ranging from $1x10^8$ to $2x10^{12}$ cfu/ day with no reported serious adverse event.

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 probiotic 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 probiotic 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® as a probiotic 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. His parents provided him with a *L. rhamnosus* LGG® capsule ($10x10^9$ cells/capsule) once daily. 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 probiotic capsule 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 probiotic 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. To restore the gut microbiota, the patient was also given *L. rhamnosus* LGG®, once daily dose 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 a fourth case, reported by Sadowska-Krawczenko et al. (2014), a 6-day-old newborn with intrauterine growth restriction (IUGR) symptoms was treated empirically with antibiotics and given *L. rhamnosus* LGG® with the aim of preventing antibiotic-associated gastrointestinal complications. The level of C-reactive protein was increased on day 5 and the blood sampled on day 6 was found to be positive for lactobacilli. The strain identity was verified as *L. rhamnosus* LGG® through PCR and 16S rRNA sequencing. After 9 days of antibiotic therapy, blood cultures became negative and laboratory tests improved on day 25. The patient was discharged from the hospital after 27 days. The author states that "IUGR with a possible link to *L. rhamnosus* LGG® bacteremia might be a new potential risk group,

beside patients with organ failure, immunocompromised status and dysfunctional gut barrier mechanisms, for which safe use of probiotics needs careful attention."

Dani et al. (2015) reported on two cases of sepsis caused by *L. rhamnosus* LGG® therapy in their neonatal intensive care units. The first case involved a female term infant affected by trisomy 18 and triple-X syndrome. Since the 9th day of life the patient was given oral drop supplementation with $5x10^9$ colony-forming unit (CFU) of *L. rhamnosus* LGG® twice daily, through the orogastric tube, for the prevention antibiotic- associated diarrhea. Among other postnatal complications, the patient had a temperature of 38.7°C and pulse of 120 beats/ min, without other signs and symptoms. After the results of a positive blood culture with *L. rhamnosus*, the probiotic supplementation was discontinued and clindamycicn was administered for 10 days until the patient's conditions normalized. The patient was discharged at 300 days of life.

In the second case, an extremely preterm male infant (23 weeks of gestation) was given daily oral drop supplementation with $5x10^9$ CFU of *L. rhamnosus* LGG® through the orogastric tube, to prevent necrotizing enterocolitis (NEC). After positive blood culture with *L. rhamnosus* appeared, probiotic supplementation was discontinued. The isolate had the same antibiotic susceptibility and resistance of the previous case. After 10 days of therapy with gentamicin, his clinical condition progressively improved. The patient was discharged at 117 days of life in good health (Dani et al. 2015).

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 received *L. rhamnosus* LGG® supplementation in an attempt to treat complications resulting from the underlying hospitalization, such as restoring gut microbiota (Meini et al. 2015) and/or preventing antibiotic-associated diarrhea and necrotizing enterocolitis (Sadowska-Krawczenko et al 2014; Dani et al. 2015). 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.

6.3.2 Deleterious Metabolic Activities and Adverse Impact on Host Nutrition

As evaluated in GRN 231, there is no scientific evidence indicating *L. rhamnosus* LGG® produces factors that might inhibit host enzymatic activity or nutrient availability. There are lines of evidence indicating that *L. rhamnosus*, LGG® does not appear to impact nutrition or growth and development in infants. Two reports on long term safety assessment in children since GRN 231 preparation are as follows.

From 2013 to 2014, Lundelin et al. (2017) performed a prospective long-term follow-up on children who had received *L. rhamnosus* LGG® alone or in combination with other probiotic strains in four separate studies conducted between 1997 and 2012. The aim was to evaluate the clinical benefit and long-term safety of specific probiotics administered during the perinatal period. All studies were double-blind, randomized, placebo-controlled trials conducted in a single tertiary center in Turku, Finland. The dose of *L. rhamnosus* LGG® administered in the four studies ranged from $10^9 - 10^{10}$ cfu/day (for reference see Kalliomaki et al. 2001; Huurre et al. 2008; Rautava et al. 2012; Luoto et al. 2014). A total of 562 children were included in the follow-up study. In addition to physical examination, data were collected by structured questionnaires on non-communicable diseases and continued probiotic use, as well as growth data from welfare clinics and school nurses.

There were no differences in growth patterns or non-communicable disease prevalence between children who had received perinatally probiotics or placebo. Children given perinatally *L. rhamnosus* LGG® alone or in combination with other defined probiotics had a lower risk of developing allergic disease (allergic rhinitis, eczema, food allergy or asthma) in long-term follow-up. There was a tendency toward a decreased risk of obesity in children who regularly consumed probiotic-containing products. The authors concluded: Perinatal probiotic administration is safe in long-term follow-up. Children receiving *L. rhamnosus* LGG® perinatally tended to have decreased allergy prevalence (Lundelin et al. 2017).

Scalabrin et al. (2017) reported a 5-year follow-up safety assessment in children who received *L. rhamnosus*, LGG® containing formula from 14 days of age through 1 year of age. The group previously demonstrated that partially hydrolyzed and extensively hydrolyzed formulas with *L. rhamnosus*, LGG® (10^8 cfu/g powder; the estimate daily intake of *L. rhamnosus*, LGG® is approximately $10^8 - 10^{10}$ cfu/day) supported normal growth in healthy term infants through 120 days of age (Scalabrin et al. 2009). Infants who completed a double-blind, randomized growth and tolerance study were eligible to continue receiving the assigned study formula through 1 year of age and participate in follow-up through 5 years of age. A total of 183 participants were eligible in the current study. Anthropometric measures (body weight and height), behavior development, and specific adverse events (allergy- and infection-related) were recorded.

Both partially and extensively hydrolyzed formulas with *L. rhamnosus* LGG® were associated with normal growth and development through 5 years of age, as well as absence of relevant infections or allergic events, or serious adverse events that could be attributed to early consumption of *L. rhamnosus* LGG®. The great majority of infants met all developmental milestones and no group differences were detected at 3 and 5 years of age, demonstrating that study formulas supported normal behavioral development through 5 years of age. The authors concluded that extensively and partially hydrolyzed formulas with *L. rhamnosus* LGG®, when consumed by healthy-term infants through 1 year of age, are associated with normal growth and development and long-term safety through 5 years of age (Scalabrin et al. 2017).

In summary, the results of long-term follow-up studies in children further supported the lines of evidence that lead to the conclusion presented in GRN 231 that "...daily

consumption of at least 10^9 cfu of LGG will not have an adverse effect on nutrient absorption that would have a negative impact on growth and development."

6.3.3 Excessive Immune Stimulation in Susceptible Individuals

As presented in GRN 231, there is no evidence that *L. rhamnosus* LGG® enhances susceptibility to infections in patients where it may be down-regulating immune responses, such as in cow's milk allergy infants, or that *L. rhamnosus* LGG® causes adverse reactions due to over-stimulation of immune responses. Reports on safety and tolerability of *L. rhamnosus* LGG® in elderly subjects age 65 – 80 years revealed that the pro-inflammatory cytokine IL-8 was decreased (Hibberd et al. 2014), and the genes involved in inflammatory response was down-regulated during *L. rhamnosus* LGG® consumption (Solano-Aguilar et al. 2016). Contrary to theoretical possibility that probiotic bacteria in general and *L. rhamnosus* LGG® in this case may cause undesirable immune stimulation, recent research has shed light on the molecular modes-of-actions of *L. rhamnosus* LGG® that provide plausible explanations for some of the clinically documented benefits of this strain. Three key examples are discussed below.

First, a component of *L. rhamnosus* LGG® cell wall, the lipoteichoic acid (LTA), has been shown to interact with the Toll-like receptors TLR2 to 6 on the surface of intestinal epithelial cells. This interaction stimulates the expression of IL-8 by the host, a chemokine that plays a role in recruiting neutrophils to the site of infection (Claes et al. 2012). Neutrophils are a type of white blood cells that fight infections by engulfing microorganisms, secreting antimicrobial substances, and generating extracellular traps that bind and kill microbes.

Second, purified pili from *L. rhamnosus* LGG® can interact with dendritic cells to increase expression of cytokines such as IL-12 (Tytgat et al. 2016). The capacity of a probiotic strain or purified bacterial component to stimulate IL-12 is an indication of their potential to stimulate Type 1 T helper (Th1) cells. An increase in Th1 responses is important for the immune defense against pathogens, and a balance of Th1/Th2 cells is important for attenuation of hypersensitivity to allergens. The ability of *L. rhamnosus* LGG® to stimulate IL-12 *in vitro* supports a model in which *L. rhamnosus* LGG® stimulates immune modulatory effects in the intestine that are beneficial for maintaining health.

Third, the *L. rhamnosus* LGG® genome contains a specific CpG (unmethylated cytosineguanine dinucleotide) motif, named ID35, which can stimulate Th1 responses *in vitro* as well as in an ovalbumin-sensitized mouse model of allergy (Iliev et al. 2005; Iliev et al. 2008). If *L. rhamnosus* LGG® are lysed in the intestine, they can release this specific DNA motif that has immune modulating potential.

In summary, the ability of *L. rhamnosus* LGG® to interact and stimulate the host immune cells in the gut may increase resistance to infections and increase tolerance, potentially decreasing allergic conditions. There is no reported evidence to indicate that *L. rhamnosus* LGG® increases risk of disease by causing excessive down regulation in hypersensitive subjects or causing over-stimulation in healthy subjects.

6.3.4 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).

6.4 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®.

6.5 Recognition of Safety by an Authoritative Group of Qualified Experts

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 probiotic 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 Common Knowledge Elements of GRAS Conclusion

All studies used to establish this GRAS status conclusion have been published in the scientific literature, thus generally available.

6.7 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 as a dietary supplement 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.

Part 7. List of Supporting Data and Information

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Appendix 1

LACTOBACILLUS RHAMNOSUS GG
Grade G

Concentrated, freeze dried bacterial grains or powder

Description of the starter

Bacterial description *Lactobacillus rhamnosus* GG, ATCC 53103

Bacterial count $\geq 5 \times 10^{11}$ cfu/g

Material no. 716417: 25 g/pouch [1]

Hygienic Quality

	Specification cfu/g
Non lactic acid bacteria	< 500
Yeasts and moulds	< 10
Enterobacteriaceae	< 10
Enterococci*	< 100
Coagulase-positive staphylococci	< 10
Salmonella ssp.**	absent/g
*Listeria monocytogenes***	absent/g

* If non lactic acid bacteria are <10 cfu/g, enterococci is not tested separately.
** *Salmonella* ssp. and *Listeria monocytogenes* are tested according to risk assessment.

Activity test

pH ≤ 4.9
Medium:
11% reconstituted skim milk
+ 2% glucose
Heat treatment: 15 min at 121 °C
Incubation:
Temperature: 37 °C
Inoculum: 5×10^7 cfu/ml
Time: 24 hours

Quality Control and release

The culture is released when it fulfils Quality Standard. Lot specific analysis certificate is supplied with deliveries. References and analytical methods are available upon request.

Starters produced by Chr. Hansen, R&D Production of Starter Cultures, including LGG® grade G, are not genetically modified, and do not contain GMO ingredients as stated at EU directives EU no. 18/2001; EU no. 1829/2003 and EU no.1830/2003

Product description

Packaging	Aluminium foil pouches, heat sealed
Storage conditions	Keep frozen at -20 °C Short-term storage or transportation at +2...+12 °C or maximum one week at room temperature
Shelf life	At least 24 months from date of manufacture when stored according to recommendations.
Physical form	Freeze dried bacterial grains or powder
Allergenic data	Contains milk-derived materials
Ingredients	Cultured lactobacilli
Chemical data	n.d
Nutritional data	n.d

n.d. = not determined

[1]The weight of the pouches may change based on the bacterial density (cfu/g) in the powder.

Appendix 2

CHR HANSEN

Improving food & health

Lactobacillus rhamnosus GG Grade G

Starter grains/powder

Lot (b) (4)

	Lot cfu/g	Specification cfu/g
***Lactobacillus* GG**	$7x10^{11}$	$\geq 5 \times 10^{11}$
Hygienic quality		
Non lactic acid bacteria	<10	< 500
Enterococci*	<10	< 100
Yeasts and moulds	<10	< 10
Enterobacteriaceae	<10	< 10
Coagulase-positive staphylococci**	**	<10
Salmonella ssp.**	**	absent/g
Listeria monocytogenes **	**	absent/g
Modified activity test in laboratory scale		
pH	4,4	≤ 4.9

* If non lactic acid bacteria are <10 cfu/g, enterococci is not tested separately

** *Coagulase positive staphylococci, Salmonella* ssp. and *Listeria monocytogenes* are tested from production according to risk assessment

Notes:

Packing size : $1\text{-}2x10^{13}$(ca 25g)

Best before : 06.10.2018
Store at –20 °C or below

Acceptance:Released

13.12.2016 Outi Kykkänen

CHR HANSEN

Improving food & health

Lactobacillus rhamnosus GG Grade G

Starter grains/powder

Lot (b) (4)

	Lot cfu/g	Specification cfu/g
***Lactobacillus* GG**	$5x10^{11}$	$\geq 5 \times 10^{11}$
Hygienic quality		
Non lactic acid bacteria	<10	< 500
Enterococci*	<10	< 100
Yeasts and moulds	<10	< 10
Enterobacteriaceae	<1	< 10
Coagulase-positive staphylococci	<10	<10
Salmonella ssp.**	**	absent/g
Listeria monocytogenes **	**	absent/g
Modified activity test in laboratory scale		
pH	4,5	≤ 4.9

* If non lactic acid bacteria are <10 cfu/g, enterococci is not tested separately

** *Salmonella* ssp. and *Listerio monocytogenes* are tested from production according to risk assessment

Notes:

Packing size: 1-2x1013 (ca 25g)

Best before: 11.08.2018
Store at –20 °C or below

Acceptance:Released

29.08.2016 Outi Kykkänen

CHR. HANSEN

Improving food & health

Lactobacillus rhamnosus GG Grade G

Starter grains/powder

Lot (b) (4)

	Lot cfu/g	Specification cfu/g
***Lactobacillus* GG**	$8x10^{11}$	$\geq 5 \times 10^{11}$
Hygienic quality		
Non lactic acid bacteria	<10	< 500
Enterococci*	<10	< 100
Yeasts and moulds	<10	< 10
Enterobacteriaceae	<1	< 10
Coagulase-positive staphylococci	<10	<10
Salmonella ssp.**	**	absent/g
Listeria monocytogenes **	**	absent/g
Modified activity test in laboratory scale		
pH	4,4	≤ 4.9

* If non lactic acid bacteria are <10 cfu/g, enterococci is not tested separately

** *Salmonella* ssp. and *Listeria monocytogenes* are tested from production according to risk assessment

Notes:

Packing size: 1-2x1013 (ca 25g)

Best before:20.09.2018
Store at –20 °C or below

Acceptance:Released

04.11.2016 Outi Kykkänen

Appendix 3

Pariza et. al. Decision Tree Analysis for Determining the Safety of Microbial Culture for Human Consumption

1. Has the strain[i] been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology?[ii] (If YES, go to 2. if NO. the strain must be characterized and unambiguously identified before proceeding).	Yes
2. Has the strain genome been sequenced? (If YES, go to 3., If No, the genome must be sequenced before proceeding to 3).[iii]	Yes
3. Is the strain genome free of genetic elements[iv] encoding virulence factors[v] and/or toxins[v] associated with pathogenicity?[vi] (If YES, go to 4. If NO, go to 15).	Yes
4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA?[vii] (If Yes, go to 5. In NO, go to 15).	Yes
5. Does the strain produce antimicrobial substances?[viii] (If NO, go to 6. If YES, go to 15).	No
6. Has the strain been genetically modified using rDNA techniques? (If YES, go to 7. If NO, go to 8).	No
7. Do the expressed product(s) that are encoded by the introduced DNA have a history of safe use in food?[ix] (If YES, go to 8. If No, the expressed product(s) must be shown to be safe before proceeding to 8).[x]	Yes
8. 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[xi] and characterizing[xii] component (not simple and 'incidental isolate')? (If Yes, go to 9. If No, go to 13).[xiii]	No
9. Has the species, to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been affirmed to be safe for food use by an authorized group of qualified scientific experts?[xiv] (If YES, go to 10. If No, go to 13).	
10. Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9 continue to support the conclusion that the species, to which the strain belongs, is safe for use in food? (If YES, go to 11. If No, go to 13).	
11. Will the intended use of the strain expand exposure to the species beyond the group(s) that typically consume the species in "traditional" food(s) in which it is typically found (for example, will a strain that was isolated from a fermented food typically consumed by healthy adults be used in food intended for an 'at risk' group)? (If NO, go to 12. If YES, go to 13).	
12. Will the intended use of the strain expand intake of the species (for example, increasing the number of foods beyond the traditional foods in which the species is typically found, or using the strain as a probiotic rather than as a fermented food starter culture, which mya significantly increase the single dose and/or chronic exposure)? (If NO, go to 14. if YES, go to 13).	
13. Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies?[xv] (If YES, go to 15. If NO, go to 14).	No
14. The strain is deemed to be safe for use in the manufacture of food probiotics, and dairy supplements for human consumption.	Yes
15. The strain is NOT APPROPRIATE for human or animal consumption.	

[i] A strain is a "population of organisms that descends from a single organism or pure culture isolate." P. 392 Prescott, Harley and Klein, 1996, Microbiology, Wiley. We recognize that the genotype and /or phenotype of a strain may change slightly when carried in culture, but such changes are irrelevant to the safety considerations because there is no mechanism or precedent for isolated strains in culture to begin spontaneously expressing pathogenic traits, unless that potential was already present in the genome at the time of isolation.

[ii] Whole Genome Sequencing provides distinct advantages for identification and characterization of microorganisms. In-depth analysis, including functional and comparative genomic studies, is afforded by sequencing the whole genome. This technology can provide a wealth of information that can be used for identification and characterization, including evidence of genetic evolution for adaption of a species to a nutrient-rich environment, such as dairy products or the gastrointestinal tract (Pfeiler, EA, Klaenhammer, TR. 2007. The genomics of lactic acid bacteria. TRENDS in Mibiol, 14(12): 546-553). Less comprehensive molecular analysis, such as RAPD, FISH, and MLST, may also provide adequate information for identification, but the characterization ability is often times limited within a bacterial species (Gosiewski, T, Chnielarczk, A, Strusm M, Brzychczy-Wlock, M, Heczko, PB. 2012. The application of genetics methods to differentiation of three *Lactobacillus* species of human origin. Ann Microbiol62:1437-1445)

[iii] The genomic sequence provides the tools to mine the genome for a number of functions, uncovering information spanning from safety to host-cell interactions (Callanan, M. 2005. Mining the Probiotic Genome: Advanced Strategies, Enhanced Benefits, Perceived Obstacles. Current Pharmaceutical Design, 11: 25-36). From a regulatory perspective, the ability to show percentage/regions of similarity and differentiation between a new strain of interest in comparison with a *type strain*, or an accepted strain with history of safe use, is beneficial (U.S. FDA; July 2011. Draft Guidance for Industry: Dietary Supplements: New Dietary Ingredient Notifications and Related Issues). The genome sequence is analogous to a chemical specification for a food ingredient, that is, it defines precisely what is being evaluated and permits a genetic assessment of pathogenic and toxigenic potential. Isolates from a type-strain culture collection, or a strain collection held by a commercial culture manufacturer, may be considered to have the same safety characteristics as, and to be *substantially equivalent* to, the original source pure culture, so in these cases the requirements for genome sequencing may be satisfied by sequencing the genome of the original source pure culture.

[iv] The term "genetic elements" refers to gene sequences encoded in the chromosome of extra-chromosomal DNA.

[v] Known genetic element sequences for virulence factors and protein toxins are searchable, e.g. the MvirDb database of microbial virulence factors (http://mvirdb.llnl.gov) [ref Nucl. Acids Res. (2007) 35 (suppl 1): D391-D394.doi: 10.1093/nar/gkl791].

[vi] In considering the issue of "pathogenicity" and the potential to produce and infection, it is important to distinguish between *true pathogens* (i.e., microbes that possess virulence factors and are therefore capable of crossing or evading non-compromised host barriers) versus *opportunistic pathogens* (i.e., microbes that do not possess the required virulence factors to produce and infection in a non-compromised host). Typically, this can be accomplished via genome analysis for known virulence factors coupled with a comprehensive search of the per-reviewed scientific literature for infectious potential.

[vii] A functional antibiotic resistance gene results in an antibiotic resistance phenotype.

[viii] in this context the tern 'antimicrobial substances' refers to antibiotics that are used in medical or veterinary applications, for example substances that are positive in the JECFA test (FAO. 1981. *FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives,* Appendix A, pp. 317-318, FAO/WHO, Geneva, Switzerland).

[xi] The use of the terms "food" and "feed" includes supplements, which are in most jurisdictions considered to be a subset of the general categories.

[x] Demonstration of the safety of the expressed product may be accomplished by testing, e.g. toxicological testing as required by various regulatory bodies such as the US FDA Redbook 2000 (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm2006826.htm) or by establishing a substantial equivalence of the test article to a substance with a safe history of use in food, or, in the case of animal feed additives, establishing a substantial equivalence of the test article to a substance with a history of safe use in target animal feeds.

[xi] Food fermentations, e.g. Cheddar cheese or yogurt, commonly result in "substantial" microbial food culture populations of $10^6 - 10^8$ colony forming units per gram of the food. Significance should be judged relative to the fermented food, i.e., numbers of different organisms in a microbial population may change during the course of the life of the fermented food, e.g., Lactobacilli counts in Cheddar cheese are routinely low in the initial stages of cheese maturation, but begin to increase in numbers while the Lactococci, responsible for initial acid production, count decreases as the cheese ripens and pH decreases. [Spatial and temporal distribution of non-starter lactic acid bacteria in Cheddar cheese. N.S. Fitzsimons, T.M. Cogan, S. Condon, T. Beresford. Journal of Applied Microbiology 90(4): 600-608, 2001; Kosikowski, F. V., and V.V. Mistry. Chees and Fermented Milk Foods. 1997. 3rd Ed. F. V. Kosikoski, L.L.D. Westport, CT.].

[xii] A species is a "characterizing" component of a food if it has a measurable impact on flavor, texture, stability or preservation properties that are characteristic of the food, e.g. typical color and flavor of "blue" cheeses derived from *Penicillium roqueforti*; or surface texture, flavor and odor of Limburger cheese resulting from *Brevibacterium linens* growth on the surface. The color and flavor of "blue" cheese and the aroma, flavor and texture of Limburger cheese are characteristic of the food and the microbial cultures that are responsible for these traits are characterizing components.

[xiii] A strain that was isolated from a type-strain or a commercial culture, with a history of safe use in food fermentations, is deemed to have satisfied this requirement and may proceed to 9.

[xiv] For example, the Qualified Presumption of Safety list (http://www.efsa.europa.eu/en/topics/topic/qps.htm) prepared and periodically updated by the European Food Safety Authority is the output from a systematic safety review of the included microorganisms by qualified experts.

[xv] Experimental evidence of safety is required. Such evidence may include, but is not necessarily limited to, studies in appropriate animal models, and clinical trials in humans.

[xvi] In some cases, the strain may be shown to be appropriate by test and re-application of the decision tree, e.g., where an undesirable genetic element has been removed from a strain's genome.

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Microbiology and Regulatory Review
U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
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Reference: Chr. Hansen GRAS Notification for
Lactobacillus rhamnosus LGG® Grn No. 845

September 5, 2019
USEMGR

Request for withdrawal of GRAS No. 845 for *Lactobacillus rhamnosus LGG®*

Dear Mr. Highbarger,

This letter is to request that our GRAS submission for *Lactobacillus rhamnosus* LGG® be withdrawn from review. The basis for this request is outlined below.

Since the submission of our GRAS notice we have new information that we feel is relevant to the accuracy and identity of the notified substance and its use in clinical trials. We are actively working on reviewing all of the references and clinical trials to ensure that they are relevant to our strain.

We expect to have completed this review by mid-October, 2019 at which time we will amend the GRAS dossier and re-submit.

Chr. Hansen first brought to FDA's attention that we had new information pertaining to our notification during a phone conversation on July 11, 2019 with Mr. Highbarger. We then spoke briefly on July 17, 2019 to discuss withdrawal vs. continuation of review by FDA. After internal discussions, Chr. Hansen sent an email requesting withdrawal of Grn

No. 845 on Sep. 3, 2019. Mr. Highbarger outlined the steps for making an official request for withdrawal on Sep. 4, 2019.

Best Regards,

Emily Gregoire
Probiotics Regulatory Affairs Manager – North America

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