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JHeimbach LLC

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March 18, 2009

Robert L. Martin, Ph.D.
Deputy Director
Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Bob:

Pursuant to proposed 21 CFR 170.36 (62 FR 18960; April 17, 1997), the Fonterra Cooperative Group of Auckland, New Zealand, through me as its agent, herby provides notice of a claim that the use of the subject probiotic bacterium in conventional foods as described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because Fonterra Coopoerative Group has determined that the intended addition of the subject probiotic to conventional foods is generally recognized as safe (GRAS) based on scientific procedures.

As required, three copies of the notification are provided. Additionally, three copies are provided of the Conclusion of the Expert Panel, including the signatures of the three members of the panel.

If you have any questions regarding this notification, please feel free to contact me at 804-742-5548 or jh@jheimbach.com.

Sincerely,

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

Encl.

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Generally Recognized as Safe (GRAS) Determination for the Use of *Lactobacillus rhamnosus* Strain HN001 (DR20™) in Conventional Foods

Prepared for
Fonterra Co-operative Group
New Zealand

Prepared by
JHeimbach LLC
Port Royal VA

January
2009

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1. GRAS Exemption Claim

1.1. Name and Address of Notifier

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Contact: Sally Collins, Regulatory Strategist, Ingredients
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Facsimile: 64 9 379 8323
E-mail: sally.collins@fonterra.com

1.2. Name of GRAS Organism

The subject of this Generally Recognized as Safe (GRAS) determination is a strain of the probiotic bacterium *Lactobacillus rhamnosus* designated HN001. The strain, which was isolated from a cheddar cheese that has been consumed in New Zealand for more than 20 years, is known commercially as *L. rhamnosus* DR20™ or simply DR20™ and as Howaru Rhamnosus™.

1.3. Intended Use and Consumer Exposure

L. rhamnosus strain HN001 is intended to be added to a variety of foods at the concentration needed, consistent with cGMP, to provide up to 10^9 colony forming units (cfu) *L. rhamnosus* per serving of the food. The foods to which *L. rhamnosus* is intended to be added are those foods that can sustain viable *L. rhamnosus* for the shelf life of the food, including but not limited to dairy products (fluid milk and milk drinks, milk-based desserts and meal replacements, dry and powdered milk, yogurt, and cheese); ready-to-eat cereals; fruit juices, nectars, ades, and drinks; confections; chewing gum; and functional/nutritional products. Anticipated consumer exposure from these intended uses is less than 10^{11} cfu/day, well within levels that have been shown to be safe.

1.4. Basis for GRAS Determination

Fonterra's GRAS determination for the intended use of *L. rhamnosus* strain HN001 is based on scientific procedures as described under 21 CFR §170.30(b). Determination of the safety and GRAS status of the intended use of *L. rhamnosus* strain HN001 was made through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D,, and Michael W. Pariza, Ph.D., who reviewed a monograph prepared by JHeimbach LLC as well as other information available to them. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients including probiotic microorganisms. They critically reviewed and evaluated the publicly available information and the potential human exposure to *L. rhamnosus* strain HN001 resulting from its intended uses and individually and collectively concluded that no evidence exists in the available information on *L. rhamnosus* strain HN001 or other *L. rhamnosus* strains that demonstrates or suggests reasonable

grounds to suspect, a hazard to adults or children under the intended conditions of use of *L. rhamnosus* strain HN001.

It is the Expert Panel's opinion that other qualified scientists reviewing the same publicly available data would reach the same conclusion. Therefore, the intended use of *Lactobacillus rhamnosus* strain HN001 is GRAS by scientific procedures.

1.5. Availability of Information

The data and information that serve as the basis for the GRAS determination will be sent to the FDA upon request, or are available for the FDA's review and copying at reasonable times at the office of James T. Heimbach, Ph.D., President, JHeimbach LLC, 923 Water Street, P.O. Box 66, Port Royal, Virginia 22535, telephone 804-742-5548 and e-mail jh@jheimbach.com.

2. Identity of the Organism

2.1. Name of the GRAS Organism

The subject of this Generally Recognized as Safe (GRAS) determination is a strain of the probiotic bacterium *Lactobacillus rhamnosus* designated HN001. The strain is known commercially as *L. rhamnosus* DR20™ and as Howaru Rhamnosus™.

2.2. Source of the GRAS Organism

L. rhamnosus strain HN001 was originally isolated from a cheddar cheese that has been consumed in New Zealand for more than 20 years. It is maintained in the culture collection of lactic acid bacteria held at the Fonterra Research Centre (formerly known as the New Zealand Dairy Research Institute) in Palmerston North, New Zealand. The strain has also been deposited with the Australian Government Analytical Laboratories (AGAL) as deposit number NM97/09513.

2.3. Description of the GRAS Organism

Lactobacillus rhamnosus is a Gram-positive bacterium that is a member of the broad classification of lactic acid bacteria (LAB). LAB comprise a group of microbes related by common metabolic functionality—the production lactic acid as the major metabolic end product of carbohydrate metabolism—and common physiological traits. LAB are Gram-positive, non-spore-forming, and catalase-negative and are devoid of cytochromes (Holzapfel et al. 2001). They are preferential nonaerobes but are aerotolerant, acid-tolerant, and strictly fermentative. Although they are not a strictly defined taxonomic grouping, LAB generally are considered to include the following phylogenetically related genera, which have several biochemical and ecological features in common (Axelsson 1998): *Aerococcus*, *Alloicoccus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenoccus*, *Vagococcus*, and *Weissella*. Due to similarities in its biochemistry, physiology, and ecology, the genus *Bifidobacterium* is often considered to be a LAB as well, even though it is phylogenetically unrelated (Axelsson 1998). With the exception of some *Streptococcus* species and possibly some *Enterococcus* strains, most LAB strains are considered to have little or no pathogenic potential (Donohue and Salminen 1996; Adams 1999). LAB have a long history of use in fermented and non-fermented foods and have been noted for their ability to inhibit other microorganisms capable of causing foodborne illness or food spoilage (Adams, 1999; Donohue and Salminen 1996). Furthermore, some LAB are ubiquitous as minor components in the intestinal epithelium and the gastrointestinal tract of humans of all ages. All of these factors lead to the reasonable conclusion that most LAB strains are safe for use in conventional foods that may be consumed by all members of the general population.

Lactobacillus is a non-pathogenic genus, comprising the rod-shaped LAB, that consists of over a hundred species. A report by the European Food Safety Authority in November 2007 (EFSA 2007b) identified 112 species, while Bernardeau et al. (2007), writing in the same year, suggested that the genus contains some 135 species and 27 subspecies. *Lactobacillus* is a heterogeneous genus with a large variety of phenotypic, biochemical, and physiological

properties; it has been suggested that the extreme diversity of the *Lactobacillus* genomes would justify recognition of new subgeneric divisions (Bernardeau et al. 2007). Lactobacilli are rod-shaped, non-motile, and non-sporulating. Lactobacilli grow under reduced oxygen conditions in habitats where ample nutrients exist. Members of the genus may be either homo- or heterofermentative. The former convert carbohydrates to lactic acid through the glycolytic pathway, while the latter convert carbohydrates using phosphoketolase to produce lactic acid, acetic acid, ethyl alcohol, and carbon dioxide. *L. rhamnosus* is a facultative hexose heterofermenter (Cogan 1996). While homofermeters are obligate homofermetative, heterofermatative strains may be either obligate or facultative. They are used in commercial applications for the fermentation of dairy products, fruits, vegetables, and meats (Aguirre and Collins 1993; Gasser 1994). Some *Lactobacillus* strains are found in the gastrointestinal tract of healthy humans of all ages, where they are among the "normal" bacteria (Saxelin et al. 1996b; Goldin et al. 1992).

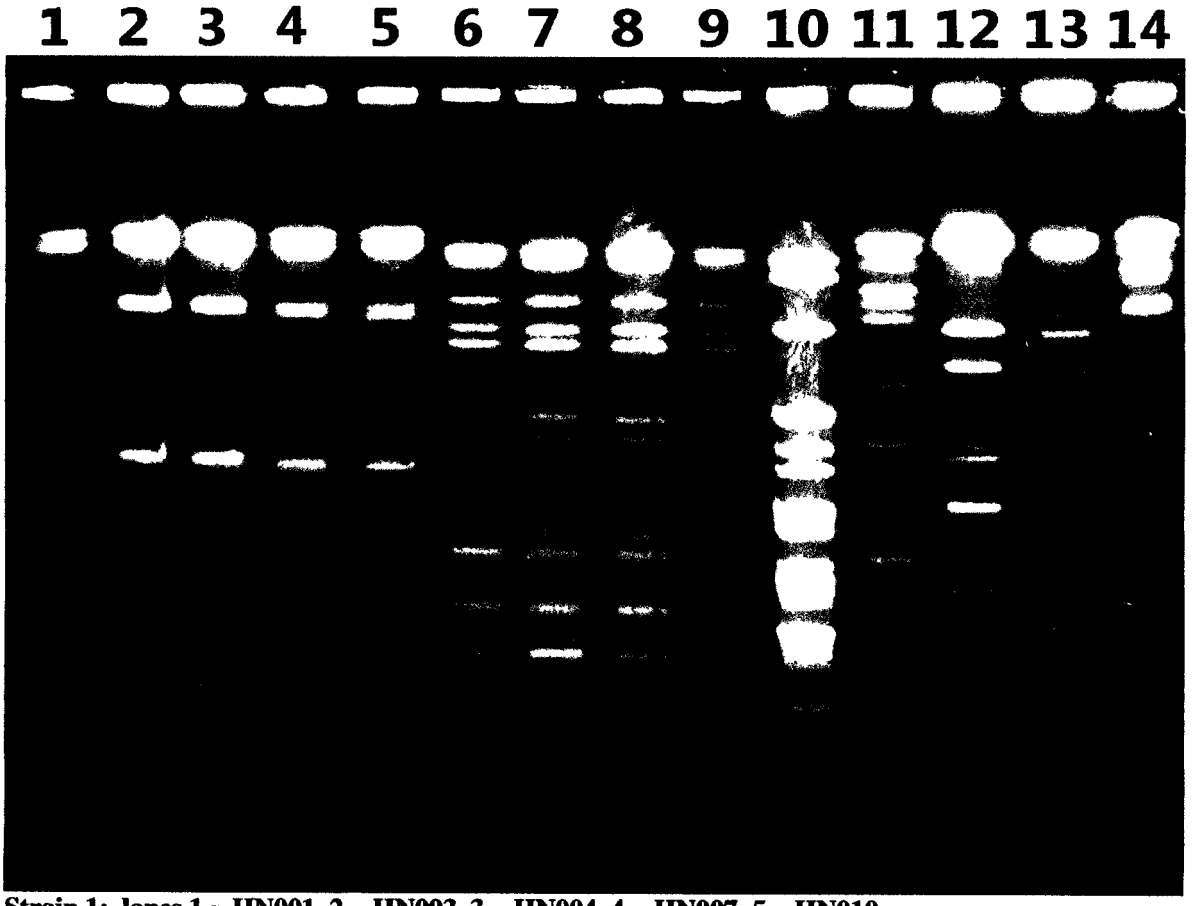
The classification of the *Lactobacillus* genus has evolved over the past several decades as a number of advances in molecular biology, particularly widespread use of 16S rDNA gene sequence analysis, have allowed further differentiation within groupings previously regarded as single species. Thus, over 100 species are now recognized while only a decade ago the number was barely over 50 (Axelsson 1998). Bernardeau et al. (2006) observed that the process of nomenclature change is continuous and ongoing. The *L. casei* group, previously regarded as a single species, is a case in point. Following a thorough molecular study of the *L. casei* complex, it was realized that *L. casei* ssp. *rhamnosus* was quite distinctive when compared with other *L. casei* strains and subspecies. Consequently, *rhamnosus* strains were denominated as a single species separate from *L. casei*, and all members of the former subspecies were transferred to a new species named *Lactobacillus rhamnosus* (Collins et al. 1989). Nevertheless, it must be borne in mind that the *rhamnosus* strains, although now classified as a species separate from *L. casei*, retain the high degree of similarity that was responsible for their original classification as a subspecies of the *L. casei* species. *L. rhamnosus* is rod-shaped and occurs in clusters, singly, or in pairs. As noted earlier, *L. rhamnosus* is a facultative hexose heterofermenter (Cogan 1996).

It has been known for some time that *L. rhamnosus*, like *L. casei*, is a commensal organism found in the healthy intestine, vagina and distal urethra (Anukam et al. 2006; Voravuthikunchai et al. 2006). The species is often used in the preparation of food (e.g., cheese production, milk fermentation). Since *L. rhamnosus* is ubiquitous in the GI tracts of both humans and infra-human mammalian species, clearly it has long been ingested during normal food-consumption activities. As a result, it may be concluded that *L. rhamnosus* strains have a long history of human ingestion with no apparent adverse effects.

In an unpublished investigation, Kelly (1997) differentiated individual strains among 41 cultures of *Lactobacillus* spp. from the New Zealand Dairy Research Institute collection using pulsed-field gel electrophoresis (PFGE) of restriction endonuclease-digested chromosomal DNA. The indentified species included *L. paracasei* (21 cultures), *L. rhamnosus* (14 cultures, including strain GG [ATCC 53103] and the *L. rhamnosus* type strain [ATCC 4356]), *L. acidophilus* (4 cultures), and *L. casei* (2 cultures). A range of restriction endonucleases were tested; for *L. rhamnosus* strains the most satisfactory restriction enzymes proved to be *Asc* I and *Sfi* I; however,

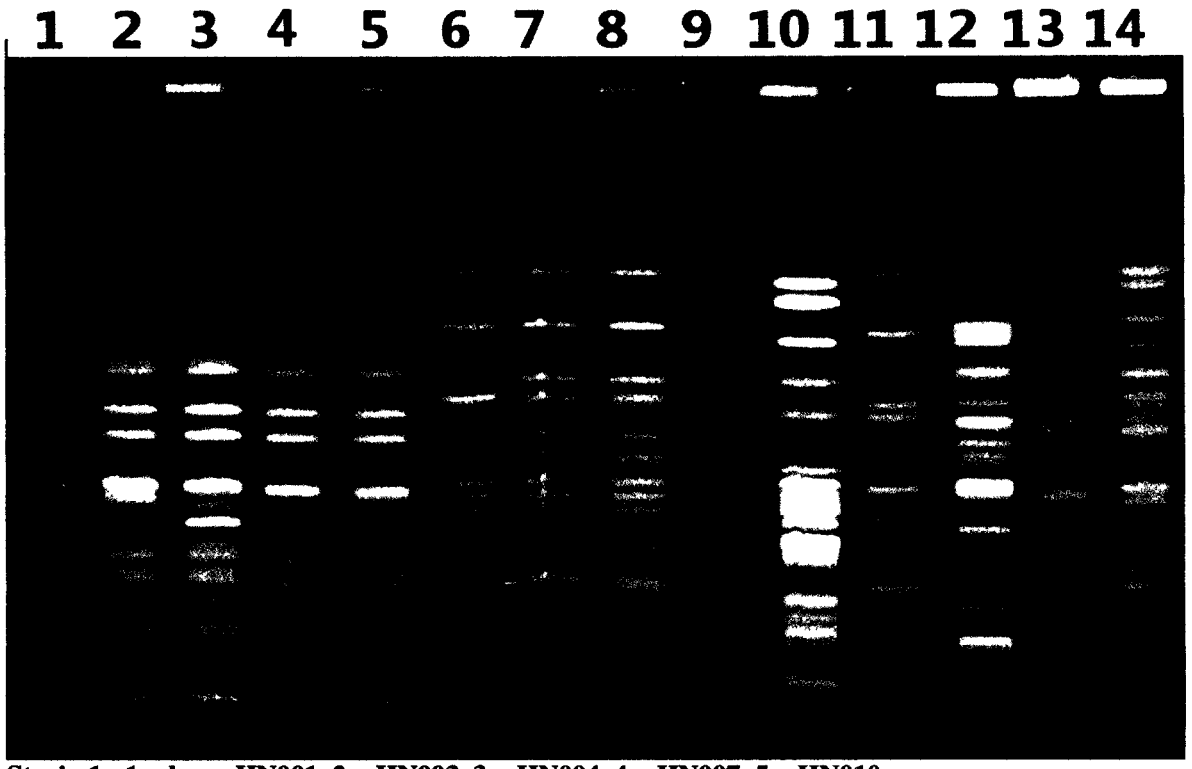
since *Sfi* I produced a small number of large fragments these strains were checked with another enzyme, *Not* I.

The PFGE patterns of the 14 *L. rhamnosus* cultures were interpreted as representing 6 distinct strains (Kelly 1997). As can be seen in Figures 1 and 2, 4 cultures (all from Cheddar cheese) had similar patterns to HN001— HN003, HN004, HN007, and HN010. All 5 of these cultures had the same *Sfi* I pattern; HN007 and HN010 had the same *Not* I pattern as HN001 while HN003 and HN004 had slight variations with *Not* I. A distinct strain was represented by isolates HN002, HN008, HN009, and HN012, all 4 strains also from Cheddar cheese. A tenth Cheddar-cheese culture, HN015, was distinct from the first 2 strains and was identified as a third strain. Culture HN031, isolated from the human gut, had a similar *Not* I pattern to HN002 but differed considerably in its *Sfi* I pattern and was regarded as a fourth strain. Two cultures of *L. rhamnosus* strain GG from different sources (HN033 [ATCC 53103] and HN033A) gave identical patterns to each other with both restriction enzymes. Finally, the *L. rhamnosus* type strain, ATCC 7469, was regarded as a sixth distinct strain.



Strain 1: lanes 1 = HN001, 2 = HN003, 3 = HN004, 4 = HN007, 5 = HN010
Strain 2: lanes 6 = HN002, 7 = HN008, 8 = HN009, 9 = HN012
Strain 3: lane 10 = HN015
Strain 4: lane 11 = HN031
Strain 5 (GG): lanes 12 = HN033 (ATCC 53103), 13 = HN033A
Strain 6: lane 14 = ATCC 7469 (type strain)

Figure 1. PFGE Patterns from *L. rhamnosus* Cultures Cut with *Sfi* I (Kelly 1997).



Strain 1: 1 = lanes HN001, 2 = HN003, 3 = HN004, 4 = HN007, 5 = HN010
Strain 2: 6 = lanes HN002, 7 = HN008, 8 = HN009, 9 = HN012
Strain 3: 10 = lane HN015
Strain 4: 11 = lane HN031
Strain 5 (GG): lanes 12 = HN033 (ATCC 53103), 13 = HN033A
Strain 6: lane 14 = ATCC 7469 (type strain)

Figure 2. PFGE Patterns from *L. rhamnosus* Cultures Cut with *Not* I (Kelly 1997).

It is clear from the PFGE patterns that strain HN001 is not very closely related to strain GG, while it is quite similar to the type strain, ATCC 7469.

Prasad et al. (1999) screened about 200 strains representing numerous species of dairy LAB (lactobacilli and bifidobacteria) for their ability to survive at low pH and high bile concentrations. The benchmark for acid tolerance was set at 80% survival after 3 hours incubation at pH 3.0 at a temperature of 37°C. Similarly, a threshold was set of 80% survival in the presence of a 1% bile solution at the same temperature. *L. rhamnosus* strains generally were more tolerant to acidity and high bile concentration than most other strains. Only 4 strains met these stringent requirements and were chosen for further testing: *L. rhamnosus* HN001, *L. rhamnosus* HN067, *L. acidophilus* HN017, and *B. lactis* HN019 (now classified as *B. animalis* ssp. *lactis*). Additionally, all strains that survived were able to proliferate in the acidic and high-bile concentration environment.

API 50CHL test strips were used to analyze the sugar fermentation patterns of the strains (Prasad et al. 1999). The pattern generated by strain HN001 was consistent with an *L. rhamnosus*

strain (as was HN067; strain HN019 was identified as a *Bifidobacterium* due to the presence of the enzyme fructose-6-phosphate phosphoketolase activity, a diagnostic feature of the genus, as well as its sugar fermentation patterns). Specifically, HN001 and HN067 fermented amygdalin, arabinose, cellobiose, esculim, galactose, glucose, lactose, maltose, mannitol, mannose, melezitose, rhamnose, sorbitol, sucrose, trehalose, fructose, and ribose, but not melibose, raffinose, or zylose. *L. rhamnosus* GG had the same fermentation pattern except it could not ferment arabinose or rhamnose.

The SDS-PAGE pattern of strain HN001 was also consistent with patterns generated by *Lactobacillus rhamnosus*, being similar to that of ATCC 7469, the type strain for *L. rhamnosus* (Prasad et al. 1999), a finding that matches the conclusion provided by Kelly's (1997) PFGE analysis Additionally, a fragment of 16S rDNA gene (bp17-bp360) containing V1 region (hp 66-hp 100) from HN001 was amplified and sequenced and found to be identical to the *L. rhamnosus* type strain. DNA-DNA homology of 94% was found between HN001 and the *L. rhamnosus* type strain ATCC 7469. Taken together, these tests provide confidence that strain HN001 is correctly assigned to the *L. rhamnosus* species.

2.4. Genomic Analysis

2.4.1. Shotgun Sequencing

The whole genome shotgun sequence of *L. rhamnosus* strain HN001 was determined and the resulting sequence was annotated and analysed for genes that may be possible safety concerns. HN001 genomic DNA was sequenced by the Anatomy Otago Genomics Sequencing Service at the University of Otago. A full picoplate was used for the sequencing reaction, resulting in 448,825 reads giving 109,063 kilobases (kb) of data. A previous estimate of the genome size based on PFGE data was about 2.99 Mb, giving at least 36-fold coverage of the genome. Assembly of these data produced 111 contigs of 2,909,284 bases (representing about 97% of the HN001 genome). Twenty-six contigs were small, 100-500 bases, and included a total of 5,783 bases. The majority of the sequence was contained in 85 contigs with an average size of 34 kb.

L. rhamnosus strain HN001 was previously shown to contain 2 plasmids, pLR001 of approximately 8.5 kb and pLR002 of greater than 16.2 kb. Several contigs in the HN001 draft genome sequence contained genes related to plasmid replication and partioning. Using these contigs as a starting point, PCR and sequencing of PCR products was used to close the gaps between these contigs and obtain the complete sequences of the 2 plasmids. The correct assemblies of these 2 plasmids were confirmed by restriction digests.

2.4.2. Annotation and Publication of the Genome

Annotation of the genome was performed using two approaches. Initially, the National Center of Biotechnology Information (NCBI) Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP, http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html) was used to annotate the genome, and subsequently Integrated Genomics, Inc., was contracted to undertake a further annotation of HN001. After completion of the work, the genome sequence and annotation

of the genome and the 2 plasmids were submitted to the NCBI. These sequences were published with the Refseq accession numbers NZ_ABWJ00000000, NC_011223, and NC_011225.

The process began with a short step to resolve conflicts of start sites, after which ribosomal RNAs were predicted by searching for sequence similarity against an RNA sequence database. In order to detect missing genes, a complete 6-frame translation of the nucleotide sequence was done and predicted proteins were masked. All predictions were then compared with all proteins from complete microbial genomes. Annotation was based on comparison to protein clusters and on the BLAST results. Conserved Domain Database and Cluster of Orthologous Group information was then added to the annotation. After frameshift detection and cleanup, the final output was analyzed and the results were submitted to GenBank.

In a parallel analysis, Integrated Genomics used a set of gene identification tools and technologies to identify open reading frames for the HN001 genome sequence. Protein encoding regions and RNAs were identified and automatically annotated using an in-house non-redundant protein database that contains >3.5 million sequences from 1,272 genomes. The automatic annotation phase was followed by manual curation to predict functions for unassigned genes and resolve cases where the automatic curation disagreed with predictions made in accordance with pattern databases. Once a function was predicted with confidence, it was connected to a specific metabolic or cellular pathway from the already existing collection of metabolic/cellular pathways in the ERGO bioinformatics suite. Once functional assignments had been reviewed and potential problem areas identified, the assignment of genes into their respective pathways was reassessed. The result of this analysis was a complete list of pathways and biochemical processes that describe the basic metabolic capacity of *L. rhamnosus* strain HN001. A total of 3,018 open reading frames was identified, with assigned functions for 2,104 (70%) of them and 1,625 (54%) in 1,031 asserted pathways.

2.4.3. Annotation of the Plasmids

Following completion of the sequence of the 2 HN001 plasmids, they were annotated using data from the annotation of contigs contained on the plasmids. For regions of the plasmids not contained in the original set of contigs, BLASTX and BLASTP searching was used to identify remaining protein coding regions. As new BLAST hits were identified these were excluded from subsequent BLAST searching so sequences with lower BLAST scores were not missed. This analysis revealed that the sequence gaps in the original plasmid contigs were mostly associated with multi-copy transposases. The majority of the sequences in these gaps were present in the original assembly, but these contigs were small in size (and not in larger contigs due to their repeated nature) and so were not among the sequences originally annotated. Final annotation of pLR001 and pLR002 gave 13 and 40 predicted protein coding regions, respectively.

2.4.4. Results of the Genomic Analysis

2.4.4.1. Antibiotic Resistance

No genes encoding for antibiotic resistance, nor any sequences showing significant homology with known antibiotic resistance genes, were identified on either of the plasmids. Although *L. rhamnosus* strain HN001, like most lactobacilli, is resistant to vacomycin, this resistance is not genetically based. While HN001 possesses some of the genes required for the

synthesis of peptidoglycans with stem pentapeptides ending in D-alanyl-D-lactate, D-alanyl-D-serine, or D-alanyl-D-alanine, these genes are scattered throughout the genome rather than clustered together as expected if they were transferable. Thus it appears that vancomycin resistance in strain HN001 is intrinsic and not transferable to other bacteria. A range of other genes possibly involved in antibiotic resistance were identified in the HN001 genome, but none were closely linked to predicted mobile genetic elements and are thus unlikely to be transferable.

2.4.4.2. Synthesis of Biogenic Amines

Microbial biogenic amine formation occurs via the decarboxylation of amino acids, and so the HN001 genome was analyzed for genes encoding amino acid decarboxylases that might catalyse the formation of biogenic amines. A gene that could possibly catalyze the conversion of ornithine to putrescine was identified, but no genes that could encode biosysnthesis of ornithine, suggesting that *L. rhamnosus* strain HN001 cannot syntheize putrescine *de novo*. Two open reading frames were also identified that showed weak similarity to genes encoding lysine decarboxylases (which would produce cadaverine), but the open reading frames lack conserved domains identified in other lysine decarboxylases, suggesting that the capability for producing decarboxylase is lacking.

2.4.4.3. Adhesion

The genomic analysis identified several possible adhesins, including a putative collagen adhesion protein located on the large plasmid, pLR002. However, this appears to be a pseudogene that has possibly arisen via insertion of a transposon followed by gene decay, and it is not clear that it is expressed at all and, if it is, wheher it would actually have collagen binding activity. Another sequence encodes for about 55 amino acids of an approximately 180-amino acid collagen-binding protein, but this is likely not adequate to result in collagen binding activity.

2.4.4.4. Virulence/Infectivity

While the genomic analysis identified several genes encoding for proteins related to known cell-invasion proteins, these same genes and their proteins are found in a range of other bacteria with no known pathogenic activity. Some of these are general housekeeping genes, encoding proteins such as enlase, an enzyme of glycolysis, and a peptidase thought to be involved in extracellular protein quality control and degradation. One sequence shows strong homology to a putative hemolysin annotated in *Enterococcus faecalis*, but the basis for the annotation is not clear as the domains present are suggestive of a magnesium and cobalt efflux transporter. A protein encoded by one identified gene shows strong identity to a cell-wall proteinase found in many LAB that is known to be involved in casein degradation. However, it is likely that many proteases are able to hydrolyze host proteins, and the possession of a cell surface protease alone is unlikely to present a significant risk factor for virulence. Finally, one sequence has homology to a 67 kDa myosin cross-reactive antigen from *Streptococcus pyogenes*. Predicted proteins that share homology to this protein are found in a range of bacteria, including lactobacilli and bifidobacteria. It is unclear whether the *S. pyogenes* protein plays a role in molecular mimicry leading to auto-antibody generation in response to *S. pyogenes*.

2.5. Production Process

Production of *L. rhamnosus* strain HN001 begins with preparation directly from the production master stock of approximately 30 batches of production working stock, which are small aliquots in cryovials held at -80°C. Renewal of the production working stock occurs approximately once every 5 years. The Fonterra Microbial Fermentation Unit at Palmerston North is certified as compliant with all requirements of standard ISO 9001:2000 for the manufacture of starters including probiotics. The culturing of *L. rhamnosus* strain HN001 is carried out through the following steps, described schematically in Figure 3. All culturing steps take place in a defined growth medium based on reconstituted skim milk (RSM), which additionally includes yeast extract, glucose, magnesium sulfate, sodium acetate, manganese sulfate, dibasic potassium phosphate, trisodium citrate, and polysorbate. The cryoprotectant (added at step 4 below) consists of maltrin, sucrose, and ascorbic acid. All components of the growth medium and the cryoprotectant are food grade.

1. Working seed vials are prepared containing 1-2 ml aliquots of viable cells and held at -80°C.
2. Each seed vial is used to inoculate a small flask which is incubated at 37°C overnight in a small inoculum fermenter until the culture reaches stationary phase. Since *L. rhamnosus* produces lactic acid, ammonium hydroxide is added as needed to maintain the pH of the broth at approximately 5.8.
3. This broth culture is added to the main production fermenter and incubated at 37°C overnight until it reaches stationary phase. Again, ammonium hydroxide is used as needed to maintain the pH at approximately 5.8.
4. The fermenter broth is chilled and concentrated approximately 15-fold through a cell-concentrating centrifuge, and the cyroprotectant is added.
5. The concentrated slurry is poured into sterilized trays which are placed in a lyophilizer at -30°C and under negative pressure.
6. After lyophilization is complete, the freeze-dried powder is milled to specification and vacuum packed under nitrogen; it is maintained at -30°C until quality-assurance is completed.
7. Finally, the frozen and freeze-dried product is tested in an internationally accredited laboratory before release.

The tests carried out in step 7 are designed to assure microbiological purity and are carried out on every lot of powder produced. These tests are listed in Table 1 on the following page.

Table 1. Tests of Microbiological Purity For Each Lot of *L. rhamnosus* HN001.

Parameter	Method
Lactobacillus counts using MRSA	NZTM2 132.72
Aerobic plate counts	NZTM2 43.2
Thermophiles	NZTM2 60.1
Bacillus cereus	NZTM2 44.1
Meso spores	NZTM2 59.1
Sulfite-reducing Clostrial spores	NZTM2 59.3
Yeast and molds	NZTM2 61.1
Enterococci (fecal *Streptococci*)	NZTM2 50.1
Clostridium perfringens count	NZTM2 46.1
Beta-hemolytic *Streptococci* detection	NZTM2 45.2
Coliforms/*E. coli*	NZTM2 48.5
Coagulase positive *Staphylococcus aureus*	NZTM2 47.2
Listeria	NZTM2 53.2
Salmonella	NZTM2 58.2
Gram stain	NZTM1 D7.2.3
Foreign matter	NSTM4 3.3
Gross contamination	Tryptic soy agar with sheep blood
1. NZTM = New Zealand Technical Manual	

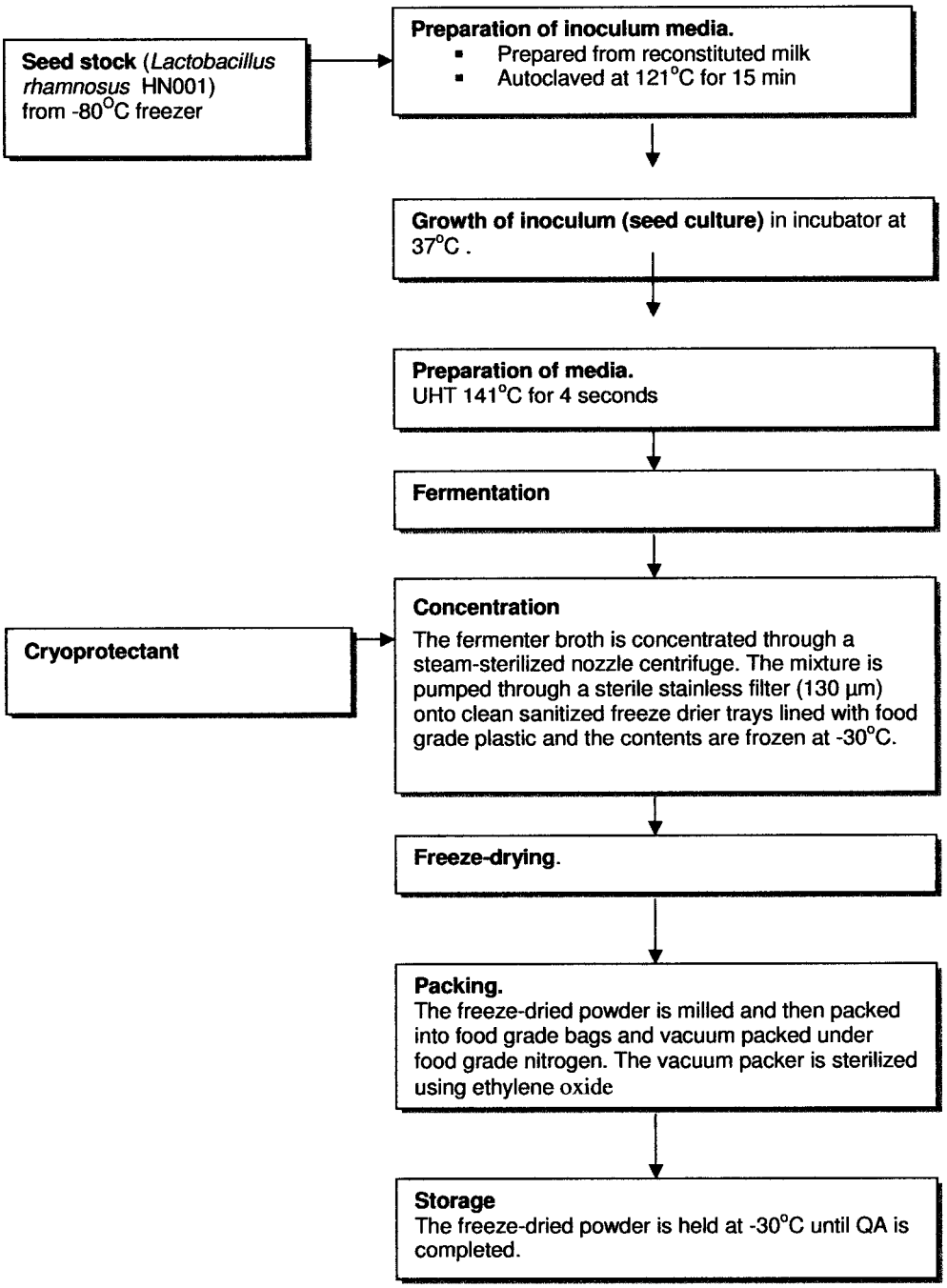


Figure 3. Production Process for the Manufacture of *L. rhamnosus* HN001

2.6. Specifications

All lots of *L. rhamnosus* HN001 meet the specifications set forth in Table 2.

Table 2. Specifications for *L. rhamnosus* HN001 Freeze-Dried Powder

Parameter	Unit	Specification	Method
Description		Freeze-dried powder	Inspection
Particle size	µm	NMT[2] 300	Sieving
Color		Light tan	Inspection
Odor		Sweet	Inspection
L. rhamnosus activity	cfu[1]/g	NLT[3] 10^{11}	NZTM2[4] No. 132.72
Bulk density	g/mL	0.75	
pH (10% solution)		4.5 – 7.2	
Ignition temperature	°C	460	
Proximates			
Carbohydrate	g/100g	30 – 40	
Protein	g/100g	60 – 70	
Moisture	g/100g	2 – 3	
Heavy metals			
Lead	mg/kg	NMT 0.1	ICP-MS[5]
Arsenic	mg/kg	NMT 0.2	ICP-MS
Cadmium	mg/kg	NMT 0.1	ICP-MS
Mercury	mg/kg	NMT 0.1	ICP-MS
Microbiological purity			
Total aerobic bacteria	cfu/g	NMT 1	NZTM2 No. 43.2
Enterococci	cfu/g	NMT 1	NZTM2 No. 50.1
Yeast and molds	cru/g	NMT 1	NZTM2 No. 61.1
Clostridium perfringens	cfu/g	NMT 1	NZTM2 No. 46.1
Bacillus cereus	cfu/g	NMT 1	NZTM2 No. 44.1
Coliforms	cfu/g	NMT 1	NZTM2 No. 48.5
Staphylococcus aureus	cfu/g	NMT 1	NZTM2 No. 47.2
Escherichia coli		Not detected in 1 g	NZTM2 No. 48.5
Listeria		Absent in 25 g	NZTM2 No. 53.2
Salmonellae		Absent in 25 g	NZTM2 No. 58.2

1. cfu = colony forming units
2. NMT = not more than
3. NLT = not less than
4. NZTM = New Zealand Technical Manual
5. ICP-MS = inductively coupled plasma mass spectrometry

2.7. Heavy Metals

Three lots of *L. rhamnosus* HN001 were analyzed for heavy metals concentrations, with the results shown in Table 3.

Table 3. Heavy Metals In Three Lots of *L. rhamnosus* HN001.

Heavy Metal	Unit	Specification	Lot Designation			Analytical Method
			643364.2	643364.3	643364.4	
Lead	mg/kg	NMT 0.1	<0.010	<0.010	0.035	ICP-MS[2]
Arsenic	mg/kg	NMT 0.2	<0.010	<0.010	<0.010	ICP-MS
Cadmium	mg/kg	NMT 0.1	0.010	0.010	0.0099	ICP-MS
Mercury	mg/kg	NMT 0.1	<0.010	<0.010	<0.010	ICP-MS

1. NMT = not more than
2. ICP-MS = inductively coupled plasma mass spectrometry

2.8. Stability

Tannock et al. (2000), in the course of performing a 6-month human study, repeatedly assayed the concentration of viable *L. rhamnosus* HN001 in a freeze dried powder stored under nitrogen at room temperature. The powder contained 5.3 x 10^7 cfu/g at the beginning of the study and still contained this concentration 6 months later; no bacterial were present other than lactobacilli and viability did not vary throughout the 6 months. A study of the stability of *L. rhamnosus* strain HN001 in infant formula held at 30°C showed a rate of decrease in viability of 0.4 log/year.

3. Intended Use and Exposure

L. rhamnosus strain HN001 is intended to be added to conventional foods at concentrations consistent with cGMP needed to provide at least 10^9 cfu/serving throughout the shelf life of the product. This addition level will usually be between 5 x 10^9 and 10^{11} cfu/serving, which provides for the loss of viability of from 80% to 99% of the bacteria added. The strain's function is to serve as a probiotic microorganism.

3.1. Food Categories for Addition of *L. rhamnosus*

The foods to which *L. rhamnosus* is intended to be added are those foods that can sustain viable *L. rhamnosus* for the shelf life of the food, including but not limited to dairy products (fluid milk and milk drinks, milk-based desserts and meal replacements, dry and powdered milk, yogurt, and cheese); ready-to-eat cereals; fruit juices, nectars, ades, and drinks; confections; chewing gum; and functional/nutritional products.

3.2. Estimated Daily Intake of *L. rhamnosus*

L. rhamnosus is expected to be present in a limited number of foods at between 10^9 and 10^{11} cfu/serving, usually at less than 10^{10} cfu/serving. It will not proliferate in the foods and beverages to which it is added, but instead will decline over the shelf-life of the food. Its likely maximum ingestion is thus less than 10^{11} cfu/day, well within levels that have been shown to be safe.

4. Safety

4.1. Safety of Lactic Acid Bacteria and *Lactobacillus* Species

The bacterial biota along the entire intestinal tract is extremely complex and includes an estimated 10^{13}-10^{14} or more bacteria representing over 400 different species (Zetterstrom et al. 1994; Edwards and Parrett 2002) or more than 2000 phylotypes (McFall-Ngai 2006). These indigenous bacteria break down some food components into more easily assimilable forms (Edwards and Parrett 2002), support local immune responses (Zetterstrom et al. 1994), and contribute to an environment that resists colonization by potential pathogens (Heavey and Rowland 1999). Probiotic strains are selected to impart beneficial effects on the host and on the composition and/or metabolism of the intestinal microbiota without causing adverse changes (e.g., invasion of the epithelial cells, degradation of the intestinal mucin layer, production of toxins, transference of antibiotic resistance) that would imperil the health or nutritional status of the host.

Lactobacilli have been consumed on a daily basis since humans started using fermented milks as food, including the probiotic use of certain *Lactobacillus* species for more than 75 years (Salminen et al. 1998), and indeed were almost certainly widely consumed even before that time since they are normal inhabitants of green plant material. Bernardeau et al. (2006) noted that, "lactobacilli are ubiquitous, being found wherever substances rich in carbohydrates are available." These authors reported that in healthy humans, "lactobacilli are normally present in the oral cavity (10^3-10^7 cfu/g), the illeum (10^3-10^7 cfu/g), and the colon (10^4-10^8 cfu/g) and they are the dominant microorganism in the vagina."

A Food and Agriculture Organization and World Health Organization expert consultation (FAO/WHO 2001) noted that, "lactobacilli have a long history of use as probiotics without established risk to humans, and this remains the best proof of their safety" (p17) and concluded that, "no pathogenic or virulence properties have been found for lactobacilli" (p17).

Discussing the use of probiotics in primary care pediatrics, Cabana et al. (2006) observed that the optimal dose of probiotics remains an area of active investigation, but noted that, "Although no specific pediatric dose has been established in general, there are no known reports of 'toxicity' associated with exceeding a specific dose in either adults or children" (p407).

Vandenplas et al. (2007) observed that lactobacilli and other probiotics "do not colonize the gastro-intestinal tract as they become undetectable a few days after stopping the administration. This results in the absence of any risk for long-term side effects" (p1212). As is discussed in more detail later, many studies have demonstrated that lactobacilli are not recovered from feces by 1-2 weeks after administration ceases. One study (Schultz et al. 2004), however, found that infants born to mothers who had received daily oral doses of 2 x 10^9 cfu *L. rhamnosus* strain GG (LGG) during the 30-36 weeks of their pregnancies had detectible LGG strains in their feces for extended periods, with strain identification confirmed by molecular methods. All of the 4 infants delivered vaginally and 1 of 2 infants delivered by Caesarian section were shedding LGG at 1 and 6 months of age. Three children still had detectible fecal LGG at 12 months and 2

at 24 months; none had detectible LGG in their feces at 36 months of age. None of the mothers, on the other hand, exhibited evidence of LGG colonization by 1 month after delivery.

In an article addressing the safety of lactobacilli and bifidobacteria, Borriello et al. (2003) suggested that "classical" approaches to evaluating safety are not appropriate for these commensal bacteria:

> "Lactobacilli and bifidobacteria are ubiquitous in the diet and in the healthy large intestine soon after birth. A classical risk assessment approach, similar to that used for pathogens, is not possible or warranted. Some studies of lactobacilli have attempted to define virulence factors. Such classical approaches, although useful for known pathogens, are inherently flawed when applied to normal commensals, lactobacilli, or bifidobacteria. In the case of the risk assessment approach for pathogens, pathogenicity is demonstrated and is normally a consequence of several properties, including colonization factors and virulence factors, acting in concert. Frequently, such factors as adhesion are considered to be virulence factors when pathogens are studied. However, mucosal adhesion and other colonization factors are essential features of most commensals. For example, there is a distinct mucosal-associated flora in the gastrointestinal tract. There is little value in screening organisms of low clinical significance and with no proven virulence determinants for such characteristics as potential virulence factors, particularly in the absence of gastrointestinal commensals as comparative controls" (p777).

Borriello et al. (2003) argued that the risk of bacteremia from probiotic lactobacilli and bifidobacteria is well under 1 in a million and concluded that, based on the overall risk from this or other adverse endpoints, "consumption of such products presents a negligible risk to consumers, including immunocompromised hosts."

In a similar vein, Bernardeau et al. (2007) suggested that, "The bibliographical data support the hypothesis that the ingestion of *Lactobacilus* is not at all hazardous since lactobacillemia induced by food, particularly fermented dairy products, is extremely rare and only occurs in predisposed patients."

The recent publication of the PROPATRIA study (Besselink et al. 2008), which reports higher mortality among subjects with acute pancreatitis treated with a combination of 6 strains of live *Lactobacillus* and *Bifidobacterium* species has caused some to question the safety of probiotics. The GRAS Expert Panel reviewed this study and determined that, for a number of reasons, the findings do not call into question the safety of the intended use of *L. reuteri* strain ATCC PTA5289.

First, the subjects in this study were acutely ill with a condition the authors indicated has a 10-30% mortality rate. Second, the route of administration of the probiotic strains in this study was naso-jejunal rather than oral. Third, the group that received the probiotics had a significantly higher rate of multiorgan failure on the first day of enrollment than did the control group (27% v. 16%), suggesting a potentially serious confounder. Finally, there was no difference between the

probiotic and control groups in the risk of developing infectious complications and no infectious complications in either group were caused by the lactobacilli or bifidobacteria used in the study.

Subsequent analysis by the study authors suggested that the increased mortality may have resulted from the infusion of the enteral nutrition solution directly into the small intestine with a large number of lactic acid-producing bacteria. Fermentation of the enteral nutrition solution by the bacteria may have led to the formation of a semi-solid mass and resulted in bowel ischemia. Patients with acute pancreatitis are characterized by inhibited transit through the small bowel. The fermented nutrition solution may thus have remained in the small bowel at 37° C with the bacteria without transiting down or being absorbed. It should be emphasized, however, that this hypothesized mechanism of effect would only be applicable to probiotics administered along with a fermentable substrate by intrajejunal tube feeding to patients with severely restricted small intestinal motility.

4.2. History of Consumption of *L. rhamnosus* Strain HN001

As previously noted, this strain of *L. rhamnosus* was isolated from cheddar cheese that has been widely consumed in New Zealand for at least 20 years. Although no documentation regarding exports exist, it is not unlikely that the cheese has been exported to other countries, including the United States. There are no records of any reported adverse events associated with consumption of this variety of cheddar cheese.

4.3. Safety Parameters

4.3.1. Ability to Adhere to Intestinal Cells

Although adherence of probiotic bacteria to intestinal surfaces is not confirmed to be required for health benefits, it has been hypothesized to be involved in establishing residence, for stimulation of the immune system, and for antagonistic activity against enteropathogens (Gopal et al. 2001). Nevertheless, some concern has been expressed that high adhesion capability—a characteristic of pathogens—may facilitate platelet aggregation and bacterial infectivity (Kirjavainen et al. 1999). *In vitro* assays of the adherence ability of bacterial strains are commonly conducted; however, their ability to predict *in vivo* adherence is uncertain. In an *in vitro* evaluation of 8 bacteremia-associated *Lactobacillus* strains Kirjavainen et al. (1999) found no relationship between adherence to Caco-2 cells, ileostomy glycoproteins, or human intestinal mucosa and either platelet aggregation or infectivity.

The adherence properties of 5 probiotic strains, including *L. rhamnosus* HN001, were evaluated *in vitro* by Gopal et al. (2001). Bacterial strains *L. rhamnosus* HN001, *L. rhamnosus* GG, *L acidophilus* LA-1, *L. acidophilus* HN017, and *B. animalis* ssp. *lactis* NH019 were grown in MRS broth to a concentration of 10^8 cfu/ml, which was then added to monolayers of cell-lines HT-29 (ATCC 38-HTB), Caco-2 (ATCC 2102-CRL), or HT29-MTX (a mucous-secreting cell-line) at 37°C. *L. bulgaricus* LB-1, known not to adhere under these conditions, was used as a negative control. Adherence was evaluated in 20 random microscopic fields and the number of adhering bacteria per 100 cells of epithelial cell-line was determined. Adhesion was also measured indirectly by radiolabeling the probiotic strains with ^{14}C-uracil and ^{14}C-adenine and measuring the radioactivity incorporated into the cell-lines. The ability of probiotic strains *L. rhamnosus* HN001, *L. acidophilus* HN017, and *B. animalis* ssp. *lactis* NH019 to inhibit

colonization of the intestinal cell monolayers by a known enterotoxigenic strain of *Escherichia coli*, O157:H7 was also tested. Finally, the effects of 2 proteolytic enzymes (trypsin and proteinase K) and of lactate dehydrogenase on antimicrobial activity were investigated

There were no significant differences in adhesion ability among the 5 probiotic strains tested; all 3 exhibited strong adhesion properties, especially with the mucous-secreting cell-line HT29-MTX. The negative control, *L. bulgaricus* LB-1, showed a complete absence of adhesion to any cell-line, providing evidence that the adhesion was more than non-specific physical entrapment. Pretreatment of the cell-lines with probiotic strains reduced both the adherence of *E. coli* O157:H7 and its invasiveness. Treatment extracts of the probiotic strains with a proteolytic enzyme significantly reduced their antimicrobial activities and lesser reductions resulted from treatment with lactate dehydrogenase, suggesting that overall inhibition may be due to a synergistic action of proteinaceous substances and lactic acid.

On the other hand, Wagner et al. (1997a), in a study discussed in detail in Section 4.4.1.2, studied probiotics in congenitally immunodeficient gnotobiotic beige-athymic (*bg/bg-nu/nu*) and beige-euthymic (*bg/bg-nu/+*) mice. *L. rhamnosus* strain GG (referred to in the article as *L. casei*) adhered poorly, as did the strain of *L. reuteri* tested, while the tested strains of *L. acidophilus* and *Bifidobacterium animalis* adhered well. Translocation generally correlated with adherence, although the bacteria that did translocate did not result in bacteremia, even in this doubly immunodeficient model. The likelihood that adherence to intestinal mucosa may also increase bacterial translocation was more recently noted by Boyle et al. (2006).

The adhesion properties of *L. rhamnosus* GG, *L. rhamnosus* LC-705, *L. rhamnosus* VTT E-800, *L. reuteri* ING1, and *L. brevis* PEL1 were assessed *in vitro* using a human intestinal mucus model (Ouwehand et al. 2001). Human intestinal mucus glycoproteins were isolated from feces of healthy adults by extraction and dual ethanol precipitation and centrifuged to remove any particulate material. The effects of exposure to acid, pepsin, and milk were tested along with differences resulting from the culture medium in which the bacteria were grown. The ability of the probiotic bacteria to reduce the adhesion of pathogenic *S. typhimurium* and *E. coli* SfaII was evaluated.

Two *L. rhamnosus* strains, GG and VTT E-800, had high mucus adhesion, but strain LC-705 adhered poorly. The adhesion capability of the *rhamnosus* strains, unlike the other species tested, was unaffected by pH. Pepsin treatment or exposure to milk significantly reduced the adhesion of all tested strains except *L. rhamnosus* LC-705, which was already close to nil. Similarly, the growth medium did not affect the adhesion of this strain but did affect that of all of the other strains, with various media being superior for different strains. None of the tested strains significantly reduced adhesion of *S. typhimurium* or *E. coli*.

A similar study of the adhesion capabilities of *L. rhamnosus* GG (LGG) and *L. rhamnosus* LC-705 and other probiotic bacteria by Collado et al. (2006) obtained similar findings: LGG was the most adhesive strain tested with about 20% adherence while LC-705 was only weakly adhesive with only 1.2% adhesion. The substrate in this study was human intestinal mucus from resected colonic tissue and immobilized on polystyrene microtitre plate wells. Collado et al. (2006) did not investigate the ability of individual probiotics to inhibit the

adherence of pathogens, but only combinations. The best combination of probiotics to inhibit pathogen adhesion was LGG + LC-705 + *Bifidobacterium breve* 99 + *Propionibacterium freudenreichii* spp. *shermanii* JS.

Ouwehand et al. (2003) studied adhesion of 6 selected lactic acid bacteria to resected colonic tissue and mucus in patients with three major intestinal diseases (diverticulitis, rectal carcinoma, and inflammatory bowel disease) and compared this adherence with adherence to healthy control tissue. The strains tested were 3 *L. rhamnosus* strains (GG [ATCC 53103], LC705, and E-800), *L. breve* PEL1, *L. reuteri* ING1, and *B. animalis* ssp. *lactis* Bb12. Tissue adhesion varied widely; the highest adhesion was 9.7% of LGG to tissue from patients with IBD while the lowest was 1.4% of LC705 to tissue from patients with diverticulitis. All strains were observed to adhere better to immobilized mucus than to whole intestinal tissue; for example, 27.6% of LGG adhered to intestinal mucus from healthy individuals while only 4.9% adhered to healthy intestinal tissue. All strains adhered better to tissue resected from IBD patients than tissue from healthy individuals although this difference was significant only for *L. rhamnosus* strain GG and *L. reuteri*. Similarly, tested strains displayed disease-specific adhesion to intestinal mucus. Adherence was not related to species; overall, the best and worst adhering strains were both *L. rhamnosus*, strain GG (best) and strain LC705 (worst).

More recently, Larsen et al. (2007) investigated the adhesion capabilities of 11 strains of *Lactobacillus in vitro* using the piglet jejunal epithelial cell line IPEC-J2; the strains included *L. rhamnosus* GG (LGG), *L. reuteri* ATCC 55730, *L. johnsonii* NCC 533, and *L. reuteri* DSM 12246 and new *Lactobacillus* isolates. The adhesions of the 11 strains tested ranged from only 1.8% to 38%; LGG was moderate with 10.9% adhesion. The presence of calcium ions significantly increased the binding capabilities of all tested strains, suggesting the advantage of milk-based matrices for the delivery of probiotic bacteria. The effect was specific to calcium; no changes in adhesion were observed in the presence of magnesium or zinc ions. LGG and the other tested strains all reduced the attachment of *E. coli* O138 by more than 2-fold both in the presence and absence of calcium ions. The efficacy of all strains in this regard was about the same, suggesting that the reduced adhesion of *E. coli* O138 was due to steric hindrance of the binding sites rather than to specific interactions.

The genomic analysis of strain HN001 (Section 2.4.4.3) identified several possible adhesins, including a putative collagen adhesion protein located on the large plasmid, pLR002. However, this appears to be a pseudogene that has possibly arisen via insertion of a transposon followed by gene decay, and it is not clear that it is expressed at all and, if it is, wheher it would actually have collagen binding activity. Another sequence encodes for about 55 amino acids of an approximately 180-amino acid collagen-binding protein, but this is likely not adequate to give collagen binding activity.

4.3.2. Ability to Degrade Mucin

As noted earlier, it has not been established whether probiotic bacteria adhere to epithelial cell surfaces, to the mucus layer covering the intestinal mucosa, or to both. Mucins, released from intestinal goblet cells, are highly complex glycoproteins that provide structure and viscosity to the mucus layer that covers the intestinal epithelial surface. The primary function of this layer is to protect the underlying epithelial cells from corrosive gastric acids, shear forces

generated by the digestive process, and invasion by pathogenic microflora. Thus, the potential for probiotic bacteria to degrade intestinal mucins is often evaluated as a potential virulence factor since damage or disturbance to the mucus layer could compromise the barrier function and lead to intestinal or other clinical infections.

Zhou et al. (2001) investigated the ability of *L. rhamnosus* HN001, *L. acidophilus* HN017, and *B. animalis* ssp. *lactis* HN019 to degrade mucin *in vitro*. The mucin source was purified hog gastric mucin. Mucin was suspended with one of the test strains, or with L. acidophilus LA-1 (as a negative control) or a fecal flora extract (as a positive control), for 24 hours at 37°C. Mucin suspensions were prepared both with 1% glucose as an energy source or without glucose, leaving the glycoproteinaceous mucin itself as the only potential source of energy. Bacterial growth was measured under each condition. Three approaches were used to assess potential mucin degradation. First, changes in the carbohydrate and protein concentrations were measured. A decrease in carbohydrate concentration indicates degradation of the oligosaccharide chains while a decrease in the protein concentration indicates degradation of the protein backbone. Second, SDS-PAGE was used to analyze the electrophoretic patterns of mucin samples before and after exposure to bacterial strains in order to detect any changes in the molecular weight of the glycoprotein. Finally, mucin was placed in a Petri dish and inoculated with a bacterial sample; after 24 hours at 37°C the size of the mucin lysis zone was measured.

Only negligible growth was detected in the probiotic cultures containing mucin as the sole carbohydrate source, while the positive-control culture (fecal extract) showed noticeable growth. All strains grew well when sucrose was provided. This finding suggests that the probiotic strains lack the enzymes needed to metabolize the components of mucin glycoprotein. Analyses of changes in carbohydrate and protein concentrations, as shown in Table 4 revealed no effect from the probiotic strains but a high level of degradation from the fecal strains. Similarly, the SDS-PAGE analyses found that the mucin exposed to probiotic strains exhibited identical electrophoretic patterns to mucin not exposed to any bacteria, while mucin exposed to the fecal bacteria exhibited patterns diagnostic of fragmentation of the glycoprotein.

Table 4. Degradation of Mucin.

Treatment	% Degradation	
	Carbohydrate	Protein
L. rhamnosus NH001	-33.9*	-7.3
L. acidophilus NH017	-8.3	5.0
L. acidophilus LA-1	3.3	-44.7
B. animalis ssp. *lactis* NH019	4.7	3.9
Fecal extract	78.1	48.7

Source : Zhou et al. (2001)
*Negative scores are artifacts of the analytical methodology indicating little or no degradation.

4.3.3. Infectivity

Cases of infection by lactic acid bacteria are extremely rare. Reid and Hammond (2005) asserted that, "The safety record of probiotics is remarkable considering that more than 20 billion doses are estimated to be used each year" (p1491). Over the past 30 years there have been about 180 published cases of bacteremia and 69 cases of endocarditis putatively caused by lactobacilli (Aguirre and Collins, 1993; Gasser, 1994; Donohue and Salminen, 1996). 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 or diabetes or therapeutic treatment (e.g., dental surgery). Boyle et al. (2006) stated firmly, "All cases of probiotic bacteremia or fungemia have occurred in patients with underlying immune compromise, chronic disease, or debilitation, and no reports have described sepsis related to probiotic use in otherwise healthy persons" (p1258).

Eleven case reports have been published on clinical infections in patients consuming probiotics, most commonly *L. rhamnosus* or *L. casei* strains. However, in only some of these cases was the strain isolated from the infection confirmed to be identical to the strain that was consumed. Kalima et al. (1996) reported on a case where *L. rhamnosus* was isolated from the blood and pericardial effusion of a 5-year-old boy following a bone marrow transplant for aplastic anemia. The boy continued to suffer from severe neutropenia and thrombocytopenia for 9 months after the transplant and was sustained with blood transfusions, but remained severely immunosuppressed. He received intravenous gamma-globulin and was placed on total parenteral nutrition. Shortly thereafter, the patient developed a fever thought to be associated with Hickman line sepsis and was given intravenous ceftazidime and vancomycin. A swab taken from the line insertion site 2 days later yielded a hemolytic streptococcus and a vancomycin-resistant *Enterococcus avium*. The therapy was changed to ampicillin, and, after 7 additional days of continued fever, ciprofloxacin and liposomal amphotericin B. This regimen was continued for 4 weeks, during which time the boy's body termperature returned to normal. The fever returned a few days after treatment was stopped, and treatment was started with ceftazidime, vancomycin, and liposomal amphotericin B. Two blood cultures yielded a coagulase-negative staphylococcus and a Gram-positive bacillus later identified as *L. rhamnosus*. The patient's febrile episodes continued, and several weeks later he suffered cardiac arrest and died. A post-mortem culture of

the pericardial fluid isolated a Gram-positive bacillus identical to the blood culture isolate. Unfortunately, the case report does not provide any information on the methods that were used to identify the Gram-positive isolate as *L. rhamnosus* and the speciation must be regarded as uncertain.

Mackay et al. (1999) reported on a 67-year-old man with long-standing mitral valve prolapse and recent multiple dental extractions who was using a supplement providing 2 x10^9 *L. rhamnosus* and other species. He presented with endocarditis, which responded to ampicillin plus gentamicin followed by pivampicillin plus probenecid. The specific cause of the endocarditis was not identified.

Rautio et al. (1999) cited the case of a 74-year-old woman with a history of diabetes and hypertension who had been chronically treated with enalapril maleate, bisoprolol fumerate, and glipizide who presented with abdominal discomfort and fever. A strain isolated from an abscess of her liver was determined via PFGE to be LGG. She recovered and was in good health after two months of antibiotic therapy. Presterl et al. (2001) reported a case involving a 23-year-old male with a bicuspid aortic valve and diabetes insipidus which was being treated with intranasal octreotid. The patient was consuming yogurt containing *L. rhamnosus* when he was hospitalized with endocarditis. *L. rhamnosus* was isolated from his blood, although it could not be identified with that in the yogurt he was consuming. He suffered acute heart failure and underwent emergency valve replacemént, leading to complete recovery. The authors concluded that the source of the endocarditis was unknown. This report was notable for the intensive testing conducted to identify the strain of *L. rhamnosus* isolated from the patient's blood. The isolates from the patient and the yogurt had identical biofermentation patterns, but differences were evident in the RAPD-PCR patterns.

Two cases of bacteremia in infants with short gut syndrome were described by Kunz et al. (2004). The first was an infant whose short gut was secondary to congenital intestinal atresia and volvulus, and who was receiving total parenteral nutrition. LGG supplementation was prescribed for cholestasis. The infant developed symptoms consistent with infection after 3 weeks, which responded to ampicillin treatment. Blood cultures grew a *Lactobacillus* species which was not further identified; LGG was suspected, but was not definitively implicated. The second infant's syndrome resulted from a severely infracted intestine at birth. A gastrostomy and a jejunostomy were performed shortly after birth and he was receiving total parenteral nutrition. Again, LGG was prescribed to treat a rapidly developing cholestasis. He developed increased temperature and tachycardia which were successfully treated with ceftriaxone and ampicillin. Blood cultures grew a *Lactobacillus* species shown by PFGE to be indistinguishable from the LGG of the supplement.

De Groote et al. (2005) reported another case of catheter-related LGG bacteremia associated with probiotic use in a child with short gut syndrome. The patient was an 11-month-old male twin born at 26 weeks of gestation, with complications including necrotizing enterocolitis and short bowel syndrome secondary to resection of approximately 80% of the small intestine. Additional complications included long-standing cholestasis, cirrhosis, secondary hyperspenism, hypothyroidism, megaloblasic anemia, chronic lung disease, and retinopathy of prematurity; the patient had undergone multiple abdominal surgical procedures. He was

maintained on parenteral nutrition and had been receiving LGG twice daily through the gastrostomy tube. While the source of the bacteremia was positively identified as *L. rhamnosus*, the blood isolate could not be determined to match the strain used as a probiotic.

A 6-week-old infant was hospitalized for repair of a double-outlet right ventricle and pulmonic stenosis (Land et al. 2005). There were postoperative problems with pacemaker placement, pulmonary artery banding, seizures, acute renal insufficiency, and prolonged respiratory support. Sepsis was suspected and a course of antibiotics was prescribed, but the infant developed severe diarrhea and 10^{10} CFU/day of LGG was administered through his gastronomy tube. When endocarditis developed, his blood was cultured and a *Lactobacillus* species was isolated that was identified by repetitive element sequence-based polymerase chain reaction DNA fingerprinting as LGG. The patient recovered after a second course of antibiotics. Land et al. (2005) also reported on a 6-year-old female with cerebral palsy, microcephaly, mental retardation, and seizure disorder who was maintained on a gastrojejunostomy tube. Following revision of a spinal rod for scoliosis she was hospitalized for a urinary tract infection, fever, and abdominal pain. Treatment with ceftriaxone and vancomycin resulted in diarrhea. After nutrition was administered through a central venous catheter, catheter-related sepsis developed and 10^{10} CFU of LGG was administered through her gastrojejunostomy tube. She developed an infection and a *Lactobacillus* species isolated from blood cultures was identified by repetitive element sequence-based polymerase chain reaction DNA fingerprinting as LGG. Following ceftriaxone and vancomycin treatment, the patient recovered fully.

A case report by Zein et al. (2008) described a 54-year-old diabetic woman who developed septicemia while using *L. rhamnosus* for oral probiotic therapy. The infection resolved after amoxicillin administration. In this report, neither the strain nor the daily dose of probiotic was described, nor was the cause of the septicemia identified by molecular methods that would allow firm attribution to the probiotic.

Brahimi et al. (2008) reported that 2 cases of *L. rhamnosus* infections that occurred within a period of 48 hours in the same hospital department. The patients were both women, one aged 35 years and the other 70 years, suffering from advanced pancreatitis. The authors reported that, "Several criteria pointed towards two independent infections but the rarity and chronology of the appearance of these infections raised the question of possible cross-contamination." *L. rhamnosus* was isolated from intra-abdominal samples from both patients and in a blood culture from one patient. The specific strain could not be identified, nor could it be shown that both women were infected with the same strain.

It is clear that all reported cases of clinical infections with suspected *Lactobacillus* involvement occurred in subjects with one or more severe underlying diseases or health conditions. While these reports indicate that *L. rhamnosus* has the potential to be an opportunistic pathogen in severely compromised subjects, it is equally clear that *L. rhamnosus* is safe in healthy subjects and those with less severe medical conditions, where adverse events have never been reported.

This conclusion is strongly supported by surveillance studies that have failed to discover any evidence of increased rates of clinical infection correlated with increased consumption of

Lactobacillus species. One of the most comprehensive such studies (Saxelin et al. 1996a; Salminen et al. 2002) showed that over a nine year period in which consumption of *L. rhamnosus* increased 10-fold in Finland (a country with an excellent reporting system for health-related events), the number of infections involving *Lactobacillus* species reported to Helsinki health authorities was unchanged.

Positive blood cultures for lactobacilli have also been regarded as indicators of serious or fatal underlying disease (Husni et al. 1997). With regard to cases of endocarditis, strains of lactobacilli are only rarely involved (0.05 – 0.4% of total) compared to bacteria shown to be most highly associated with endocarditis (e.g., >79% by the *Streptococcus-Staphylococcus* group). Cases of lactobacilli endocarditis are typically associated with serious underlying health conditions, such as structural heart disease, that predisposed the patient to opportunistic infections (Donohue and Salminen, 1996). These observations suggest that lactobacilli are much less capable of adhering to intact cardiac valves than other bacteria and only become involved in infections when a predisposing circumstance exists. Although lactobacilli play a minor etiologic role in the context of all cases of endocarditis, in cases where etiologic strains were identified at the species level (a procedure that is not always done), the majority of cases were caused by vancomycin-resistant strains of *L. rhamnosus*, *L. plantarum*, and *L. casei* (Gasser, 1994; Donohue and Salminen, 1996). Saxelin et al. (1996a) studied the prevalence of bacteremia due to *Lactobacillus* species during the period 1989-1992. Among 3,317 blood culture isolates, lactobacilli were identified in 8 patients, 5 of whom had severe diseases predisposing to bacteremic complications.

No case has been described of a *Lactobacillus* infection derived from food or feed fermented with *Lactobacillus* cultures (Adams and Marteau 1995). The participants in the 2007 EU-PROSAFE project (Vankerckhoven et al. 2007a) observed, "It was argued that clinical cases of LAB endocarditis were so rare that they were more medical exceptions, or even curiosities, than a genuine public health issue, especially with regard to the huge worldwide daily consumption of LAB in regular food intake (p111)."

The interaction between platelets and bloodborne bacteria is likely involved both in the pathophysiological mechanisms of septicemia and bacterial infective endocarditis (Zhou et al. 2005b); aggregation of platelets is thought to be a contributory factor in the progression of *Lactobacillus*-associated endocarditis (Adams 1999; Gasser 1994). Isolates of certain bacteria, including *L. rhamnosus*, from patients with infective endocarditis have been reported to uniformly induce irreversible platelet aggregation *in vitro* (Harty et al. 1993). Thus, a lack of platelet aggregation potential may be an important criterion in assessing the safety of potential probiotic bacteria (Donohue and Salminen 1996; Kirjavainen et al. 1999).

For this reason, Zhou et al. (2005b) studied the ability of *L. rhamnosus* HN001 and strain GG as well as *B. animalis* ssp. *lactis* HN019 to induce human platelet aggregation *in vitro*. The three probiotic strains, along with *Streptococcus sanguis* 133-79, used as a positive control, were cultured, purified, and concentrated into a suspension containing approximately 10^9 cfu/ml. Blood samples were taken from 6 healthy volunteers age 24 to 45, centrifuged, exposed to the bacterial cells at a 1:1 ratio of platelets to cells (the optimum ratio for inducing platelet aggregation), and incubated at room temperature for 30 minutes. Platelet samples were analyzed

by flow cytometry for mean fluorescence intensity and the percentage of FITC-CD41a and PE-CD62p (markers for normal and activated platelets, respectively) double-positive cells. Additionally, the following hematologic parameters were measured: total red blood cell and platelet counts, hemoglobin concentration, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration.

None of the hematologic markers was affected by exposure to bacterial cells. *S. sanguis* induced a high degree of platelet activity; more than 91% of the cells were CD62p-positive. The probiotic bacterial strains, on the other hand, had no effect on platelet activity; platelet activation was not significantly different between those exposed to the probiotic strains and those not exposed. Similarly, the platelets exposed to *S. sanguis* exhibited significant increases in the size of the platelet particles—i.e., aggregates. The probiotic bacteria had no effect on the size of the platelet particles, demonstrating that *L. rhamnosus* HN001 and the other tested probiotic strains do not induce or exacerbate platelet aggregation.

While the genomic analysis of strain HN001 discussed in Section 2.4.4.4 identified several genes encoding for proteins related to known cell-invasion proteins, these same genes and their proteins are found in a range of other bacteria with no known pathogenic activity. Some of these are general housekeeping genes encoding proteins such as enlase, an enzyme of glycolysis, and a peptidase thought to be involved in extracellular protein quality control and degradation. One sequence shows strong homology to a putative hemolysin annotated in *Enterococcus faecalis*, but the basis for the annotation is not clear as the domains present are suggestive of a magnesium and cobalt efflux transporter. A protein encoded by one identified gene shows strong identity to a cell-wall proteinase found in many LAB that is known to be involved in casein degradation. However, it is likely that many proteases are able to hydrolyze host proteins, and the possession of a cell surface protease alone is unlikely to present a significant risk factor for virulence. Finally, one sequence has homology to a 67 kDa myosin cross-reactive antigen from *Streptococcus pyogenes*. Predicted proteins that share homology to this protein are found in a range of bacteria including lactobacilli and bifidobacteria. It is unclear whether the *S. pyogenes* protein plays a role in molecular mimicry leading to auto-antibody generation in response to *S. pyogenes*.

4.3.4. Undesirable Metabolic Activity

Some metabolic products of lactobacilli may have adverse effects on human safety. For example, the production of D-lactate by some species of *Lactobacillus*, although not *rhamnosus*, has been identified as a possible safety issue (Mack 2004). Similarly, it has been questioned whether a safety issue may arise in fermented dairy products due to production by lactobacilli of biogenic amines, primarily histamine or tyramine, through amino acid decarboxylase activity. However, Bernardeau et al. (2006) observed that, although biogenic amines may be harmful to consumers, "no such potentially harmful compounds have been found in fermented milk prepared with probiotic lactobacilli." In a later article, Bernardeau et al. (2007) noted that, if production of biogenic amines is indeed a safety issue, it is linked to spoilage or to long fermentation processes and pertains to the use of *Lactobacillus* species in the production of fermented dairy products and not to their use as probiotics. Indeed, Naidu et al. (1999) pointed out that, by producing lactic acid, probiotic lactobacilli reduce intestinal pH, which in turn limits the growth of many potential putrefactive bacteria that produce harmful biogenic amines.

As discussed in Section 2.4.4.2, the genome of strain HN001 was analyzed for genes encoding amino acid decarboxylases that could catalyze the formation of biogenic animes. A gene that could possibly catalyze the conversion of ornithine to putresine was identified, but no genes that could encode biosysnthesis of ornithine, suggesting that strain HN001 cannot syntheize putrescine *de novo*. Two open reading frames were also identified that showed weak similarity to genes encoding lysine decarboxylases (which would produce cadaverine), but both open reading frames lack conserved domains identified in other lysine decarboxylases, suggesting that the capability of producing decarboxylase is lacking.

4.3.5. Presence of Antibiotic Resistance Genes and Likelihood of Transference

In a detailed evaluation of the safety of the *Lactobacillus* genus, Bernardeau et al. (2007) addressed all issues pertaining to safety and concluded that, "transferable antibiotic resistance is the only relevant cause for caution . . . Safety assessment requirements for *Lactobacillus* strains of technological interest should be limited to an antibiotic profile and a study to determine whether any antibiotic resistance(s) of medical interest detected is (or are) transferable."

Ammor et al. (2007) observed that "lactobacilli are usually sensitive to penicillins and β-lactamase inhibitors, but more resistant to oxacillin and cephalosporins; cell wall impermeability seems to be the main mechanism of resistance." With respect to genetically encoded antibiotic resistance, tests of 43 *L. rhamnosus* isolates identified 2 resistance genes, *tet*(W) and *erm*(B), encoding for tetracycline and erythromycin resistance, respectively.

Like all bacteria, LAB are prone to gene exchange to enhance their survival in antibiotic-containing environments (Teuber et al. 1999). The primary concern with the presence of phenotypic resistance to antibiotics in probiotic bacteria is the potential for transfer of this resistance to pathogenic or potentially pathogenic organisms *in vivo*.

Salminen et al. (1998) reviewed the safety of lactic acid bacteria, noting that these bacteria have a long history of safe use in foods. Lactic acid bacteria are intrinsically resistant to many antibiotics. In many cases resistances are not, however, transmissible, and the species are also sensitive to many clinically used antibiotics even in the case of a lactic acid bacteria-associated opportunistic infection. Therefore no particular safety concern is associated with intrinsic type of resistance.

Many strains of lactobacilli are intrinsically resistant to vancomycin; however, it is accepted that antibiotic nonsusceptibility or resistance is not, in itself, a hazard unless it renders the probiotic untreatable in rare cases of infection or unless it can be transferred to potential pathogens for which resistance could have therapeutic consequences (Borriello et al. 2003). Similarly, work by Temmerman et al. (2003) found resistance to kanamycin in all strains of *L. rhamnosus* tested, along with all strains of *L. acidophilus, L. reuteri, L. casei, L. johnsonii, L. lactis, Bifidobacterium longum*, and *B. lactis*; no genetic basis for kanamycin resistance was identifiable and thus it is presumptively intrinsic.

Arthur and Courvalin (1993), in a review of antibiotic resistance of enterococci, noted that plasmid-mediated resistance to the glycopeptide antibiotics vancomycin and teicoplanin was

first detected in 1986 (Leclercq et al. 1988; Uttley et al. 1989) and that inducible resistance to high levels of vancomycin and teicoplanin defines the *van(A)* phenotype. They concluded that nucleotide sequences related to the *van(A)* gene have not been detected in Gram-positive organisms with intrinsic resistance to glycopeptides, including *Lactobacillus* spp., indicating that the resistance genes are not part of the chromosomes of these species and are not transferable.

Although transfer of antibiotic resistance genes within LAB by bacteriophages and prophages seems theoretically possible, this has not actually been observed; further, the host range of such transfers would be limited to closely related strains within a single species (Teuber et al. 1999). As a result, the only recognized mechanism of horizontal gene transfer in bacteria outside the microbiological laboratory is conjugation based on conjugative plasmids and transposons (Teuber et al. 1999).

The antibiotic susceptibility of *L. rhamnosus* HN001, as well as *L. rhamnosus* strains HN067 and GG; *L. acidophilus* strains HN017 and LA1; *L. plantarum* strain HN045; and *B. animalis* ssp. *lactis* strains HN019, HN049, HN098, and Bb12, was studied by Zhou et al. (2005a), using the disk-diffusion method. Four sterilized paper disks were placed aseptically on the surface of agar to which fresh culture had been added. Five µl of the test antibiotic solution was applied to each disk and the agar plates were incubated for 24 hours, following which the diameters of the inhibition zones were measured. Based on the average of 5 readings, results were expressed as sensitive, intermediate, or resistant using the standards reported by Acar and Goldstein (1991), Isenberg (1992), and Woods and Washington (1995). The 18 tested antibiotics, their concentrations, and the resistance of *L. rhamnosus* HN001 are displayed in Table 5, which also summarizes the results of susceptibility testing of the other 9 strains[1].

The resistances shown by *L. rhamnosus* HN001 were also exhibited by all or nearly all of the other 9 probiotic strains tested and are unlikely to be acquired resistances. In order to further explore whether antibiotic resistance traits are intrinsic or acquired, Zhou et al. (2005a) also determined whether each tested strain contained plasmids. No plasmids were detected in *L. rhamnosus* HN001 (or in NH017, HN019, or HN067). However, preliminary work with strain HN001 had determined that the strain actually contained 2 plasmids, a small plasmid of 9 kb and a large one of approximately 40 kb, that are easily lost on repeated subculturing. The same antibiotic resistance tests were conducted with plasmid-containing *L. rhamnosus* HN001 and identical results were obtained, suggesting that the antibiotic resistance traits seen in this strain are not plasmid-based.

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[1] The list of 18 antibiotics tested by Zhou et al. (2005a) is similar but not identical to the list of 13 antibiotics suggested for resistance testing by EFSA (2005). Both lists include ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, neomycin, erythromycin, tetracycline, and chloramphenicol. The EFSA list also includes clindamycin, quinupristin+dalfopristin, trimethoprim, and linezolid, not tested by Zhou et al. (2005), while the latter did test penicillin G, cephalothin, cloxacillin, novobiocin, fusidic acid, nalidixic acid, polymyxin B, rifampin, and spectinomycin, not listed by EFSA (2005).

Table 5. Antimicrobial Agents Tested and *L. rhamnosus* HN001 Resistances

Antibiotic	Concentration (µ/disk)	HN001 Resistance	Resistance of Other Strains[1]
β-Lactams			
Penicillin G	10	susceptible	all S
Ampicillin	10	susceptible	all S
Cephalothin	30	susceptible	all S
Cloxacillin	1	susceptible	3 I, 6 R
Gram-positive spectrum			
Erythromycin	15	susceptible	all S
Novobiocin	5	susceptible	all S
Vancomycin	30	resistant	5 S, 4 R
Gram-negative spectrum			
Fusidic acid	10	resistant	2 S, 7 R
Nalidixic acid	30	resistant	all R
Polymyxin B	300	resistant	all R
Broad spectrum			
Chloramphenicol	30	susceptible	all S
Rifampin	5	susceptible	all S
Spectinomycin	100	susceptible	all S
Tetracycline	30	susceptible	all S
Aminoglycosides			
Kanamycin	30	resistant	all R
Gentamicin	10	resistant	1 S, 1 I, 7 R
Neomycin	30	resistant	1 S, 8 R
Streptomycin	10	resistant	1 I, 8 R

Source: Zhou et al. 2005a
1 Number of strains that were susceptible (S), intermediate (I), or resistant (R).

Klein et al. (2000) investigated whether glycopeptide resistance in lactobacilli has a similar genetic basis to that found in enterococci. *L. rhamnosus* strain GG (ATCC 53103), as well as 5 *L. reuteri* strains and 4 *Enterococcus* control strains, were probed for the *van(A)* gene cluster, the *van(B)* gene and the *van(C)* gene by PCR and Southern hybridization and DNA/DNA hybridization. Their resistance and plasmid patterns were also investigated. All *Lactobacillus* strains were resistant to some degree to vancomycin, oxacillin, gentamicin, ciprofloxacin, and trimethoprim/sulphamethoxazol, but susceptible to a broad range of antibiotics, including clindamycin, imipenem, chloramphenicol and rifampin. Klein et al. (2000) noted that the glycopeptide resistance of *L. rhamnosus* and *L. reuteri* is a natural trait in these species and there has been no evidence of transmission of the *Lactobacillus* glycopeptide resistance to other bacterial strains. None of the *Lactobacillus* strains possessed the *van(A)*, *van(B)*, or *van(C)* gene.

These findings indicated that the glycopeptide resistance of the *Lactobacillus* strains analyzed is different from the enterococcal type, and the authors concluded that the study provides reassurance regarding the safety with regard to vancomycin resistance of the *Lactobacillus* strains tested.

Kastner et al. (2006) conducted a survey of starter and probiotic cultures to determine the current antibiotic resistance situation in microbial food additives in Switzerland. Two hundred isolates from 90 different sources were typed by molecular and other methods to belong to the genera *Lactobacillus* (74 samples), *Staphylococcus* (33 samples), *Bifidobacterium* (6 samples), or *Pediococcus* (5 samples), or were categorized as lactococci or streptococci (82 samples). These bacteria were screened for phenotypic resistances to 20 antibiotics by the disk diffusion method. *L. rhamnosus* GG (the only *rhamnosus* strain tested) exhibited resistance to 8 antibiotics (fusidic acid, kanamycin, nalidixic acid, tobramycin, nitrofurantoin, oxacillin, streptomycin, and vancomycin) and) but not to the other antibiotics tested (cefotaxime, clindamycin, ofloxacin, penicillin G, tetracycline, lincomycin, methicillin, erythromycin, chloramphenicol, rifampicin, novobiocin, and gentamicin). This last result—the susceptibility of LGG to genamicin—differs from the findings of Zhou et al. (2005a), discussed above. Under the conditions tested by Zhou et al. (2005a), both LGG and HN001 were resistant to gentamicin, as were most of the other strains tested.

Twenty-seven isolates exhibiting resistances that are not intrinsic features of the respective genera were further analyzed by microarray hybridization to trace phenotypic resistances to specific genetic determinants. Their presence was verified by PCR amplification or Southern hybridization. These studies resulted in the detection of the tetracycline resistance gene tet(K) in 5 *Staphylococcus* isolates used as meat starter cultures, the tetracycline resistance gene tet(W) in the probiotic cultures *Bifidobacterium lactis* DSM 10140 and *Lactobacillus* (residing on a plasmid), and the lincosamide resistance gene *lnu(A)* (formerly *lin(A)*) in *L. reuteri* SD2112 (Kastner et al. 2006), but no antibiotic resistance genes were identified in *L. rhamnosus* GG.

Klare et al. (2007) studied a variety of *Lactobacillus* as well as *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic use. A total of 416 isolates of *Lactobacillus* representing 21 species, including 131 isolates of *L. rhamnosus* strains, were tested against 16 antimicrobial agents encompassing nearly all important classes to determine the distribution of MIC for each isolate. The goal was to determine tentative species- or group-specific epidemiological cut-off (ECOFF) values to allow differentiation between wild-type isolates lacking acquired antibiotic resistance traits and non-wild-type isolates containing one or more acquired antibiotic resistance traits. ECOFF values could only be determined for those 12 species that were represented by at least 10 isolates. A surprisingly small number of acquired antibiotic resistances were found; the authors suggested that this might be due to the fact that all isolates tested were of well known and generally recognized as safe strains. Acquired resistances were found only to streptomycin, erythromucin, clindamycin, and oxytetracycline—3 isolates each for the first 3 antimicrobials and 12 isolates for oxytetracycline. Five of the 131 *L. rhamnosus* isolates exhibited putatively acquired resistance, but in none of these cases was a known resistance gene found. Two strains had high MICs for streptomycin, but neither harbored the *aad*(E) gene encoding this resistance. Similarly, 1 strain was resistant to erythromycin but tested negative for *erm*(A), *erm*(B), and *erm*(C), the known genes encoding for this resistance,

while the 3 isolates exhibiting tetracycline resistance did not harbor any known tetracycline-resistance genes. While a great number of other resistances were identified, they were classified as intrinsic and therefore less capable of conjugation.

Klare et al. (2007) also carried out conjugation experiments, using non-wild-type isolates containing 1 or 2 antibiotic resistance genes as donors and rifampicin- and fusidic-acid-resistant isolates of the corresponding *Lactobacillus* species, as well as strains *E. faecium* 64/3 and *E. faecalis* JH 2-2, as recipients. None of the intra- or interspecies donor-recipient combinations tested produced transconjugants under the experimental conditions applied.

As discussed earlier (Section 2.4.4.1), a genomic analysis of strain HN001 found that, while the genes required for the synthesis of a peptidoglycan with a stem pentapeptide ending in D-alanyl-D-lactate, D-alanyl-D-serine, or D-alanyl-D-alanine dipeptide, these genes are scattered throughout the HN001 genome rather than clustered together as expected if they were transferable. Thus it appears that vancomycin resistance in strain HN001 is intrinsic and is not transferable to other bacteria. Several other genes possibly involved in antibiotic resistance were identified in the HN001 genome, but none of these are closely linked to predicted mobile genetic elements and it thus appears unlikely that they are transferable. Of particular significance is that no known antibiotic resistance genes were identified in either of the 2 plasmids.

4.4. *In Vivo* Studies of *L. rhamnosus*

4.4.1. Animal Studies

A number of animal studies of *L. rhamnosus* strain HN001 as well as several other *L. rhamnosus* strains were conducted in order to demonstrate safety and to investigate potential beneficial effects, including protection from *Salmonella typhimurium, Clostridium difficile, Candida albicans* and other infective microbes, prevention or amelioration of colitis, and reduction of bacterial translocation following liver injury. As discussed below, the evidence for such benefits is generally supportive but variable. Most important, though, is that these studies—in which *L. rhamnosus* was given orally to mice, hamsters, rats, and rabbits—have consistently found an absence of any observable adverse effects due to administration of *L. rhamnosus* at daily doses as high as 5×10^{12} cfu/kg bw/day, 10,000 to 100,000 times the exposure likely to occur from its intended use in foods.

4.4.1.1. Studies of *L. rhamnosus* HN001 in Animals

The experimental studies discussed in this section, all in the mouse model, are summarized in Table 7 at the end of the section.

Shu et al. (1999) assessed the safety of *L. rhamnosus* HN001, as well as *L. acidophilus* HN017 and *Bifidobacterium animalis* ssp. *lactis* HN019, in BALB/c mice. Male mice age 6-8 weeks and weighing about 25 g were acclimatized for 14 days on a skim-milk powder-based diet and then randomized into 13 groups with n = 12 mice/group. The 3 probiotic strains, along with commercially obtained *L. acidophilus* LC1 as a probiotic control, were administered orally to mice at 5×10^7, 10^9, or 5×10^{10} cfu/day; a 13th group of mice served as a non-probiotic control. Mice received their assigned treatment for 7 days. Half of the mice in each group were killed

immediately at the termination of treatment while the other half remained on the basal diet (without probiotics) for another 7 days before sacrifice. Clinical observations were conducted daily; feed and water intake and body weight were recorded weekly. After sacrifice, spleens and kidneys were removed to be weighed and examined for any indication of bacterial translocation or infection. Any organisms isolated from tissue samples were subjected to randomly amplified polymorphic DNA (RAPD) finger-printing to identify them. Small sections of ileum, cecum, and colon were excised for histological examination. Blood was drawn after euthanasia and analyzed for plasma glucose level as well as several hematological parameters: erythrocyte and platelet numbers, hemoglobin, mean corpuscular volume, hematocrit, and mean corpuscular hemoglobin concentration.

No adverse clinical signs were noted in any animals, and the mean general appearance scores did not differ among the groups. There was no effect of any treatment on feed or water consumption or on body-weight gain. No viable bacteria were detected in the spleens of any mice. Bacteria were isolated from one mouse in the HN001 high-dose group, one mouse in the HN019 low-dose group, and one mouse in the LC1 mid-dose group, but these isolates produced different DNA finger prints than the test strains and likely came from the mice's intestinal microbiota. The histological analysis found no effects of any treatment on the villi height, mucosal thickness, or epithelial cell height of the intestinal samples. No effects were seen in blood glucose or any hematological measures. Both absolute and relative spleen weights were unaffected, indicating an absence of splenic inflammation such as would result from bacterial infection.

The authors noted that the high dose of 5×10^{10} cfu/day shown to be without adverse effect in this study corresponds to a level of 2×10^{12} cfu/kg bw/day; equivalent to 1.5×10^{13} cfu/day for a 75-kg adult human.

Zhou et al. (2000a) again used male BALB/c mice to assess the safety of *L. rhamnosus* HN001, as well as *L. acidophilus* HN017 and *Bifidobacterium animalis* ssp. *lactis* HN019. Mice age 6-8 weeks and weighing about 20 g were acclimatized for 14 days on a skim-milk powder-based diet and then randomized into 13 groups with n = 6 mice/group. The 3 probiotic strains, along with commercially obtained *L. acidophilus* LA-1 as a probiotic control, were administered orally to mice at 5×10^7, 10^9, or 5×10^{10} cfu/day; a 13th group of mice served as a non-probiotic control. Mice received their assigned treatment for 21 days. Clinical observations were conducted daily; feed and water intake and body weight were recorded weekly. After sacrifice, spleens, livers, and mesenteric lymph nodes were removed to be weighed and examined, along with blood samples, for any indication of bacterial translocation or infection. Any organisms isolated from tissue samples were subjected to randomly amplified polymorphic DNA (RAPD) finger-printing to identify them. Small sections of ileum, cecum, and colon were excised for histological examination. Blood was drawn after euthanasia and analyzed for both hematological and biochemical parameters. Hematology measures included erythrocyte and platelet numbers, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and both total and differential leukocyte counts (lymphocytes, neutrophils, monocytes, and eosinophils). Biochemistry measures were total plasma protein, albumin, plasma glucose, and total plasma cholesterol.

There was no mortality and no adverse clinical signs were noted in any animals. There was no effect of any treatment on feed or water consumption or on body-weight gain. There was no effect on any of the hematological or biochemical parameters assessed. The histological analysis found no sign of hepatomegaly, splenomegaly, or cecal enlargement. Both absolute and relative spleen weights were unaffected. Mucosal examination found no indication of inflammation, degeneration, or necrosis or any effect on the villi height, mucosal thickness, or epithelial cell height of the intestinal samples. No viable bacteria were detected in the spleens of any mice except 1 mouse receiving LA-1. The mesenteric lymph nodes revealed bacteria in 1 of the 6 control mice and 10 of the 72 mice receiving probiotics. Bacteria were isolated from the livers of 1 control mouse and 2 mice receiving LA-1, but no mice receiving HN001, HN017, or HN019. Bacteria were isolated from the kidneys of one mouse receiving HN017 and 2 mice receiving LA-1. No bacteria were isolated from the blood of any mouse. There was no correspondence between bacterial translocation and probiotic administration v. non-administration or with probiotic strain or dose. Further, no RAPD pattern from the isolated bacteria matched any of the test strains. Zhou et al. (2000a) concluded that HN001, HN017, and HN019 are non-pathogenic and safe for human consumption.

Zhou et al. (2000b) performed a similar study using these same bacterial strains but administered them at far higher doses, 10^{11} cfu/day. *L. rhamnosus* HN001, *L. acidophilus* HN017, *B. animalis* ssp. *lactis* HN019, *L. rhamnosus* GG, *L. acidophilus* LA-1, or placebo were administered twice a day at doses of 5×10^{10} via oral pipette to 48 male BALB/c mice aged 6-8 weeks and weighing about 20 g (8 mice/group). Mice were acclimatized for 7 days on a skim-milk powder-based diet and then randomized to receive one of the 6 treatments for 8 days. Clinical observations were conducted twice daily and activity level was recorded daily; feed intake and body weight were recorded weekly. After sacrifice, spleens, livers, and mesenteric lymph nodes were removed to be weighed and examined, along with blood samples, for any indication of bacterial translocation or infection. Prior to excision of tissue samples the surfaces of viscera were swiped with sterile swabs that were subsequently cultured to test for bacterial contamination. Any organisms isolated from visceral surfaces or tissue samples were subjected to randomly amplified polymorphic DNA (RAPD) finger-printing to identify them. Small sections of ileum, cecum, and colon were excised for histological examination.

There was no mortality and no effects of any of the treatments were observed in the animals' behavior, hair luster, or activity level. There were no effects on feed intake or body-weight gain. Neither macro- nor microscopic examination of the excised organs revealed any effect of any of the treatments; there was no sign of inflammation, degeneration, or necrosis of intestinal mucosa; no significant differences were detected in villus height, epithelial cell height, crypt depth, or mucosa thickness. There was no indication of hepatomegaly, splenomegaly, or cecal enlargement. Neither absolute nor relative spleen weight was affected. There was no bacterial growth in the visceral swab cultures or in the blood of any animal. Although bacteria were detected in excised tissues, especially the mesenteric lymph nodes, the prevalence was the same in all groups and RAPD testing established that no isolated bacteria matched any of the probiotic strains administered.

In summary, Zhou et al. (2000b) concluded that the tested probiotic strains, administered at daily doses of 10^{11} cfu (about 700 times the likely human dose), had very low acute toxicity, no effect upon mucosa, little likelihood of translocation, and no indication of potential infectivity.

Gill et al. (2000) studied the effect of feeding *L. rhamnosus* HN001, *L. acidophilus* HN017, and *B. animalis* ssp. *lactis* on indices of natural and acquired immunity in mice. In the first experiment, 80 male BALB/c mice age 6-7 weeks were fed a milk-based diet for 1 week and then randomized into 4 groups (n = 20 mice/group) receiving 10^9 cfu/day of 1 of the 3 probiotic strains in skim milk or skim milk alone for 10 days. On days 0 and 7, mice were orally administered cholera toxin. At termination on day 10, peritoneal macrophages, blood, spleens, and intestinal washings were taken for examination. In the second experiment, 72 mice were treated as in the first experiment (with 18 mice/group) but maintained on the diets for 28 days and inoculated subcutaneously with tetanus vaccine or days 7 and 21. Feed intake and body weights were measured weekly. At termination on day 28, peritoneal macrophages, blood, spleens, and intestinal washings were taken for examination. Spleens were forced into single-cell suspensions and their proliferation measured *in vitro* in the presence or absence of T- and B-cell mitogens (concanavalin A and lipopolysaccharide, respectively). Spleen cells were also cultured and the presence of interleukin (IL)-4 and interferon (IFN)-γ was determined using ELISA. Serum or intestinal fluid samples were exposed to cholera toxin or tetanus toxoid and ELISA was used to determine antibody response. The expression of CD4, CD8, and CD40 antigens on peripheral blood leucocytes was monitored by flow cytometric analysis. The phagocytic capacity of peripheral blood leucocytes and peritoneal macrophages as well as the natural killer (NK)-cell activity of spleen cells were also assessed using flow cytometry-based assays.

None of the probiotic strains had any effect on feed intake, body-weight gain, production of IL-4, or expression of CD4, CD8, or CD40 cells in the blood, but all 3 produced significant increases in the phagocytic activity of peripheral blood leucocytes (monocytes and polymorphonuclear cells) and peritoneal macrophages as well as the proliferative responses of spleen cells to both T- and B-cell mitogens. The spleen cells of mice given *L. rhamnosus* HN001 or *L. acidophilus* HN017 produced higher amounts of IFN-γ in response to stimulation with concanavalin A, indicating an effect on Th1 T-cells but apparently not on Th2 cells. Serum antibody responses to antigens were enhanced by oral administration of all 3 probiotic strains, but NK-cell activity was increased only in the mice given *L. rhamnosus* HN001.

A set of 3 experiments was conducted to assess the ability of *L. rhamnosus* HN001 to protect against *Salmonella typhimurium* (ATCC 1772) infection (Gill et al. 2001b). All 3 studies used 6-8-week-old conventional male BALB/c mice that were individually caged with *ad libitum* access to water and a skim milk powder-based diet. In the first experiment, 36 mice received 10^9 cfu/day *L. rhamnosus* for 7 days while 35 mice served as controls. All mice then were challenged with a single oral dose of 10^7 cfu *S. typhimurium*. The mice continued to receive their assigned treatment for 21 additional days, although 6 mice from each group were euthanized 6 days after the challenge. General health scores, based on observation, were recorded daily; at termination intestinal washings and blood samples were taken for antibody analysis. The general health scores, as well as feed and water intake and weight gain, were significantly higher among the mice receiving probiotics, and mortality was much lower; only 2 of the 29 control mice survived to the end of the study while 27 of 30 HN001-fed mice survived. The anti-*S. typhimurium*

antibody titers were significantly higher in both blood and intestinal fluid samples among the probiotic-fed mice.

In Experiment 2, Gill et al. (2001b) fed 21 mice 10^9 cfu/day *L. rhamnosus* HN001 for 14 days while 21 mice served as controls. All mice were challenged on days 7, 8, 9, 10, and 11 with 8 x 10^5 cfu/day *S. typhimurium.* All mice were monitored for general health and mortality and 6 mice were killed from each group at the end of the dosing period (day 14). Blood and peritoneal and visceral tissue samples were taken for bacteriological and immunological analysis. Both general health scores and survival were lower among the control mice than the HN001-fed mice; at day 14 16/21 HN001-fed mice and 12/21 controls survived. Significantly higher counts of salmonellae were recovered from the liver and spleen of the control mice than the probiotic-fed mice, and the phagocytosis responses of the blood and peritoneal leucocytes were significantly higher among the HN001-fed mice than the controls.

Gill et al.'s (2001b) Experiment 3 included 3 groups of mice: 2 test groups (n = 5 mice each) and a control group with 4 mice. Test group 1 received 10^9 cfu/day *L. rhamnosus* HN001 for 7 days before the *S. typhimurium* challenge and for 5 additional days after it began while test group 2 received the same dose of probiotic at the same time as the challenge and also received it for 5 further days. The control group received no probiotic. The *Salmonella* challenge was 5 x 10^7 cfu/day *S. typhimurium* each day for 5 days. All mice were killed 5 days after initiation of the challenge and visceral tissue samples were taken for bacteriological investigation. Mice pretreated with HN001 showed significantly lower counts (about 100-fold) of *Salmonellae* in the spleen and liver compared with controls. Mice that received the probiotic simultaneously with the challenge also had slightly lower salmonella counts than did the controls, but the difference was significant only in splenic tissue.

Together, these experiments demonstrated that ingestion of *L. rhamnosus* HN001 significantly reduces the ability of *S. typhimurium*, a highly infective species, to translocate to the visceral organs. This effect was seen with both high and low doses of *Salmonella*. Further, *L. rhamnosus* HN001 also enhanced the murine innate immune response to pathogenic challenge among different host cells and at different somatic sites, with neutrophils in blood and macrophages in peritoneal leucocytes showing enhanced activity (Gill et al. 2001b). No adverse effects due to the probiotic treatment were reported in any of the 3 experiments.

Because of the possibility that intestinal microbiota could contribute to the pathogenesis of autoimmune diseases, Zhou and Gill (2005) tested the effect of administration of *L. rhamnosus* HN001 and *B. animalis* ssp. *lactis* HN019 on pathological inflammation in a mouse model of experimental autoimmune thyroiditis. Mouse thyroglobulin was obtained through processing of frozen thyroids from 300 inbred BALB/c and CBA/CaH mice and its purity confirmed by SDS-PAGE analysis. Female CBA/CaH (n = 114) mice 4-6 weeks old and possessing the H-2^k haplotype (which renders them more susceptible to the induction of experimental autoimmune thyroiditis) were acclimated to the housing conditions for 2 weeks and then randomized to receive saline solution, *L. rhamnosus* HN001, *B. animalis* ssp. *lactis* HN019, or *L. rhamnosus* GG once a day via pipette. The daily probiotic dose was 6 x 10^7 to 1.5 x 10^8 cfu. The mice were randomized to be inoculated subcutaneously with thyroglobulin after 7 days and again after 14 days or to be inoculated with thyroglobulin + lipopolysaccharide precipitated from

E. coli 055:B5, intended to exacerbate the development of autoimmune response; this second treatment also included another booster dose after 42 days. The health status and behavior of the mice was observed daily, body temperatures were taken every other day, and body weights were measured weekly. Animals were euthanized after receiving probiotics or placebo for 5, 8, or 9 weeks and blood, spleen, and thyroid glands were collected. ELISA was used to determine levels of anti-thyroglobulin IgG1 and IgG2a antibodies in serum in order to confirm the establishment of an autoimmune reaction. Spleens were weighed and spleen lymphocyte proliferative response to thyroglobulin was assessed; and the extent of mononuclear cell infiltration in the thyroid tissues was determined.

There was no mortality, and there were no differences in clinical observations, body temperatures, or body weights of the mice receiving saline or probiotics. All mice inoculated with thyroglobulin produced measurable titers of IgG1 and IgG2a antibodies against thyroglobulin, demonstrating that an autoimmune response had been induced. The mice also showed inflammatory responses in increased relative spleen weights and increased splenic lymphocyte proliferation. Probiotic feeding had no effect on these responses. Histological changes in thyroid tissue were mild after inoculation with thyroglobulin alone but greater with the addition of *E. coli* lipopolysaccharide. As shown in Table 6, administration of probiotic bacteria did not affect the incidence of experimental autoimmune thyroiditis.

Table 6. Incidence of Experimental Autoimmune Thyroiditis.

Group	Thyroglobulin Alone		Thyroglobulin + Lipopolysaccharide	
	N	% EAT*	N	% EAT
Control (no treatment)	12	0	12	0
Saline	12	17	12	50
HN001	12	17	12	50
HN019	12	25.00	12	42
LGG	11	18	6	50

Source: Zhou and Gill 2005

% EAT = the percentage of mice that exhibited symptoms of experimental autoimmune thyroiditis

Zhou and Gill (2005) concluded that long-term daily ingestion of the tested probiotics had no adverse effect on the induction or progression of thyroglobulin-induced experimental autoimmune thyroiditis in CBA/CaH mice. Because this murine model mimics the cellular and pathological manifestations of Hashimoto's thyroiditis (a human autoimmune disease), it is widely used as a model to study human autoimmunity. Thus, the findings suggest that immunostimulatory probiotic *L. rhamnosus* HN001 (as well as the 2 other strains tested) does not induce or enhance autoimmune response in humans.

In summary, *L. rhamnosus* HN001 has been orally administered to BALB/c mice for up to 21 days at doses as high as 10^{11} cfu/day with no indication of toxicity or adverse effects on

immune, gastrointestinal, hematological, or biochemical parameters. The highest dose administered, 10^{11} cfu/day, is equivalent to 5 x 10^{12} cfu/kg bw/day. For a 65-kg human, a daily dose of 5 x 10^{12} cfu/kg is equivalent to 3 x 10^{14} cfu/day, 10,000 to 100,000 times the intake estimated to result from the intended use of HN001.

Additionally, there was no suggestion of infectivity in any of the research; there was no sign of inflammation, hepatomegaly, or splenomegaly, and no viable bacteria matching the test strains were isolated in spleens or blood of any mouse. Furthermore, the probiotic did not affect the incidence of experimental autoimmune thyroiditis in highly susceptible female CBA/CaH mice possessing the H-2^k haplotype.

Table 7. Studies of *L. rhamnosus* HN001 in Animals

Reference	Objective	Study Design	Animal Model	*L. rhamnosus* Dose	Duration	Safety-Related Results
Gill et al. (2000) (2 reported experiments)	Investigate the effect of feeding *L. rhamnosus* HN001, *L. acidophilus* HN017, or *B. animalis* ssp. *lactis* on indices of natural and acquired immunity in mice.	1. Mice were acclimatized for 1 week and randomized into 4 groups receiving 1 of the 3 probiotic strains or placebo for 10 days. On days 0 and 7, mice were orally administered cholera toxin 2. Same, but maintained on the diets for 28 days and inoculated subcutaneously with tetanus vaccine or days 7 and 21	1. 80 male BALB/c mice age 6-7 weeks 2. 72 male BALB/c mice age 6-7 weeks	1. 10^9 cfu/day 2. 10^9 cfu/day	1. 10 days 2. 28 days	None of the probiotic strains had any effect on feed intake, body-weight gain, production of IL-4, or expression of CD4, CD8, or CD40 cells in the blood, but all 3 produced significant increases in the phagocytic activity of peripheral blood monocytes and polymorphonuclear cells, peritoneal macrophages, and proliferative responses of spleen cells to T- and B-cell mitogens. The spleen cells of mice given *L. rhamnosus* HN001 or *L. acidophilus* HN017 produced higher amounts of IFN-γ in response to stimulation with concanavalin A, indicating an effect on Th1 T-cells but not on Th2 cells. Serum antibody responses to antigens were enhanced by oral administration of all 3 probiotic strains, but NK-cell activity was increased only in the mice given *L. rhamnosus* HN001. No adverse effects were observed.
Gill et al. (2001b) (3 reported experiments)	Assess the ability of *L. rhamnosus* HN001 to protect against *Salmonella typhimurium* infection in mice.	1. Mice received *L. rhamnosus* or placebo for 28 days, challenged on day 8 with a high dose of *S. typhimurium.* 2. Mice received *L. rhamnosus* or placebo for 14 days, challenged on days 7, 8, 9, 10, and 11 with low doses of *S. typhimurium.*	1. 71 6-8-week-old male BALB/c mice 2. 42 6-8-week-old male BALB/c mice	1. 10^9 cfu/day 2. 10^9 cfu/day	1. 28 days (7 pre-infection, 21 post infection) 2. 14 days	1. General health scores, feed and water intake, and weight gain were higher among mice receiving probiotics and mortality was lower. Anti-*S. typhimurium* antibody titers were higher in blood and intestinal fluid samples among the probiotic-fed mice. 2. General health scores and survival were lower among the control mice than the HN001-fed mice. Higher counts of salmonellae were recovered from the liver and spleen of the control mice than the probiotic-fed mice, and the phagocytosis responses of the blood and peritoneal leucocytes were significantly higher among the HN001-fed mice than the controls.

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Table 7. Studies of *L. rhamnosus* HN001 in Animals

Reference	Objective	Study Design	Animal Model	*L. rhamnosus* Dose	Duration	Safety-Related Results
Gill et al. (2001b), continued	Assess the ability of *L. rhamnosus* HN001 to protect against *Salmonella typhimurium* infection in mice.	3. One group of mice received *L. rhamnosus* or placebo for 12 days before and during the *S. typhimurium* challenge on days 8, 9, 10, 11, and 12 while the other group received *L. rhamnosus* only for 5 days simultaneously with the challenge	3. 14 6-8-week-old male BALB/c mice	3. 10^9 cfu/day	3. 12 days and 5 days	3. Mice pretreated with HN001 showed significantly lower counts (about 100-fold) of salmonellae in the spleen and liver compared with controls. Mice that received the probiotic simultaneously with the challenge also had slightly lower salmonella counts than did the controls, but the difference was significant only in splenic tissue. No adverse effects were reported in any of the 3 experiments.
Shu et al. (1999)	Assess the safety of *L. rhamnosus* HN001, *L. acidophilus* HN017, and *Bifidobacterium animalis* ssp. *lactis* HN019	Mice were acclimatized and then randomized into 13 groups. The 3 probiotic strains and commercially obtained *L. acidophilus* LC1 as a probiotic control were administered orally to mice at 3 dose levels; a 13th group of mice served as a non-probiotic control. Half of the mice were killed at the termination of treatment (7 days) while the other half remained on the basal diet (without probiotics) for another 7 days before sacrifice	156 male BALB/c mice aged 6-8 weeks and weighing about 25 g	0, 5×10^7, 10^9, or 5×10^{10} cfu/day	7 days	No adverse clinical signs were noted in any animals, and the mean general appearance scores did not differ among the groups. There was no effect of any treatment on feed or water consumption or on body-weight gain. No viable bacteria were detected in the spleens of any mice. The histological analysis found no effects of any treatment on the villi height, mucosal thickness, or epithelial cell height of the intestinal samples. No effects were seen in blood glucose or any hematological measures. Both absolute and relative spleen weights were unaffected, indicating an absence of splenic inflammation such as would result from bacterial infection.

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Table 7. Studies of *L. rhamnosus* HN001 in Animals

Reference	Objective	Study Design	Animal Model	*L. rhamnosus* Dose	Duration	Safety-Related Results
Zhou et al. (2000a)	Assess the safety of *L. rhamnosus* HN001, *L. acidophilus* HN017, and *Bifidobacterium animalis* ssp. *lactis* HN019	Mice were acclimatized and then randomized into 13 groups. The 3 probiotic strains and commercially obtained *L. acidophilus* LC1 as a probiotic control were administered orally to mice at 3 dose levels; a 13th group of mice served as a non-probiotic control.	78 male BALB/c mice aged 6-8 weeks and weighing about 20 g	0, 5 x 10^7, 10^9, or 5 x 10^{10} cfu/day	21 days	There was no mortality and no adverse clinical signs were noted in any animals. There was no effect of any treatment on feed or water consumption, body-weight gain, any of the hematological or biochemical parameters assessed, or on histological parameters. Both absolute and relative spleen weights were unaffected. Mucosal examination found no indication of inflammation, degeneration, or necrosis or any effect on the villi height, mucosal thickness, or epithelial cell height of the intestinal samples. No viable test-strain bacteria were detected in the spleens, kidneys, or blood of any mice.
Zhou et al. (2000b)	Assess the safety of *L. rhamnosus* HN001, *L. acidophilus* HN017, and *Bifidobacterium animalis* ssp. *lactis* HN019 with *L. rhamnosus* GG and *L. acidophilus* LA-1 as comparison strains	Mice were acclimatized for 7 days and randomized to receive one of the 5 treatments or placebo	48 male BALB/c mice aged 6-8 weeks and weighing about 20 g	10^{11} cfu/day (twice a day at doses of 5 x 10^{10} via oral pipette)	8 days	There was no mortality and no effects of any of the treatments were observed in the animals' behavior, hair luster, activity level, feed intake, or body-weight gain. Macro- and microscopic examination of the excised organs revealed no effect or any sign of inflammation, degeneration, or necrosis of intestinal mucosa; no difference was seen in villus height, epithelial cell height, crypt depth, or mucosa thickness. No hepatomegaly, splenomegaly, or cecal enlargement was seen. Neither absolute nor relative spleen weight was affected. There was no bacterial growth in the visceral swab cultures or in the blood of any animal. No test-strain bacteria were detected in excised tissues. The tested probiotic strains had very low acute toxicity, no effect on mucosa, little likelihood of translocation, and no sign of infectivity.

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Table 7. Studies of *L. rhamnosus* HN001 in Animals

Reference	Objective	Study Design	Animal Model	*L. rhamnosus* Dose	Duration	Safety-Related Results
Zhou and Gill (2005)	Investigate the effect of administration of *L. rhamnosus* HN001 and *B. animalis* ssp. *lactis* HN019 on pathological inflammation in a mouse model of experimental autoimmune thyroiditis	Mice were acclimated for 2 weeks and randomized to receive one of the test strains, *L. rhamnosus* strain GG, or placebo. The mice were also randomized to be inoculated subcutaneously with thyroglobulin after 7 days and again after 14 days or to be inoculated with thyroglobulin + lipopolysaccharide with a booster at 42 days	114 female CBA/CaH mice 4-6 weeks old possessing the H-2^k haplotype for susceptibility to experimental autoimmune thyroiditis	6×10^7 to 1.5×10^8 cfu/day via pipette	5, 8, or 9 weeks	There was no mortality and no differences in clinical observations, body temperatures, or body weights of the mice receiving saline or probiotics. All mice inoculated with thyroglobulin produced measurable titers of IgG1 and IgG2a antibodies against thyroglobulin and inflammation was shown in increased relative spleen weights and increased splenic lymphocyte proliferation. Administration of probiotic bacteria did not affect the incidence of experimental autoimmune thyroiditis. Long-term daily ingestion of the tested probiotics had no adverse effect on the induction or progression of thyroglobulin-induced experimental autoimmune thyroiditis in CBA/CaH mice. The findings suggest that *L. rhamnosus* HN001 (as well as the 2 other strains tested) does not induce or enhance autoimmune response in humans.

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4.4.1.2. Studies of Other *L. rhamnosus* Strains in Animals

The experimental studies discussed below are summarized in Table 9 at the end of the section.

4.4.1.2.1. Mouse

In 2 experiments, Dong et al. (1987) studied the effect of *L. rhamnosus* strain GG (LGG) on mice subjected to lethal radiation. For each experiment, 40 CD1 white Swiss mice (21-28 days old) were randomized into groups that were fed either 0 or 3.5 -- 4.0 x 10^{10} cfu LGG/ day via suspension in drinking water available *ad libitum*. Ten days after beginning ingestion of the bacteria, the mice were irradiated with 1400 rads; feeding of LGG was continued. The only difference in the 2 experiments was whether the mice were fed chow or meat. Both experiments showed a statistically significant reduction in the time to mortality for the probiotic-fed animals relative to controls. All of the animals had lactobacilli in their stools, but only the test mice had lactobacilli that morphologically resembled those administered. Although bacteremia was observed *post mortem* in all of the mice, both in the experimental groups and in the controls, none of the isolates were identified as lactobacilli. The *L. rhamnosus*-fed mice yielded more streptococcal isolates and fewer Gram-negative enteric organisms than controls. No adverse effects of the probiotic administration were reported. The authors concluded that the *L. rhamnosus* strain tested "is a noninvasive organism that may reduce Gram-negative bacteremia and prolong survival in irradiated mice."

Donohue et al. (1993), using healthy adult male Swiss mice, conducted acute toxicity studies with a number of strains of probiotic bacteria belonging to the genera *Lactobacillus* (including a *rhamnosus* strain identified as GG), *Streptococcus*, and *Bifidobacterium*. Mice were randomized into groups (n = 5 mice/group) to receive doses of 0, 1.5 x 10^{11}, 3 x 10^{11}, 6 x 10^{11}, or 9 x 10^{11} cfu/kg via gavage; the mice were observed twice daily for 7 days prior to sacrifice and necropsy. No significant changes were observed in behavior, feed intake, or body weight. No treatment-related deaths or signs of toxicity were observed, and no remarkable changes were reported in the gross appearance of any organs. The authors concluded that the acute oral LD_{50} for male Swiss mice of each of the bacterial strains tested is >9 x 10^{11} cfu/kg bw.

Wagner et al. (1997a) assessed the capacity of *L. rhamnosus* strain GG (referred to at the time as *L. casei* strain GG), *L. reuteri*, *L. acidophilus*, and *Bifidobacterium animalis* to colonize, stimulate immune responses in, and affect the growth and survival of congenitally immunodeficient gnotobiotic beige-athymic (*bg/bg-nu/nu*) and beige-euthymic (*bg/bg-nu/+*) mice. The bacteria were introduced by means of swabs of the oral and rectal cavities of the mice with a solution containing 10^8 cfu/ml of the specific strain. The bacteria colonized and persisted at high concentrations (10^8 to 10^{10} cfu/g) in the alimentary tracts of both mouse strains for the entire study period (12 weeks). It was not clear why such persistent colonization occurred since it is not seen in humans or other animal models, but likely results from the doubly immunodeficient status of this model.

Adherence to epithelial surfaces was widely different across the various strains tested, with *L. acidophilus* and *B. animalis* showing 86% and 82% adherence, respectively, compared with 2% and 5%, respectively, for *L. reuteri* and *L. rhamnosus* GG. Translocation closely

correlated with adherence — such translocation to internal organs was detected in 50% or more of the mice colonized with *L. acidophilus* or *B. animalis*, 26% of those colonized with *L. rhamnosus*, and none of the mice colonized with *L. reuteri*. The translocation did not result in any signs of morbidity, mortality, or pathologic changes in the immunodeficient mice, and the probiotic bacteria neither retarded nor enhanced the growth of male or female athymic or euthymic mice.

Although all adult and neonatal beige-euthymic mice survived probiotic colonization, some infant mortality occurred in beige-athymic pups born to mothers colonized with pure cultures of *L. rhamnosus* or *L. reuteri*. Six of 28 pups born to *L. reuteri* colonized dams and 19 of 53 pups born to *L. rhamnosus* colonized dams died; there was no mortality among pups of any other group. This mortality was evident by 4 weeks of age, and no further mortality occurred after that time up to 12 weeks. There was no evidence of translocation and histological examination of tissue from 4 pups treated with each strain did not show any evidence of pathologic changes in the gastrointestinal tract or internal organs; thus, the reason for the infant mortality remains unknown. The authors offered the thought that although the probiotic species were innocuous for adults, these results suggest that caution and further studies are required to assess the safety of probiotic bacteria for immunodeficient neonates. It should be noted, however, that the EU-PROSAFE project (Vankerckhoven et al. 2007a) considered that use of the immunocompromised mouse model is premature and recommended against it, and thus it might be appropriate to regard the results of Wagner et al. (1997a) as suggestive rather than determinative. Indeed, the homozygote *bg/bg nu/nu* mouse is doubly immunodeficient in that it lacks both NK-cells and functional T-cells and has deficient phagocytosis. It is not scientifically valid to use data from such immunocompromised animals to depict circumstances applicable to immunocompetent humans.

The same 4 strains, *L. rhamnosus* strain GG (referred to as *L. casei* strain GG), *L. reuteri*, *L. acidophilus*, and *Bifidobacterium animalis*, were assessed for their ability to protect athymic *bg/bg-nu/nu* and euthymic *bg/bg-nu/+* mice from mucosal and systemic candidiasis (Wagner et al. 1997b). Each bacterial strain and *Candida albicans* colonized the gastrointestinal tracts of both strains of mice. The presence of probiotic bacteria in the gastrointestinal tracts prolonged the survival of adult and neonatal *bg/bg-nu/nu* mice compared to that of isogenic mice colonized with *C. albicans* alone. The incidence of systemic candidiasis in *bg/bg-nu/nu* mice was significantly reduced by each of the four probiotic bacterial strains. None of the probiotic bacteria strains completely prevented mucosal candidiasis, but the prolonged survival time, decreased severity of mucosal and systemic candidiasis, modulation of immune responses, decreased number of *C. albicans* in the alimentary tract, and reduced numbers of orogastric infections demonstrated that these probiotic bacteria have biotherapeutic potential for prophylaxis against this fungal disease, with no harmful side effects. Notably, no bacterial infection occurred in this doubly immunodeficient animal model.

Nineteen 6-week-old female mice homozygous for colitic severe combined immunodeficiency (scid/scid) were treated for 1 week with the antibiotics vancomycin and meropenem, followed by a 3-week administration of $2x10^{10}$ cfu/day *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246 or placebo (Moller et al. 2005). After 12 weeks, the rectums were removed for histology and CD4 T cells from the mesenteric lymph nodes (MLN) were polyclonally

activated for cytokine measurements. All mice treated with antibiotics but not fed probiotics showed severe gut inflammation, whereas only 2 of the 7 mice fed probiotics showed signs of severe colitis ($p<0.05$). MLN-derived CD4 T cells from this latter group of mice showed lower levels of interleukin-4 secretion ($p<0.05$) and a tendency to higher interferon-γ production than mice not fed probiotics. No adverse effects resulted from the administration of *L. rhamnosus* and *L. reuteri* to these severely immunodeficient animals.

Starter cultures used in Chinese fermented milk products were tested for acute toxicity in an unreported number of an unidentified strain of mice (Xu et al. 2008). The studied bacteria included LGG and an unspecified *L. rhamnosus* strain, *Bifidobacterium animalis* ssp. *lactis* Bb-12, and a strain of *L.reuteri*. None showed any toxic effects when administered orally over 3 days. One *L. rhamnosus* strain, after intraperitoneal injection at the highest dose of 5.0×10^9 cfu/ml, led to toxic symptoms and lethality in most of the mice. These toxic effects were not observed with lower doses (5×10^7 cfu/ml or 5×10^8 cfu/ml) of this strain or with any dose of the three other strains studied.

4.4.1.2.2. Hamster

As part of a larger study, Naaber et al. (1998) observed the effects of ingestion of *L. rhamnosus* strain GG (LGG) on 10 adult Syrian hamsters that received a single 3-mg dose of ampicillin and were inoculated with *Clostridium difficile* 24 hours later. The hamsters were given 0 or 0.5 ml LGG in broth culture (the cfu content was not specified) once daily starting 20 hours prior to the *C. difficile* challenge and continuing for 5 days until sacrifice. LGG was present in intestinal cultures from all treated animals, but it did not become predominant in the intestinal microbiota and was not observed among translocating lactobacilli. Four of the 5 hamsters receiving LGG remained healthy and the inflammatory changes in their intestinal mucosa were milder than those receiving no probiotic. No adverse effects were noted.

4.4.1.2.3. Rat

Adawi et al. (1997) investigated the ability of a variety of lactobacillus strains to reduce bacterial translocation after acute liver injury. One of 5 lactobacillus strains, including 1 strain of *L. rhamnosus* (DSM 6594) as well as 2 strains of *L. reuteri*, 1 *L. plantarum*, and 1 *L. fermentum*, was administered rectally for 8 days to 78 male Sprague-Dawley rats (weight 200-300 g) which were then injected with D-galactosamine to induce acute liver injury. Twelve rats received each probiotic treatment, 6 with supplementary arginine and 6 without. Three additional groups of 6 rats served as a control, an acute liver injury control, and a group that received only arginine. Samples of the liver caudate lobe, mesenteric lymph nodes, cecal and colonic contents, and aortic and portal blood were obtained 24 and 48 hours after D-galactosamine administration. Liver function tests for alkaline phosphatase, aspartate transaminase, alanine transaminase, and bilirubin found lower levels, indicating improved liver function, in the groups treated with *L. rhamnosus* or the other probiotic strains than in the controls. Bacterial translocation was evaluated by bacterial culture from portal and arterial blood, mesenteric lymph nodes, and liver tissue after 24 and 48 hours. The probiotic treatment reduced bacterial translocation without any reported adverse effects.

Pessi et al. (1998) studied the effects of *L. rhamnosus* GG (LGG) on macromolecular degradation in the gut mucosa of neonatal rats. Wistar rat dams gave birth to pups that were

winnowed to a density of 8-10 pups/dam. At postnatal day 14 the pups were randomized into 5 feeding groups (n = 10 pups/group) which received both maternal milk and a daily gavage of bicarbonate buffer (control), cow's milk, cow's milk + 10^{10} cfu LGG, extensively hydrolyzed whey formula, or extensively hydrolyzed whey formula + 10^{10} cfu LGG. After 7 days on these regimens (postnatal day 21), the pups were sacrificed, a laparotomy was performed, and 8-19 jejunal segments were excised. Transmural protein transfer was assessed, as well as β-lactoglobulin and horseradish peroxidase, chosen to represent the molecular weight and intestinal transport properties of dietary antigens. The degree of macromolecular degradation was studied by means of HPLC gel filtration. Intestinal function and tissue damage was assessed by monitoring various electrical parameters.

The rate of absorption of horseradish peroxidase across the jejunum was significantly higher in the milk and hydrolysate groups than the controls, but supplementation with LGG restored absorption rate to the control level. For β-lactoglobulin, a greater absorption rate was observed in the milk group relative to controls, while the milk-LGG, hydrolysate, and hydrolysate-LGG groups did not differ from controls. The addition of LGG to milk was observed to increase the transport of degraded horseradish peroxidase relative to milk alone, while the addition of LGG to hydrolysate was observed to reduce the transport of degraded horseradish peroxidase relative to hydrolysate alone. During the study, absorption of intact proteins and electrical parameters were unchanged, which indicated that LGG supplementation did not result in tissue damage or dysfunction of the gut mucosa. No adverse effects were reported, and the authors concluded that, "probiotics affect mucosal permeability by restoring aberrant macromolecular transport and may thus be beneficial in reversing disturbances induced by mucosal inflammation increased permeability, immune response to antigen, and composition of the microflora."

Holma et al. (2001) compared the effects of *L. rhamnosus* GG (LGG) and rat-derived *L. reuteri* R2LC on acetic acid-induced colitis in rats. Forty male outbred HY:WIST rats weighing 180-280 g were housed individually and randomized into 5 groups of 8 rats each. One group was normal control rats and a second group was rats with induced colitis. The third group received 10^9-10^{10} cfu LGG/day in lactose-free cows' milk, the fourth received the same amount of *L. reuteri* strain R2LC, and the fifth group received 100 mg sulphasalazine/kg bw/day. Supplementation was begun 7 days before colitis induction and continued through the study. The rats were sacrificed and colitis was assessed 72 hours after induction with acetic acid. *L. reuteri* significantly antagonized body weight loss caused by inflammation compared with LGG or sulphasalazine, and edema formation in the colon compared with sulphasalazine. *L. reuteri* reduced the median value of macroscopic ulceration and the protein content of inducible nitric oxide synthase by 50% and the median of the protein content of inducible cyclooxygenase by 30% compared with that of the colitis control group. The authors concluded that *L. reuteri* R2LC, but not LGG, is of benefit in reducing the severity of acetic acid-induced colitis in rats. They also noted that neither LGG nor *L. reuteri* produced any observable side effects.

Effects of *L. rhamnosus* and *L. reuteri* administration on hematological parameters were studied in Sprague-Dawley rats by Anukam et al. (2004). The strains tested were *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (classified at the time of the study as *L. fermentum*). Daily doses of 10^9 cfu of the strains were fed via orogastric tube for 21 days to 20 male SD rats weighing

between 110 and 170 g; control rats received a daily gavage with the same quantity of bicarbonate buffer. The animals had *ad libitum* access to rat chow and water. After 21 days the rats were anesthetized with ether and blood samples were collected by carotid artery cannulation for analysis of 12 hematological parameters. As shown in Table 8, no effects were observed in any hematological measure; no differences between test and control rats were significant and all values were within the normal range. The absence of any effect on the total white cell count indicates that there was no induction of the peripheral inflammatory response associated with pathogens. Similarly, the lack of any change in lymphocyte count indicates that the probiotic bacteria did not evoke any peripheral lymphocytosis.

Table 8. Hematological Parameters Of Rats Given *L. rhamnosus* and *L. reuteri*

Parameter	Probiotic-Fed Rats	Control Rats
Total white blood cells x $10^3/\mu l$	5.18	8.46
Lymphocytes (%)	84.00	85.62
Neutrophils (%)	5.18	6.66
Monocytes, eosinophils, basophils (%)	10.26	7.72
Red blood cells x $10^6/\mu l$	5.74	6.01
Hemoglobin (g/dl)	12.20	12.36
Hematocrit concentration (%)	34.26	36.02
Mean cell volume	59.56	60.38
Mean cell hemoglobin	17.80	17.30
Mean cell hemoglobin concentration	29.92	28.68
Red blood cell distribution width	12.90	14.48
Platelets x $10^3/\mu l$	448.8	347.2
Source: Anukam et al. (2004)		

Vankerckhoven et al. (2007) studied the potential infectivity of 10 isolates of *L. rhamnosus* and 7 isolates of *L. paracasei* in a rat model of experimental infective endocarditis. All test organisms were obtained from the PROSAFE strain collection. Four of the *L. rhamnosus* strains were commonly used probiotics, 4 were strains isolated from human endocarditis events, 1 was a strain used for fermentation processes, and 1 strain was under investigation as a potential probiotic. The authors argued that the rat model is appropriate in that it simulates the pathophysiology of human infective endocarditis, requiring a pre-existing valve lesion that becomes colonized during transient bacteremia, followed by bacterial proliferation and dissemination of septic emboli. Lesions were induced in female Wistar rats weighing 180-200 g by insertion of a catheter across the aortic valve; 24 hours later, the rats were intravenously

injected with 10^4-10^8 cfu of each test organism, using groups of 7-11 animals per strain and per inoculum. The rats were sacrificed 72 hours later and the heart and infected valves were dissected for bacterial enumeration. Positive and negative controls of infectivity were provided by *Staphylococcus aureus* Newman, *Streptococcus gordonii* Challis, and *Lactococcus lactis* 1363. In addition, adhesion of lactobacilli to immobilized host matrix proteins was assessed as well as susceptibility to platelet microbiocidal proteins.

The 90% infective doses (ID_{90}) of *S. aureus* and *S. gordonii* were 10^4 and 10^5 cfu, respectively, corresponding to the high infectivity of true endocarditis pathogens, while the negative control *Lactococcus lactis* strain had an ID_{90} of 10^7 cfu. The ID_{90} of the *L. rhamnosus* endocarditis isolates ranged between 10^6 and 10^7 cfu, between the true pathogens and the negative control. On the other hand, 2 of the probiotic isolates, the fermentation isolate, and the strain under investigation exhibited ID_{90} of 10^8 cfu, 10-fold higher than even the negative control. However, the remaining 2 *L. rhamnosus* probiotic isolates (including LGG) had ID_{90} of 10^6-10^7, similar to the lower range of the clinical *L. rhamnosus* isolates. The *L. paracasei* strains were less potentially infective than the endocarditis *L. rhamnosus* strains. The densities of lactobacilli in infected tissues were significantly lower than those of *S. aureus* and *S. gordonii*, in the range of 3.9-6.8 $\log_{10}$ cfu/g tissue vs. 7-9 $\log_{10}$ cfu/g tissue. None of the *L. rhamnosus* strains exhibited more than weak adhesion to fibrinogen, fibronectin, collagen, or laminin. Anionic platelet protein FP-A had no activity against any *Lactobacillus* isolates, but cationic peptide CTAP-3 was highly lethal to all strains.

The authors concluded that "probiotic, nutritional, and probiotic research isolates of *Lactobacillus* are less able to cause experimental endocarditis than isolates commonly responsible for human endocarditis; it required a 100- to 1000-fold greater number of lactobacilli than *S. aureus* or *S. gordonii* to infect ≥90% of the animals." They further suggested that the fact that lactobacilli have been implicated in endocarditis may be due to host factors, as *Lactobacillus* endocarditis has occurred mostly in patients with severe underlying disease or decreased immunocompetence.

4.4.1.2.4. Rabbit

Bacterial translocation of ampicillin-resistant *Escherichia coli* K1 (K1) and the effect of *L. rhamnosus* GG (LGG) was studied in 77 newborn New Zealand white rabbit pups (Lee et al. 2000). On the day of delivery the pups were separated from their dams and assigned to receive twice daily via gavage placebo (n = 10), 10^8 cfu LGG (n = 8), 10^8 cfu K1 (n = 26), or a combination of the 10^8 cfu of both bacteria (n = 33) for 2 days. The pups were sacrificed on day 3; tissue specimens were excised and aerobically incubated for 48 hours, and LGG and K1 colonies were enumerated. Neonatal rabbits fed LGG had significantly reduced (25% less) small bowel colonization by K1 than did those not receiving LGG, as well as significantly reduced bacterial translocation of K1 to the mesenteric lymph nodes, spleen, and liver. No mucosal damage was detected in the small bowel of any pups. Bacterial translocation of LGG to these extraintestinal sites was observed in 1 of 8 pups in the LGG group and 4 of 33 pups in the LGG + K1 group. The authors concluded that LGG inhibited the colonization of *E. coli* K1 and its translocation to extraintestinal sites. However, although the neonatal rabbit display a propensity for immature immune function, mucosa and mucosal gel layer development, and intestinal epithelial development, and although there was no apparent clinical distress among the pups

infected with LGG, the authors regarded the evidence of even rare translocation of LGG as a matter of concern.

4.4.1.3. Conclusions from Studies of *L. rhamnosus* in Animals

In conclusion, several strains of *L. rhamnosus* were administered to a variety of animal species in a total of 19 studies, with dose levels as high as 10^{12} cfu/day. *L. rhamnosus*. No adverse effects were seen in immune or hematological parameters. Translocation was observed in 2 studies, Wagner et al (1997a) with congenitally immunodeficient gnotobiotic athymic mice and Lee et al. (2000) with neonatal rabbit pups also administered *E. coli* K1. In the latter study, LGG inhibited translocation of K1 and its ability to colonize extraintestinal sites, but some location of LGG was observed. No clinical distress was evident as a result of this translocation, and the neonatal rabbit model is recognized as having immature development of mucosa, mucosal gel layer, and epithelial barrier function. As was noted previously with regard to Wagner et al. (1997a), there is doubtful validity in extrapolating to healthy humans observations made in immunodeficient mice; use of this model was rejected by the EU-PROSAFE committee (Vankerckhoven et al. 2007a). There are no findings from the animal studies that would bring into question the safety of the administration of *L.rhamnosus* HN001 under the intended conditions of use.

Table 9. Studies of Other *L. rhamnosus* Strains in Animals

Reference	Objective	Study Design	Animal Model	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Adawi et al. (1997)	Investigate the ability of a variety of lactobacillus strains (including *L. rhamnosus* DSM 6594) to reduce bacterial translocation after acute liver injury	Five lactobacillus strains were administered rectally to rats with later acute liver injury. Bacterial translocation was evaluated by bacterial culture from portal and arterial blood, mesenteric lymph nodes, and liver tissue.	78 male Sprague-Dawley rats	Rectal dose of *L. rhamnosus* DSM 6594 or other strains not reported;	8 days	The probiotic treatment reduced bacterial translocation without any reported adverse effects.
Anukam et al. 2004	Determine the effect of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 administration on hematological parameters	Rats were fed the test strains of lactobacillus species, after which their blood was analyzed for any effects on hematology.	20 male Sprague-Dawley rats	10^9 cfu/day each of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 via orogastric tube	21 days	No effects were observed in any of the 12 hematological parameters tested; no differences between test and control rats were significant and all values were within the normal range. The absence of effect on the total white cell count indicates that there was no induction of the peripheral inflammatory response associated with pathogens. Similarly, the lack of any change in lymphocyte count indicates that the probiotic bacteria did no evoke any peripheral lymphocytosis.

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Table 9. Studies of Other *L. rhamnosus* Strains in Animals

Reference	Objective	Study Design	Animal Model	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Dong et al. (1987) (2 reported experiments)	Evaluate the ability of *L. rhamnosus* GG to prolong the lives of mice subjected to lethal radiation	Test mice were fed u *L. rhamnosus* via suspension in drinking water; control mice were not. Mice were irradiated; feeding of LGG was continued. (In Experiment 1 the mice were fed chow; in Experiment 2 they were fed meat.)	40 21-28-day-old CD1 mice in each experiment	3.5-4.0 x 10^{10} cfu LGG/day	10 days before irradiation and then until death	Although bacteremia was observed *post mortem* in all of the irradiated mice in both the experimental and control groups, none of the isolates were identified as lactobacilli. The LGG-fed mice yielded more streptococcal isolates and fewer Gram-negative enteric organisms than controls. No adverse effects of the probiotic administration were reported.
Donohue et al. (1993)	Assess acute toxicity of several strains *Lactobacillus* (including a *rhamnosus* strain identified as GG), *Streptococcus*, and *Bifidobacterium*	Probiotic bacteria were administered by gavage; mice were observed twice daily for 7 days prior to sacrifice and necropsy.\	25 healthy male adult Swiss mice with each probiotic strain	0, 1.5, 3, 6, or 9 x 10^{11} cfu/kg of a strain identified as LGG administered by gavage	Single dose	No significant changes were observed in behavior, feed intake, or body weight. No treatment-related deaths or signs of toxicity were observed, and no remarkable changes were reported in the gross appearance of any organs. The authors concluded that the acute oral LD_{50} for male Swiss mice of each of the bacterial strains tested is >9 x 10^{11} cfu/kg bw.

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Table 9. Studies of Other *L. rhamnosus* Strains in Animals

Reference	Objective	Study Design	Animal Model	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Holma et al. 2001	Compare the effects of *L. rhamnosus* GG (LGG), *L. reuteri* R2LC, and sulphasalazine on acetic acid-induced colitis in rats.	Colitis was induced by colonic instillation of acetic acid 7 days after beginning oral administration of *L. reuteri*, LGG, or sulphasalazine; ulceration was measured.	40 male HY:WIST rats	10^9-10^{10} cfu/day of LGG or *L. reuteri* R2LC	10 days	The authors concluded that *L. reuteri* R2LC, but not LGG, reduces the severity of acetic acid-induced colitis in rats and noted that neither LGG nor *L. reuteri* produced any observable side effects.
Lee et al. 2000	Investigate the effect of LGG on bacterial translocation of ampicillin-resistant *Escherichia coli* K1	Neonatal pups were assigned to receive twice daily via gavage placebo, LGG, K1, or a combination of both bacteria. Tissue specimens were excised and bacteria were identified and enumerated.	77 newborn New Zealand white rabbit pups	10^8 cfu LGG twice a day	2 days	Neonatal rabbits fed LGG had significantly reduced (25% less) small bowel colonization by K1 than did those not receiving LGG, as well as significantly reduced bacterial translocation of K1 to the mesenteric lymph nodes, spleen, and liver. No mucosal damage was detected in the small bowel of any pups. Bacterial translocation of LGG to these extraintestinal sites was observed in 1 of 8 pups in the LGG group and 4 of 33 pups in the LGG + K1 group. The authors concluded that LGG inhibited the colonization of *E. coli* K1 and its translocation to extraintestinal sites. The authors regarded the evidence of even rare translocation of LGG as a matter of concern.

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Table 9. Studies of Other *L. rhamnosus* Strains in Animals

Reference	Objective	Study Design	Animal Model	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Moller et al. 2005	Investigate if colitis is influenced in colitic scid mice treated with antibiotic and fed *Lactobacillus* spp.	Mice were treated for 1 week with vancomycin and meropenem, then *L. reuteri* or *L. rhamnosus* or placebo for 3 weeks. After 12 weeks, rectums were removed for histology, and CD4 T cells from the mesenteric lymph node were activated for cytokine measurements.	19 6-week-old female colitic scid mice	$2x10^{10}$ cfu/day *L.rhamnosus* 19070-2 *and L. reuteri* DSM 12246	3 weeks	All mice treated with antibiotics but not fed probiotics showed severe gut inflammation, whereas only 2 of the 7 mice fed probiotics showed signs of severe colitis. MLN-derived CD4 T cells from this latter group of mice showed lower levels of interleukin-4 secretion and a tendency to higher interferon-γ production than mice not fed probiotics. No adverse effects resulted from the administration of *L. reuteri* and *L. rhamnosus*.
Naaber et al. (1998)	Determine the ability of LGG to reduce the effects of *Clostridium difficile* in Syrian hamsters	Hamsters received ampicillin and then were inoculated with *Clostridium difficile*. They were given 0 or 0.5 ml LGG/day starting 20 hours prior to the *C. difficile* challenge.	10 adult Syrian hamsters	LGG; dose not reported	6 days total	Four of the 5 hamsters receiving LGG remained healthy and the inflammatory changes in their intestinal mucosa were milder than those receiving no probiotic. LGG was present in intestinal cultures from all treated animals, but it did not become predominant in the intestinal microbiota and was not observed among translocating lactobacilli. No adverse effects were noted.
Pessi et al. (1998)	Study the effects of(LGG on macro-molecular degradation in the gut mucosa of neonatal rats	5 feeding groups received 1) maternal milk and bicarbonate buffer, 2) cow's milk, 3) cow's milk + LGG, 4) extensively hydro-lyzed whey formula, 5) hydrolyzed whey formula + LGG.	50 2-week-old Wistar rat pups	10^{10} cfu LGG/day	7 days	Absorption of intact proteins and electrical parameters were unchanged, indicating that LGG supplementation did not result in tissue damage or dysfunction of the gut mucosa. No adverse effects were reported.

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Table 9. Studies of Other *L. rhamnosus* Strains in Animals

Reference	Objective	Study Design	Animal Model	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Vankerck-hoven et al. (2007)	Study the potential infectivity of *L. rhamnosus* and *L. paracasei* in a rat model of experimental infective endocarditis	Lesions were induced by insertion of a catheter across the aortic valve; the rats were inoculated with each test strain and sacrificed 72 hours later. Positive and negative controls of infectivity were provided by *Staphylococcus aureus* Newman, *Streptococcus gordonii* Challis, and *Lactococcus lactis* 1363.	7-11 female Wistar rats per strain and per inoculum	10 isolates of *L. rhamnosus* (4 probiotics, 4 strains isolated from human endocarditis events, 1 strain used for fer-mentation, 1 strain under investigation as a potential probiotic); 10^4-10^8 cfu i.v.	Single dose	The ID_{90} of the *L. rhamnosus* endocarditis isolates was 10^6-10^7 cfu, between the true pathogens and the negative control. 2 of the probiotic isolates, the fermentation isolate, and the strain under investigation had ID_{90} of 10^8 cfu, 10-fold higher than even the negative control. 2 probiotic isolates (including LGG) had ID_{90} of 10^6-10^7, similar to the lower range of the clinical *L. rhamnosus* isolates. Densities of lactobacilli in infected tissues were lower than those of *S. aureus* and *S. gordonii*. No strain had more than weak adhesion to fibrinogen, fibronectin, collagen, or laminin. Anionic platelet protein FP-A had no activity against any *Lactobacillus* isolates, but cationic peptide CTAP-3 was lethal to all strains. The authors concluded that "probiotic, nutritional, and probiotic research isolates of *Lactobacillus* are less able to cause experimental endocarditis than isolates commonly responsible for human endocarditis; it required a 100- to 1000-fold greater number of lactobacilli than *S. aureus* or *S. gordonii* to infect ≥90% of the animals." They further suggested that the fact that lactobacilli have been implicated in endocarditis may be due to host factors.

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Table 9. Studies of Other *L. rhamnosus* Strains in Animals

Reference	Objective	Study Design	Animal Model	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Wagner et al. (1997a)	Assessed the ability of LGG, *L. reuteri*, *L. acidophilus*, and *Bifidobacterium animalis* to colonize, stimulate immune responses in, and affect growth and survival of congenitally immunodeficient mice	The bacteria were introduced by means of swabs of the oral and rectal cavities of the mice. Adherence to epithelial surfaces was tested.	Congenitally immunodeficient gnotobiotic beige-athymic (*bg/bg-nu/nu*) and beige-euthymic (*bg/bg-nu/+*) mice	10^8 cfu/ml introduced into the oral and rectal cavities by swabs	12 weeks	The bacteria colonized and persisted at high concentrations in the alimentary tracts of both mouse strains for the entire study period. Adherence to epithelial surfaces by LGG was only 5%. Translocation to internal organs was detected in 26% of the mice colonized with LGG, but did not result in any signs of morbidity, mortality, pathologic changes, or effects on growth. 19 of 53 pups born to LGG-colonized dams died. There was no evidence of translocation and histological examination did not show any evidence of pathologic changes in the gastrointestinal tract or internal organs; thus, the reason for the infant mortality remains unknown.
Wagner et al. (1997b)	Study the ability of LGG, *L. reuteri*, *L. acidophilus*, and *Bifidobacterium animalis* to protect congenitally immunodeficient mice from mucosal and systemic candidiasis	The bacteria were introduced by means of swabs of the oral and rectal cavities of the mice. Adherence to epithelial surfaces was tested.	Congenitally immunodeficient gnotobiotic beige-athymic (*bg/bg-nu/nu*) and beige-euthymic (*bg/bg-nu/+*) mice	10^8 cfu/ml introduced into the oral and rectal cavities by swabs	Single administration	Each bacterial strain and *Candida albicans* colonized the gastrointestinal tracts of both strains of mice. The presence of probiotic bacteria in the gastrointestinal tracts prolonged the survival of adult and neonatal *bg/bg-nu/nu* mice compared to that of isogenic mice colonized with *C. albicans* alone. The incidence of systemic candidiasis in *bg/bg-nu/nu* mice was significantly reduced by each of the four probiotic bacterial strains. No bacterial infection occurred in this doubly immunodeficient animal model.

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Table 9. Studies of Other *L. rhamnosus* Strains in Animals

Reference	Objective	Study Design	Animal Model	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Wagner et al. (2000).	Study the ability of LGG, *L. reuteri*, *L. acidophilus*, and *Bifidobacterium animalis* to protect congenitally immuno-deficient mice from mucosal and systemic candidiasis	The bacteria were introduced by means of swabs of the oral and rectal cavities of the mice. Colonized mice and uncolonized controls were challenged with *Candida albicans*	Germ-free beige-nude (*bg/bg-nu/nu*) and beige-heterozygous (*bg/bg-nu/+*) mice	10^8 cfu/ml introduced into the oral and rectal cavities by swabs	Single administration	No infections or other adverse effects were seen as a result of the administration of any of the probiotic strains of bacteria.
Xu et al. (2008)	Test acute toxicity of starter cultures used in Chinese fermented milk products.	Bacteria (LGG and an unspecified *L. rhamnosus* strain, *Bifidobacterium animalis* ssp. *lactis* Bb-12, or a strain of *L.reuteri*) were administered either orally or by i.p. injection.	Mice (number and strain not reported)	$5x10^7$, $5x10^8$, or $5x10^9$ cfu/ml LGG or an unidentified *L. rhamnosus* strain administered orally or by i.p. injection	3 days	No bacteria showed toxic effects when administered orally over 3 days. One *L. rhamnosus* strain, after i.p. injection at the highest dose of $5x10^9$ cfu/ml, led to toxic symptoms and lethality in most of the mice. These toxic effects were not observed with lower doses ($5x10^7$ cfu/ml or $5x10^8$ cfu/ml) of this strain or with any dose of the three other strains studied.

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4.4.2. Human Studies

4.4.2.1. Studies in Adults

4.4.2.1.1. Studies of L. rhamnosus *HN001 in Adults*

The research studies discussed below are summarized in Table 11 at the end of the section.

Tannock et al. (2000) investigated the effect of ingestion of *L. rhamnosus* HN001 on the fecal microbiota of 10 healthy human subjects, 5 males and 5 females, age 25 to 55 years. The participants consumed 32 g/day low-lactose, low-fat milk powder dissolved in 250 ml water once a day for 6 months, during which fecal samples were taken monthly. For the next 6 months *L. rhamnosus* HN001 was added to the milk-powder solution to provide 1.6 x 10^9 cfu/day; fecal samples were again obtained monthly. Fecal samples continued to be taken monthly during a 3-month washout period during which both milk-powder and probiotic administration were stopped. Fecal samples were analyzed for bacteria, lactobacilli, and the test strain as well as for short-chain fatty acids and the fecal enzymes azoreductase and β-glucuronidase.

Administration of probiotics had no effect on short-chain fatty acids or fecal enzymes (both of which were highly variable across individuals) or on counts of total aerobic, bifidobacteria, lactose-fermenting enterobacteria, clostridia, *Bacteroides*, or yeast cfu/g feces. Both the frequency of detection of lactobacilli and the concentration of lactobacilli (cfu/g) in the feces increased during the period of administration of HN001, but declined to baseline soon after administration ceased; the majority of the isolates belonged to the *L. casei* group (at the time defined as *L. casei, L paracasei, L. rhamnosus*, and *L. zeae*). Enterococci also increased in both frequency of detection and concentration in the feces during the administration of the probiotic. During the period in which it was ingested, the test strain of *L. rhamnosus*, HN001, was consistently detected as a numerically dominant member of the *Lactobacillus* population in 6 subjects, inconsistently detected in 3 subjects, and detected only as a minor component for the remaining subject. It was not detected after administration ceased. Tannock et al. (2000) concluded that relatively long-term administration of *L. rhamnosus* HN001 does not alter the biochemistry or permanent bacteriology of the fecal microbiota in humans.

In a study of the effect of *L. rhamnosus* HN001 on cellular immune function in older individuals, Gill and Rutherfurd (2001) conducted a 9 week study with 13 healthy volunteers (5 male, 8 female) aged 62-77 years. All participants received reconstituted low-fat milk powder in 200 ml water twice a day for 3 weeks as a run-in, then the same beverage with added *L. rhamnosus* HN001 at a dose of 5 x 10^{10} cfu/day for another 3 weeks, and then the probiotic-free beverage for a 3-week washout period. Peripheral blood samples were drawn at 4 time points: baseline, after the run-in, after the test diet, and after the washout. Immune function was assessed by measurement of blood leukocyte phagocytosis, differentiating between polymorphonuclear and mononuclear phagocytes. As shown in Table 10, the activity levels of both types of phagocytes were unaffected by the milk-powder alone but were significantly elevated by the probiotic; levels returned toward baseline during the washout. Gill and Rutherfurd (2001) suggested that the transient immune-stimulating effect of *L. rhamnosus* offers an effective means

of boosting immunity in a controlled fashion. The authors noted that there were no reports of adverse effects on health and no general health problems were recorded during the study.

Table 10. Leukocyte Phagocytosis In Response to *L. rhamnosus* HN001

Trial Stage	Cells Displaying Phagocytic Activity (%)	
	Polymorpho-nuclear Cells	Monocytes
Week 0 (baseline)	57.0	15.3
Week 3 (post-run-in)	56.0	15.1
Week 6 (post-treatment)	76.4	21.4
Week 9 (post-washout)	59.9	18.1
Source: Gill and Rutherfurd 2001		

Gill et al. (2001a) investigated the ability of LAB to enhance declining immune function in the elderly. In a comparative study, 27 healthy individuals age 60-84 years (11 males, 16 females; median age 69.5 years) consumed milk for 3 weeks as a run-in, after which 13 participants were randomly assigned to consume 5 x 10^{10} cfu/day *L. rhamnosus* HN001 for 3 weeks while 14 others consumed 5 x 10^{9} cfu/day *B. animalis* ssp. *lactis* strain HN019 for the same period. All subjects then consumed unsupplemented milk for a 3-week washout period. Peripheral blood samples were taken by venipuncture on day 0, day 21 (after the run-in but before probiotic supplementation), day 42 (after supplementation), and day 63 (after the washout). Mononuclear cells were separated from the blood samples and the proportions staining positive for CD56 or CD3/CD56 were determined. Their tumoricidal (natural killer [NK]-cell) activity was assessed *in vitro* by lysis of target K562 cells, an NK-sensitive erythroleukemic cell line.

The run-in diet had no effect on immune parameters, but enhancement of NK-cell activity was seen during administration of both probiotics. Additionally, the proportion of peripheral blood cells staining for CD56 increased. Levels of both immune-system parameters returned to baseline levels by the end of the washout period. No adverse effects were reported.

Gill et al. (2001c) conducted a second study to further investigate the effect of dietary consumption of 5x10^{9} cfu *L.rhamnosus* HN001/day on the cellular immune capacity of elderly subjects. Fifteen healthy elderly individuals were enrolled, but 2 withdrew early for non-ptrobiotic related reasons, and 13 individuals (5 males and 8 females aged 65-85 years; median age = 70 years) completed the study. In a 3-stage single-blind crossover trial, all participants consumed 200 ml low-lactose/low-fat milk twice a day for 3 weeks. In stsge 2 lasting for 3 weeks,, the milk was supplemented with a daily total of 5 x 10^{9} cfu *L.rhamnosus* HN001. Finally, the participants again consumed unsupplemented milk for 3 weeks. At enrollment and at the end of each stage, *in vitro* immunological assays were conducted on whole-blood samples or mononuclear leucocytes isolated from peripheral blood. Phagocytosis was measured by cellular *in vitro* updake of *E. coli* following co-culture of fluoroscein-isothiocyanate-labeled *E. coli* with whole blood, while tumoricidal activity was assessed by monitoring the killing of K562 erythroleukemic cells during co-culture with leucocytes.

The control dietary run-in treatment of unsupplemented milk had no significant effect on subjects' *ex vivo* cellular immune function. In contrast, the phagocytic capacities of monocytes and polymorphonuclear (PMN) cells were both significantly elevated following consumption of *L.rhamnosus* HN001-supplemented milk. Assays of function returned to near pre-treatment levels following washout. A similar pattern, again statistically significant, was recorded for NK-cell tumorocidal activity. The authors noted that no adverse gastrointestinal disturbances were reported and that, "both the supplemented and non-supplemented milk diets were well tolerated," and concluded that, "*L. rhamnosus* HN001 represents a safe and effective way of enhancing immune function in the elderly by dietary means" (Gill et al. 2001c).

A similar study of the effect of *L. rhamnosus* HN001 on cellular immune function was conducted by Sheih et al. (2001). Fifty-two healthy middle-aged and elderly volunteers (age range 44-80 years; median age 63.5 years; 17 males, 35 females) consumed 25 g reconstituted low-fat milk powder in 200 ml water twice a day for 3 weeks as a run-in, then a milk beverage with added *L. rhamnosus* HN001 at a dose of 5×10^{10} cfu/day for another 3 weeks, and then the probiotic-free beverage for a 3-week washout period. During the probiotic phase, half of the participants received *L. rhamnosus* dissolved in the same low-fat milk powder beverage as they consumed during the run-in and washout periods while the other half received a lactose-hydrolyzed low-fat milk powder beverage. Peripheral blood samples were drawn at 4 time points—baseline, after the run-in, after the test diet, and after the washout—and analyzed for polymorphonuclear and mononuclear phagocytes. Immune function was assessed by measurement of blood leukocyte phagocyic activity, reflected for polymorphonuclear cells in membrane-bound NADPH-oxidase activity, and for monocytes by specific target lysis against chromium-labeled K562 tumor cells.

The activity levels of both types of phagocytes were unaffected by the milk-powder alone but were significantly elevated by the probiotic; levels returned toward baseline during the washout. The low-fat milk powder matrix—regular v. lactose-hydrolyzed—did not affect probiotic efficacy. The authors noted that there were no reports of adverse effects on health throughout the study and no general health problems were recorded.

Table 41. Studies of *L. rhamnosus* HN001 in Adults

Reference	Objective	Study Design	Subjects	NH001 Dose	Duration	Safety-Related Results
Gill and Rutherfurd (2001)	Investigate the effect of *L. rhamnosus* HN001 on cellular immune function in older individuals	Single-blind uncontrolled study; participants consumed reconstituted low-fat milk for 3 weeks followed by the same milk with probiotic for 3 weeks followed by a 3-week washout; blood was drawn at 4 time points and analyzed for phagocytic activity	13 healthy volunteers, 5 males and 8 females aged 62-77 years	5×10^{10} cfu/day	3 weeks	Activity levels of both polymorphonuclear and mononuclear phagocytes were unaffected by the milk-powder alone but were significantly elevated by the probiotic; levels returned toward baseline during the washout. The authors noted that there were no reports of adverse effects on health and no general health problems were recorded during the study.
Gill et al. (2001a)	Investigate the ability of LAB (including *L. rhamnosus* HN001) to enhance declining immune function in the elderly	Double-blind comparative study; participants consumed reconstituted low-fat milk for 3 weeks followed by the same milk with probiotic (either *L. rhamnosus* HN001or *B. animalis* ssp. *lactis* strain HN019 for 3 weeks followed by a 3-week washout; blood was drawn at 4 time points and analyzed for phagocytic activity	27 healthy individuals aged 60-84 years, 11 males and 16 females	5×10^{10} cfu/day	3 weeks	The run-in diet had no effect on immune parameters, but enhancement of NK-cell activity was seen during administration of both probiotics. Additionally, the proportion of peripheral blood cells staining for CD56 increased. Levels of both immune-system parameters returned to baseline levels by the end of the washout period. No adverse effects were reported.
Gill et al. (2001c)	Investigate the effect of *L..rhamnosus* HN001 on the immune capacity of the elderly	3-stage single-blind crossover trial; 3-week run-in of base diet, 3-week test period, 3-week washout.	13 heaalthy elderly subjects; 5 males and 8 females aged 65 -85 years (median = 70 years)	5×10^{9} cfu/day	3 weeks	Phagocytic capacity of monocytes and PMN cells was significantly elevated and NK-cell tumorocidal activity was significant increased. No adverse effects were reported and the authors concluded that administration of *L. rhamnosus* HN001 is a safe and effective means of ehancing immune function in the elderly.

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Table 41. Studies of *L. rhamnosus* HN001 in Adults

Reference	Objective	Study Design	Subjects	NH001 Dose	Duration	Safety-Related Results
Sheih et al. (2001)	Investigate the effect of *L. rhamnosus* HN001 on cellular immune function in middle-aged and elderly populations	Single-blind uncontrolled study; participants consumed reconstituted low-fat milk for 3 weeks followed by the same milk with *L. rhamnosus* HN001 for 3 weeks followed by a 3-week washout; blood was drawn at 4 time points and analyzed for phagocytic activity; during the probiotic phase half of the subjects received regular low-fat milk and the others received lactose-hydrolyzed low-fat milk	52 healthy middle-aged and elderly volunteers aged 44-80 years; median age 63.5 years; 17 males, 35 females	5×10^{10} cfu/day	3 weeks	Activity levels of both polymorphonuclear and mononuclear phagocytes were unaffected by the milk-powder alone but were significantly elevated by the probiotic; levels returned toward baseline during the washout. The authors noted that there were no reports of adverse effects on health throughout the study and no general health problems were recorded.
Tannock et al. (2000)	Investigate the effect of ingestion of *L. rhamnosus* HN001 on the fecal microbiota	Single-blind uncontrolled study; participants consumed reconstituted low-fat milk for 6 months followed by the same milk with probiotic for 6 months followed by a 3-month washout; fecal samples were taken monthly	10 healthy human subjects, 5 males and 5 females, aged 25 to 55 years	1.6×10^{9} cfu/day	6 months	*L. rhamnosus* had no effect on short-chain fatty acids, fecal enzymes, or counts of total aerobic, bifidobacterial, lactose-fermenting enterobacterial, clostridial, bacteroides, or yeast cfu/g feces. Both the frequency of detection of lactobacilli and the concentration of lactobacilli (cfu/g) in the feces increased during the period of administration of HN001, but declined to baseline soon after administration ceased. HN001 was not detected after administration ceased. *L. rhamnosus* HN001 did not alter the biochemistry or permanent bacteriology of the fecal microbiota in humans.

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4.4.2.1.2. Studies of Other L. rhamnosus *Strains in Adults*

A number of strains of *L. rhamnosus* other than HN001 have been clinically investigated. Most of the earlier studies, and a high proportion of the total, focused on *L. rhamnosus* strain GG (LGG), formerly identified as *L. casei* GG. This strain is properly regarded as the most studied probiotic strain in the world. A number of studies of *L. rhamnosus* strain GR-1 have also appeared in the literature. The studies discussed in this section are summarized in Table 12 at the end of the section.

Siitonen et al. (1990) studied the efficacy of supplementing yogurt with LGG to reduce erythromycin-associated diarrhea in 16 healthy adult males. Participants were given 400 mg of erythromycin acistrate 3 times/day for 7 days; half of them consumed 125 mL of regular yogurt twice a day (control group) while the test group received yogurt supplemented with LGG. Stool samples were taken on days 1 and 7 and self-rated symptoms were recorded daily. Individuals receiving the LGG-supplemented yogurt had significantly less diarrhea, abdominal distress, stomach pain, and flatulence than did those consuming the regular yogurt. Although the numbers of total fecal lactobacilli were unchanged in either the treated or control groups, colonies morphologically consistent with LGG were found in the feces of the LGG-treated subjects but not in the feces of the controls. The authors did not report any adverse effects.

Saxelin et al. (1991) performed a dose-response study to determine the fecal colonization of LGG. Forty healthy adult volunteers ingested lyophilized LGG powder once a day for 7 days at doses of 1.5×10^6, 1.5×10^7, 1.5×10^8, 1.5×10^9, 1.5×10^{10}, and 1.1×10^{11} cfu/day. Fecal samples taken on day 0 and daily during the treatment period were analyzed for total lactobacilli and LGG. No LGG was detected in fecal samples prior to the dosing period, and none was detected in the fecal samples of the 10^6-10^8 dosing groups during administration. Two of 7 subjects in the 10^9 dosing group were colonized with LGG at a detectable level sporadically during the test period, while all subjects in the 10^{10} and 10^{11} test groups were colonized at fecal levels of 10^5 - 10^7 cfu LGG/g. No level of LGG ingestion tested influenced the total number of lactobacilli found nor resulted in LGG becoming the dominant strain in the feces. No adverse effects were reported.

In a study of the survival of LGG in the gastrointestinal tract, Goldin et al. (1992) had 76 healthy male and female volunteers aged 21-55 years consume a frozen concentrate for 28 days, a fermented milk for 7 days, or a fermented whey drink for 35 days. These food matrices were supplemented with strain LGG to provide daily doses of 4×10^{10} cfu, 3.6×10^{11} cfu, and 1.6×10^{11} cfu, respectively. Survival of bacteria in the gut was determined by culturing fecal specimens during the dosing periods as well as 3 and 7 days after the feeding was discontinued. LGG was recovered in the feces of all subjects receiving the fermented milk or whey and in 86% of those receiving the frozen concentrate. LGG was found to persist in the feces of 87% of the study subjects 3 days after the feeding was discontinued and in 33% of subjects after 7 days. No adverse effects were reported.

Ling et al. (1992) provided a whey drink fermented with LGG to elderly nursing home residents complaining of difficulties in defecation for 2 weeks, preceded and followed by 2-week run-ins and washouts. All participants had detectable LGG in their feces, and levels of

glycocholic acid hydrolase activity and tryptic activity were significantly decreased. A mild normalization of the consistency of the stools took place, but there was no effect on fecal frequency, weight, or pH. The authors concluded that LGG can change the bacterial metabolism, but has no significant effect on bowel function.

Saxelin et al. (1993) studied the fecal concentrations of LGG in 44 healthy human adults who were fed either enterocoated tablets providing daily doses of 1×10^9, 4×10^9, or 8×10^9 cfu LGG or fermented milk providing daily doses of 2.1×10^9 or 1.2×10^{10} cfu LGG for 7 days. Fecal specimens were collected on day 0 and daily during the test period. By day 3, LGG was found in the feces of all subjects at all dose levels, but neither the dose levels nor the carrier significantly influenced the numbers of LGG cells found. No adverse effects were reported.

In an investigation of the ability of *Lactobacillus* strains to colonize the human intestinal mucosa, Johansson et al. (1993) administered 19 strains of *Lactobacillus* to 13 healthy adult volunteers age 31 to 56 years for 10 days. The tested strains included 2 *L. rhamnosus* strains—LCR 98 (originally isolated from the human rectum) and LCR 271 (originally derived from the human colon). All probiotic strains were added to an oatmeal soup base at concentrations of 2.7×10^7 cfu/g soup; 18.4 g/day of soup was ingested daily providing about 5×10^8 cfu/day of each test strain. Rectal and jejunal biopsies were taken at baseline and 1 and 11 days after the end of administration and bacteria were genotypically identified and enumerated.

L. rhamnosus strain LCR 271 successfully established temporary residence in the mucosa, but strain LCR 98 did not. A significant increase in *Lactobacillus* spp. was seen in the jejunum but not in the rectum 1 day after administration, but had disappeared by day 11. No tolerance problems or adverse events were reported.

Twenty healthy human adults aged 20-55 years (sex not reported) consumed gelatin capsules providing daily doses of 1.6×10^8 and 1.2×10^{10} cfu freeze-dried LGG once a day for 7 days (Saxelin et al. 1995). Fecal samples were collected on day 0 and on test days 3, 5, and 7. LGG was identified after culturing based on cell morphology and lactose fermentation, confirmed based on carbohydrate profile. None of the participants had detectable LGG in the feces prior to administration. By day 3, LGG was recovered in the feces of all subjects receiving the higher dose, but only 1 of 10 subjects in the lower dose group had detectable LGG in the feces by the end of the test period. No effect was observed on the total number of fecal lactobacilli. No adverse effects were reported.

Jacobsen et al. (1999) investigated the probiotic potential of 47 selected strains of *Lactobacillus* spp., including 7 *L. rhamnosus* strains (not including HN001). The strains were examined for resistance to acidity (pH 2.5), adhesion to Caco-2 cells, and antimicrobial activity against enteric pathogenic bacteria in model systems. From the results obtained *in vitro*, five strains were selected for *in vivo* studies: *L. rhamnosus* DSM 12246, *L. rhamnosus* 19070-2, *L. rhamnosus* GG, *L. delbrueckii* ssp. *lactis* CHCC 2329, and *L. casei* ssp. *alactus* CHCC 3137. The daily consumption by 12 healthy male volunteers of two doses of 10^{10} cfu freeze-dried bacteria of the selected strains for 18 days was followed by a washout period of 17 days. Fecal samples were taken at days 0 and 18 and during the washout period at days 5 and 11. Two of the 7 *L. rhamnosus* strains tested, strains 19070-2 and GG, showed the highest adhesion to Caco-2

cells of all strains tested, but 4 other *rhamnosus* strains showed only moderate adhesion and 1 strain (Lc705) exhibited poor adhesion. None of the 7 tested *L. rhamnosus* strains was able to grown at pH 2.5, although all were able to survive. Similarly, all of them survived a 0.3% oxgall solution, but only 2 strains were able to exhibit even inhibited growth. All rhamnosus strains exhibited broad but generally moderate inhibition of the growth of pathogens. Among the strains selected for further testing, *L. rhamnosus* DSM 12246, *L. rhamnosus* 19070-2, and LGG were identified most frequently in fecal samples; they were found in 8, 10, and 7 of the 12 samples tested during the intervention period, respectively. Re-isolations were less frequent in the washout period; *L. rhamnosus* was found in only 1 sample at day 5 and none at day 11. The bacteria were re-isolated in concentrations from 10^5 to 10^8 cfu/g of feces. Survival and re-isolation of the bacteria *in vivo* appeared to be linked to pH tolerance, adhesion, and antimicrobial properties *in vitro*, confirming the validity of the *in vitro* testing. The authors made no mention of any adverse effects from any of the treatments.

Alander et al. (1999) studied the ability of *L. rhamnosus* GG (LGG) to adhere to human colonic mucosa. Twenty-one healthy adults scheduled for routine diagnostic colonoscopy consumed a drink comprising lactose-hydrolyzed whey fermented with LGG twice daily for 12 days, ingesting 6 x 10^{10} cfu LGG/day. Six individuals (1 male and 5 females, age 34-78 years) underwent the colonoscopy immediately at the end of the 12-day consumption of LGG; 8 people (5 males and 3 females, age 42-68 years) had their colonoscopies 1 week later; and 7 patients (4 males and 3 females, age 27-73 years) were examined 2 weeks later. Prior to the colonoscopies, evacuation of the colon was induced by 3 doses of a laxative; fecal samples were taken during this process. Three parallel biopsies were taken from the descending colon. LGG colonies were identified by morphology and confirmed by PCR analysis.

The results from the patients undergoing colonoscopy immediately after the period of LGG ingestion showed the ability of the strain to adhere to colonic mucosa; LGG was identified in the biopsies of all 6 individuals. Colonies were found in the biopsies of 7 of the 8 patients who had colonoscopies a week later, but in only 2 of the 7 patients who had them 2 weeks later. Alander et al. (1999) concluded that LGG adheres well to colonic mucosa but is unable to establish itself as a permanent resident of the colon. No adverse effects were noted during this study.

Fang et al. (2000) randomized 30 healthy adults (15 of each sex, age 20-50 years) into 3 groups to receive orally 4 x 10^{10} cfu freeze-dried LGG/day, 3.4 x 10^{10} cfu *B. lactis*/day, or placebo for 7 days. They consumed an attenuated *Salmonella typhimurium* TY21A oral vaccine capsule on days 1, 3, and 5. Blood samples were drawn on days -1 and +14 and analyzed for immunoglobulin-secreting cells and cells secreting anti-*S. typhimurium* antibodies.

No significant differences between groups were seen in numbers of IgA-, IgG-, or IgM-secreting cells. The authors did not report any adverse events.

In a study of bacterial vaginosis, 42 healthy women were randomized to receive orally up to 6 x 10^9 cfu/day of a combination of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 or 10^{10} cfu/day of LGG alone for 28 days (Reid et al. 2001). While the data were difficult to interpret, it appeared that the probiotic combination had a generally beneficial effect on vaginal microbiota

populations. None of the patients reported symptomatic vaginitis or urinary tract infections or any adverse side effects during or following the study. Fecal shedding of all tested bacterial strains ceased by day 14 after supplementation terminated.

A prospective, randomized, double-blind, placebo-controlled study of LGG was undertaken by Armuzzi et al. (2001) to investigate its use during *Helicobacter pylori* eradication therapy. Sixty healthy asymptomatic patients (25 males and 35 females, mean age 40 years) who screened positive for *H. pylori* infection received rabeprazole, clarithromycin, and tinidazole for 7 days, either with placebo or 1.2 x 10^{10} cfu LGG/day in the form of a sachet containing a freeze-dried powder. The treatment with LGG or placebo continued for 7 days after completion of the eradication therapy. Patients completed questionnaires during the week of treatment and the following 3 weeks to assess tolerability and any side effects.

The patients who received LGG reported significantly less nausea and diarrhea than did the placebo group and had significantly higher assessments of the tolerability of the treatment. There was no effect of the LGG v. placebo on the success of the eradication of *H. pylori* and no indication of any adverse effects resulting from ingestion of LGG.

Thomas et al. (2001) studied the efficacy of LGG in preventing antibiotic-associated diarrhea in adults. In a prospective, randomized, double-blind, placebo-controlled trial, 302 hospitalized patients (143 males and 159 females, mean age 55.8 years with range 18 to 90 years) receiving intravenous or oral antibiotics were randomized to receive capsules containing placebo or a daily total of 2 x 10^{10} cfu LGG twice a day for 14 days. The primary outcome was the proportion of patients experiencing diarrhea in the first 21 days after enrollment, defined as watery or liquid stools for 2 consecutive days or 3+ bowel movements more than normal for that individual. Patients were also asked to record adverse effects such as nausea, abdominal cramping, and gas or bloating.

Treatment with LGG had no effect on the incidence of diarrhea; 29-30% of the patients in both the test and control groups developed diarrhea. There were no differences between the test and placebo groups in the number, type, or severity of reported adverse effects.

In a crossover study (Elmadfa et al. 2001), 12 healthy adults (6 males and 6 females aged 25-36 years) consumed 500 grams of yogurt providing 2.5 x 10^{10} cfu LGG/day for 4 weeks. The yogurt also contained *Streptococcus thermophilus* and *Lactobacillus acidophilus*. During the first two weeks, the yogurt cultures were thermally inactivated. Urine was collected daily and blood samples were drawn on days 1, 15, and 29; urine and blood samples were analyzed for B vitamins

Vitamin B_1 levels in plasma decreased significantly and progressively over the course of the study, and a slight but significant decrease of this vitamin was found in the urine as well; these changes were attributed to a 25% reduction of bioavailable vitamin B_1 in the diets of the test subjects. The levels of the B_2 and B_6 vitamins in plasma and urine differed slightly throughout the study, with most of them decreasing, but all were considered to be within normal ranges. The authors concluded that, "the bacterial flora of the examined yoghurt does not

influence the vitamin B_1, B_2 and B_6 status of man." (Elmadfa *et al.*, 2001). No adverse effects were reported.

Ouwehand et al. (2002) enrolled 28 elderly subjects in an open parallel study of the effects of probiotic bacteria on constipation. The subjects were divided into 3 groups: the control group received fruit juice; the *rhamnosus* group received fruit juice supplemented with *L. rhamnosus* LC705 and *Propionibacterium freudenreichii* ssp. *shermanii* JS, and the *reuteri* group received fruit juice supplemented with *L. reuteri* ING1. During the first 3 weeks all subjects consumed unsupplemented juice. In the subsequent 4 weeks, the subjects received their designated juice. During the last 3 weeks, all subjects again received unsupplemented juice. Defecation frequency, laxative use, fecal pH, mucin content, and azoreductase activity were assessed during the last week of each period. The treatment with *L. reuteri* had no significant effect, but the subjects receiving the *L. rhamnosus/P. freudenreichii*-supplemented juice had a 24% increase in defecation frequency; no reduction was observed in laxative use. Fecal azoreductase activity was also significantly reduced in this group. No changes in fecal pH or mucin excretion were observed in any group. The tested probiotics did not affect the mucosal barrier, and no adverse effects were observed; the authors concluded that use of the tested bacterial strains is safe.

Cremonini et al. (2002) investigated the effect of probiotics on anti-*Helicobacter pylori* therapy-related side effects, studying the effect of LGG, *Saccharomyces boulardii*, and a combination of *Lactobacillus* spp. and bifidobacteria. A total of 97 asymptomatic *H. pylori*-positive patients (43 males and 54 females aged 18-61 years) were randomized to receive one of the probiotic treatments or placebo for 2 weeks. The test period was preceded by a 3-week run-in period during which 12 individuals were excluded due to occurrence of symptoms or use of drugs associated with gastrointestinal side effects. All patients received eradication therapy consisting of rabeprazole, clarythromicin, and tinidazole for 7 days and the probiotic or placebo twice a day for the same 7 days and the following week. The daily dose of LGG was 1.2×10^{10} cfu. Patients completed questionnaires assessing side effects at the end of the antibiotic week and at the end of each of the next 3 weeks.

There were no side effects resulting in study discontinuation. All 3 probiotic treatments reduced reported side effects as compared with the placebo group; the difference was significant only during the first week and decreased monotonically through week 4. The most notable effect was a significant reduction in diarrhea.

In a randomized, prospective, double-blind, placebo-controlled trial, Prantera et al. (2002) evaluated the efficacy of LGG in preventing recurrence of symptoms after curative resection for Crohn's disease. Forty-five Crohn's patients who had undergone complete resection of all diseased intestine within the previous 10 days (29 males and 16 females aged 22-71 with mean 37 years) were randomized to consume 2 sachets a day containing a total of 1.2×10^{10} cfu LGG or placebo for 1 year. Follow-up visits were performed 13, 26, 39, and 52 weeks after initiation of treatment; urine samples were taken and blood was drawn and analyzed for blood count, serum iron and ferritin, creatinine, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase. An ileocolonoscopy was performed at the end of the trial.

LGG ingestion for 1 year had no effect on recurrence of Crohn's disease nor on the severity of recurrent lesions. Overall, 13 participants failed to complete the study; none of the drop-outs terminated due to adverse events and the reasons for the termination did not differ between the treatment and control groups. Symptoms such as diarrhea, bloating, and meteorism did not differ between the groups. No effects were noted on liver enzymes. Adverse events were suffered by 2 LGG patients and 6 placebo patients; none of the events were regarded as trial-related and they did not cause interruption of the study.

In a study of the immunomodulatory effects of LGG (Schultz et al. 2003), 10 healthy adults (6 males and 4 females) aged 21-43 years (mean age = 29.9 years) ingested a single daily oral dose of 2 x 10^9 cfu lyophilized LGG in capsule form for 35 days. Stool samples were collected 3 weeks prior to the study, the day before the first dose, and the day after the last dose. Blood was also drawn on these 2 latter occasions. Peripheral blood cells were tested for response to "self" v "non-self" fecal preparations; *Bacteroides fragilis* group organisms, *Escherichia coli*, and pro- and anti-inflammatory cytokines (IL-10, IL-4, IL-6, IFN-γ, TNF-α) were measured in the culture supernatant, as was CD4 T-lymphocyte activation.

Lactobacilli indistinguishable from LGG were isolated from the feces of all individuals at the end of the administration period. Three participants had mild bloating, but no other adverse effects were reported by the participants. A mild modulation of the cellular immune response was seen, primarily in increased CD4 T-lymphocyte activity and decreased secretion of TNF-α and IL-6 cytokines.

Reid et al. (2003a) used a double-blind, placebo-controlled study to investigate the effect of oral probiotic treatment on vaginal microbiota during and after antibiotic therapy. Twenty-four female patients who had been diagnosed with respiratory or oral infections received antibiotic treatment and were randomized to receive either placebo or 2 x 10^9 cfu/day of a combination of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 for 21 days, beginning on the same day as the antibiotic therapy. No cases of vaginitis or diarrhea were reported in the probiotic group v. 3 cases in the placebo group; this difference was not statistically significant. There were no adverse effects of the probiotic therapy.

The effect of probiotic administration on women with asymptomatic bacterial vaginitis was studied in a randomized, placebo-controlled trial in which 64 women averaging 35 years of age received either placebo or 2 x 10^9 cfu/day of a combination of *L. reuteri* RC-14 and *L. rhamnosus* for 60 days (Reid et al. 2003b). Examination of vaginal swabs on days 0, 7, 28, 60, and 90 showed improvement in vaginal lactobacilli populations and reduced presence of pathogenic bacteria and yeast. There were no adverse effects of the probiotic treatment.

Hatakka et al. (2003) performed a randomized, double-blind, placebo-controlled pilot study on the effect of LGG on mild rheumatoid arthritis. A total of 21 patients aged 18-64 years were randomized to receive 2 capsules of LGG or placebo twice daily for 12 months; the daily dose of LGG was 10^{10} cfu. Fecal analysis was used to verify compliance. Arthritis activity was assessed based on clinical examination and laboratory tests. The treatment had no effect on any arthritis parameter, but the LGG group reported significantly greater general well-being. There were no study withdrawals due to adverse effects.

In a double-blind, placebo-controlled study, 10 women received either placebo or a combination of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 for 14 days at a dose of 5×10^9 cfu/day of each strain (Morelli et al. 2004). No adverse effects were observed, and both fecal and vaginal sampling and repetitive extragenic palindromic polymerase chain reaction (rep-PCR) strain identification showed that neither strain of bacteria persisted beyond 14 days after cessation of administration.

Salminen et al. (2004) evaluated the efficacy and safety of LGG ingestion in ameliorating gastrointestinal symptoms in HIV-infected patients receiving antiretroviral therapy. In a prospective, randomized, double-blind, placebo-controlled crossover trial, 17 HIV patients (14 males and 3 females, average age 44.5 years) with chronic diarrhea received either placebo or a twice-daily juice-milk drink providing approximately 10^{11} cfu/day LGG. Patients were excluded if they were suffering from bacterial infection, undergoing antibiotic therapy, or allergic to bovine milk proteins. Following a 2-week run-in period, patients were randomly assigned to a treatment of 2 weeks, followed by a 2-week washout period, and 2 weeks on the alternative treatment. Gastrointestinal symptoms were assessed daily by a self-administered questionnaire, including bowel movements, incontinence, diarrhea, flatulence, bloating, pain, and fever. Stool samples were taken at the beginning of the study and after each intervention period and cultured for total bacteria, pathogens, lactobacilli, and LGG. CD4 cell counts were performed prior to enrollment and at the end of the study, along with plasma HIV viral load levels and C-reactive protein. Additionally, the microbiological laboratory was asked to look specifically for lactobacilli from all clinical samples coming from any patient in the hospital ward during and after the study.

There was no effect of LGG on stool frequency diarrhea, or other gastrointestinal parameters. No adverse events were reported during the treatment periods and no patient withdrew due to side effects. No effects were seen on CD4 cell counts or on HIV load levels. No clinical infections due to lactobacilli were observed in study patients during this study or during a half-year follow-up at the outpatient clinic. The authors concluded that "LGG-containing products are not likely to have any major health risks among HIV-positive patients."

In a study to assess whether LGG consumption by pregnant women results in LGG colonization of their infants (Schultz et al. 2004), 6 women ingesting 2×10^9 cfu LGG/day during weeks 30-36 of pregnancy were identified. Four infants were delivered vaginally and 2 by caesarean section. LGG (identified by DNA sequence analysis) was isolated from the feces at 1 and 6 months of age from all 4 of the vaginally delivered infants and 1 of the 2 delivered by section. LGG could still be detected in the feces of 3 children at 12 months and in the feces of 2 children at 24 months. LGG was not isolated at 36 months. The authors concluded that temporary colonization of an infant with LGG may be possible by colonizing the pregnant mother before delivery.

Di Caro et al. (2005) studied the effects of *L. rhamnosus* GG (LGG) on gene expression patterns in the small bowel mucosa. Six male patients (average age 38) with endoscopically proven esophagitis were treated for 1 month with esomeprazole with either placebo or 1.2×10^{10} cfu LGG/day provided as freeze-dried powder in sachets to be taken twice a day. Biopsies of the

distal duodenal mucosa were taken during endoscopies before and after treatment. Total DNA and RNA were extracted and the expressions of more than 22,000 genes were analyzed. A total of 316 genes were up-regulated and 78 down-regulated in the LGG group but not the placebo group. The genes most affected involved immune response and inflammation (e.g., TGF-β, TNF family members, cytokines, nitric oxide synthase), apoptosis, cell growth and differentiation (e.g., cyclins and caspases, oncogenes), cell:cell signaling, cell adhesion, signal transcription and transduction. The authors did not report any adverse effects due to LGG administration.

In a randomized, double-blind, placebo-controlled study, Anukam et al. (2006) studied the effect of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 on bacterial vaginitis. Premenopausal sub-Saharan women (n = 125), age 18-44 years, suffering from bacterial vaginitis, were given 2 daily doses containing a total of 5 x 10^9 cfu each of *L. rhamnosus* and *L. reuteri* or placebo for 30 days. At the conclusion, 88% of the probiotic-treated group had normal Nugent scores v. 40% in the control; none of the probiotic group but 30% of the controls were determined still to have bacterial vaginitis. No adverse effects were noted from the administration of the two probiotics to this compromised population, and the participants did not report any side-effects when they were questioned.

The efficacy of a combination of 4 probiotic strains—*L. rhamnosus* GG, *L. rhamnosus* LC-705, *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *Bifidobacterium breve* Bb99—in alleviating symptoms of irritable bowel syndrome (IBS) was studied by Kajander and Korpela (2006). In a randomized, double-blind, placebo-controlled trial, 103 IBS patients were randomized to receive either placebo or a daily capsule providing 8-9 x 10^9 cfu of each strain for 6 months. The patients maintained a diary of abdominal symptoms and bowel habits.

Eighty-six patients completed the study and the probiotic treatment was successful in significantly reducing IBS symptoms—a reduction of 42% in the probiotic group v. 6% in the placebo group. There were no changes in bowel habits or quality of life. The reasons for the drop-outs were not reported, nor was it clear whether equal numbers discontinued from each group of patients. There was no discussion of any observed adverse effects of the probiotic treatment.

Baroja et al. (2007) conducted an open-label study (with a side control group) in which 20 adults with symptomatic inflammatory bowel disease (IBD; 15 with Crohn's disease and 5 with ulcerative colitis) and 20 healthy controls consumed 125 g/day of yogurt containing 2 x 10^7 cfu/ml of *L. rhamnosus* GR-1 and 10^3 cfu/ml *L. reuteri* RC-14, providing a daily intake of 2.5 x 10^9 cfu of *L. rhamnosus* and 1.25 x 10^5 cfu of *L. reuteri*. The probiotic yogurt intake resulted in beneficial anti-inflammatory effects in the IBD patients that were associated with expansion of the peripheral blood pool of regulatory T-cells. No anti-inflammatory effect was seen when the yogurt alone was given to 8 other individuals with IBD. The authors concluded, "Short-term consumption of yogurt supplemented with *Lactobacillus* strains GR-1 and RC-14 promoted the formation of a desirable anti-inflammatory environment in the peripheral blood of IBD patients, and showed no harmful effects in these patients." Of particular importance is that no effects were seen that would indicate that the probiotic bacteria had crossed the severely compromised GI barrier of these IBD patients, supporting other evidence indicating the lack of potential for infectivity of these strains.

In a prospective, randomized, double-blind, placebo-controlled pilot study in intensive care unit (ICU) patients, Forestier et al. (2008) administered either placebo or 10^9 cfu "*Lactobacillus casei rhamnosus*[1]" strain 35 twice a day from the 3rd day after admission to the ICU until discharge; the number of days was not reported. A total of 208 patients (146 males and 62 females, mean age = 58.4 years) was enrolled, 102 in the probiotic group and 106 in the placebo group. The objective of the study was to determine if probiotic treatment would reduce the incidence of respiratory infection by *Pseudomonas aeruginosa*. Most patients were post-surgical or suffering from trauma or respiratory distress. The incidence of *P. aeruginosa* infection was significantly reduced and the time to infection was significantly increased by the probiotic treatment; there was no effect on the incidence of infections by other microorganisms. No cases of *Lactobacillus*-related sepsis were observed.

Anukam et al. (2008), in a prospective, double-blind, placebo-controlled trial, studied the effect of consumption of yogurt containing 10^7 cfu/ml *L. rhamnosus* GR-1 and 10^7 cfu/ml *L. reuteri* RC-14 on premenopausal women with HIV/AIDS. Twelve women consumed 100 ml/day of placebo yogurt (containing normal levels of *L. delbrueckii* var *bulgaricus* and *Streptococcus thermophilus*), while 12 women consumed 100 ml/day of this same yogurt supplemented with *L. rhamnosus* and *L. reuteri*, a daily ingestion of 10^9 cfu of each probiotic bacterium for 15 days. A structured questionnaire was designed to evaluate clinical history and quality of life, including any gastrointestinal discomfort, water stools, nausea, flatulence, or diarrhea, at baseline and at day 15, 30, and 90. Urine samples were taken at baseline and on days 15 and 30 and analyzed for color, bilirubin, urobilinogen, proteins, ketones, nitrite, glucose, blood, leukocyte esterase, motile bacteria, and white blood cell counts. Blood samples taken on the same days were analyzed for CD4 count (a marker of immune status of HIV-infected patients) and the following hematologic parameters: red blood cells, total white blood cells, differential white blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red blood cell distribution width, and platelets.

The CD4 counts improved significantly in the probiotic-treated group while they deteriorated among the controls. The women treated with probiotics also had nonsignificantly improved quality-of-life parameters as compared with the controls. The urinalysis showed that the women treated with the probiotics experienced statistically significant reductions in urine leukocyte esterase, motile bacteria, and white blood cell counts as compared to those consuming the placebo yogurt. There was no significant alteration in any of the hematologic parameters tested. No side effects were noted and no bacteremia was detected in any of these highly immunocompromised women.

In an investigation of the effects of 3 probiotics on immune parameters, Kekkonen et al. (2008) conducted a prospective, randomized, double-blind, placebo-controlled parallel-group intervention study with 62 healthy adults (17 males and 45 females aged 23-58 years; mean age = 44 years). After a 3-week run-in, participants were randomly assigned to 1 of 4 groups to receive the specified intervention (in a milk-based fruit drink) for 3 weeks: placebo (n = 16), 1.6

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[1] As noted previously, *rhamnosus* was previously regarded as a subspecies of *L. casei*, but is now classified as a separate species.

x 10^{10} cfu LGG/day (n = 13), 3.5 x 10^{10} cfu *Bifidobacterium animalis* ssp. *lactis* Bb12/day (n = 16), or 3.3 x 10^{10} cfu *Propionibacterium freudenreichii* ssp. *shermanii* JS/day (n = 17). Venous blood samples were taken at baseline, on days 1, 7, and 21, and after a 3-week washout, and analyzed for leukocytes, monocytes, neutrophils, basophils, eosinophils, and lymphocytes; IgA, IgG, and IgM; C-reactive protein; cytokines (TNF-α, IL-6, IFN-γ, and IL-10). An unstimulated saliva sample was taken at each time point for analysis of secretory IgA. Fecal samples were also collected at baseline and on day 21 and probiotic strains were identified and enumerated. Participants maintained a diary of any respiratory, GI, or other symptoms and the use of any medication.

Feces levels of studied probiotics increased significantly from baseline values in each probiotic intervention group. LGG, but not the other probiotics, significantly reduced C-reactive protein. There were no significant differences between the groups in white blood cell counts, immunoglobulins, or cytokine levels. No adverse effects were reported.

4.4.2.1.3. Conclusions from Studies of L. rhamnosus *in Adults*

Thirty-five studies with over 1600 participants have appeared in the literature in which one or more strains of *L. rhamnosus* were orally administered to adults under controlled conditions. The strain HN001 was ingested in 4 studies, LGG in 20, strain GR-1 in 7, and other *L. rhamnosus* strains in 5 studies. Daily doses of probiotic exceeded 10^{10} cfu in 17 studies and 10^{11} cfu in 3 of these. Most of the studies were short term, lasting only a month or less, but 1 study included probiotic feeding for 6 months and 2 studies included a full year of probiotic administration. Participants in these studies included healthy adults, women suffering from bacterial vaginitis, IBS sufferers, patients with *H. pylori* infection, Crohn's disease patients and patients with ulcerative colitis, adults with rheumatoid arthritis HIV/AIDS patients, and patients confined to an ICU for a variety of causes.

In none of these studies were any adverse effects reported due to the probiotic treatment; many study reports specifically noted the absence of such effects. Several studies in at-risk subjects such as ICU patients reported that no cases of *Lactobacillus* infections occurred. This extensive body of human research strongly supports the conclusion that *L. rhamnosus*, and specifically the HN001 strain, is safe for adults under its intended conditions of use.

Table 52. Studies of Other *L. rhamnosus* Strains in Adults

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Dura-tion	Safety-Related Results
Alander et al. (1999)	Determine the ability of LGG to adhere to human colonic mucosa	Open-label study	21 healthy adults (10 males and 11 females, age 27-78 years) scheduled for routine diagnostic colonoscopy	6×10^{10} cfu LGG/day	12 days	The results from the patients undergoing colonoscopy immediately after the period of LGG ingestion showed the ability of the strain to adhere to colonic mucosa; LGG was identified in the biopsies of all 6 individuals. Colonies were found in the biopsies of 7 of the 8 patients who had colonoscopies a week later, but in only 2 of the 7 patients who had them 2 weeks later. Alander et al. (1999) concluded that LGG adheres well to colonic mucosa but is unable to establish itself as a permanent resident of the colon. No adverse effects were noted during this study.
Anukam et al. (2006)	Study the effect of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 on bacterial vaginitis.	Prospective, randomized, double-blind, placebo-controlled study	125 premeno-pausal sub-Saharan women age 18-44 years suffering from bacterial vaginitis	5×10^{9} cfu/day each of *L. rhamnosus* GR-1 and *L. reuteri* RC-14	30 days	No adverse effects were noted from the administration of the two probiotics to this compromised population, and the participants did not report any side-effects when they were questioned.
Anukam et al. (2008)	Study the effect of consumption of yogurt containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 on women with HIV/AIDS	Prospective, randomized, double-blind, placebo-controlled trial	24 women being treated for HIV/AIDS	10^{9} cfu/day each of *L. rhamnosus* GR-1 and *L. reuteri* RC-14	15 days	No effects were seen in urine color, bilirubin, urobilinogen, proteins, ketones, nitrite, glucose, or blood. No changes were seen in hematology parameters: red blood cells, total white blood cells, differential white blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red blood cell distribution width, and platelets. Serum CD4 counts improved significantly in the probiotic-treated group while deteriorating among the controls. Urine leukocyte esterase, motile bacteria, and white blood cell counts also improved in the test but not the control group. The women treated with probiotics also had nonsignificantly improved quality-of-life parameters as compared with the controls. No side effects were noted and no bacteremia was detected in any of these highly immunocompromised women.

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Table 52. Studies of Other *L. rhamnosus* Strains in Adults

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Dura-tion	Safety-Related Results
Armuzzi et al. (2001)	Investigate use of LGG as adjunct therapy during *Helicobacter pylori* eradication treatment with rabeprazole, clarithromycin, and tinidazole	Prospective, randomized, double-blind, placebo-controlled study	60 healthy asymptomatic *H. pylori* infection patients (25 males and 35 females, mean age 40 years)	1.2×10^{10} cfu LGG/day	14 days (7 days during therapy and 7 days after)	The patients who received LGG reported less nausea and diarrhea and had higher assessments of the tolerability of the treatment. There was no effect of the LGG v. placebo on the success of the eradication of *H. pylori* and no indication of any adverse effects resulting from ingestion of LGG.
Baroja et al. (2007)	Evaluate the anti-inflammatory effect of yogurt supplemented with *L. rhamnosus* GR-1 and *L. reuteri* RC-14	Open-label study with both IBD and healthy participants	20 adults with symptomatic inflammatory bowel disease (IBD; 15 with Crohn's disease and 5 with ulcerative colitis) and 20 healthy controls	2.5×10^{9} cfu of *L. rhamnosus* GR-1 and 1.25×10^{5} cfu of *L. reuteri* RC-14	1 month	Short-term consumption of yogurt with *Lactobacillus* promoted the formation of an anti-inflammatory environment in the peripheral blood of IBD patients and showed no harmful effects in these patients. No effects were seen indicating that the probiotic bacteria had crossed the severely compromised GI barrier of these IBD patients, indicating the lack of potential for infectivity of these strains.
Cremonini et al. (2002)	Investigate the effect of LGG and other probiotics on side-effects during anti-*Helicobacter pylori* therapy with rabeprazole, clarythromicin, and tinidazole	Prospective, randomized, double-blind, placebo-controlled trial	97 asymptomatic *H. pylori*-positive patients (43 males and 54 females aged 18-61 years)	1.2×10^{10} cfu LGG/day	14 days (7 days during therapy and 7 days after)	There were no side effects resulting in study discontinuation. All 3 probiotic treatments reduced reported side effects as compared with the placebo group; the difference was significant only during the first week and decreased monotonically through week 4. The most notable effect was a significant reduction in diarrhea. No adverse reactions were reported.

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Table 52. Studies of Other *L. rhamnosus* Strains in Adults

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Di Caro et al. (2005)	Study the effects of LGG on gene expression patterns in the small bowel mucosa	Open-label study with biopsies of the distal duodenal mucosa	6 male esophagitis patients (average age 38 years)	1.2×10^{10} cfu LGG/day	1 month	316 genes were up-regulated and 78 down-regulated in the LGG group but not the placebo group. The genes most affected involved immune response and inflammation, apoptosis, cell growth and differentiation, cell:cell signaling, cell adhesion, signal transcription and transduction. The authors did not report any adverse effects due to LGG administration.
Elmadfa et al. (2001)	Study the effects of viable v. head deactivated LGG in yogurt	Prospective single-blind crossover study	12 healthy adults (6 males and 6 females aged 25-36 years)	2.5×10^{10} cfu LGG/day	4 weeks (2 weeks inactivated and 2 weeks viable	The authors concluded that, "the bacterial flora of the examined yoghurt does not influence the vitamin B_1, B_2 and B_6 status of man." No adverse effects were reported.
Fang et al. (2000)	Study the effect of LGG or *B. lactis* on the immune system of adults exposed to *Salmonella typhimurium*	Prospective, randomized, double-blind, placebo-controlled trial	30 healthy adults (15 males and 15 females, age 20-50 years)	4×10^{10} cfu LGG/day	7 days	No significant differences between groups were seen in numbers of IgA-, IgG-, or IgM-secreting cells. The authors did not report any adverse events.
Forestier et al. (2008)	Determine if probiotic treatment would reduce the incidence of respiratory infection by *Pseudomonas aeruginosa.*	Prospective, randomized, double-blind, placebo-controlled pilot study	208 ICU patients (146 males and 62 females, mean age = 58.4 years)	10^9 cfu *L. rhamnosus* strain 35 twice a day	Until discharge from the ICU; number of days not reported	The incidence of *P. aeruginosa* infection was significantly reduced and the time to infection was significantly increased by the probiotic treatment; there was no effect on the incidence of infections by other microorganisms. No cases of *Lactobacillus*-related sepsis were observed.

000083

Table 52. Studies of Other *L. rhamnosus* Strains in Adults

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Dura-tion	Safety-Related Results
Goldin et al. (1992)	Study the survival of LGG in the gastrointestinal tract	Open-label parallel study of dosages and food matrices	76 healthy male and female adults aged 21-55 years	4×10^{10}, 1.6×10^{11}, and 3.6×10^{11} cfu LGG/day	7, 28, or 35 days	LGG was found to persist in the feces of 87% of the study subjects 3 days after the feeding was discontinued and in 33% of subjects after 7 days. No adverse effects were reported.
Hatakka et al. (2003)	Investigate the effect of LGG on mild rheumatoid arthritis	Prospective, randomized, double-blind, placebo-controlled pilot study	21 rheumatoid arthritis patients aged 18-64 years	10^{10} cfu LGG/day	12 months	The treatment had no effect on any arthritis parameter, but the LGG group reported significantly greater general well-being. There were no study withdrawals due to adverse effects.
Jacobsen et al. (1999)	Evaluate the probiotic potential of 47 strains of *Lactobacillus* spp. in resis-tance to acidity, adhesion to Caco-2 cells, and antimicro-bial activity	Preliminary open-label *in vitro* testing to identify 5 candidate strains.	12 healthy adults	10^{10} cfu of *L. rhamnosus* DSM 12246 twice a day	18 days	Among the tested strains, was identified most frequently in fecal samples. The bacteria were re-isolated in concentrations from 10^5 to 10^8 cfu/g of feces. *L. rhamnosus* DSM 12246 showed strong inhibition of all of the pathogenic bacteria tested, but the normal intestinal flora tested was unaffected by DSM 12246 *in vitro*. Even strong adhesive properties and pronounced pH tolerance did not result in colonization and persistence of the lactobacilli after administration of the cultures was terminated.

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Table 52. Studies of Other *L. rhamnosus* Strains in Adults

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Dura-tion	Safety-Related Results
Johansson et al. (1993)	Study the ability of *Lactobacillus* strains to colonize human intestinal mucosa.	Open-label parallel study; gave 19 different strains of multiple species of Lactobacilli, including LGG and *L. rhamnosus* 108	13 healthy adult volunteers	$5x10^8$ cfu/day of LGG or *L. rhamnosus* 108 and other strains	10 days	Colonization was shown for both *L. rhamnosus* strains tested. No adverse effects or tolerance issues were reported.
Kajander and Korpela (2006)	Test the efficacy of a combination of 4 probiotic strains—LGG, *L. rhamnosus* LC-705, *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *Bifidobacterium breve* Bb99—in alleviating symptoms of irritable bowel syndrome	Prospective, randomized, double-blind, placebo-controlled trial	103 IBS patients	8-9 x 10^9 cfu of combined LGG, *L. rhamnosus* LC-705, and the other probiotics	6 months	86 patients completed the study. The reasons for the drop-outs were not reported, nor was it clear whether equal numbers discontinued from each group of patients. There was no discussion of any observed adverse effects of the probiotic treatment.

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Table 52. Studies of Other *L. rhamnosus* Strains in Adults

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Dura-tion	Safety-Related Results
Kekkonen et al. (2008)	Investigate the effects of 3 probiotics on immune parameters	Prospective, randomized, double-blind, placebo-controlled parallel-group intervention study	62 healthy adults (17 males and 45 females aged 23-58 years; mean age = 44 years).	1.6×10^{10} cfu LGG/day	3 weeks	LGG, but not the other probiotics, significantly reduced C-reactive protein. There were no significant differences between the groups in white blood cell counts, immunoglobulins, or cytokine levels. No adverse effects were reported.
Ling et al. (1992)	Study the effect of LGG on intestinal enzymes and GI function	Open-label study	Elderly nursing home residents complaining of difficulties in defecation	Not reported	2 weeks	Levels of glycocholic acid hydrolase activity and tryptic activity were significantly decreased. A mild normalization of the consistency of the stools took place, but there was no effect on fecal frequency, weight, or pH.
Morelli et al. (2004)	Study the ability to reach the vagina and the persistence of orally administered probiotic bacteria for the treatment of bacterial vaginitis.	Double-blind, placebo-controlled	10 healthy women with no history of orogenital infection	5×10^{9} cfu/day each of *L. rhamnosus* GR-1 and *L. reuteri* RC-14	14 days	No adverse effects were observed, and both fecal and vaginal sampling showed that neither strain of bacteria persisted beyond 14 days after cessation of administration.
Ouwehand et al. (2002)	Investigate the effects of probiotic bacteria on constipation	Open-label parallel study	28 healthy elderly adults	$2\text{-}4 \times 10^{10}$ cfu/day of *L. rhamnosus* LC705	4 weeks	Fecal azoreductase activity was reduced in the LGG group. No changes in fecal pH or mucin excretion were observed in any group. The tested probiotics did not affect the mucosal barrier, and no adverse effects were observed; the authors concluded that use of the tested bacterial strains is safe.

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Table 52. Studies of Other *L. rhamnosus* Strains in Adults

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Dura-tion	Safety-Related Results
Prantera et al. (2002)	Evaluate the efficacy of LGG in preventing recurrence of symptoms after curative resection for Crohn's disease	Randomized prospective, double-blind, placebo-controlled trial	45 Crohn's patients (29 males and 16 females aged 22-71 years) who had resection of diseased intestine within the past 10 days	1.2×10^{10} cfu LGG/day	1 year	13 participants failed to complete the study; none of the drop-outs terminated due to adverse events and the reasons for the termination did not differ between the treatment and control groups. Symptoms such as diarrhea, bloating, and meteorism did not differ between the groups. No effects were noted on liver enzymes. Adverse events were suffered by 2 LGG patients and 6 placebo patients; none of the events were regarded as trial-related and they did not cause interruption of the study.
Reid et al. (2001)	Comparison of the effect on bacterial vaginitis of a combination of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 v. LGG	Randomized intervention trial	42 healthy women	6×10^{9} cfu/day of a combination of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 or 10^{10} cfu/day of LGG	28 days	The probiotic combination had a beneficial effect on vaginal microbiota populations. None of the patients reported symptomatic vaginitis, urinary tract infections, or any adverse side effects during or following the study. No further fecal shedding of the test bacteria was detected 14 days after the end of treatment.
Reid et al. (2003a)	Investigate the effect of oral probiotic treatment on vaginal microbiota during and after antibiotic therapy	Double-blind, placebo-controlled	24 female patients who had been diagnosed with respiratory or oral infections and were receiving antibiotic treatment	2×10^{9} cfu/day of a combination of *L. rhamnosus* GR-1 and *L. reuteri* RC-14	21 days	No cases of vaginitis or diarrhea were reported in the probiotic group v. 3 cases in the placebo group. There were no adverse effects of the probiotic therapy.

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Table 52. Studies of Other *L. rhamnosus* Strains in Adults

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Dura-tion	Safety-Related Results
Reid et al. (2003b)	Determine the effect of probiotic administration on women with asymptomatic bacterial vaginitis	Randomized, placebo-controlled trial	64 women averaging 35 years with asymptomatic bacterial vaginitis	2×10^9 cfu/day of a combination of *L. rhamnosus* GR- 1 and *L. reuteri* RC-14	60 days	Improvement was seen in vaginal lactobacilli populations and reduced presence of pathogenic bacteria and yeast. There were no adverse effects of the probiotic treatment.
Salminen et al. (2004)	Assess the efficacy and safety of LGG ingestion in ameliorating gastrointestinal symptoms in HIV-infected patients receiving antiretroviral therapy	Prospective, randomized, double-blind, placebo-controlled crossover trial	17 HIV patients (14 males and 3 females, average age 44.5 years) with chronic diarrhea	10^{11} cfu LGG/day	2 weeks	The microbiological laboratory looked specifically for lactobacilli from all clinical samples coming from any patient in the hospital ward during and after the study. There was no effect of LGG on stool frequency diarrhea, or other gastrointestinal parameters. No adverse events were reported during the treatment periods and no patient withdrew due to side effects. No effects were seen on CD4 cell counts or on HIV load levels. No clinical infections due to lactobacilli were observed in study patients during this study or during a half-year follow-up at the outpatient clinic. The authors concluded that "LGG-containing products are not likely to have any major health risks among HIV-positive patients."
Saxelin et al. (1991)	Study the effect of dose on colonization by LGG	Open-label dose-response study	40 healthy adults	1.5×10^6, 1.5×10^7, 1.5×10^8, 1.5×10^9, 1.5×10^{10}, and 1.1×10^{11} cfu LGG/day	7 days	No LGG was detected in fecal samples of the 10^6-10^8 dosing groups. 2 of 7 subjects in the 10^9 group were colonized with LGG sporadically, while all subjects in the 10^{10} and 10^{11} test groups were colonized. No level of LGG ingestion tested influenced the total number of lactobacilli found nor resulted in LGG becoming the dominant strain in the feces. No adverse effects were reported.

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Table 52. Studies of Other *L. rhamnosus* Strains in Adults

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Dura-tion	Safety-Related Results
Saxelin et al. (1993)	Study fecal concentrations of LGG after ingestion of either entero-coated tablets or fermented milk containing LGG	Open-label parallel study	44 healthy adults	Enterocoated tablets with 1 x 10^9, 4 x 10^9, or 8 x 10^9 cfu LGG/ day or fermented milk with 2.1 x 10^9 or 1.2 x 10^{10} cfu LGG/day	7 days	By day 3, LGG was found in the feces of all subjects at all dose levels, but neither the dose levels nor the carrier influenced the numbers of LGG cells found. No adverse effects were reported.
Saxelin et al. (1995)	Study the effect of dose on LGG colonization of healthy adults	Open-label study	20 healthy adults aged 20-55 years	1.6 x 10^8 and 1.2 x 10^{10} cfu LGG/day	7 days	By day 3, LGG was recovered in the feces of all subjects receiving the higher dose, but only 1 of 10 subjects in the lower dose group had detectable LGG in the feces by the end of the test period. No effect was observed on the total number of fecal lactobacilli. No adverse effects were reported.
Schultz et al. (2003)	Study the immunomodul atory effects of LGG in healthy adults	Open-label study	10 healthy adults (6 males and 4 females aged 21-43 years)	2 x 10^9 cfu LGG/day	35 days	Lactobacilli indistinguishable from LGG were isolated from the feces of all individuals at the end of the administration period. A mild modulation of the cellular immune response was seen, primarily in increased CD4 T-lymphocyte activity and decreased secretion of TNF-α and IL-6 cytokines. 3 participants had mild bloating, but no other adverse effects were reported by the participants.
Schultz et al. (2004)	Determine whether LGG consumption by pregnant women results in LGG colonization of their infants	Open-label study	6 healthy pregnant women	2 x 10^9 cfu LGG/day	Weeks 30-36 of preg-nancy	LGG was isolated from the feces at 1 and 6 months of age from all 4 of the vaginally delivered infants and 1 of the 2 delivered by section. LGG could still be detected in the feces of 3 children at 12 months and in the feces of 2 children at 24 months. LGG was not isolated at 36 months. The authors concluded that temporary colonization of an infant with LGG may be possible by colonizing the pregnant mother before delivery.

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Table 52. Studies of Other *L. rhamnosus* Strains in Adults

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Dura-tion	Safety-Related Results
Siitonen et al. (1990)	Study the efficacy of LGG to reduce erythromycin-associated diarrhea in adults	Prospective, randomized, double-blind, placebo-controlled study	16 healthy adult males	Not reported	7 days	Individuals receiving the LGG-supplemented yogurt had less diarrhea, abdominal distress, stomach pain, and flatulence than did those consuming the regular yogurt. The authors did not report any adverse effects.
Thomas et al. (2001)	Study the efficacy of LGG in preventing antibiotic-associated diarrhea in adults	Prospective, randomized, double-blind, placebo-controlled trial	302 hospitalized patients (143 males and 159 females aged 18 to 90 years) receiving intravenous or oral antibiotics	2×10^{10} cfu LGG	14 days	Treatment with LGG had no effect on the incidence of diarrhea; 29-30% of the patients in both the test and control groups developed diarrhea. There were no differences between the test and placebo groups in the number, type, or severity of reported adverse effects.

000090

4.4.2.2. Studies in Children

4.4.2.2.1. Studies of L. rhamnosus *HN001 in Children*

In a prospective, randomized, double-blind, placebo-controlled trial, Sistek et al. (2006) studied the effect of administration of daily doses of 2 x 10^{10} cfu of both *L. rhamnosus* strain HN001 and *B. animalis* ssp. *lactis* strain HN019 on children with atopic dermatitis. A total of 60 children ranging in age from 1 to 11 years (median = 4 years), 32 males and 28 females, previously diagnosed with eczema and with stable SCORADs of 10 or more, were randomized to receive either probiotic or placebo. The probiotic was administered once daily as a powder mixed with drink or food, as was the microcrystalline cellulose placebo. Two weeks before beginning treatment, the study participants' parents completed questionnaires about their children's health and the family history of allergic disease. The parents were also interviewed when treatment began (week 0), after 2 weeks, at the termination of treatment at week 12, and 2 weeks after treatment ceased (week 16). During the 12-week treatment period and the 4-week washout period the parents maintained a diary of any health problems, medication use, and the severity of the atopic dermatitis.

Five children withdrew from the probiotic group and 6 from the placebo group during the course of the study, none for reasons related to treatment. The probiotic treatment slightly reduced SCORAD scores relative to the placebo group among children with food-related allergies, but not among those with environmental allergies. No adverse effects were reported from the probiotic treatment.

4.4.2.2.2. Studies of Other L. rhamnosus *Strains in Children*

All of the studies involving the administration of strains of *L. rhamnosus* other than HN001 to children discussed below are summarized in Table 13 at the end of the section.

Kaila et al. (1992) studied the efficacy of LGG in assisting in the recovery of children suffering from acute rotavirus diarrhea in a prospective, randomized, double-blind, placebo-controlled trial. After rehydration, 39 well-nourished children aged 7-37 months (mean = 16 months) who had suffered from diarrhea for less than 7 days received 125 grams of either pasteurized regular yogurt (n = 17) or a fermented milk product containing LGG (n = 22) twice daily (10^{10} to 10^{11} cfu/day) for 5 days. The patients were weighed daily, their parents filled out questionnaires on stool quality throughout the study and follow-up, and blood was drawn on days 1 and 21 for immunological assessment.

The duration of diarrhea was significantly reduced in the test group compared to controls, and no recurrences of diarrhea occurred during the three-week follow-up period. Based on the IgG, IgA, and IgM-secreting cell numbers, those receiving LGG had significantly enhanced non-specific humoral response during the acute phase of the infection; by the end of the follow-up period an IgA-specific antibody-secreting cell response to rotavirus was present in significantly more test than control patients (90% v. 46%). No adverse events were reported by the authors.

In a prospective, randomized, double-blind, placebo-controlled study (Isolauri et al. 1994), 42 well-nourished children aged 5-28 months (mean = 14 months) who suffered from

acute diarrhea ingested doses of 10^{10} cfu of LGG in a freeze-dried powder or placebo twice a day for 5 days. Treatment began immediately after oral rehydration was completed. All study participants were diagnosed with rotavirus-induced acute gastroenteritis and fewer than 7 days of symptoms. The children were weighed daily and their stools were recorded as watery, loose, or solid. LGG counts and bacterial enzyme activities in the feces were assessed within six hours of enrollment, 30-36 hours after enrollment, and 21-24 days after enrollment.

No LGG was found in the feces of any study participant prior to treatment. At 30-36 hours after the beginning of LGG administration the strain was found in the feces of 15/18 patients from the study group, but in none of the control group. The reduction in the duration of diarrhea was significantly reduced in the test group (mean = 1.5 days v. 2.3 days among the controls). Urease activity during diarrhea transiently increased in the control group but not in the test group, while no differences were found in fecal levels of ß-glucuronidase, ß- glucosidase, or glycocholic acid hydrolase. There was no report regarding any adverse effects.

Kaila et al. (1995) performed a randomized, double-blind study assessing the effect of viable or heat-inactivated LGG on the immune response to rotavirus-induced acute diarrhea in children. Forty-one well-nourished children aged 1-38 months (mean = 13 months) who experienced acute gastroenteritis of less than 7 days' duration received 10^{10}-10^{11} cfu of either viable (n = 20) or heat-inactivated LGG (n = 21) in water twice daily for 5 days. The patients were weighed daily, stool quality was recorded throughout the study and follow-up period, and blood was drawn 1 and 30 days after enrollment and analyzed for immunological assessment.

There were no significant differences in diarrhea recovery between the two groups. However, the viable LGG stimulated rotavirus-specific IgA antibody responses to a significantly greater extent than did the heat-inactivated cells. No safety-related endpoints were reported by the authors.

In a prospective, randomized, double-blind, placebo-controlled study, Majamaa et al. (1995) tested the effect on the immune response in children with acute rotavirus-induced gastroenteritis of several probiotics, including LGG, another strain of *L. rhamnosus* designated Lactophilus, and a combination of *Streptococcus thermophilus*, and *Lactobacillus delbrueckii* ssp. *bulgaricus* referred to as Yalacta. Forty-nine children aged 6-35 months who were admitted to the hospital for acute gastroenteritis of less than seven days duration were randomized to receive daily either a placebo (n = 5) or 5 ml water (either orally or via nasalgastric tube) containing doses of 6.25×10^9 cfu LGG (n = 16), 2.75×10^8 cfu Lactophilus (n = 14), or 3.5×10^9 cfu Yalacta (n = 19) for 5 days. Stool samples were taken on day 1, the number and quality of stools and the number of vomiting episodes were monitored during the hospital stay, incidence of recurring diarrhea was noted through the follow-up period, and blood samples were taken to assess rotavirus antibodies on days 1 and 21 during a medical examination.

The children receiving LGG had significantly shorter duration of diarrhea than the controls or those receiving either Lactophilus or Yalacta; the remaining groups did not differ from each other. The total number of immunoglobulin-secreting cells was comparable in the three treated groups, as were the levels of IgM and IgG rotavirus-specific antibody secreting cells. Relative to the other treated groups, the LGG-treated group had enhanced IgA-specific

antibody secreting cells to rotavirus and serum IgA antibody levels during the convalescent stage. No adverse effects were reported.

Raza et al. (1995) studied the effect of LGG on the course of acute non-bloody diarrhea in a prospective, randomized, double-blind, placebo-controlled study in malnourished hospitalized children in Pakistan. Forty children aged 1-24 months (mean = 13 months) who were admitted with acute diarrhea and at least moderate dehydration participated in the study. The control group (n = 19) received 10 ml oral rehydration solution twice daily for 2 days while the test group (n = 21) received 10^{10}-10^{11} cfu LGG dissolved in the same quantity of oral rehydration solution. Subjects were discharged from the hospital after 48 hours. Treatment prior to admission, frequency of diarrhea and vomiting, and description of stools were recorded; fecal samples were taken at admission and at hospital discharge.

Rotavirus was found in 22% of subjects' stools. The children in the test group had significantly less frequent vomiting than did the control-group patients. No significant difference in the frequency of diarrhea was observed in those presenting with bloody diarrhea but, among those presenting with non-bloody diarrhea (n = 32), the percentage of children with persistent watery diarrhea was significantly less in the *Lactobacillus* group: 31% vs. 75%. No safety-related endpoints were discussed, although the authors stated that the LGG treatment was well tolerated.

In a prospective, randomized, controlled trial, Guarino et al. (1997) studied the effect of LGG on the duration of diarrhea in hospitalized children aged 3-36 months (mean = 19 months). A total of 100 patients (51 males and 49 females) were enrolled and randomly assigned to the test (n = 52) or control (n = 48) groups. After initial oral rehydration, further solution was administered alone or in combination with twice daily feedings of 3 x 10^9 cfu of LGG in 200 ml milk or formula for up to 5 days. Full age-appropriate feeding was introduced soon after initial rehydration. Stools were collected on enrollment and 6 days after the onset of diarrhea and were analyzed by ELISA for rotavirus. A slight majority (61%) of the children tested positive for rotavirus at the time of enrollment. Diarrhea was defined as 3 or more watery stools per day. Recovery from diarrhea was defined as the time since the last loose or liquid stools. The outcome of diarrhea was evaluated by the mothers of enrolled children, who were provided instructions and interviewed daily.

The duration of diarrhea was significantly reduced (by ~50%) in both rotavirus-positive and rotavirus-negative children receiving LGG compared with controls. Among the children who were initially positive for rotavirus, the number of patients excreting rotavirus at the end of therapy was significantly reduced in the LGG group relative to the control group (4 treated children v. 26 untreated children). No adverse effects were reported.

Shornikova et al. (1997) investigated the effects of adding LGG to oral rehydration for treatment of acute diarrhea in a prospective, randomized, double-blinded, placebo-controlled study in the Karelian Republic of Russia. On hospital admission, 123 children aged 1-36 months suffering from acute diarrhea of less than five days' duration were randomized to receive twice a day either intravenous fluids for rehydration (n = 26) or 1 of 2 oral hydration solutions differing in osmolarity and sodium concentration and again randomized to the solution supplemented with

either LGG (5 x 10^9 cfu per dose) or a cellulose powder placebo for 5 days. The normal full diet for age was started after rehydration, but yogurt, fermented milk and sour cream were excluded. Upon admission, the subjects were weighed and clinically examined, and the severity of dehydration was estimated. The number and characteristics of stools were recorded by attending nurses, and the duration of diarrhea (based on the last appearance of watery stools) was determined. Stool samples were cultured for *Salmonella* and *Shigella*, as well as tested for rotavirus antigen.

Thirty-four children tested positive for rotavirus antigen, and 26 had a confirmed bacterial etiology, including 11 cases of *Salmonella enteriditis*, 13 cases of *Shigella sonnei*, and two cases of *Shigella flexneri*. The children receiving intravenous fluids had a significantly longer mean duration of diarrhea compared to those receiving oral rehydration solution. Patients receiving LGG had a significantly shorter mean duration of diarrhea (2.7 days) compared to those receiving placebo (3.8 days). The frequency of watery stools began to decrease significantly in the LGG group from the second treatment day, and the cumulative number of diarrheal stools was significantly lower in the LGG group on days 2 and 3 after initiation of treatment. LGG was found to significantly decrease the number of watery stools in rotavirus-positive subjects (n = 13) on days 0-5 of treatment compared to controls (n = 21), while no effect was observed between those subjects with confirmed bacterial diarrhea who received LGG (n = 11) v. placebo (n = 15). No safety-related endpoints were discussed, but they reported that LGG was administered "without difficulties."

In a prospective, randomized, placebo-controlled, double-blind study, Arvola et al. (1999) evaluated the effects of LGG on the incidence of diarrhea in infants and children following 7 to 10 days of oral antibiotic treatment with amoxicillin, penicillin, kephalosporins, erythromycin, or trimetoprim-sulpha for acute respiratory infections. The ages of the 119 study participants ranged from 2 weeks to 12.8 years (mean = 4.5 years). The children were randomized to receive capsules containing either placebo (microcrystalline cellulose) or 2 x 10^{10} cfu LGG twice daily during antibiotic treatment. Fecal samples were taken from the children at enrollment and on day 7 of antibiotic therapy. Their parents maintained a daily record of defecation frequency and consistency for 3 months. The primary outcome measure was diarrhea during the first 2 weeks after the beginning of antibiotic treatment; secondary outcome measures were activities of fecal urease, β-glucuronidase, and β-glucosidase.

Fecal samples from 23 randomly selected LGG-group patients were cultured and tested for LGG; 21 were positive and 2 were negative. Although no effect was noted on severity of antibiotic-induced diarrhea, LGG significantly reduced its incidence in this study: in the 2-week period following the introduction of antibiotics, only 5% of the children who received LGG v. 16% of the placebo group developed diarrhea. The groups did not differ in the activity levels of fecal enzymes. Questioning of the parents of the children elicited no reports of adverse effects of LGG.

Vanderhoof et al. (1999) explored the ability of LGG to reduce the incidence of antibiotic-induced diarrhea in children with acute infections of the respiratory tract, urinary tract, soft tissues, or skin. In a prospective, randomized, placebo-controlled, double-blind trial, 202 children aged 6 months to 10 years (median = 4 years) received a 10-day course of oral

antibiotics (e.g., amoxicillin, amoxicillin/clavulanate potassium, cefprozil, or clarithomycin) along with capsules containing either placebo or LGG at 10^9 (for children weighing less than 12 kg) or 2 x 10^9 cfu (for those over 12 kg) per day. Children's parents were interviewed by investigators every 3 days about stool frequency and consistency, blood in the stood, appetite suppression, and gastrointestinal symptoms such as abdominal pain, nausea, vomiting, or bloating.

Fourteen children failed to complete the study for a number of non-treatment-related causes. The authors noted that, "There were no failures [to complete the study] resulting from untoward effects of either LGG or placebo." The incidence of diarrhea was significantly reduced in the children receiving LGG (8%) compared to control patients (26%) and the mean duration of diarrhea was significantly shortened to 4.7 days v. 5.9. No differences were seen between the LGG and placebo groups in any of the other parameters assessed and no adverse effects related to LGG consumption were noted by the authors.

In a prospective, randomized, double-blind, placebo-controlled, multi-center study, Guandalini et al. (2000) studied the effect of LGG administered in oral rehydration solution on acute diarrhea in children aged 1-36 months (mean = 12.3 months). A total of 287 children hospitalized with acute diarrhea from all causes received during the first 4-6 hours oral rehydration solution containing 10^{10} cfu LGG per 250 ml (n = 147) or placebo (n = 140). Anthropometric measurements including weight as well as stool samples were taken at admission; stool samples were also taken 48 hours later and, as possible, 7 days and 30 days later. Fluid intake, number and characteristics of stools, and occurrence of vomiting were monitored throughout the observation period.

Rotavirus was the most commonly detected pathogen in both the test and control groups (38% and 32%, respectively) and 15% of the children in both groups suffered from invasive enteritis caused by *Salmonella*, *Campylobacter*, *Yersinia enterocolitica*, or *Shigella*. Mean consumption of oral rehydration solution by the LGG-group patients was 382 ml in the first 4 hours, 459 ml during the following 20 hours, and 1194 ml overall, although the variability was large. Consumption of oral rehydration solution in the control group was similar. The duration of diarrhea was significantly reduced in the test group compared to controls (mean of 58 hours v.72 hours). The incidence of diarrhea lasting longer than 7 days was significantly lower in the test group than among to controls (2.7% v. 10.7%) as was the mean duration of hospitalization. There was no difference in weight gain of the 2 groups over the first 24 hours of rehydration. Although there was no specific discussion of adverse effects, the authors concluded that, "Administering oral rehydration solution containing *Lactobacillus* GG to children with acute diarrhea is safe and results in shorter duration of diarrhea, less chance of a protracted course, and faster discharge from the hospital."

Hatakka et al. (2001) examined whether long term consumption of LGG in cow's milk affected the incidence or severity of gastrointestinal and respiratory infections in children in day-care centers. In a prospective, randomized, double-blind, placebo-controlled, multi-center study, 571 children aged 1-6 years received milk either with LGG supplementation (n = 282) or without LGG (n = 289). Supplemented milk contained 5-10 x 10^5 cfu LGG/ml , and the average daily milk consumption (5 days/week) was 260 milliliters for both the treatment and control groups

over the course of the 18 month study; thus, the mean daily dose of LGG in the test groups was 1.3-2.6 x 10^7 cfu. During the study, parents kept a daily record of respiratory symptoms (fever, runny nose, sore throat, cough, chest wheezes, earache) and gastrointestinal symptoms (diarrhea, vomiting, stomach ache) as well as absences due to illness. Fecal samples were collected at the beginning, middle, and end of the study to measure LGG recovery and assess compliance. An eight-month follow-up period was included, although 58 children did not complete this phase of the study.

LGG was recovered in the feces of 97% of the treated subjects (v. 9% of controls) by the end of the study period. Children in the LGG treatment group had a statistically significant reduction in days of absence from the day-care centers (mean = 4.9 v. 5.8 days for controls). The treatment group also had a significant relative reduction in incidences of respiratory tract infections with complications and lower respiratory tract infections and a significant relative reduction in antibiotic treatments for respiratory infection. No differences in adverse events – listed as including stool frequency or consistency, abdominal pain, allergic symptoms, or side effects – between the LGG treatment group and the controls were observed.

Szajewska et al. (2001) evaluated the efficacy of LGG in the prevention of nosocomial diarrhea in young children admitted to pediatric hospitals. In a prospective, randomized, double-blind, placebo-controlled study, 81 children aged 1-36 months (mean = 10.8 months) hospitalized for reasons other than diarrhea received either placebo (n = 36) or 6 x 10^9 cfu LGG suspended in water (n = 45) with regular feedings twice a day for the duration of their hospital stay (2-23 days; mean = 9.0 days). Patients were evaluated daily for stool number and consistency and stool samples were collected weekly and during episodes of diarrhea and were analyzed for bacteria and rotavirus antigen.

The patients who received LGG had a significantly reduced incidence of nosocomial diarrhea (6.7% v. 33.2% among the control group). Rotavirus was the most common cause of nosocomial diarrhea, being found in the stool of 1 of the 3 cases of nosocomial diarrhea observed in the LGG group and in 6 of the 12 cases seen in the control group. The incidence of gastroenteritis was also significantly reduced in the treatment group compared to the control group (2.2% v. 16.7%). Szajewska et al. (2001) reported that, "LGG was well tolerated, and no adverse effects of the treatment were noted."

Rosenfeldt et al. conducted a series of 4 prospective, randomized, double-blind, placebo-controlled trials (2002a, 2002b, 2003, 2004) using combined *L. rhamnosus* 19070-2 and *L. reuteri* DSM 122460. In all 4 studies, doses of 10^{10} cfu each of *L. rhamnosus* and *L. reuteri* were given twice a day; the placebo control was skim milk powder and dextrose anhydrate. In the first study (Rosenfeldt et al. 2002a), 86 young children aged 6-36 months hospitalized with acute diarrhea with duration not more than 7 days were enrolled and given either the probiotic or placebo beverage for 5 days. No safety-related endpoints were tested, but the authors did not report observing any adverse effects.

In the second trial by Rosenfeldt et al. (2002b), 43 children aged 9-44 months attending child-care centers and identified by their parents as having diarrhea were given either placebo or

the probiotic beverage for 5 days. The authors stated that "no serious adverse effects were registered."

In the following year, Rosenfeldt et al. (2003) investigated the effect of *L. rhamnosus* 19070-2 and *L. reuteri* DSM 122460 on children with atopic dermatitis. Forty-three children aged 1-13 years (mean age = 5.2 years) were enrolled and randomly assigned to receive the probiotic strains or placebo for 6 weeks; in a crossover design, they were assigned to the other condition after a 6-week washout. No significant differences were found in the production of cytokines IL-2, IL-4, IL-10, or IFN-γ although the severity of eczema was reduced. Neither of the test strains could be isolated from feces 5 days after administration had stopped. The authors did not report the occurrence of any adverse effects.

In Rosenfeldt et al. (2004), the two *Lactobacillus* strains were tested for their effects on gastrointestinal symptoms and small intestinal permeability of children with atopic dermatitis. A crossover design was again employed, in which 41 children aged 1-13 years (median age = 4.0 years) with moderate or severe atopic dermatitis were given each of the two treatments for 6 weeks with a 6-week washout period. The frequency of reporting of "any GI symptom" was reduced during probiotic administration as compared to control, including fewer reports of diarrhea, vomiting, and abdominal pain. Administration of the probiotic bacteria resulted in significantly improved intestinal mucosal barrier function (as measured by the ratio of the absorption of lactulose v mannitol), but the investigators did not report on any other safety-related endpoints or events.

In all four of the studies by Rosenfeldt et al. (2002a, 2002b, 2003, 2004), over 200 young children ranging in age from 6 months to 13 years were administered probiotic bacteria for as long as 6 weeks. All of these children were suffering from acute diarrhea or moderate to severe atopic dermatitis and were likely to have impaired mucosal barrier function. Not a single case of bacteremia was reported, indicating a low potential for infectivity of these bacterial strains.

Salazar-Lindo et al. (2004) studied the effect of LGG suspended in a milk formula on the duration and severity of non-rotavirus-induced acute diarrhea in young boys in Peru. A total of 179 male children aged 3-36 months were rehydrated with oral rehydration solution and randomly assigned to receive a milk formula with 10^9 cfu LGG/ ml (n = 90) or without LGG (n = 89). The first dose of formula was given immediately after rehydration was completed and subsequent feedings were given every four hours until cessation of diarrhea or for a maximum of five days. Each child was given 150 ml formula/kg bw/day to a maximum of 1000 ml/day. The dose of LGG in the study was estimated at 6-8 x 10^{11} cfu LGG/day." A clinical history, physical examination, and blood draw was completed before starting treatment, and blood was taken again after 24 hours of treatment. Stool volume was measured periodically and the duration of diarrhea was estimated based on the physical characteristics of the stools.

Eight boys from the LGG group and 11 from the control group were withdrawn from the study for a variety of non-treatment-related conditions (bloody stools within the first 24 hours after admission, parental non-compliance, etc.). Stool output was significantly larger in the LGG group than in the control group, although no significant differences were found in duration of diarrhea. About 12% of patients had unresolved diarrhea and an additional 20% were classified

as treatment failures, mostly due to severe diarrhea; these proportions did not differ between the 2 groups. The authors concluded that this study did not show a positive effect of LGG on the clinical course of acute watery diarrhea, but they noted that, "No adverse effects due to the study formula were noticed in either group during the study."

In a randomized, double-blind, placebo-controlled trial, Bausserman and Michail (2005) administered $2x10^{10}$ cfu LGG/day or placebo (inulin) for 6 weeks to 50 children suffering from irritable bowel syndrome (IBS). A total of 64 children (12 male, 52 female; mean age 12 years with a range of 6-20 years) with diagnosed IBS were entrolled. Patients were clinically examined at enrollment and at termination. Six participants (4 LGG, 2 control) withdrew before study initiation, 3 patients from each group were lost to follow-up, and 2 patients in the placebo group had unsatisfactory compliance, leaving 50 patients (25 in each group) for analysis. The analyzed group ranged from 6-17 years of age (mean = 12 years) and included 10 males and 40 females. No significant differences in reduction of abdominal pain were observed between the LGG and placebo groups. The authors noted that, "No adverse events with the use of *Lactobacillus* GG were seen."

Banaszkiewcz and Szajewska (2005) employed a randomized, double-blind, placebo-controlled study to determine if LGG is an effective adjunct to lactulose for treating constipation in children. A total of 84 children aged 2-16 years (mean = 7.3 years; sex not reported) suffering from constipation received 1 ml of a 70% solution of lactulose/kg bw along with either placebo (n = 41) or 10^9 cfu LGG/day in 2 daily doses (n = 43) for 12 weeks. The LGG and placebo were both presented as powder-containing capsules. The children were evaluated clinically at baseline and at 4, 8, and 12 weeks. The primary outcome measure was the number of bowel movements per week. Addition of LGG to lactulose provided no benefit in reducing constipation. The incidence of adverse effects was non-significantly lower in the LGG group than in the placebo group, and the authors observed that, "The LGG was well tolerated, and the incidence of adverse events associated with this therapy was no higher than that associated with the placebo."

Clinically healthy Malawian children at elevated risk of tropical enteropathy participated in a prospective, randomized, double-blind, placebo-controlled trial of the effect of LGG on intestinal integrity (Galpin et al. 2005). Tropical enteropathy is an aspymptomatic villous atrophy of the small bowel prevalent in the developing world. A total of 164 children were enrolled in the study and 161 completed it. The enrollment criteria required the children to be aged 36-60 months; the average age of the 76 boys and 88 girls at entry was 46.4 months. At entry, each child was clinically examined and the parents completed a demographic and sanitation-data questionnaire. Children consumed 2 capsules/day containing either $5x10^{10}$ cfu LGG (daily dose = 10^{11} cfu) or placebo for 30 days. Children were examined twice a week during the study period. The primary study outcome was the absorption of mannitol, lactulose, and sucrose, based on urinalysis.

The administration of LGG had no effect on absorption of mannitol, lactulose, or sucrose. No vomiting or other adverse effects were reported by caretakers during the study and there were no differences in rates of growth or the incidence of fever, cough, or diarrhea.

In a prospective, randomized, double-blind, placebo-controlled trial, Szymanski et al. (2006) studied the efficacy of *L. rhamnosus* strains 573L/1, 573L/2, and 573L/3 in shortening the duration of acute infectious diarrhea in infants and children. A freeze-dried powder containing a combination of these 3 strains is sold under the trade name Lakcid L by Biomed of Lublin, Poland. A total of 87 children aged 2 months to 6 years (mean = 25.8 months; 45 males and 42 females) with acute diarrhea (defined as 3+ loose stools/day for 2-4 days) were weighed and orally or intravenously rehydrated and randomized to receive either 1.2 x 10^{10} cfu Lakcid L suspended in water (n = 46) or placebo (n = 41) twice daily for 5 days. The stools of patients were checked on admission and on days 5 and 14 for the presence of *L. rhamnosus* as well as rotavirus or adenovirus, *Salmonella*, *Shigella*, and enteropathogenic strains of *Escherichia coli*. Hospitalized patients were examined for clinical parameters at hours 6 and 24 after initiation of treatment and daily during their stay in the hospital; outpatients were examined on their first visit and on days 6 and 14. The children's parents maintained diaries on the number of stools and vomiting. Bacterial isolates were speciated using polymerase chain reaction (PCR) and the strain identified with pulsed-field gel electrophoresis (PFGE).

The reduction in the overall duration of diarrhea from treatment with *L. rhamnosus* was not statistically significant, but rotavirus-induced diarrhea was significantly shortened compared with the control group (mean of 76 hours v. 115 hours) and the duration of parenteral rehydration was also significantly shorted (mean of 15 hours v. 38 hours in the control group). The administered strains were recovered in the feces of 80% of the test-group children at 5 days and in 41% at 14 days. The authors reported that no adverse events of the probiotic treatment were noted during the study.

Honeycutt et al. (2007) initiated a prospective, randomized, double-blind, placebo-controlled trial in a 16-bed pediatric intensive-care unit to evaluate the efficacy of LGG to reduce the rate of nosocomial infection. Sixty-one pediatric patients (40 male and 21 female) were enrolled; the study population included 12 neonates under 1 month of age, 24 infants between 1 month and 2 years old, and 25 young children more than 2 years old. Participants were randomized into a treatment group (n = 31) that received one capsule daily of LGG (10^{10} cfu/capsule) or a control group (n = 30) that received an identical capsule containing insulin as a placebo until discharge from the hospital. Pertinent clinical data were collected daily by the study investigators until discharge and for 48 hours after discharge.

Six patients in the LGG group developed 11 infections, while only 3 patients in the control group developed only 4 infections; differences between the number of patients developing infections and the number of infections per infected patient were not statistically significant. Of the 15 infections, 6 were caused by Gram-positive bacteria and 6 by Gram-negative bacteria while 3 were caused by *Candida* species. There were 6 deaths during the study (2 in the LGG group and 4 in the control group), all from non-infectious causes and none apparently related to treatment. There were no cases of *Lactobacillus* bacteremia in the study population and no known treatment-related serious adverse effects occurred in any patient during the study period. Nevertheless, due to the absence of any observed benefit to LGG treatment and concern regarding the safety of the treatment in the light of published reports of sepsis associated with LGG administration (discussed above in Section 4.3.3), the investigators terminated the study. (A review of this study by EFSA [2008] concluded that "The results are most likely due to

small study sample size and more severely ill patients in the treatment group [longer hospital and intensive care unit stay] as compared to the control group."

A large (571 participants) but only single-blinded clinical trial was performed by Canani et al. (2007) to investigate the use of probiotics for the treatment of acute diarrhea in children aged 3-36 months. Five probiotic preparations were tested: 1) LGG; 2) *Saccharomyces boulardii*; 3) *Bacillus clausii*; 4) mix of *L. delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *L. acidophilus*, and *Bifidobacterium bifidum*; and 5) *Enterococcus faecium* strain SF68. Eligible children (282 males and 289 females; age 8-28 months with median = 17.5 months) with acute diarrhea of less than 48 hours duration were given oral rehydration solution for 3-6 hours and then fed formula containing lactose or cows' milk, depending on age. Children were also randomized to receive for 5 days oral rehydration alone (n = 92), 6 x 10^9 cfu/day LGG (n = 100), or 1 of the other 4 probiotic treatments (n = 91, 100, 97, and 91, respectively). On enrollment, each child was evaluated for the duration and severity of diarrhea and associated clinical features, while parents received a reporting form for daily recording of the number of fecal outputs and their consistency, the presence of vomiting and fever, and any adverse events. The children's parents purchased the probiotics at a pharmacy; the family physicians were in charge of treatment allocation, gave written instructions to the parents, and verified compliance, while the investigators collecting the reporting forms were blinded to the assigned treatment.

Two of the experimental treatments, LGG and the mix of *L. delbrueckii* and other bacteria, significantly reduced the duration and severity of diarrhea; the other 3 treatments had no significant effect. No adverse effects were reported by the parents of children receiving any of the experimental probiotic treatments.

Ruszczynski et al. (2008) studied the safety, tolerability, and effectiveness of a combination of 3 strains of *L. rhamnosus* in preventing antibiotic-associated diarrhea in children in a prospective, randomized, double-blind, placebo-controlled trial. A total of 240 children, 130 males and 110 females, ranging in age from 3 months to 14 years, receiving standard antibiotic treatment for common infections, were also given 2 x 10^{10} cfu of a combination of *L. rhamnosus* strains E/N, Oxy, and Pen[1] twice a day (n = 120) or placebo of saccharose in nonfat milk n = 120) throughout the antibiotic treatment. Children or their parents recorded the frequency of daily bowel movements as well as any symptoms they considered important. Stool numbers and consistency were recorded daily. All diarrheal stool samples were tested for viral pathogens.

The children received antibioic therapy (and *L. rhamnosus* in the test group) for 3-30 days, with a mean of 8 days. The addition of *L. rhamnosus* to the antibiotic therapy significantly reduced the incidence of diarrhea. Two control children and one child in the probiotic group required intravenous rehydration. No side effects or adverse effects were observed. The authors noted that, "*Lactobacillus rhamnosus* was well tolerated, and no adverse event associated with this therapy (or with the use of placebo) was reported."

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[1] These strains are deposited at the Polish Institute of Biochemistry and Biophysics under the numbers 2593 (Pen), 2594 (E/N), and 2595 (Oxy).

Piirainen et al. (2008) investigated whether the addition of galactooligosaccharides (GOS) to LGG administration would result in enhanced bifidogenesis in school-aged children. In a randomized, double-blind, 2-period crossover study, 30 healthy children (16 boys, 14 girls) aged 6.7 to 11.1 years (mean = 8.8 years), after a 4-week run-in, consumed either LGG alone or LGG + GOS for 3 weeks, followed by the alternative treatment after a 4-week washout period. The LGG was given at a dosage of 6.5 x 10^9 cfu/day in 65 ml milk-based fruit juice, with or without 2 g GOS. Children and their parents completed a daily questionnaire about their defecation frequency, stool consistency, ease of defecation, flatulence, abdominal pain, abdominal distension, and heartburn. Fecal samples were collected on the first and last days of each study period and evaluated for enumeration of bifidobacteria, lactobacilli, and *L. rhamnosus*.

Both treatments significantly increased fecal counts of lactobacilli and *L. rhamnosus*, but only the LGG+GOS treatment significantly increased bifidobacteria. Neither treatment significantly affected defecation frequency, stool consistency, ease of defecation, or frequency or severity of gastrointestinal symptoms.

4.4.2.2.3. Conclusions from Studies of L. rhamnosus *in Children*

Twenty-five studies have been reported in the literature in which one or more strains of *L. rhamnosus* have been orally administered to children ranging in age from 2 weeks to 17 years, although nearly all of the studies involved children aged less than 36 months. A total of 3,463 children participated in these studies. Nineteen studies included LGG in at least one arm and 8 included other strains of *L. rhamnosus*, 1 with strain HN001. The tested doses were generally high: 19 studies used doses exceeding 10^{10} cfu/day and 5 of these studies included doses above 10^{11} cfu/day. The great majority of these studies were short term with durations of less than a month, but one study included probiotic administration for 18 months and several had durations of 6 weeks or more.

One reason why study durations were generally short is that the published studies were all concerned with treatment or prevention of disease or disorder; 11 studies were in children with acute diarrhea, 3 in children with atopic dermatitis, 3 in children receiving antibiotic therapy, and 4 in children with gastrointestinal disturbances. Four studies were conducted in children at elevated risk of infection, most often those confined to a pediatric ICU, and only 1 study involved healthy free-living children.

It is notable that no adverse effects associated with the probiotic treatment were reported, even with administration to what one article referred to as young patients in "fragile" medical condition. No cases of *Lactobacillus* infection were observed in any study. This body of evidence confirms the safety of oral administration of *L. rhamnosus* strains, including HN001, to young children.

Table 63. Studies of Other *L. rhamnosus* Strains in Children

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Arvola et al. (1999)	Evaluate the effects of LGG on the incidence of diarrhea in infants and children following oral antibiotic treatment	Prospective, randomized, placebo-controlled, double-blind study	119 children aged 2 weeks to 12.8 years (mean = 4.5 years) who had received oral antibiotic treatment	2×10^{10} cfu LGG/day	3 months	In the 2-week period following the introduction of antibiotics, only 5% of the children who received LGG v. 16% of the placebo group developed diarrhea. The groups did not differ in the activity levels of fecal enzymes. Questioning of the parents of the children elicited no reports of adverse effects of LGG.
Banaszkiewcz and Szajewska (2005)	Determine if LGG is an effective adjunct to lactulose for treating constipation in children	Randomized, double-blind, placebo-controlled study	84 children aged 2-16 years (mean = 7.3 years) with constipation	10^9 cfu LGG/day in 2 doses	12 weeks	The incidence of adverse effects was non-significantly lower in the LGG group than in the placebo group, and the authors observed that, "The LGG was well tolerated, and the incidence of adverse events associated with this therapy was no higher than that associated with the placebo."
Bausserman and Michail (2005)	Determine if LGG is safe and effective in treating IBS in children	Randomized, double-blind, placebo-controlled trial	50 children with IBS; 6-17 years old (mean = 12 years); 10 boys and 40 girls	2×10^{10} cfu LGG/day	6 weeks	The authors noted that, "No adverse events with the use of *Lactobacillus* GG were seen."

000102

Table 63. Studies of Other *L. rhamnosus* Strains in Children

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Canani et al. (2007)	Investigate the effectiveness of probiotics in the treatment of acute diarrhea in children aged 3-36 months	Randomized single-blinded comparison trial of LGG; *Saccharomyces boulardii; Bacillus clausii*; mix of *L. delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, *L. acidophilus*, and *Bifidobacterium bifidum*; and *Enterococcus faecium* strain SF68	571 children (282 males and 289 females; age 8-28 months; median = 17.5 months) with acute diarrhea of less than 48 hours duration	6×10^9 cfu LGG/day	5 days	Two of the experimental treatments, LGG and the mix of *L. delbrueckii* and other bacteria, significantly reduced the duration and severity of diarrhea; the other 3 treatments had no significant effect. No adverse effects were reported by the parents of children receiving any of the experimental probiotic treatments.
Galpin et al. (2005)	Evaluate the effect of LGG on intestinal integrity of children at elevated risk of tropical enteropathy	Prospective, randomized, double-blind, placebo-controlled trial	164 Malawian children at elevated risk of tropical enteropathy; 76 boys and 88 girls; average age = 46.4 months	10^{11} cfu LGG/day in two doses	30 days	No vomiting or other adverse effects were reported by caretakers during the study and there were no effects in rates of growth or the incidence of fever, cough, or diarrhea.
Guandalini et al. (2000)	Study the effect of LGG on acute diarrhea in children hospitalized with acute diarrhea	Prospective, randomized, double-blind, placebo-controlled, multi-center study	287 children aged 1-36 months (mean = 12.3 months) hospitalized with acute diarrhea from all causes	$4.8\ 10^{10}$ cfu LGG	Single admin-istration	The duration of diarrhea was reduced in the test group compared to controls. Although there was no specific discussion of adverse effects, the authors concluded that, "Administering oral rehydration solution containing *Lactobacillus* GG to children with acute diarrhea is safe and results in shorter duration of diarrhea, less chance of a protracted course, and faster discharge from the hospital."

000103

Table 63. Studies of Other *L. rhamnosus* Strains in Children

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Guarino et al. (1997)	Study the effect of LGG on the duration of diarrhea in hospitalized children	Prospective, randomized, placebo-controlled trial	100 diarrhea patients age 3-36 months (mean = 19 months), 51 males and 49 females	3×10^9 cfu of LGG twice a day	Up to 5 days	The duration of diarrhea was reduced in both rotavirus-positive and rotavirus-negative children receiving LGG compared with controls. No adverse effects were reported.
Hatakka et al. (2001)	Assess if long term ingestion of LGG reduces the incidence or severity of gastrointestinal and respiratory infections in children in day-care centers	Prospective, randomized, double-blind, placebo-controlled, multi-center study	571 children aged 1-6 years in day-care	$5\text{-}10 \times 10^5$ cfu LGG/ml of milk, equivalent to $1.3\text{-}2.6 \times 10^7$ cfu LGG/day	18 months	Children in the LGG treatment group had a reduction in days of absence. The treatment group also had a relative reduction in incidences of respiratory tract infections with complications and lower respiratory tract infections and a significant relative reduction in antibiotic treatments for respiratory infection. No differences in adverse events – listed as including stool frequency or consistency, abdominal pain, allergic symptoms, or side effects – between the LGG treatment group and the controls were observed.
Honeycutt et al. (2007)	Evaluate the efficacy of LGG to reduce the rate of nosocomial infection in a pediatric ICU	Prospective, randomized, double-blind, placebo-controlled trial	61 pediatric patients (40 male and 21 female), including 12 neonates under 1 month of age, 24 infants between 1 month and 2 years, and 25 young children more than 2 years old	10^{10} cfu LGG/day	Until discharge from the ICU; number of days was not reported	Patients in the LGG group developed more infections than the controls, but the difference was not significant. 6 infections were caused by Gram+ bacteria, 6 by Gram- bacteria, and 3 by *Candida*. There were 2 deaths in the LGG group and 4 in the control group, all from non-infectious causes and not related to treatment. There were no cases of *Lactobacillus* bacteremia and no serious adverse effects in any patient. Nevertheless, due to the absence of any observed benefit to LGG treatment and concern regarding the safety of the treatment in the light of published reports of sepsis associated with LGG administration, the investigators terminated the study.

000104

Table 63. Studies of Other *L. rhamnosus* Strains in Children

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Isolauri et al. (1994)	Study the efficacy of LGG in assisting in the recovery of children suffering from acute rotavirus-induced diarrhea	Prospective, randomized, double-blind, placebo-controlled study	42 well-nourished children aged 5-28 months (mean = 14 months) who suffered from acute diarrhea	10^{10} cfu LGG twice a day	5 days	Urease activity during diarrhea transiently increased in the control group but not in the test group, while no differences were found in fecal levels of ß-glucuronidase, ß- glucosidase, or glycocholic acid hydrolase. There was no report regarding any adverse effects.
Kaila et al. (1992)	Study the efficacy of LGG in assisting in the recovery of children suffering from acute rotavirus diarrhea	Prospective, randomized, double-blind, placebo-controlled trial	39 well-nourished children aged 7-37 months (mean = 16 months) who had suffered from diarrhea for less than 7 days	10^{10} to 10^{11} cfu LGG/day	5 days	Those receiving LGG had enhanced non-specific humoral response during the acute phase of the infection; by the end of the follow-up period an IgA-specific antibody-secreting cell response to rotavirus was present in more test than control patients. No adverse events were reported by the authors.
Kaila et al. (1995)	Assess the effect of viable or heat-inactivated LGG on the immune response to rotavirus-induced acute diarrhea in children	Prospective, randomized, double-blind comparative study	41 well-nourished children aged 1-38 months (mean = 13 months) who experienced acute gastroenteritis of less than 7 days' duration	10^{10}-10^{11} cfu of either viable or heat-inactivated LGG twice daily	5 days	There were no significant differences in diarrhea recovery between the two groups. However, the viable LGG stimulated rotavirus-specific IgA antibody responses to a significantly greater extent than did the heat-inactivated cells. No safety-related endpoints were reported by the authors.

000105

Table 63. Studies of Other *L. rhamnosus* Strains in Children

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Majamaa et al. (1995)	Investigate the effect of LGG, another strain of *rhamnosus*, and other strains on the immune response of children with acute rotavirus-induced gastroenteritis	Prospective, randomized, double-blind, placebo-controlled study	49 children aged 6-35 months hospitalized for acute gastroenteritis of less than seven days duration	6.25×10^9 cfu LGG/day or 2.75×10^8 cfu/day of the other *L. rhamnosus* strain	5 days	Relative to the other groups, the LGG-treated group had enhanced IgA-specific antibody secreting cells to rotavirus and serum IgA antibody levels during the convalescent stage. No adverse effects were reported.
Piirainen et al. (2008)	Determine if the addition of GOS to LGG results in enhanced bifidogenesis in school-aged children	Randomized, double-blind, 2-period crossover study	30 healthy children (16 boys, 14 girls) aged 6.7 to 11.1 years (mean = 8.8 years)	6.5×10^9 cfu LGG/day	3 weeks with LGG alone, 3 weeks LGG with GOS	Both treatments significantly increased fecal counts of lactobacilli and L. rhamnosus, but only the LGG+GOS treatment significantly increase bifidobacteria. Neither treatment significantly affected defecation frequency, stool consistency, ease of defecation, or frequency or severity of gastrointestinal symptoms.

000106

Table 63. Studies of Other *L. rhamnosus* Strains in Children

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Raza et al. (1995)	Study the effect of LGG on the course of acute non-bloody diarrhea in malnourished hospitalized children	Prospective, randomized, double-blind, placebo-controlled study	40 children aged 1-24 months (mean = 13 months) who were hospitalized with acute diarrhea and dehydration	10^{10}-10^{11} cfu LGG twice a day	2 days	No safety-related endpoints were discussed. by the authors although they stated that the LGG treatment was well tolerated.
Rosenfeldt et al. 2002a	Assess the value of combined *L. rhamnosus* and *L. reuteri* treatment on acute diarrhea	Randomized placebo-controlled trial; measures of diarrhea severity	86 children age 6-36 months hospitalized with acute diarrhea with duration ≤7 days	10^{10} cfu 2x/day *L. rhamnosus* 19070-2 and *L. reuteri* DSM 122460	5 days	No safety-related endpoints were tested, but the authors did not report observing any adverse effects.
Rosenfeldt et al. 2002b	Assess the value of combined *L. rhamnosus* and *L. reuteri* treatment on mild diarrhea	Randomized placebo-controlled trial; measures of diarrhea severity	43 children age 9-44 months attending child-care centers and identified by their parents as having diarrhea	10^{10} cfu 2x/day *L. rhamnosus* 19070-2 and *L. reuteri* DSM 122460	5 days	The authors stated that "no serious adverse effects were registered."

000107

Table 63. Studies of Other *L. rhamnosus* Strains in Children

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Rosenfeldt et al. 2003	Assess the value of combined *L. rhamnosus* and *L. reuteri* treatment on atopic dermatitis	Randomized placebo-controlled crossover trial; measured production of cytokines	43 children age 1-13 years (mean age = 5.2 years) with atopic dermatitis	10^{10} cfu 2x/day *L. rhamnosus* 19070-2 and *L. reuteri* DSM 122460	6 weeks with 6-week washout	Although the severity of eczema was reduced, no significant differences were found in the production of cytokines IL-2, IL-4, IL-10, or IFN-γ. The authors did not report the occurrence of any adverse effects.
Rosenfeldt et al. 2004	Assess the value of combined *L. rhamnosus* and *L. reuteri* treatment on gastrointestinal symptoms and intestinal permeability in children with atopic dermatitis	Randomized placebo-controlled crossover trial; measured intestinal permeability	41 children age 1-13 years (median age = 4.0 years) with moderate or severe atopic dermatitis	10^{10} cfu 2x/day *L. rhamnosus* 19070-2 and *L. reuteri* DSM 122460	6 weeks with 6-week washout	Administration of the probiotic bacteria resulted in a significant reduction in the incidence of GI symptoms and significantly improved intestinal mucosal barrier function, but the investigators did not report on any other safety-related endpoints or events.
Ruszczynski et al. (2008)	Assess the safety, tolerability, and effectiveness of a combination of 3 strains of *L. rhamnosus* in preventing antibiotic-associated diarrhea in children	Prospective, randomized, double-blind, placebo-controlled trial	240 children, 130 boys and 110 girls, aged 3 months to 14 years, receiving antibiotic treatment for common infections	2×10^{10} cfu of a combination of *L. rhamnosus* strains E/N, Oxy, and Pen twice a day	Throughout antibiotic treatment (3-30 days, mean = 8 days)	Two control children and one child in the probiotic group required intravenous rehydration. No side effects or adverse effects were observed. The authors noted that, "*Lactobacillus rhamnosus* was well tolerated, and no adverse event associated with this therapy (or with the use of placebo) was reported."

000108

Table 63. Studies of Other *L. rhamnosus* Strains in Children

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Salazar-Lindo et al. (2004)	Study the effect of LGG on the duration and severity of non-rotavirus-induced acute diarrhea in young boys	Prospective, randomized, double-blinded, placebo-controlled study	179 male children aged 3-36 months with non-rotavirus-induced acute diarrhea	10^9 cfu LGG/ml in formula every 4 hours; estimated dose = 6-8 x 10^{11} cfu LGG/day	Until cessation of diarrhea or up to 5 days	8 boys from the LGG group and 11 from the control group were withdrawn from the study for a variety of non-treatment-related conditions The authors noted that, "No adverse effects due to the study formula were noticed in either group during the study."
Shornikova et al. (1997)	Investigate the effects of adding LGG to oral rehydration for treatment of acute diarrhea	Prospective, randomized, double-blinded, placebo-controlled study	123 children aged 1-36 months suffering from acute diarrhea of less than five days' duration upon hospital admission	5 x 10^9 cfu LGG twice a day along with oral or intravenous rehydration solution	5 days	Patients receiving LGG had a significantly shorter mean duration of diarrhea compared to those receiving placebo. No safety-related endpoints were discussed, but the authors reported that LGG was administered "without difficulties."
Szajewska et al. (2001)	Evaluate the efficacy of LGG in the prevention of nosocomial diarrhea in young children admitted to pediatric hospitals	Prospective, randomized, double-blind, placebo-controlled study	81 children aged 1-36 months (mean = 10.8 months) hospitalized for reasons other than diarrhea	6 x 10^9 cfu LGG twice a day	Duration of hospital stay (2-23 days; mean = 9.0 days)	Patients who received LGG had a reduced incidence of nosocomial diarrhea and gastroenteritis. Szajewska et al. (2001) reported that, "LGG was well tolerated, and no adverse effects of the treatment were noted."

000109

Table 63. Studies of Other *L. rhamnosus* Strains in Children

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose and Source	Duration	Safety-Related Results
Szymanski et al. (2006)	Study the efficacy of *L. rhamnosus* strains 573L/1, 573L/2, and 573L/3 in shortening the duration of acute infectious diarrhea in infants and children	Prospective, randomized, double-blind, placebo-controlled trial	87 children age 2 months to 6 years; (mean = 25.8 months); 45 males and 42 females with acute diarrhea	1.2×10^{10} cfu of a combination of the tested strains twice daily	5 days	The administered strains were recovered in the feces of 80% of the test-group children at 5 days and in 41% at 14 days. The authors reported that no adverse events of the probiotic treatment were noted during the study.
Vanderhoof et al. (1999)	Investigate the ability of LGG to reduce the incidence of antibiotic-induced diarrhea in children with acute infections of the respiratory tract, urinary tract, soft tissues, or skin	Prospective, randomized, placebo-controlled, double-blind trial	202 children aged 6 months to 10 years (median = 4 years) with acute infections of the respiratory tract, urinary tract, soft tissues, or skin	10^9 cfu/day for children weighing less than 12 kg and 2×10^9 cfu/day for those over 12 kg	10 days while receiving antibiotics	14 children failed to complete the study for a number of non-treatment-related causes. The authors noted that, "There were no failures [to complete the study] resulting from untoward effects of either LGG or placebo." Other than reduced incidence and duration of diarrhea, no differences were seen between the LGG and placebo groups in any parameter assessed and no adverse effects related to LGG consumption were noted by the authors.

000110

4.4.2.3. Studies in Infants

4.4.2.3.1. *Studies of* L. rhamnosus *HN001 in Infants*

In a multi-center, prospective, randomized, placebo-controlled, double-blind trial, Dekker et al. (2009) investigated the ability of *L. rhamnosus* HN001, as well as *Bifidobacterium animalis* ssp. *lactis* HN019, to reduce the risk of infant eczema and atopy[1]. A number of endpoints related to safety were assessed and these data were published separately from the report of the efficacy measures. A total of 512 pregnant women with a familial history of diagnosed asthma, hay fever, or eczema were enrolled and randomized to receive 6 x 10^9 cfu HN001/day (n = 170), 9 x 10^9 cfu HN019/day (n = 171), or placebo (n = 171) beginning at gestational week 35 and continuing for up to 6 months postnatally, while their infants received the same regimen from birth to 2 years of age. The inclusion criteria were at least 37 weeks gestation and treated asthma, hay fever, or eczema in at least one biological parent. Exclusion criteria were intention to move from the area within 2 years, infant birth weight under the 3rd percentile for sex and gestation, infant admission to a neonatal unit for more than 48 hours, serious congenital abnormalities, long-term probiotic use in mother or intention to treat the infant with probiotics, and the mother receiving less than 2 weeks of the treatments prior to giving birth.

The probiotics and placebo were provided in capsules, each of which provided one daily dose. Breastfeeding mothers continued to take capsules until postnatal month 6. Infants received one capsule per day starting at birth with the contents dissolved in 2 ml of breast milk or water and given orally via a syringe or teaspoon; after weaning, the babies received the contents of the capsule sprinkled on food. Each study participant received 7 visits from trained research nurses: mother's recruitment, infant's birth, and postnatal months 3, 6, 12, 18, and 24. The nurses completed standardized questionnaires that addressed illnesses, hospitalizations, medications including antibiotics, and gastrointestinal events (such as loose or watery stools, stooling patterns, reflux, vomiting, abdominal pain). Any serious adverse events were recorded and forwarded to the Wellington Ethics Committee for review. Head circumference was measured immediately after birth and at 3, 12, and 24 months; length and weight were measured after birth and at 12 and 24 months.

Seven mothers withdrew from the study prior to the birth of their babies, 2 from the HN001 group and 5 from the HN019 group; none of these withdrawals was due to adverse events. Thirty-one infants (11 in the HN001 group, 8 in the HN019 group, and 12 in the placebo group) did not meet the inclusion/exclusion criteria and were dropped from the study. Finally, during the 24-month intervention phase 13 mothers from the HN001 group, 6 from the HN019 group, and 9 from the placebo group withdrew from the study, most often due to moving from the area. There were no differences among the treatment groups in the incidence of or reasons for withdrawal.

At the end of the two-year period, there were 144 infants in the HN001 group, 152 in the HN019 group and 150 in the placebo group. There were no statistically-significant differences between the treatment groups in reasons for study withdrawal, incidence of adverse events, or

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[1] The study was approved by the Wellington Ethics Committee and the Auckland Ethics Committee.

antibiotic use over the 2-year feeding period, nor were any effects observed on height, weight, or head circumference. The incidence of reported adverse effects is shown below in Table 14. The authors concluded that *L. rhamnosus* HN001 and *B. animalis* ssp. *lactis* HN019 are safe and well-tolerated in an infant population fed from 0 to 24 months of age and has no effect on normal growth.

Table 14. Adverse Events Reported in Dekker et al. (2009).

	HN001 (n = 154) %	HN019 (n = 155) %	Placebo (n = 156) %
Diarrhea[1] after antibiotic therapy	31.8	36.1	32.1
Other diarrhea	76.0	76.1	75.0
Reflux or spilling	61.7	65.8	68.0
Abdominal pain	48.7	52.3	49.4
Vomiting	70.1	78.7	73.1
Source: Wickens et al. (2008)			

In a companion publication (Wickens et al. 2008), it was reported that *L. rhamnosus* strain HN001 significantly reduced the risk of eczema although it had no significant impact on atopy. *B. animalis* ssp. *lactis* HN019 had no significant effect on either atopy or eczema.

4.4.2.3.2. Studies of Other L. rhamnosus *Strains in Infants*

The studies discussed in this section are summarized in Table 15 at the end of the section.

LGG colonization of the intestinal tract of term neonates was investigated by Sepp et al. (1993) along with the effect of LGG on the intestinal microbiota. A total of 25 infants less than 1 week old were randomized to receive breast milk and/or formula with 10^{10}-10^{11} cfu freeze-dried LGG/g suspended in water (n = 15), or breast milk alone (n = 10) for 2 weeks. The meconium (first stool resulting from *in utero* nutrition) of 21 infants was collected, along with the feces of 15 infants on days 3-4, 17 infants on days 5-7, and 23 infants on days 28-32.

The microbiota content of the meconium was similar in the LGG and control-group infants, and did not include LGG. LGG was isolated from the feces of a majority of infants receiving LGG (and 1 control infant) during administration and from about half of them 2 weeks after administration ended. The feces of the infants receiving LGG had higher counts of total lactobacilli than did those of the controls, but the overall patterns of intestinal microbiota were similar both during and after the test period. The authors concluded that, "The study shows that 2 wk administration of Lactobacillus GG, which starts right after birth, increases intestinal lactobacilli concentrations and does not impair the establishment of normal fecal microbiota." No adverse effects were reported related to the administration of high doses of LGG to neonates.

Millar et al. (1993) evaluated the ability of LGG to colonize the immature bowel of premature infants, the effect of LGG colonization on the intestinal reservoir of nosocomial

[1] The term "diarrhea" was not defined clinically for adverse event data collected by interview and reported diarrhea includes such episodes as loose or watery stools.

pathogens such as enterobacteriaceae, enterococci, yeasts, or staphylococci, and effects on clinical progress and outcome. Twenty preterm infants with gestational ages of 33 weeks or less were studied from the initiation of feeding (term formula, preterm formula, or expressed breast milk) until discharge from the neonatal unit. Infants received either formula or milk alone (n = 10) or with 10^8 cfu LGG (n = 10) twice a day for 14 days. Some infants also received antibiotic treatment with cefotaxime or flucloxacillin and netilimicin. Fecal samples were collected daily and analyzed for viable LGG. (These samples were also evaluated for fermentation products as discussed in Stansbridge et al. [1993], reported below.) Daily records were kept of general well being; any abdominal distension, vomiting or regurgitation, feed intolerance, perineal rash; frequency and consistency of stools, number of suppositories used, and fluid intake. Other clinical variables evaluated included weight gain, energy intake, evidence of sepsis, antibiotic use or other concomitant medication, oxygen and ventilatory requirements, and the duration of hospital stay.

LGG was found in the feces of 9 of the 10 treated infants and 1 of the 10 infants in the control group. Although concentrations declined over time, 4 of the 7 infants for whom fecal samples were available 3 weeks after LGG administration ended still harbored detectable LGG in their feces. No significant differences were observed between treatment and control groups in either the numbers of nosocomial pathogens and anaerobes in the feces or the clinical parameters recorded: general well being, signs of abdominal distension, vomiting or regurgitation, feed intolerance, the incidence of perineal rash, the frequency and consistency of stools, the number of suppositories used, weight gain, or fluid intake. No adverse effects related to LGG were reported. There were no episodes of infection attributable to LGG.

Additional measures on the same preterm infant population as was reported in Millar et al. (1993), described above, were described in Stansbridge et al. (1993). Fecal samples were analyzed for SCFA, ethanol, and urinary 2,3-butanediol. Colonization with LGG had little impact on fecal SCFA; SCFA from treated infants did not differ significantly from controls from days 1-28. Ethanol excretion was significantly increased in the treated infants in both the proportion of samples testing positive (65% from infants fed LGG v. 37% from control infants) and in concentration in positive samples (median for LGG-fed infants = 6.3 μmol/g and for control infants = 3.3 μmol/g). This small increase in ethanol secretion was judged unlikely to have clinical significance.

Marini et al. (1997) fed 10 preterm neonates an oral dose of 10^9 cfu LGG for 15 days beginning on the first day of life while a control group of 5 infants of similar gestational age and weight received unsupplemented formula. Intestinal colonization of LGG was evaluated by enumerating the microbial composition of their feces in samples collected on days 0, 2, 5, 10, and 15. LGG was isolated at detectable levels in the feces of all treated infants, with peak levels at 5 days of initiation of LGG feeding. After 5 days, the concentration of LGG declined monotonically over the remaining 10 days of administration. The ratio of aerobic to anaerobic microorganisms in the feces of treated infants also decreased significantly relative to controls. The authors concluded that LGG quickly establishes temporary residence in preterm neonates, but this residence is transient even with continued administration. No adverse effects were reported.

In a randomized, prospective, double-blind, placebo-controlled study, Majamaa and Isolauri (1997) investigated the clinical and immunological effects of LGG in hydrolyzed whey formula on infants allergic to cow's milk and suffering from atopic eczema. Thirty-one infants aged 2.5 to 15.7 months who met the Hanifin criteria for atopic eczema received extensively hydrolyzed whey formula with or without $5x10^8$ cfu LGG/g formula for 1 month followed by the same whey formula without LGG for the following month. The infants then received a cow's milk challenge; only those with positive reactions to the cow's milk challenge (13 test infants and 14 controls) were included in the final study population. All infants were examined by a physician after 1 month of study participation and again after 2 months; blood and stool samples were collected at the beginning of the study and during both examinations.

Prior to treatment, the test and control groups did not differ significantly in the severity of atopic dermatitis (SCORAD = 26 in the test group and 21 in the controls). After the 1-month administration of LGG, the atopic dermatitis improved significantly in the LGG group as compared to the controls (SCORAD = 15 v. 19). However, there were no significant differences between the test and control groups 1 month after the end of LGG supplementation (SCORAD = 16 in the test group and 14 in the control group). The concentrations of α_1-antitrypsin and fecal tumor necrosis factor-α (TNF-α) decreased significantly in the LGG group but not in the controls. No adverse effects of the LGG administration were reported.

After 2 preterm infants died within 2 weeks in a neonatal intensive care unit from necrotizing enterocolitis caused by *Klebsiella oxytoca*, and *K. oxytoca* was isolated from 10 of the 20 neonates remaining in the unit, Grönlund et al. (1997) began to provide all neonates entering or already present in the NICU (number not reported) twice-daily doses of $2.5x10^8$ cfu LGG for two weeks or until discharge. The mean duration of LGG supplementation was 7.8 days. Fecal samples were taken both before and after LGG supplementation, and the NICU colonization rate of *K. oxytoca* was assessed at 1, 2.5, 6, and 7 months after the supplementation. Two of the 30 infants already in the NICU at the beginning of the study but no infants entering the NICU tested positive for *K. oxytoca* prior to LGG supplementation. After supplementation, LGG was detected in 82% of the fecal samples and no new clinical infections caused by *K. oxytoca* were observed, nor were there any infections caused by LGG. No adverse effects were reported.

Biadaioli et al. (1998) carried out a randomized, double-blind, placebo-controlled, multi-center center study with premature infants in neonatal intensive care units to assess the ability of LGG to reduce the enteric infections commonly found in premature neonates receiving antibiotic treatment. Infants less than 33 weeks gestational age or weighing less than 1500 grams were eligible; 224 infants were enrolled and randomly assigned to the treatment group (n = 119) or the control group (n = 105). Neonates in the treatment group were given 10^9 cfu LGG/day in their food from NICU admittance to discharge, an average of 49.7 days. Control group neonates received a placebo in place of LGG.

No statistically significant differences were found in the number of infections observed in the test and treatment groups—the incidence was 18.4% in the test group and 15.2% among the controls. The authors stated that "there were no negative clinical effects of a relevant nature to be reported."

In a randomized, prospective, double-blind, placebo-controlled study (Oberhelman et al. 1999), modertely undernourished Peruvian infants aged 6 to 24 months (mean = 14.3 months) received either $3.7x10^{10}$ cfu LGG/day (n = 99) or placebo (n = 105) in flavored gelatin 6 days/week for up to 15 months. Incidences of diarrhea were recorded. A subset of 40 infants from the 2 groups provided stool specimens 3, 6, and 13 months after the beginning of treatment; samples were analyzed for LGG and pathogens. A total of 954 episodes of diarrhea were reported over the 15 months of the study, 490 in the LGG group and 464 among the controls; these rates of total incidence were not significantly different. However, the individual diarrhea frequencies were significantly lower in the LGG than in the control group, 5.21 v. 6.02 episodes/infant/year. In 58.2% of the episodes among the 40 infants for whom stool samples were available, a pathogen was isolated; enterotoxigenic *E. coli* in 96 cases, *Campylobacter jejuni* in 33 cases, rotavirus in 26 cases, and *Shigella* spp. in 16 cases. No differences in the duration of diarrhea were observed and no adverse effects were reported.

A prospective, randomized, double-blind, placebo-controlled study designed to assess the ability of LGG or *Bifidobacterium animalis* ssp. *lactis* strain Bb12 to control allergic inflammation in infants with atopic eczema was conducted by Isolauri et al. (2000). Infants with a mean age of 4.6 months who had developed atopic eczema during breast feeding and had not been exposed to infant formula prior to enrollment were randomly assigned (n = 9 per group) to extensively hydrolyzed whey formulas with or without supplementation with $3x10^8$ cfu LGG or 10^9 cfu Bb12/gram of formula for at least 6 months. The exposure to the 2 probiotics was about 3 to $8x10^{10}$ cfu/day. The severity of the atopic eczema was assessed prior to introduction of the whey formulas and at 2 and 6 months, and body measurements and samples of stool and blood were taken at the same times. The primary endpoints were the severity of atopic eczema and the growth of the infants. The mean SCORAD at the beginning of the study was 16, and the infants given LLG or Bb12 showed significant improvement as compared to the starting levels and to the controls; after 2 months of feeding the LGG group's mean SCORAD was 1.0 and that of the Bb12 group was 0.0, the mean SCORAD of the controls was 13.4. Soluble CD4 in blood and eosinophilic protein X in urine were also significantly reduced in the probiotic groups. There was no effect on growth, which was normal in all infants studied. No adverse events were reported.

Kalliomaki et al. (2001) conducted a prospective, randomized, double-blind, placebo-controlled study in which capsules containing 10^{10} cfu LGG or a placebo were given once a day over from 2 to 3 weeks to 159 pregnant women with a family history of atopic eczema, allergic rhinitis, or asthma. After delivery, breastfeeding mothers continued to ingest the capsules for 6 months while formula-fed infants were given the same dose of LGG or placebo orally for 6 months. The infants were examined shortly after birth and at ages 3, 6, 12, 18, and 24 months, including inspection of eyes, ears, nose, and skin, auscultation of heart and lungs, palpitation of the abdomen, and measurement of growth and neurological development. Skin-prick tests were done at 6, 12, and 24 months, and antigen-specific IgE assays were performed in umbilical cord blood and at ages 3, 12, and 24 months. The primary endpoint of interest was atopic disease at age 2 years. A total of 132 infants completed the study. At 2 years of age, the incidence of atopic eczema in the LGG-treated group was only 23% (15 of 64 infants), significantly lower than in the control group (46%; 31 of 68 infants). However, the severities of atopic eczema (as measured by SCORAD) among those with the disease did not differ between the LGG and control groups. Similarly, no differences were seen in concentrations of IgE or in frequencies of

increased antigen-specific IgE concentrations or positive reactions to skin-prick tests. There were no differences between infants who received LGG by oral administration and the breastfeeding infants whose mothers consumed the capsules. No study withdrawals were due to adverse effects and there were no reports of any adverse events.

In a follow-up of these infants when they reached the age of 4 years, Kalliomaki et al. (2003) found that the incidence of atopic eczema in the LGG group continued to be significantly lower than in the placebo group, and no adverse effects had appeared. The authors concluded that, "the probiotic approach was promising and safe."

In a prospective, randomized, double-blind, placebo-controlled, multi-center trial, Dani et al. (2002) assessed the effect of LGG on the incidence of urinary tract infections, bacterial sepsis, and necrotizing enterocolitis among preterm infants confined to an NICU. All infants were less than 33 weeks in gestational age or weighed under 1500 grams, and most of them had received at least one course of antibiotic therapy. Infants in the test group (n = 295) received standard milk providing $6x10^9$ cfu LGG/day while the control-group infants (n = 290) received the standard milk alone. The study continued until discharge from the NICU, an average period of 47.3 days. The LGG treatment significantly reduced the incidence of urinary tract infections and necrotizing enterocolitis, but there was no statistically significant effect on bacterial sepsis. No adverse effects were reported due to the LGG treatment.

In an evaluation of the ability of LGG to colonize the gut and modify the microbial ecology of low-birth-weight preterm infants (Agarwal et al. 2003), 71 preterm infants with birth weights under 2000 g received expressed breast milk either unsupplemented or supplemented with 10^9 cfu LGG twice a day for either 8 or 21 days. The study subjects included 39 infants with birth weights under 1500 g (24 in the LGG group and 15 controls) and 32 infants with birth weights between 1500 and 2000 g (23 in the LGG group and 9 controls). Stool samples were collected before treatment and on day 7 or 8 and, for the infants under 1500 g, on days 14 and 21. Data were also collected on any adverse effects.

LGG was detected in the stool samples from 5 of the LGG infants who weighed less than 1500 g (21%) and 11 of those weighing 1500 – 2000 g (47%), but not in stool samples from any of the controls. The stools of infants weighing less than 1500 g who received LGG showed an increase in microbial species, especially Gram-positive bacteria and anaerobes, but a similar effect was not found in the 1500 – 2000 g LGG group nor in the controls of either weight range. The authors noted that LGG was well tolerated in all infants and that, "No side effects were observed in babies either fed with or colonized by LGG."

Kankaanpää et al. (2002) performed a pilot study for a randomized, double-blind, placebo-controlled study to investigate the effect of LGG and *Bifidobacterium animalis* ssp. *lactis* strain Bb12 on the composition of plasma lipids in atopic infants. Fifteen breastfed infants (mean age 5.2 months) referred to a pediatric clinic for treatment of atopic eczema were given an extensively hydrolyzed infant formula alone or supplemented with either $3x10^8$ cfu LGG or 10^9 cfu Bb12/g formula. The average duration of feeding was 5.5 months. Blood samples were taken at outset and 2 months later. The average formula intake was 75 ml/kg bw/day, thus providing daily doses of $2.25x10^{10}$ cfu LGG/kg bw or $7.5x10^{10}$ cfu Bb12/kg bw, approximately equivalent

to 1.8×10^{11} cfu LGG or 6×10^{11} cfu Bb12 per infant. LGG (and Bb12) administration reduced the proportion of α-linolenic acid in plasma neutral lipids, but LGG had no effect on phospholipids. The authors noted that all formulas were well tolerated by the infants, and no adverse effects were reported.

Petschow et al. (2003) investigated dose-response relationships between LGG administration and intestinal colonization in healthy full-term infants. Forty-nine infants under the age of 3 months were fed Nutramigen®, a hydrolyzed casein-based infant formula, for 14 days, then randomly assigned to received the same formula supplemented with 0 (n = 12), 10^8 (n = 12), 10^9(n = 13), or 10^{10} (n = 12) cfu LGG/day for 14 days, followed by a 14-day washout period during which they received unsupplemented formula. Tolerance measures and stool characteristics were recorded daily and viable LGG were enumerated in stool samples obtained on days 3, 6, 9, 12, 15, 21, and 28. LGG was detected in significantly higher percentages of infants fed LGG than infants in the control group (67% of the 10^8 group, 85% of the 10^9 group, 83% of the 10^{10} group, and 17% of the controls), although there was no clear dose-response relationship. The authors concluded that, "Feeding LGG at 10^8 to 10^{10} cfu/day was well tolerated and led to transient colonization in healthy term infants. LGG colonization was apparently not related to level of LGG administered and tended to decrease 2 weeks after feeding supplemented formula." Stool consistency, flatulence, and fussiness were similar in all groups and no adverse effects were reported.

A randomized, double-blind, placebo-controlled study was performed to evaluate the effects of LGG alone or in combination with other probiotics on treatment of cow's milk allergy and IgE-associated dermatitis in infants (Pohjavuori et al. 2004). After beginning a cows-milk-free diet and treatment for dermatitis, 230 infants (mean age = 6.5 months) were randomized to receive extensively hydrolyzed whey formula containing 5×10^9 cfu LGG; a combination of 5×10^9 cfu LGG, 5×10^9 cfu *L. rhamnosus* LC705, 2×10^8 cfu *Bifidobacterium breve* Bbi99, and 2×10^9 cfu *Propionibacterium freudenreichii* ssp. *shermanii* JS; or placebo twice a day for 4 weeks, followed by 4 weeks during which unsupplemented formula was fed to the infants. Clinical improvement was evaluated prior to probiotic administration, after the 4-week treatment period, and after the 4-week washout period. At the end of the washout period, concentrations of serum cow's milk and wheat-specific IgE were measured and a double-blind, placebo-controlled, crossover cow's milk challenge was performed. A total of 119 infants were studied out of the 230 infants with atopic dermatitis and suspected cow's milk allergy who completed the feeding phase.

The cow's milk challenge was negative in 54 of the 119 infants, but cow's milk allergy was diagnosed in the remaining 65 infants. IgE-associated cow's milk allergy was diagnosed in 42 infants and non-IgE-associated cow's milk allergy in 32 infants. IgE-associated dermatitis was diagnosed in 72 infants. Secretion of interferon- γ (IFN-γ) before the probiotic treatment was significantly lower in infants with cow's milk allergy than in those without, but LGG feeding increased the level of secreted IFN-γ in those infants with cow's milk allergy or IgE-associated dermatitis. Consumption of the probiotic combination significantly increased secretion of IL-4 in infants with cow's milk allergy, but LGG alone had no effect on IL-4 secretion. No safety-related effects were reported.

Viljanen et al. (2005) investigated the effects on treatment of atopic eczema of LGG alone or as part of a combination of 4 probiotics in food-allergic infants. After cow's milk was eliminated from the diets of 230 infants under 12 months old (mean age = 6.4 months) with atopic eczema, the infants were randomly assigned to receive 2 doses a day of extensively hydrolyzed whey formula supplemented with 5 x 10^9 cfu LGG; a combination of 5 x 10^9 cfu LGG, 5 x 10^9 cfu *L. rhamnosus* LC705, 2 x 10^8 cfu *Bifidobacterium breve* Bbi99, and 2 x 10^9 cfu *Propionibacterium freudenreichii* ssp. *shermanii* JS; or placebo for 4 weeks, followed by a 4-week washout period. Eczematous lesions were treated with hydrocortisone as needed during the study period. Clinical signs were evaluated prior to administration of probiotics, at the end of the 4-week treatment period, and after the 4-week washout. Parents completed a daily diary in which they were asked to record any skin, gastrointestinal, or respiratory system symptoms. Following the washout period, a double-blind, placebo-controlled, crossover cow's milk challenge was performed.

There were no significant differences between the placebo group or either treatment group in reduction of the severity of atopic eczema (mean SCORADs decreased by 65% in all groups) except among IgE-sensitized infants, for whom the LGG treatment reduced SCORADs significantly as compared to the control infants. There were no specific measures of safety-related endpoints, but no adverse effects were reported.

Romeo et al. (2006) studied the ability of the probiotics LGG and *L. reuteri* (strain not reported) to reduce the incidence of bacterial infections and *Candida* in neonatal intensive care. In a randomized, prospective, double-blind, placebo-controlled trial, 184 newborns with average gestational age 34.9 weeks admitted to a NICU (107 boys, 77 girls) were randomly assigned to receive 3x10^9 cfu LGG/day (n = 55), 10^8 cfu *L. reuteri*/day (n = 67), or placebo (n = 62). The probiotics were delivered dispersed in water or milk once a day via mouth or nasogastric tube. Most infants had been delivered by Caesarean section and more than half of them had central venous catheters. They remained on treatment throughout their residence in the NICU, an average of 10 days.

Infants receiving the probiotics had significantly reduced incidence of *Candida* and of bacterial infections, as well as shortened duration of required parenteral nutrition and of total hospital stay. The probiotic-treated infants exhibited higher rates of growth than did the control group. No adverse effects were noted due to the treatment with either *L. reuteri* or LGG.

In a randomized, prospective, double-blind, placebo-controlled trial, Brouwer et al. (2006) evaluated the clinical and immunological effects of LGG or a different (unspecified) strain of *L. rhamnosus*. Fifty infants under 5 months of age who met the Hanafin criteria for atopic dermatitis were given extensively hydrolyzed whey formula (Nutrilon Pepti) during 3-5-week baseline period and then randomly assigned to receive hydrolyzed formula supplemented with 5 x 10^9 cfu LGG (n = 16) or *L. rhamnosus* (n = 17) per 100 ml formula or to receive unsupplemented formula (n = 17) for 3 months. Atopic dermatitis was clinically evaluated both before and after the baseline period and after each month of consumption of study formula. Allergic sensitization was measured by total IgE, a battery of food-specific IgE, and a skin-prick assay of cow's milk. Inflammatory measures included were blood eosinophils; production of IL-4, IL-5, and IFN-γ by peripheral blood mononuclear cells after polyclonal stimulation; eosinophil

protein X in urine; and fecal α-1-antitrypsin. Infants who showed improvement were given a cow's milk formula challenge, and those who showed a reaction to the cow's milk were given a randomized, double-blind, placebo-controlled cow's milk challenge for 2-7 days.

No statistically significant differences between the groups were observed for SCORAD, sensitization, inflammatory parameters or cytokine production. No adverse events were reported.

Vendt et al. (2006) performed a prospective, randomized, double-blind, placebo-controlled trial to study the effect of LGG on the growth and fecal microbiota of healthy Estonian infants. Sixty boys and 60 girls aged 0-2 months (average age 39.8 days) were randomly assigned to receive cows-milk-based formula supplemented with 10^7 cfu LGG/g (n = 60) or unsupplemented formula (n = 60) up to the age of 6 months. Breast-fed infants (over 50% of feedings) were excluded. The infants were examined monthly and any gastrointestinal or respiratory symptoms were noted; mothers provided monthly impressions of their infant's fecal consistency; and a general health questionnaire was completed at the end of the study.

The mean daily intake of formula increased during the study from 623 ml at the beginning and 803 ml at 3 months to 933 ml at 6 months; thus, the daily dose of LGG increased from 8.1×10^8 to 1.2×10^9 cfu. After removal of breast-fed infants and discontinuations, only 105 infants remained in the study at completion, 51 in the LGG group and 54 controls. The reasons for discontinuation were similar in the LGG and control groups. There were no differences between the groups in total duration of crying or in infectious episodes. The frequency of bowel movements was significantly higher in the LGG group than in the controls and the stools were significantly looser.

The authors concluded that the LGG-enriched formula was well-tolerated, and no adverse effects were reported. Infants receiving LGG formula showed significantly greater increases in length and weight at the end of study compared to infants receiving regular formula, but this might have been due to the fact that the infants receiving LGG had been slightly smaller at the beginning of the study than the control infants. Vendt et al. (2006) concluded, "The results of this randomized, controlled trial suggest that LGG-enriched formula was safe and well tolerated and resulted in normal growth."

In a prospective, randomized, double-blind, placebo-controlled, multi-center trial (Chouraqui et al. 2008), healthy full-term infants were exclusively fed one of 3 test formulas or a control formula from less than 2 weeks of age until they reached the age of at least 16 weeks; each infant was thus treated for 14 to 16 weeks. The test formulas contained (1) 6.4×10^7 cfu/ml of *L. rhamnosus* strain LPR + 1.3×10^7 cfu/ml of *Bifidobacterium longum* strain BL888, or (2) test formula 1 + 4 mg/ml of oligosaccharides (90% galactooligosaccharide and 10% fructooligosaccharide) or (3) 2.6×10^7 cfu/ml *L. paracasei* strain ST11 + 2.6×10^7 cfu/ml *B. longum* strain BL888 + 4 mg/ml of oligosaccharide. Parents kept records of the infants' formula consumption, any supplementary foods, and any medication. Parent also recorded stool characteristics, frequency of flatulence, and behavior for 3 days before and after each visit, which occurred at approximately 2, 5, 8, 12, 16, and 52 weeks of age. At each visit anthropomorphic measures were taken and any incidents of morbidity were assessed.

The average daily consumption of formula in all groups was about 700 ml, and thus the dose of *L. rhamnosus* was equivalent to approximately 4.5 x 10^{10} cfu/day. A total of 284 infants were enrolled and 70 were randomly assigned to each group with 74 assigned to experimental formula 3. Seventeen infants withdrew or were lost to follow-up in the control group, 10 in test group 1, 16 in test group 2, and 14 in test group 3. The 227 infants included in the data analysis consisted of 110 boys and 117 girls with mean birth weights of 3.4 kg in each of the 4 groups. The reasons for withdrawal were not related to treatment. Infants in all 4 groups consumed similar amounts of formula and there were no significant differences in weight gain, length, head circumference, or BMI. There were no differences among the groups in the incidence of diarrhea, frequency of antibiotic treatment, or hospitalization. Serious adverse events were reported in 24 infants and lesser adverse events (mostly respiratory and GI problems and infections) in 184 infants, but there were no differences in frequencies among the different groups, and the adverse events were not regarded as study-product related. The authors concluded that, "this study confirms the safety of different mixtures of probiotics and synbiotics."

Kopp et al. (2008) conduced a randomized, prospective, double-blind, placebo-controlled trial to study the preventive effect of LGG on the development of atopic dermatitis, which was the primary endpoint of the study. Pregnant women (n = 105) from families with one or more members with an atopic disease were randomly assigned to receive capsules containing either 5 x 10^9 cfu LGG (n = 54) or placebo (n = 51) twice a day for the 4-6 weeks remaining before delivery. Breastfeeding women continued to take the capsules postnatally for 3 months, after which the contents of the capsules were given directly to the infants for 3 additional months. Non-breastfeeding infants (2 in the LGG group and 3 in the control group) received the capsule contents for the full 6 postnatal months. Parents completed questionnaires prenatally and a postnatal months 6, 12, and 24. Infants with suspected eczema were examined at 12 months and all infants were examined at 24 months. In addition to a general physical examination, the children were evaluated for the presence and severity of atopic dermatitis, incidence of recurrent wheezing bronchitis, total immunoglobulin E (IgE), and sensitization to an inhalent allergen.

Ninety-four women and their children completed the study, 50 in the LGG group and 44 in the placebo group. The children included 42 boys and 52 girls with a mean birthweight of 3440 g. There were no significant differences in drop-out rates or in the reasons for withdrawal. No significant differences were reported between the groups in sex, birth weight, gestational age, of method of delivery of the infants. The incidence of atopic dermatitis at age 2 was nearly identical in the 2 groups, and no significant difference was reported in the cumulative incidence of atopic symptoms during the first 2 years or in the severity of atopic dermatitis at age 2. No differences were reported in episodes of fever, antibiotic use, allergen sensitization, or IgE concentrations. One secondary endpoint, the incidence of wheezing bronchitis, was significantly ($p = 0.03$) more frequent in the LGG group than in the placebo group. Despite this finding, the authors reported that, "The administration of LGG was well tolerated without any notable adverse effects attributable to the supplementation of probiotics."

Kukkonen et al. (2008) conducted a prospective, randomized, double-blind, placebo-controlled trial of the safety and impact on infection rates of feeding synbiotics to infants. The synbiotic tested was a combination of 4 probiotic species (LGG, *L. rhamnosus* LC705, *Bifidobacterium breve* Bb99, and *Propionibacterium freudenreichii* ssp. *shermanii*) along with

galactooligosaccharides (GOS). Pregnant women (n = 1223) carrying infants at high risk for allergy were randomly assigned to ingest capsules containing either 8-9 x 10^9 cfu of the 4 probiotic strains or a placebo (microcrystalline cellulose + sugar syrup) twice a day for the last 4 weeks before delivery. For the first 6 months after birth their 1018 eligible infants (504 boys, 514 girls) received 1 capsule a day equivalent to the capsules their mothers had received; those infants receiving the probiotics also received 0.8 g GOS/day. The parents were instructed to mix the contents of the capsule and the GOS with breast milk or formula and spoon-feed it to the infants. Infants were regarded as eligible unless they were preterm (less than 37 weeks gestation), a B twin, or had a major malformation. The parents were interviewed and the infants examined at ages 3, 6, and 24 months, while the parents completed questionnaires at 3, 6, 12, and 24 months covering the previous period. Interviews and questionnaires covered neonatal morbidity, feeding behaviors, nutrition, environment, and numbers of infections, antibiotic use, and other diseases.

Of the 1018 intention-to-treat infants, 939 (468 synbiotic group, 471 placebo group) completed the 6-month follow-up evaluation and 925 (461 synbiotic group, 464 placebo group) completed the 2-year follow-up evaluation. There were no differences in parent-reported neonatal morbidity or in reasons for withdrawal between the synbiotic and placebo groups, nor in the incidence of infantile colic. Vomiting, constipation, excessive crying, diarrhea, and abdominal discomfort occurred with equal frequencies in the 2 groups. There were no differences between groups in the incidence of respiratory or middle-ear infections or in gastroenteritis during the intervention, although the infants receiving synbiotics significantly less often required antibiotic therapy. During the follow-up period, the infants that had received synbiotics had a significantly lower incidence of respiratory infections. Growth measures (weight, length, and head circumference) at 6 and 24 months were similar in the test and control group infants. The authors stated that, "We showed that treatment of mothers with probiotics during late pregnancy and treatment of their healthy allergy-prone infants with synbiotics for 6 months after birth were safe."

4.4.2.3.3. Conclusions from Studies of L. rhamnosus *in Infants*

A total of 23 trials have been reported in the literature in which one or more strains of *L. rhamnosus* were administered to infants. In most, although not all, of these studies, feeding of the probiotic strain commenced immediately after birth; indeed, in several studies ingestion of the probiotic by the neonate followed previous ingestion by the mother during the later stages of her pregnancy. The single study of strain HN001 was of this type: each mother in the HN001 group ingested 6x10^9 cfu *L. rhamnosus*/day and then the infant consumed the same dose daily from birth through age 2 years. Confirming the status of *L. rhamnosus* strain GG (LGG) as the most widely studied probiotic, 21 of the 22 reported trials included LGG in at least one arm of the study. Two studies were of other strains (including HN001) and 4 studies included *L. rhamnosus* strains in addition to LGG.

The daily doses of probiotic in these studies were at or above 10^{10} cfu/day in 8 studies and above 10^{11} cfu/day in 1 study. The 2-year study of HN001 was the longest duration, but 5 other studies had durations of 6 months or longer and 4 more lasted for 3 months or longer. The participants in the studies included healthy full-term infants (8 studies), premature infants (7 studies), and infants with health issues ranging from mild to life-threatening (9 studies). In all, about 4,000 infants were enrolled in these studies.

With the exception of a single finding of increased incidence of recurrent wheezing bronchitis among infants at risk of atopic dermatitis receiving LGG (Kopp et al. 2008), in no case was there any indication of any adverse effect due to the administration of *L. rhamnosus* to the infant. Reasons for withdrawal from the studies did not differ between test and control groups, nor did the incidence, character, or severity of adverse effects (except where adverse effects were lower in the probiotic group than in the test group). Notably, no a single case of *Lactobacillus* bacteremia was observed, even in severely compromised infants with central lines and histories of surgical interventions.

The evidence from this large body of data supports the conclusion that the tested strains of *L. rhamnosus* are safe for administration to term and preterm neonates, as well as older infants, under the conditions of the studies.

Table 15. Studies of Other *L. rhamnosus* Strains in Infants

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose	Duration	Safety-Related Results
Agarwal et al. (2003)	Evaluate the ability of LGG to colonize the gut and modify the microbial ecology of low-birth-weight preterm infants	Randomized, double-blind, placebo-controlled study	71 preterm infants with birth weights under 2000 g (39 with birth weights under 1500 g)	10^9 cfu LGG twice a day	8 or 21 days	The stools of infants weighing less than 1500 g who received LGG showed an increase in microbial species, especially gram-positive bacteria and anaerobes, but a similar effect was not found in the 1500 – 2000 g LGG group nor in the controls of either weight range. The authors noted that LGG was well tolerated in all infants and that, "No side effects were observed in babies either fed with or colonized by LGG."
Biadaioli et al. (1998)	Assess the ability of LGG to reduce enteric infections in premature neonates receiving antibiotic treatment	Randomized, double-blind, placebo-controlled, multi-center study	224 infants less than 33 weeks gestational age or weighing under 1500 g and confined to a NICU	10^9 cfu LGG/day	Until discharge; mean = 49.7 days	No statistically significant differences were found in the number of infections observed in the test and treatment groups. The authors stated that "there were no negative clinical effects of a relevant nature to be reported."
Brouwer et al. (2006)	Evaluated the clinical and immunological effects of LGG or a different (unspecified) strain of *L. rhamnosus* .on infants with atopic dermatitis	Randomized, prospective, double-blind, placebo-controlled trial	50 infants under 5 months of age with atopic dermatitis	5×10^9 cfu LGG or *L. rhamnosus*/ 100 ml formula, approximately equivalent to 3×10^{10} cfu LGG/day	3 months	No statistically significant differences between the groups were observed on SCORAD, sensitization, inflammatory parameters or cytokine production. No adverse events were reported.

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Table 15. Studies of Other *L. rhamnosus* Strains in Infants

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose	Duration	Safety-Related Results
Chouraqui et al. (2008)	Assess the safety and tolerance of mixtures of probiotics and prebiotics and their protective effect against infant diarrhea	Prospective, randomized, double-blind, placebo-controlled, multi-center trial	227 healthy full-term infants (110 boys and 117 girls) less than 2 weeks old at enrollment	6.45×10^{10} cfu/l of *L. rhamnosus* strain LPR, equivalent to approximately 4.5×10^{10} cfu/day	14 to 16 weeks	Infants in all 4 groups exhibited no significant differences in weight gain, length, head circumference, or BMI. There were no differences among the groups in the incidence of diarrhea, frequency of antibiotic treatment, or hospitalization. There were no differences in frequencies of adverse events among the different groups, and the adverse events were not regarded as study-product related. The authors concluded that, "this study confirms the safety of different mixtures of probiotics and synbiotics."
Dani et al. (2002)	Assessed the effect of LGG on the incidence of urinary tract infections, bacterial sepsis, and necrotizing enterocolitis among preterm infants confined to an NICU	Prospective, randomized, double-blind, placebo-controlled, multi-center trial	585 infants less than 33 weeks gestational age or weighing under 1500 g; most had received antibiotic therapy	6×10^{9} cfu LGG/day	Until discharge from the NICU, mean = 47.3 days	The LGG treatment significantly reduced the incidence of urinary tract infections and necrotizing enterocolitis, but there was no statistically significant effect on bacterial sepsis. No adverse effects were reported due to the LGG treatment.
Grönlund et al. (1997)	Test the ability of LGG to reduce the risk of infection by *Klebsiella oxytoca* among infants in a neonatal ICU	Open-label study, no control group	All neonates entering or already present in the NICU (number not reported)	2.5×10^{8} cfu LGG twice a day	Up to 2 weeks; mean 7.8 days	No clinical infections caused by *K. oxytoca* were observed, nor were there any infections caused by LGG. No adverse effects were reported.

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Table 15. Studies of Other *L. rhamnosus* Strains in Infants

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose	Duration	Safety-Related Results
Isolauri et al. (2000)	Assess the ability of LGG or *Bifido-bacterium animalis* ssp. *lactis* strain Bb12 to control allergic inflammation in infants with atopic eczema	Prospective, randomized, double-blind, placebo-controlled study	27 infants with a mean age of 4.6 months who had developed atopic eczema during breast feeding and had not been exposed to infant formula	$3x10^8$ cfu LGG/g formula, approximately equivalent to $1.8x10^{11}$ cfu LGG/day	6 months or longer	SCORAD, soluble CD4 in blood, and eosinophilic protein X in urine were significantly reduced in the probiotic groups. There was no effect on growth, which was normal in all infants in all groups. No adverse events were reported.
Kalliomäki et al. (2001)	Study the effect of LGG administration to mothers and infants on the development of atopic eczema at age 2 years	Prospective, randomized, double-blind, placebo-controlled study	159 pregnant women with a family history of atopic eczema, allergic rhinitis, or asthma and their infants	10^{10} cfu LGG/day	2 to 3 weeks to mothers prenatally and 6 months to infants	At 2 years of age, the incidence of atopic eczema in the LGG-treated group was significantly lower than in the control group. No differences were seen in concentrations of IgE or in frequencies of increased antigen-specific IgE concentrations or positive reactions to skin-prick tests. No study withdrawals were due to adverse effects and there were no reports of any adverse events.
Kankaanpää et al. (2002)	Investigate the effect of LGG and *Bifido-bacterium animalis* ssp. *lactis* strain Bb12 on the composition of plasma lipids in atopic infants	Pilot for a randomized, double-blind, placebo-controlled study	15 breastfed infants (mean age = 5.2 months) referred to a pediatric clinic for treatment of atopic eczema	$3x10^8$ cfu LGG/g formula, approximately equivalent to $1.8x10^{11}$ cfu LGG/day or $2.25x10^{10}$ cfu LGG/kg bw/day	average 5.5 months	LGG had no effect on phospholipids. The authors noted that all formulas were well tolerated by the infants, and no adverse effects were reported.

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Table 15. Studies of Other *L. rhamnosus* Strains in Infants

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose	Duration	Safety-Related Results
Kopp et al. (2008)	Study the preventive effect of LGG administration to mother and infant on the development of atopic dermatitis	Randomized, prospective, double-blind, placebo-controlled trial	105 pregnant women and their infants; 94 mothers and infants completed the study	5×10^9 cfu LGG twice a day	4-6 weeks prenatally and 6 months postnatally	One secondary endpoint, the incidence of wheezing bronchitis, was significantly ($p = 0.03$) more frequent in the LGG group than in the placebo group. Despite this finding, the authors reported that, "The administration of LGG was well tolerated without any notable adverse effects attributable to the supplementation of probiotics."
Kukkonen et al. (2008)	Evaluate the safety and impact on infection rates of feeding synbiotics to healthy full-term infants at risk of allergy	Prospective, randomized, double-blind, placebo-controlled trial.	1018 healthy full-term infants (504 boys, 514 girls)	$8\text{-}9 \times 10^9$ cfu of a mixture of LGG, *L. rhamnosus* LC705, *B. breve* Bb99, and *Propioni-bacterium freudenreichii* ssp. *shermanii* along with galactooligo-saccharides	6 months (following 4-week dosing of their pregnant mothers at double the infant dosage)	There were no differences in parent-reported neonatal morbidity or in reasons for withdrawal between the synbiotic and placebo groups, nor in the incidence of infantile colic. Vomiting, constipation, excessive crying, diarrhea, and abdominal discomfort occurred with equal frequencies in the 2 groups. There were no differences between groups in the incidence of respiratory or middle-ear infections or in gastroenteritis during the intervention, although the infants receiving synbiotics significantly less often required antibiotic therapy. During the follow-up period, the infants that had received synbiotics had a significantly lower incidence of respiratory infections. Growth measures (weight, length, and head circumference) at 6 and 24 months were similar in the test and control group infants. The authors stated that, "We showed that treatment of mothers with probiotics during late pregnancy and treatment of their healthy allergy-prone infants with synbiotics for 6 months after birth were safe."

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Table 15. Studies of Other *L. rhamnosus* Strains in Infants

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose	Duration	Safety-Related Results
Majamaa and Isolauri (1997)	Investigate the clinical and immunological effects of LGG in hydrolyzed whey formula on infants allergic to cow's milk and suffering from atopic eczema	Randomized, prospective, double-blind, placebo-controlled study	31 infants aged 2.5 to 15.7 months who met the Hanifin criteria for atopic eczema	$5x10^8$ cfu LGG/g formula, approximately equivalent to $3x10^{11}$ cfu LGG/day	1 month	The concentrations of α_1-antitrypsin and fecal tumor necrosis factor-α (TNF-α) decreased significantly in the LGG group but not in the controls. No adverse effects of the LGG administration were reported.
Marini et al. (1997)	Study intestinal colonization of LGG among healthy neonates	Prospective, randomized, double-blind, placebo-controlled study	10 preterm neonates	10^9 cfu LGG/day	15 days	The authors concluded that LGG quickly establishes temporary residence in preterm neonates, but this residence is transient even with continued administration. No adverse effects were reported.
Millar et al. (1993)	Evaluate the ability of LGG to colonize the immature bowel of premature infants, the effect of LGG colonization on the intestinal reservoir of nosocomial pathogens, and effects on clinical progress and outcome	Prospective, randomized, double-blind, placebo-controlled study	20 preterm infants with gestational ages of 33 weeks or less	10^8 cfu LGG twice a day	14 days	No significant differences were observed between treatment and control groups in either the numbers of nosocomial pathogens and anaerobes in the feces or the clinical parameters recorded: general well being, signs of abdominal distension, vomiting or regurgitation, feed intolerance, the incidence of perineal rash, the frequency and consistency of stools, the number of suppositories used, weight gain, or fluid intake. No adverse effects related to LGG were reported. There were no episodes of infection attributable to LGG.

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Table 15. Studies of Other *L. rhamnosus* Strains in Infants

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose	Duration	Safety-Related Results
Oberhelman et al. (1999)	Determine the effects of feeding LGG to under-nourished Peruvian infants	Randomized, prospective, double-blind, placebo-controlled study	204 under-nourished Peruvian infants aged 6 to 24 months (mean = 14.3 months)	3.7×10^{10} cfu LGG/day	Up to 15 months	Diarrhea frequencies were lower in the LGG than in the control group. No differences in the duration of diarrhea were observed and no adverse effects were reported.
Petschow et al. (2003)	Assess dose-response relationships between LGG administration and intestinal colonization in healthy full-term infants.	Randomized, placebo-controlled, double-blind study	49 healthy infants under the age of 3 months	10^8, 10^9, or 10^{10} cfu LGG/day	14 days	The authors concluded that, "Feeding LGG at 10^8 to 10^{10} cfu/day was well tolerated and led to transient colonization in healthy term infants." Stool consistency, flatulence, and fussiness were similar in all groups and no adverse effects were reported.
Pohjavuori et al. (2004)	Evaluate the effects of LGG alone or in combination with other probiotics on treatment of cow's milk allergy and IgE-associated dermatitis in infants	Randomized, double-blind, placebo-controlled study	230 allergic infants (mean age = 6.5 months) on a cows-milk-free diet and receiving treatment for dermatitis	5×10^9 cfu LGG twice a day or 5×10^9 cfu LGG, 5×10^9 cfu *L. rhamnosus* LC705, 2×10^8 cfu *B. breve* Bbi99, and 2×10^9 cfu *Propi-onibacterium freudenreichii* ssp. *shermanii* JS twice a day	4 weeks	LGG feeding increased the level of secreted IFN-γ in those infants with cow's milk allergy or IgE-associated dermatitis. Consumption of the probiotic combination significantly increased secretion of IL-4 in infants with cow's milk allergy, but LGG alone had no effect on IL-4 secretion. No safety-related effects were reported.

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Table 15. Studies of Other *L. rhamnosus* Strains in Infants

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose	Duration	Safety-Related Results
Romeo et al. (2006)	Study the ability of LGG and *L. reuteri* to reduce the incidence of bacterial infections and Candida in a NICU	Randomized, prospective, double-blind, placebo-controlled trial	184 newborns with average gestational age 34.9 weeks admitted to a NICU (107 boys, 77 girls)	$3x10^9$ cfu LGG/day	Duration of NICU stay, an average of 10 days	Infants receiving the probiotics had significantly reduced incidence of Candida and of bacterial infections, as well as shortened duration of required parenteral nutrition and of total hospital stay. The probiotic-treated infants exhibited higher rates of growth than did the control group. No adverse effects were noted due to the treatment with either *L. reuteri* or LGG.
Sepp et al. (1993)	Assess the effect of LGG on the establishment of the intestinal microbiota in neonates	Prospective randomized placebo-controlled study	25 neonates under 1 week of age	10^{10}-10^{11} cfu LGG/day	14 days	While the fecal concentrations of lactobacilli in the group administered LGG was consistently greater than that of the controls, the predominance pattern of the intestinal microbiota in the test group did not change. The authors concluded that, "The study shows that 2 wk administration of Lactobacillus GG, which starts right after birth, increases intestinal lactobacilli concentrations and does not impair the establishment of normal fecal microbiota."
Stansbridge et al. (1993)	Evaluate the effect of LGG colonization on GI function in the immature bowel of premature infants (follow-up to Millar et al. [1993])	Randomized prospective placebo-controlled trial	20 preterm infants with gestational ages of 33 weeks or less	10^8 cfu LGG twice a day	14 days	SCFA from treated infants did not differ from controls. Ethanol excretion was increased in treated infants. This small increase in ethanol secretion was judged unlikely to have clinical significance.

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Table 15. Studies of Other *L. rhamnosus* Strains in Infants

Reference	Objective	Study Design	Subjects	*L. rhamnosus* Dose	Duration	Safety-Related Results
Vendt et al. (2006)	Study the effect of LGG on the growth and fecal biota of healthy infants	Prospective, randomized, double-blind, placebo-controlled trial	60 boys and 60 girls aged 0-2 months (average age 39.8 days)	Increasing over the study from 8.1×10^8 to 1.2×10^9 cfu/day	up to age 6 months (average 4.7 months)	There were no differences between the groups in total duration of crying or in infectious episodes. No adverse effects were reported. Vendt et al. (2006) concluded, "The results of this randomized, controlled trial suggest that LGG-enriched formula was safe and well tolerated and resulted in normal growth."
Viljanen et al. (2005)	Investigate the effects on treatment of atopic eczema of LGG alone or as part of a combination of 4 probiotics in food-allergic infants.	Randomized placebo-controlled intervention study	230 infants under 12 months old (mean age = 6.4 months) with atopic eczema	5×10^9 cfu LGG or combination of 5×10^9 cfu LGG, 5×10^9 cfu *L. rhamnosus* LC705, 2×10^8 cfu *Bifidobacterium breve* Bbi99, and 2×10^9 cfu *Propionibacterium freudenreichii* ssp. *shermanii* JS	4 weeks	Parents completed a daily diary in which they were asked to record any skin, gastrointestinal, or respiratory system symptoms. There were no specific measures of safety-related endpoints, but no adverse effects were reported.

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4.5. Review Articles Regarding the Safety of *L. rhamnosus*

A large number of articles have appeared in the literature reviewing the history of use of *Lactobacillus* species and citing research data, and in most cases concluding that, except in exceptional circumstances, the entire genus poses little risk of harm to humans at any reasonable level of oral exposure. Some of these articles were cited earlier in support of the general safety of lactobacilli. This section deals with review articles focusing on *L. rhamnosus* and basing their conclusions on research from controlled human studies rather than history of use.

Two Cochran Collaboration reviews, Allen et al. (2003) and Johnston et al. (2007), addressed the safety of probiotics. In the first of these reviews, which evaluated the use of probiotics for treating infectious diarrhea, only randomized controlled trials comparing a specified probiotic agent with placebo in people with diagnosed infectious acute diarrhea were included; 23 studies including 1917 participants were reviewed. The participants included 1449 infants and children, 740 of whom received probiotics (while the others received placebo). Two studies included only malnourished children, while 2 others included such children among their study population. Eight of these studies included *L. rhamnosus*, 6 with strain GG and 2 with strain 19070-2. Of the 23 studies reviewed, 12 reported that clinical observation revealed no adverse events while 3 studies reported the occurrence of adverse events but determined that they were not related to the ingestion of probiotics. The 3 reported adverse events were vomiting, and in all cases the vomiting was less prevalent in the probiotic group than in the placebo group. The reviewers concluded that probiotics appear to be a safe and useful adjunct to rehydration therapy in treating acute infectious diarrhea in adults and children.

The Johnston et al. (2007) review focused on the use of probiotics for the prevention of antibiotic-associated diarrhea. Ten studies met the inclusion criteria; they included 1986 participants of whom 1015 received probiotic treatment with *Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus* spp., or *Saccharomyces boulardii*. Two trials involving *L. rhamnosus* were included, both with strain GG. Five of the 10 trials (including 647 patients) included specific monitoring for adverse events; of these, 3 reported that no adverse events had occurred and 2 reported that observed adverse events were equally prevalent among test and control groups and were not attributable to the probiotic intervention.

In 2006, after reviewing the safety of exposure to lactobacilli by animals, workers, consumers, and in the environment, Bernardeau et al. (2006) urged EFSA to accord *Lactobacillus* "Long-standing Presumption of Safety" status, a recommendation which was followed by EFSA. Bernardeau et al. (2006) suggested that new strains of *Lactobacillus* should be subjected to a "single-criterion safety assessment" with the single criterion being transferable antibiotic resistance.

With regard to pediatric applications, Lee et al. (2008) reviewed 21 randomized, placebo-controlled human studies of probiotics for the prevention and treatment of pediatric atopic dermatitis, involving 1898 infants and children aged 0 to 13 years (more than half of them involving *L. rhamnosus* strain GG), and concluded that "prenatal and postnatal administratation of probiotics appears to be a safe intervention."

4.6. Previous Safety Evaluations of *L. rhamnosus* By Authoritative Bodies

Noting that a wide variety of microbial species are used in food, some with a long history of apparent safe use, and facing the need to set priorities for risk assessment, the European Food Safety Authority (EFSA) proposed a system referred to as "Qualified Presumption of Safety" (QPS; EFSA 2007a). This system proposed basing the safety assessment of a defined taxonomic group (e.g., a genus or a species) on 4 pillars: established identity, body of knowledge, possible pathogenicity, and end use. If the taxonomic group did not raise safety concerns or, if safety concerns existed, but could be defined and excluded, the grouping could be granted QPS status. Thereafter, "any strain of microorganism the identity of which could be unambiguously established and assigned to a QPS group would be freed from the need for further safety assessment other than satisfying any qualifications specified" (EFSA 2007a, p1).

EFSA's Scientific Committee was asked to recommend organisms regarded as suitable for QPS status. The list of such organisms proposed by the Committee included *L. rhamnosus*. In listing *L. rhamnosus* and other species of *Lactobacillus* as suitable for QPS status, the Committee stated, "Where QPS status is proposed, the Scientific Committee is satisfied that the body of knowledge available is sufficient to provide adequate assurance that any potential to produce adverse effects in humans, livestock or the wider environment is understood and capable of exclusion" (EFSA 2007a, p8) and that the recommendations are "based on a thorough review of the available scientific literature and the knowledge and experience of the scientists involved" (EFSA 2007a, p8). With regard to *L. rhamnosus* the Committee noted the rare cases of infection of individuals with severe health issues and stated that, "The Scientific Committee took the view that the at-risk population is not placed at added risk by the use of *L. rhamnosus* in food/feed and so confirmed the proposed QPS status of this bacterium. The committee considers that this is a decision which should be reviewed at regular intervals" (EFSA 2007a, p11).

In 2006, the Norwegian Food Safety Authority determined that Mead Johnson's Nutramigen with *L. rhamnosus* GG could not be marketed in Norway as a medical food for infants. In response to an appeal by Mead Johnson Nutritionals, the Authority requested a risk assessment from the Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for Mattrygghet; VKM 2007). The VKM review concluded that, "Since there is no documented prophylactic effect of LGG on any diseases in children, there is currently no medical indication for supplementing milk substitutes or children's food with LGG" (VKM 2007, p 7). Further, the VKM argued that conclusive evidence of long-term safety of LGG supplementation during infancy was lacking with respect to development of the colonic microbiota and maturation of the immune system, and concluded that, "Our lack of knowledge necessitates the application of precautionary principles" (VKM 2007, p 11). In the opinion of the Expert Panel, this precautionary-principle based conclusion is of only tangential relevance to *L. rhamnosus* strain HN001 and does not call into question the reasonable certainty of no harm from the intended use of this strain.

In 2008, FDA, responding to a GRAS notice submitted by Mead Johnson & Company, indicated that the agency had no questions regarding Mead Johnson's determination that *L. rhamnosus* strain GG is GRAS for use as an ingredient in exempt hypoallergenic infant formula powder at a level of 10^8 cfu/g. FDA recognized that this formula powder is intended for consumption by term infants from the time of birth and that the estimated daily intake of LGG

from this use would be 10^8-10^{10} cfu/day. Mead Johnson noted that LGG is nonpathogenic, nontoxigenic, and not known to produce exotoxins; that analysis of the complete genome of LGG demonstrates that there is no evidence of a safety hazard; and that LGG has shown no adverse impact on nutritional, metabolic, or immune parameters at the level intended for use in infant formula powder. FDA did not disagree with or express any reservations regarding these assertions.

In December 2008, EFSA's Panel on Biological Hazards released an opinion reassessing the QPS status of *L. rhamnosus* strains. After reviewing published data that had become available since the species was originally assigned QPS status, the Panel concluded:

> "In conclusion, isolation of *L. rhamnosus* in clinical cases remains a rare event, but within the *Lactobacillus* group it is the most frequently isolated species (Salminen et al., 2004 and 2006). It should be highlighted, that on the one hand clinical studies prove the safety of oral application of strains of the species, on the other hand in sporadic cases, an infection is possible after oral administration (Zein et al., 2008) under specific circumstances, e.g. enteral administration of the probiotic to seriously ill, hospitalised patients, which is outside the domain of QPS. The increased consumption of the probiotic strain *L. rhamnosus* GG in a population was not found to lead to increased numbers of bacteraemia due to lactobacilli, suggesting that the consumption of *L. rhamnosus* outside the most vulnerable patient groups, is generally safe (Salminen et al., 2002). Therefore at the moment no change to the QPS status of *L. rhamnosus* is necessary' (EFSA 2008, p 9).

5. Safety Assessment and GRAS Determination

5.1. Introduction

This section presents an assessment that demonstrates that the addition of *Lactobacillus rhamnosus* strain HN001 to conventional foods as probiotic bacteria under the conditions of use described is safe and is GRAS.

This safety assessment and GRAS determination entail two steps. In the first step, the safety of the intended use of *L. rhamnosus* HN001 is demonstrated. Safety is established by demonstrating a reasonable certainty that the exposure of adults, infants, and children to *L. rhamnosus* HN001 under its intended conditions of use is not harmful. In the second step, the intended use of *L. rhamnosus* HN001 is determined to be GRAS by demonstrating that the safety of this product under its intended conditions of use is generally recognized among qualified scientific experts and is based on publicly available and accepted information.

The regulatory framework for establishing whether the intended use of a substance (or organism) is GRAS, in accordance with Section 201(s) of the Federal Food Drug and Cosmetic Act, is set forth under 21 CFR §170.30. This regulation states that general recognition of safety may be based on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. A GRAS determination may be made either: 1) through scientific procedures under §170.30(b); or 2) through experience based on common use in food, in the case of a substance used in food prior to January 1, 1958, under §170.30(c). This GRAS determination employs scientific procedures established under §170.30(b).

A scientific procedures GRAS determination requires the same quantity and quality of scientific evidence as is needed to obtain approval of the substance as a food additive. In addition to requiring scientific evidence of safety, a GRAS determination also requires that this scientific evidence of safety be generally known and accepted among qualified scientific experts. This "common knowledge" element of a GRAS determination consists of two components:

1. data and information relied upon to establish the scientific element of safety must be generally available; and

2. there must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a scientific-procedures GRAS determination are applied below in an analysis of whether the addition of *L. rhamnosus* HN001 to foods is safe and is GRAS.

5.2. Safety Evaluation

The body of evidence supporting the safety of oral administration of *Lactobacillus rhamnosus* strains in general, and strain HN001 in particular, is large and convincing. Numerous commentators—in addition to authoritative bodies such as EFSA—have noted the safe history of human ingestion of *L. rhamnosus* over many hundreds of years. *L. rhamnosus* produces no deleterious metabolites and is not destructive of mucin. Any effects that this probiotic microorganism has on intestinal permeability appear to be beneficial in strengthening barrier function. While it is theoretically possible for biogenic amines to be produced as a result of fermentation of dairy products by lactobacilli, this phenomenon has not been observed and, when *Lactobacillus* strains are ingested as probiotics, they produce lactic acid, lowering the intestinal pH and reducing the opportunity for production of harmful biogenic amines by putrefactive bacteria.

Lactobacilli are not regarded as pathogens, although some strains—including at least the GG strain of *L. rhamnosus*—are capable of opportunistic infection in extremely favorable circumstances invariably involving severe underlying disease states and most often also involving a facilitated pathway such as surgical intervention or the presence of central lines. Documented cases of *Lactobacillus* bacteremia are so rare, in comparison to the widespread use of *Lactobacillus* strains in the environment, in food production, and in probiotic applications, that the participants in the 2007 EU-PROSAFE project (Vankerckhoven et al. 2007a) suggested that "they are more medical exceptions, or even curiosities, than a genuine public health issue."

The safe history of human exposure to *L. rhamnosus* strains is strongly supported by an extremely large body of published research. In addition to *in vitro* work, the published literature includes 19 experimental studies in a variety of animal species (normal and immunodeficient mice, hamsters, rats, and rabbits) as well as 83 separate studies in humans: 35 in adults, 25 in children, and 23 in infants. These studies have included about 1700 adults, 3400 children, and 4100 infants, a total of well over 9000 individuals. Adverse effects were seen in only 2 animal studies and in no human studies, with the arguable exception of an increased incidence of recurrent wheezing bronchitis in infants at risk of atopic dermatitis receiving LGG. The 2 animal studies in which adverse events were observed both involved animal models of questionable application to human beings. In one case, the animals were doubly immunodeficient athymic mice, a model rejected by the EU-PROSAFE team. In the other case, the model was the neonatal New Zealand white rabbit; LGG was given along with the highly invasive *E. coli* K1 strain. Although LGG reduced the invasiveness of K1, and prevented mucosal damage, some translocation of LGG was observed. It is recognized, however, that the neonatal rabbit displays a propensity for immature immune function, mucosa and mucosal gel layer development, and intestinal epithelial development, and it is notable that the LGG translocation produced no apparent clinical distress. No other animal study, and no animal study with HN001, reported any adverse effects.

The human data are even more confirmatory of the safety of *L. rhamnosus* for oral ingestion. Despite being given to over 9000 adults, children, and infants at doses up to and exceeding 10^{11} cfu/day for periods as long as 2 years, no adverse effects were reported in any of the 83 published studies with the exception of the increased incidence of wheezing noted above. It is particularly significant that no *Lactobacillus* infectivity was observed even in medically fragile patients, including infants confined to a NICU following surgery and often possessing central lines.

The final issue in evaluating the safety of the intended probiotic use of *L. rhamnosus* strain HN001 is its freedom from transferable antibiotic resistance. As noted previously, many reviewers have argued that this is the attribute of most importance with regard to safety of *Lactobacillus* exposure. Two lines of evidence demonstrate that transferable antibiotic resistance is not present in the HN001 strain.

First, the research by Zhou et al. (2005a) found that *L. rhamnosus* HN001 exhibits no phenotypic resistance not widely found in other *Lactobacillus* strains. This is evidence that the strain is of the "wild type" with regard to commonly used antibiotics. The principle behind the phenotypic test is that the wild-type strains of a species have a normal distribution of minimum inhibitory concentrations (MIC) when the number of strains is plotted against the MIC for a given antibiotic. If all strains fall within this distribution, then it is likely that the susceptibility or resistance of the species is intrinsic since it is found in all strains. On the other hand, if a separate cluster of strains is found with higher resistance, it is likely that the resistance of these strains is acquired. This is important, because acquired resistance is far more likely to be transferable than intrinsic resistance (Vankerckhoven et al. 2007a). The usefulness of susceptibility testing for indication of acquired resistance is increased as the numbers of both susceptible strains and resistant strains tested are increased. The number of *L. rhamnosus* strains tested by Zhou et al. (2005a) is insufficient to make this study determinative when taken alone, but it does provide strong evidence of the absence of transferable antibiotic resistance.

The second line of evidence is more direct, and that is that the published genomic analysis of *L. rhamnosus* HN001 found no genetic basis for antibiotic resistance that is even potentially transferable in either the genome or in either of the 2 plasmids.

All of the available evidence demonstrates clearly that there is no reason to suspect harm to individuals consuming conventional foods supplemented with *Lactobacillus rhamnosus* strain HN001.

5.3. General Recognition of the Safety of *L. rhamnosus* Strain HN001

The proposed use of *L. rhamnosus* strain HN001, to be added as a probiotic to a variety of conventional foods, 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* HN001 by adults and children 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.

Determination of the safety and GRAS status of the addition of *L. rhamnosus* to food has been made through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., and Michael W. Pariza, Ph.D., who reviewed a monograph prepared by JHeimbach LLC as well as other information available to them. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients, including probiotic bacteria. They critically reviewed and evaluated the publicly available information and the potential human exposure to *L. rhamnosus* HN001 anticipated to result from its intended uses, and individually and collectively concluded that no evidence exists in the available information on *L. rhamnosus* HN001 or other *L. rhamnosus* strains, that demonstrates, or suggests reasonable grounds to suspect, a hazard to either adults or children under the intended conditions of use of *L. rhamnosus* HN001.

It is the Expert Panel's opinion that other qualified scientists reviewing the same publicly available data would reach the same conclusion. Therefore, the intended use of *Lactobacillus rhamnosus* strain HN001 is GRAS by scientific procedures.

6. References

Acar JF and FW Goldstein. 1991. Disk susceptibility test. In Lorian V (Ed.), *Antibiotics in laboratory medicine, 3rd edition*. New York: Williams & Wilkins, 17-52.

Adams MR. 1999. Safety of industrial lactic acid bacteria. *J Biotechnol* 68:171-178.

Adams MR and P Marteau. 1995. On the safety of lactic acid bacteria from food. *Int J Food Microbiol* 27:263-264.

Adawi D, FB Kasravi, G Molin, B Jeppsson. 1997. Effect of lactobacillus supplementation with and without arginine on liver damage and bacterial translocation in an acute liver injury model in the rat. *Hepatol* 25:642-647.

Agarwal R, N Sharma, R Chaudhry, A Deorari, VK Paul, IH Gewolb, P Panigrahi. 2003. Effects of oral *Lactobacillus* GG on enteric microflora in low-birth-weight neonates. *J Pediatr Gastroenterol Nutr* 36:397-402.

Aguirre M and MD Collins. 1993. Lactic acid bacteria and human clinical infection. *J Appl Bacteriol* 75:95-107.

Alander M, R Satokari, R Korpela, M Saxelin, T Vilpponen-Salmella, T Mattila-Sandholm, A von Wright. 1999. Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. *Appl Environ Microbiol* 65:351-354.

Allen SJ, B Okoko, E Martinez, G Gregorio, LF Dans. 2003. Probiotics for treating infectious diarrhoea. *Cochrane Database of Systematic Reviews* 4:CD003048.

Ammor MS, AB Florez, B Mayo. 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiol* 24:559-570.

Anukam KC, EO Osazuwa, G Reid, RI Ozolua. 2004. Feeding probiotic strains *Lactobacillus rhamnosus* GR-1 and *Lactobacillus fermentum* RC-14 does not significantly alter haematological parameters of Sprague-Dawley rats. *Haema* 7:497-501.

Anukam K, E Osazuwa, I Ahonkhai, M Ngwu, G Osemene, AW Bruce, G Reid. 2006. Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: randomized, double-blind, placebo-controlled trial. *Microbes Infect* 8:1450-1454.

Anukam KC, EO Osazuwa, HB Osadolor, AW Bruce, G Reid. 2008. Yogurt containing probiotic *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 helps resolve moderate diarrhea and increases CD4 count in HIV/AIDS patients. *J Clin Gastroenterol* 42:239-243.

Armuzzi A, F Cremonini, F Bartolozzi, F Canducci, M Candelli, V Ojetti, G Cammarota, M Anti, A De Lorenzo, P Pola, G Gasbarrini, A Basbarrini. 2001. The effect of oral

administration of *Lactobacillus* GG on antibiotic-associated gastrointestinal side-effects during *Helicobacter pylori* eradication therapy. *Aliment Pharmacol Ther* 15:163-169.

Arthur M and P Courvalin. 1993. Genetics and mechanisms of glycopeptide resistance in enterococci. *Antimicrob Agents Chemother* 37:1563-1571.

Arvola T, K Laiho, S Torkkeli, H Mykkäneh, S Salminen, L Maunula, E Isolauri. 1999. Prophylactic *Lactobacillus* GG reduces antibiotic-associated diarrhea in children with respiratory infections: A randomized study. *Pediatrics* 104:e64.

Axelsson L. 1998. Lactic acid bacteria: classification and physiology. In Salminen S and A von Wright, eds. *Lactic acid bacteria, microbiology and functional aspects*. New York: Marcel Dekker, Inc, 1-73.

Banaszkiewicz A and H Szajewska. 2005. Ineffectiveness of *Lactobacillus* GG as an adjunct to lactulose for the treatment of constipation in children: a double-blind, placebo-controlled randomized trial. *J Pediatr* 146:364-369.

Baroja ML, PV Kirjavainen, S Hekmat, G Reid. 2007. Anti-inflammatory effects of probiotic yogurt in inflammatory bowel disease patients. *Clin Exp Immunol* 149:470-479.

Bausserman M and S Michail. 2005. The use of *Lactobacillus* GG in irritable bowel syndrome in children: a double-blind randomized control trial. *J Pediatr* 147:197-201.

Bernardeau, M., Gueguen, M., Vernoux, J.P., 2006. Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. *FEMS Microbiol Rev* 30:487–513.

Bernardeau M, JP Vernoux, S Henri-Dubernet, M Gueguen. 2007. The *Lactobacillus* genus. *Int J Food Microbiol* E-pub ahead of print.

Besselink MGH, HC van Santvoort, E Buskens, MA Boermeester, H van Goor, HM Timmerman, VB Nieuwenhuijs, TL Bollen, B van Ramshorst, BJM Witteman, C Rosman, RJ Ploeg, MA Brink, AFM Schaapherder, CHC Dejong, PJ Wahab, CJHM van Laarhoven, E van der Harst, CHJ van Eijck, MA Cuesta, LMA Akkermans, HG Gooszen. 2008. Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. *Lancet* 371:651-659.

Biadaioli R, R Bagna, P Murru, P Popolo, D Rosatelli, A Pinna. 1998. An attempt to reduce premature infant infections in intensive care units by means of *Lactobacillus* GG. *Gastroenterol Int* 11(Suppl 1):136.

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

Boyle RJ, RM Robins-Browne, MLK Tang. 2006. Probiotic use in clinical practice: what are the risks? *Am J Clin Nutr* 83:1256-1264.

Brahimi M, P Mathern, P Fascia, JM Afchain, F Lucht. 2008. Two cases of *Lactobacillus rhamnosus* infection and pancreatitis. *Med Mal Infect* 38:29-31. [Article in French with English abstract]

Brouwer ML, SAA Wolt-Plompen, AEJ Dubois, S van der Heide, DF Jansen, MA Hoijer, HF Kauffman, EJ Duiverman. 2006. No effects of priobiotics on atopic dermatitis in infancy: a randomized placebo-controlled trial. *Clin Exper Allergy* 36:899-906.

Cabana MD, AL Shane, C Chao, M Oliva-Hemker. 2006. Probiotics in primary care pediatrics. *Clin Pediatr* 45:405-410.

Canani RB, P Cirillo, G Terrin, L Cesarano, MI Spagnuolo, A De Vincenzo, F Albano, A Passariello, G De Marco, F Manguso, A Guarino. 2007. Probiotics for treatment of acute diarrhoea in children: randomised clinical trial of five different preparations. *Br Med J* 335:340-345.

Chouraqui JP, D Grathwohl, JM Labaune, JM Hascoet, I de Montgolfier, M Leclaire, M Giarre, P Steenhout. 2008. Assessment of the safety, tolerance, and protective effect against diarrhea of infant formulas containing mixtures of probiotics or probiotics and prebiotics in a randomized controlled trial. *Am J Clin Nutr* 87:1365-1373.

Cogan TM 1996. History and taxonomy of starter cultures. In *Dairy Starter Cultures*. TM Cogan and JP Accolas, editors. VCH Publishers: New York. 1-23.

Collado MC, L Jalonen, J Meriluoto, S Salminen. 2006. Protection mechanism of probiotic combination against human pathogens: *in vitro* adhesion to human intestinal mucus. *Asia Pac J Clin Nutr* 15:570-575.

Collins MD, BA Phillips, P Zanoni. 1989. Deoxyribonucleic acid homology studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans*, and *Lactobacillus rhamnosus* sp. nov., comb. nov. *Int J Syst Bacteriol* 39:105-108.

Cremonini F, S Di Caro, M Covina, A Armuzzi, M Gabrielli, L Santarelli, EC Nista, G Cammarota, G Gasbarrini, A Gasbarrini. 2002. Effect of different probiotic preparations on anti-*Helicobacter pylori* therapy-related side effects: a parallel group, triple blind, placebo-controlled study. *Am J Gastroenterol* 97:2744-2749.

Dani C, R Biadaioli, G Bertini, E Martelli, FF Rubaltelli. 2002. Probiotics feeding in prevention of urinary tract infection, bacterial sepsis and necrotizing enterocolitis in preterm infants. *Biol Neonate* 82:103-108.

Dekker JW, K Wickens, PN Black, TV Stanley, EA Mitchell, P Fitzharris, GW Tannock, G Purdie, J Crane. 2009. Safety aspects of probiotic bacterial strains *Lactobacillus rhamnosus* HN001 and *Bifidobacterium animalis* ssp. *lactis* HN019 in human infants aged 0 to 2 years. *Int Dairy J* 19:149-154.

De Groote MA, DN Frank, E Dowell, MP Glode, NR Pace. 2005. *Lactobacillus rhamnosus* GG bacteremia associated with probiotic use in a child with short gut syndrome. *Pediatr Infect Dis J* 24:278-280.

Di Caro S, H Tao, A Grillo, C Elia, G Gasbarrini, AR Sepulveda, A Gasbarrini. 2005. Effects of *Lactobacillus* GG on genes expression pattern in small bowel mucosa. *Digest Liver Dis* 37:320-329.

Dong MY, TW Chang, SL Gorbach. 1987. Effect of feeding *Lactobacillus* GG on lethal irradiation in mice. *Diagn Microbiol Infect Dis* 7:1-7.

Donohue D, M Deighton, JT Ahokas, S Salminen. 1993. Toxicity of Lactic acid bacteria. In S Salminen and A von Wright (Eds): *Lactic Acid Bacteria*: Marcel Dekker Inc., 307-313.

Donohue DC and S Salminen. 1996. Safety of probiotic bacteria. *Asia Pacific J Clin Nutr* 5:25-28.

Edwards CA and AM Parrett. 2002. Intestinal flora during the first months of life: new perspectives. *Brit J Nutr* 88(Suppl 1):S11-18.

Elmadfa I, CO Heinzle, D Majchrzak, H Foissy. 2001. Influence of a probiotic yoghurt on the status of vitamins B1, B2, and B6 in the healthy adult human. *Ann Nutr Metabol* 45:13-18.

European Food Safety Authority (EFSA). 2005.Opinion of the Scientific Panel on additives and products or substances used in animal feed on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance. *EFSA J* 223:1-12.

European Food Safety Authority (EFSA). 2007a. Introduction of a qualified presumption of safety (QPS) approach for assessment of selected microorganisms referred to EFSA: opinion of the scientific committee. *EFSA J* 587:8-16.

European Food Safety Authority (EFSA). 2007b. Introduction of a qualified presumption of safety (QPS) approach for assessment of selected microorganisms referred to EFSA: opinion of the scientific committee. Appendix A: Assessment of gram-positive non-sporulating bacteria. *EFSA J* 587.

European Food Safety Authority (EFSA). 2008. The maintenance of the list of QPS microorganisms intentionally added to food or feed. Scientific opinion of the Panel on Biological Hazards. *EFSA J* 923:1-48.

Fang H, T Elina, A Heikki, S Seppo. 2000. Modulation of humoral immune response through probiotic intake. *FEMS Immunol Med Microbiol* 29:47-52.

Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO). 2001. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Report of a Joint FAO/WHO Expert Consultation. October.

Food and Drug Administration (FDA). 2006. *Estimating Dietary Intake of Substances in Food.* Guidance document released August 2006, available on the FDA website at http://www.cfsan.fda.gov/~dms/opa2cg8.html#upper

Forestier C, D Guelon, V Cluytens, T Gillart, J Sirot, C De Champs. 2008. Oral probiotic and prevention of *Pseudomonas aeruginosa* infections: a randomized, double-blind, placebo-controlled pilot study in intensive care unit patients. *Critical Care* 12 [e-pub].

Galpin L, MJ Manary, K Fleming, C-N Ou, P Ashorn, RJ Shulman. 2005. Effect of *Lactobacillus* GG on intestinal integrity in Malawian children at risk of tropical enteropathy. *Am J Clin Nutr* 82:1040-1045.

Gasser F. 1994. Safety of lactic acid bacteria and their occurrence in human clinical infections. *Bull Inst Pasteur* 92:45-67.

Gill HS, KJ Rutherfurd, J Prasad, PK Gopal. 2000. Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019. *Br J Nutr* 83:167-176.

Gill HS and KJ Rutherfurd. 2001. Probiotic supplementation to enhance natural immunity in the elderly: effects of a newly characterized immunostimulatory strain *Lactobacillus rhamnosus* HN001 (DR20™) on leucocyte phagocytosis. *Nutr Res* 21:183-189.

Gill HS, KJ Rutherfurd, ML Cross. 2001a. Dietary probiotic supplementation enhances natural killer cell activity in the elderly: an investigation of age-related immunological changes. *J Clin Immunol* 21:264-271.

Gill HS, Q Shu, H Lin, KJ Rutherfurd, ML Cross. 2001b. Protection agains translocating *Salmonella typhimurium* infection in mice by feeding the immuno-enhancing probiotic *Lactobacillus rhamnosus* strain HN001. *Med Microbiol Immunol* 190:97-104.

Gill HS, ML Cross, KJ Rutherfurd, PK Gopal. 2001c. Dietary probiotics supplementation to enhance cellular immunity in elderly. *Br J Biomed Sci* 58:94-96.

Goldin BR, SL Gorbach, M Saxelin, S Barakat, L.Gualtieri, S Salminen. 1992. Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract. *Dig Dis Sci* 37:121-128.

Gopal PK, J Prasad, J Smart, HS Gill. 2001. In vitro adherence properties of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escerichia coli. Int J Food Microbiol* 67:207-216.

Grönlund MM, OP Lehtonen, P Kero, M Saxelin, S Salminen. 1997. *Lactobacillus* GG supplementation does not reduce faecal colonization of *Klebsiella oxytoca* in preterm infants. *Acta Paediatr* 86:785-786.

Guandalini S, L Pensabene, MA Zikri, JA Dias, LG Casali, H Hoekstra, S Kolacek, K Massar, D Micetic-Turk, A Papadopoulou, JS de Sousa, B Sandhu, H Szajewska, Z Weizman. 2000. *Lactobacillus* GG administered in oral rehydration solution to children with acute diarrhoea: A multicenter European trial. *J Pediatr Gastroenterol Nutr* 30:54-60.

Guarino A, RB Canani, MI Spagnuolo, F Albano, L Di Benedetto. 1997. Oral bacterial therapy reduces the duration of symptoms and of viral excretion in children with mild diarrhea. *J Pediatr Gastroenterol Nutr* 25:516-519.

Harty DWS, M Patrikakis, EBH Hume, HJ Oakey, KW Knox. 1993. The aggregation of human platelets by *Lactobacillus* species. *J Gen Microbiol* 139:2945-2951.

Hatakka K, E Savilahti, A Pönkä, JH Meurman, T Poussa, L Näse, M Saxelin, R Korpela. 2001. Effect of long term consumption of probiotic milk on infections in children attending day care centres: double blind, randomised trial. *Br Med J* 322:1327-1329.

Hatakka K, J Martio, M Korpeia, M Herranen, T Poussa, T Laasanen, M Saxelin, H Vapaatalo, E Moilanen, R Korpela. 2003. Effects of probiotic therapy on the activity and activation of mild rheumatoid arthritis—a pilot study. *Scand J Rheumatol* 32:211-215.

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

Holma R, P Salmenpera, J Lohi, H Vapaatalo, R Korpela. 2001. Effects of *Lactobacillus rhamnosus* GG and *Lactobacillus reuteri* R2LC on acetic acid-induced colitis in rats. *Scand J Gastroenterol* 36:630-635.

Holzapfel WH, P Haberer, R Geisen, J Bjorkroth, U Schillinger. 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am J Clin Nutr* 73(suppl): 365S-373S.

Honeycutt TC, M El Khashab, RM Wardrop, K McNeal-Trice, AL Honeycutt, CG Christy, K Mistry, BD Harris, JN Meliones, KC Kocis. 2007. Probiotic administration and the incidence of nosocomial infection in pediatric intensive care: a randomized placebo-controlled trial. *Pediatr Crit Care Med* 8:452-458.

Husni RN, SM Gordon, JA Washington, DL Longworth. 1997. *Lactobacillus* bacteremia and endocarditis: review of 45 cases. *Clin Infect Dis* 25:1048-1055.

Isenberg HD. 1992. Antimicrobial susceptibility testing. In Isenberg HD (Ed.), *Clinical microbiology procedures handbook, vol. 1*. New York: ASM Press, 5.1.1-5.6.14.

Isolauri E, M Kaila, H Mykkänen, WH Ling, S Salminen. 1994. Oral bacteriotheraphy for viral gastroenteritis. *Dig Dis Sci* 39:2595-2600.

Isolauri E, T Arvola, E Moilanen, S Salminen. 2000. Probiotics in the management of atopic eczema. *Clin Experiment Allergy* 30:1604-1610.

Jacobsen CN, VR Nielsen, AE Hayford, PL Moller, KF Michaelsen, A Paerregaard, B Sandstrom, M Tvede, M Jakobsen. 1999. Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. *Appl Environ Microbiol* 65:4949-4956.

Johansson ML, G Molin, B Jeppsson, S Nobaek, S Ahrne, S Bengmark. 1993. Administration of different *Lactobacillus* strains in fermented oatmeal soup: *in vivo* colonization of human intestinal mucosa and effect on the indigenous flora. *Appl Environ Microbiol* 59:15–20.

Johnston BD, AL Supina, M Ospina, S Vohra. 2007. Probiotics for the prevention of pediatric antibiotic-associated diarrhea. *Cochrane Database of Systematic Reviews* 2:CD004827.

Kaila M, E Isolauri, E Soppi, E Virtanen, S Laine, H Arvilommi. 1992. Enhancement of the circulating antibody secreting cell response in human diarrhea by a human *Lactobacillus* strain. *Pediatr Res* 32:141-144.

Kaila M, E Isolauri, M Saxelin, H Arvilommi, T Vesikari. 1995. Viable versus inactivated *Lactobacillus* strain GG in acute rotavirus diarrhoea. *Arch Dis Child* 72:51-53.

Kajander K and R Korpela. 2006. Clinical studies on alleviating the symptoms of irritable bowel syndrome with a probiotic combination. *Asia Pac J Clin Nutr* 15:576-580.

Kalima P, RG Masterton, PH Roddie, AE Thomas. 1996. *Lactobacillus rhamnosus* infection in a child following bone marrow transplant. *J Infect* 32:165-167.

Kalliomäki M, S Salminen, H Arvilommi, P Kero, P Koskinen, E Isolauri. 2001. Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* 357:1076-1079.

Kalliomaki M, S Salminen, T Poussa, H Arvilommi, E Isolauri. 2003. Probiotics and prevention of atopic disease: 4-year follow-up of a randomised placebo-controlled trial. *Lancet* 361:1869-1871.

Kankaanpaa P BYang, H Kallio, E Isolauri, S Salminen. 2002. Influence of probiotic supplemented infant formula on composition of plasma lipids in atopic infants. *J Nutr Biochem* 13:364-369.

Kastner S, V Perreten, H Bleuler, G Hugenschmidt, C Lacroix, L Meile. 2006. Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food. *Systemat Appl Microbiol* 29:145-155.

Kekkonen RA, N Lummela, H Karjalainen, S Latvala, S Tynkkynen, S Jarvenpaa, H Kautiainen, I Julkunen, H Vapaatalo, R Korpela. 2008. Probiotic intervention has strain-specific anti-inflammatory effects in healthy adults. *World J Gastroenterol* 14:2029-2036.

Kirjavainen, P., E.M. Tuomola, R.G. Crittenden, A.C. Ouwehand, D.W.S. Harty, L.F. Morris, H. Rautelin, M.J. Playne, D.C. Donohue, and S.J. Salminen. 1999. *In vitro* adhesion and platelet aggregation properties of bacteremia-associated lactobacilli. *Infect. Immun.* 67: 2652-2655.

Klare I, C Konstabel, G Werner, G Huys, V Vankerckhoven, G Kahlmeter, B Hildebrandt, S Muller-Bertling, W Witte, H Goossens. 2007. Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. *J Antimicrob Chemother* 59:900-912.

Klein G, C Hallmann, IA Casas, J Abad, J Louwers, G Reuter. 2000. Exclusion of *van*A, *van*B, and *van*C type glycopeptide resistance in strains of *Lactobacillus rhamnosus* and *Lactobacillus rhamnosus* used as probiotics by polymerase chain reaction and hybridization methods. *J Appl Microbiol* 89:815-824.

Kopp MV, I Hennemuth, A Heinzmann, R Urbanek. 2008. Randomized, double-blind, placebo-controlled trial of probiotics for primary prevention: no clinical effects of *Lactobacillus* GG supplementation. *Pediatrics* 121:e850-e856.

Kukkonen K, E Savilahti, T Haahtela, K Juntunen-Backman, R Korpela, T Poussa, T Tuure, M Kuitunen. 2008. Long-term safety and impact on infection rates of postnatal probiotic and prebiotic (synbiotic) treatment: randomized, double-blind, placebo-controlled trial. *Pediatrics* 122:8-12.

Kunz AN, JM Noel, MP Fairchok. 2004. Two cases of *Lactobacillus* bacteria during probiotic treatment of short gut syndrome. *J Pediatr Gastroenterol Nutr* 38:457-458.

Land, M.H., K. Rouster-Stevens, C.R. Woods, M.L. Cannon, J.Cnota, and A.K. Shetty. 2005. *Lactobacillus* sepsis associated with probiotic therapy. *Pediatrics* 2005;115;178-181.

Larsen N, P Nissen, WGT Willats. 2007. The effect of calcium ions on adhesion and competitive exclusion of *Lactobacillus* ssp. and *E. coli* O138. *Int J Food Microbiol* 114:113-119.

Leclercq R, E Derlot, J Duval, P Courvalin. 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium. N Engl J Med* 319:157-161.

Lee DJ, RA Drongowski, AG Coran, CM Harmon. 2000. Evaluation of probiotic treatment in a neonatal animal model. *Pediatr Surg Int* 16:237-242.

Lee J, D Seto, L Bielory. 2008. Meta-analysis of clinical trials of probiotics for prevention and treatment of pediatric atopic dermatitis. *J Allergy Clin Immunol* 121:116-121.

Ling WH, O Hanninen, H Mykkanen, M Heikura, S Salminen, A Von Wright. 1992. Colonization and fecal enzyme activities after oral *Lactobacillus* GG administration in elderly nursing home residents. *Ann Nutr Metab* 36:162-166.

Mackay AD, MBN Taylor, CC Kibbler, JMT Hamilton-Miller. 1999. *Lactobacillus* endocarditis caused by a probiotic organism. *Clin Microbiol Infect* 5:290-92.

Mack DR. 2004. D(-)-lactic acid-producing probiotics, D(-)-lactic acidosis and infants. *Can J Gastroenterol* 18:671-675.

Majamaa H and E Isolauri. 1997. Probiotics: A novel approach in the management of food allergy. *J Allergy Clin Immunol* 99:179-185.

Majamaa H, E Isolauri, M Saxelin, T Vesikari. 1995. Lactic acid bacteria in the treatment of acute rotavirus gastroenteritis. *J Pediatr Gastroent Nutr* 20:333-338.

Marini A, D Clerici-Bagozzi, T Maglia, P Casetta, F Negretti. 1997. Microbiological and immunological observations in the stools of preterm neonates orally treated with probiotic products. Note III: treatment with *Lactobacillus* GG. *Dev Physiopath Clin* 7:87-94.

McFall-Ngai M. 2006. Love the one you're with: vertebrate guts shape their microbiota. *Cell* 127:247-249

Millar MR, C Bacon, SL Smith, V Walker, MA Hall. 1993. Enteral feeding of premature infants with *Lactobacillus* GG. *Arch Dis Child* 69:483-487.

Moller PL, A Paerregaard, M Gad, NN Kristensen, MH Claesson. 2005. Colitic scid mice fed *Lactobacillus* spp. show an ameliorated gut histopathology and an altered cytokine profile by local T cells. *Inflamm Bowel Dis* 11:814-819.

Morelli L, D Zonenschain, M Del Piano, P Cognein. 2004. Utilization of the intestinal tract as a delivery system for urogenital probiotics. *J Clin Gastroenterol* 38:S107-S110.

Naaber P, S Mikelsaar, S Salminen, M Mikelsaar. 1998. Bacterial translocation, intestinal microflora and morphological changes of intestinal mucosa in experimental models of *Clostridium difficile* infection. *J Med Microbiol* 47:1-8.

Naidu AS, WR Bidlack, RA Clemens. 1999. Probiotic spectra of lactic acid bacteria (LAB). *Critical Rev Food Sci Nutr* 38:13-126.

Oberhelman RA, RH Gilman, P Sheen, DN Taylor, RE Black, L Cabrera, AG Lescano, R Meza, G Madico. 1999. A placebo-controlled trial of *Lactobacillus* GG to prevent diarrhea in undernourished Peruvian children. *J Pediatr* 134:15-20.

O'Sullivan MA and CA O'Morain. 2000. Bacterial supplementation in the irritable bowel syndrome. A randomized double-blind placebo-controlled crossover study. *Dig Liver Dis* 32:294-301.

Ouwehand AC, EM Tuomola, S Tolkko, S Salminen. 2001. Assessment of adhesion properties of novel probiotic strains to human intestinal mucus. *Int J Food Microbiol* 64:119-126.

Ouwehand AC, H Lagstrom, T Suomalainen, S Salminen. 2002. Effect of probiotics on constipation, fecal azoreductase activity and fecal mucin content in the elderly. *Ann Nutr Metabol* 46:159-162.

Ouwehand AC, S Salminen, PJ Roberts, J Ovaska, E Salminen. 2003. Disease-dependent adhesion of lactic acid bacteria to the human intestinal mucosa. *Clin Diag Lab Immunol* 10:643-646.

Pessi T, Y Sütas, A Martinnen, E Isolauri. 1998. Probiotics reinforce mucousal degradation of antigens in rats: Implications for therapeutic use of probiotics. *J Nutr* 128:2313-2318.

Petschow BW, R Figueroa, CL Harris, LB Beck, E Ziegler, B Goldin. 2005. Effects of feeding an infant formula containing Lactobacillus GG on colonization of the intestine: a dose-response study in healthy infants. *J Clin Gastroenterol* 39:786-790.

Piirainen L, RA Kekkonen, K Kajander, T Ahlroos, S Tynkkynen, R Nevala, R Korpela. 2008. In school-aged children a combination of galacto-oligosaccharides and *Lactobacillus* GG increases bifidobacteria more than *Lactobacillus* GG on its own. *Ann Nutr Metab* 52:204-208.

Pohjavuori E, M Viljanen, R Korpela, M Kuitunen, M Tittanen, O Varala, E Savilahti. 2004. *Lactobacillus* GG effect in increasing IFN-γ production in infants with cow's milk allergy. *J Allergy Clin Immunol* 114:131-136.

Prantera C, ML Scribano, G Falasco, A Andreoli, C Luzi. 2002. Ineffectiveness of probiotics in preventing recurrence after curative resection for Crohn's disease: a randomised controlled trial with *Lactobacillus* GG. *Gut* 51:405-409.

Prasad J, H Gill, J Smart, PK Gopal. 1999. Slection and characterisation of *Lactobacillus* and B*ifidobacterium* strains for use as probiotics. *Int Dairy J* 8:993-1002.

Presterl E, W Kneifel, HK Mayer, W Kneifel, HK Mayer, M Zehetgruber, A Makristathis, W Grainger. 2001. Endocarditis by *Lactobacillus rhamnosus* due to yogurt ingestion? *Scand J Infect Dis* 33:710-714.

Rautio M, H Jousimies-Somer, H Kauma, I Pietarinen, M Saxelin, S Tynkkynen, M Koskela. 1999. Liver abscess due to *Lactobacillus rhamnosus* indistinguishable from *Lactobacillus* GG. *Clin Infect Dis* 28:1159-60.

Raza S, SM Graham, SJ Allen, S Sultana, L Cuevas, CA Hart. 1995. *Lactobacillus* GG promotes recovery from acute nonbloody diarrhea in Pakistan. *Pediatr Infect Dis J* 14:107-111.

Reid G, D Beuerman, C Heinemann, AW Bruce. 2001. Probiotic *Lactobacillus* dose required to restore and maintain a normal vaginal flora. *FEMS Immunol Med Microbiol* 32:37-41.

Reid G, J-A Hammond, AW Bruce. 2003a. Effect of *Lactobacilli* oral supplement on the vaginal microflora of antibiotic treated patients: randomized, placebo-controlled study. *Neutraceuticals Food*: 8:145-148.

Reid G, D Charbonneau, J Erb, B Kochanowski, D Beuerman, R Poehner, AW Bruce. 2003b. Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. *FEMS Immunol Med Microbiol* 35:131-134.

Reid G and J-A Hammond. 2005. Probiotics—some evidence of their effectiveness. *Can Fam Physician* 51:1487-1493.

Romeo MG, P Betta, L Trovato, S Oliveri. 2006. The role of probiotics in the prevention of bacterial infections and Candida in neonatal intensive care; prospective study with control groups. 12th National SIN Congress (Italian Neonatology Society), May.

Rosenfeldt V, KF Michaelsen, M Jakobsen, CN Larsen, PL Moller, P Pedersen, M Tvede, H Weyrehter, NH Valerius, A Paerregaard. 2002a. Effect of probiotic *Lactobacillus* strains in young children hospitalized with acute diarrhea. *Pediatr Infect Dis J* 21:411-416.

Rosenfeldt V, KF Michaelsen, M Jakobsen, CN Larsen, PL Moller, M Tvede, H Weyrehter, NH Valerius, A Paerregaard. 2002b. Effect of probiotic *Lactobacillus* strains on acute diarrhea in a cohort of nonhospitalized children attending day-care centers. *Pediatr Infect Dis J* 21:417-419.

Rosenfeldt V, E Benfeldt, SD Nielsen, KF Michaelsen, DL Jeppesen, NH Valerius, A Paerregaard. 2003. Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis. *J Allergy Clin Immunol* 111:389-395.

Rosenfeldt V, E Benfeldt, NH Valerius, A Paerregaard, KF Michaelsen. 2004. Effect of probiotics on gastrointestinal symptoms and small intestinal permeability in children with atopic dermatitis. *J Pediatr* 145:612-616.

Ruszczynski M, A Radzikowski, H Szajewska. 2008. Clinical trial: effectiveness of *Lactobacillus rhamnosus* (strains E/N, Oxy and Pen) in the prevention of antibiotic-associated diarrhoea in children. *Aliment Pharmacol Ther* 28:154-161.

Salazar-Lindo E, P Miranda-Langschwager, M Campos-Sanchez, E Chea-Woo, RB Sack. 2004. *Lactobacillus casei* strain GG in the treatment of infants with acute watery diarrhea: A randomized, double-blind, placebo controlled clinical trial. *BMC Pediatrics* 4:18.

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

Salminen MK, S Tynkkynen, H Rautelin, M Saxelin, M Vaara, P Ruutu, S Sarna, V Valtonen, A Järvinen. 2002. *Lactobacillus* bacteremia during a rapid increase in probiotic use of *Lactobacillus rhamnosus* GG in Finland. *Clin Infect Dis* 35:1155-1160.

Salminen MK, S Tynkkynen, H Rautelin, T Poussa, M Saxelin, M Ristola, V Valtonen, A Jarvinen. 2004. The efficacy and safety of probiotic *Lactobacillus rhamnosus* GG on prolonged, noninfectious diarrhea in HIV patients on antiretroviral therapy: a randomized, placebo-controlled, crossover study. *HIV Clin Trials* 5:183-191.

Saxelin M, S Elo, S Salminen, H Vapaatalo. 1991. Dose response colonization of faeces after oral administration of *Lactobacillus casei* strain GG. *Microb Ecol Health Dis* 4:209-214

Saxelin M, M Ahokas, S Salminen. 1993. Dose response on the faecal colonization of *Lactobacillus* strain GG administered in two different formulations. *Microb Ecol Health Dis* 6:119-122.

Saxelin M, T Pessi, S Salminen. 1995. Fecal recovery following oral administration of *Lactobacillus* strain GG (ATCC 53103) in gelatine capsules to healthy volunteers. *Int J Food Microb* 25:199-203

Saxelin M, H Rautelin, B Chassy, SL Gorbach, S Salminen, P Mäkelä. 1996a. Lactobacilli and bacteremia in Southern Finland during 1989-1992. *Clin Infect Dis* 22:564-566.

Saxelin M, H Rautelin, S Salminen, P Mäkelä. 1996b. Safety of commercial products with viable *Lactobacillus* strains. *Infect Dis Clin Pract* 5:331-335.

Schultz M, HJ Linde, N Lehn, K Zimmermann, J Grossmann, W Falk, J Scholmerich. 2003. Immunomodulatory consequences of oral administration of *Lactobacillus rhamnosus* strain GG in healthy volunteers. *J Dairy Res* 70:165-173.

Schultz M, C Gottl, RJ Young, P Iwen, JA Vanderhoof. 2004. Administration of oral probiotic bacteria to pregnant women causes temporary infantile colonization. *J Pediatr Gastroenterol Nutr* 38:293-297.

Sepp E, M Mikelsaar, S Salminen. 1993. Effect of administration of *Lactobacillus casei* strain GG on the gastrointestinal microbiota of newborns. *Microb Ecol Health Dis* 6:309-314.

Sheen P, RA Oberhelman, RH Gilman, L Cabrera, M Verastegui, G Madico. 1995. Short report; a placebo-controlled study of *Lactobacillus* GG colonization in one-to-three-year-old Peruvian children. *Am J Trop Med Hyg* 52:389-392.

Sheih Y-H, B-L Chiang, L-H Wang, C-K Liao, HS Gill. 2001. Systemic immunity-enhancing effects in healthy subjects following dietary consumption of the lactic acid bacterium *Lactobacillus rhamnosus* HN001. *J Am Coll Nutr* 20:149-156.

Shornikova A-V, E Isolauri, L Burkanova, S Lukovnikova, T Vesikari. 1997. A trial in the Karelian Republic of oral rehydration and *Lactobacillus* GG for treatment of acute diarrhoea. *Acta Pædiatr* 86:460-465.

Shu Q, JS Zhou, KJ Rutherfurd, MJ Birtles, J Prasad, PK Gopal, HS Gill. 1999. Probiotic lactic acid bacteria (*Lactobacillus acidophilus* HN017, *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019) have no adverse effects on the health of mice. *Int Dairy J* 9:831-836.

Siitonen S, H Vapaatalo, S Salminen, NA Gordin, M Saxelin, R Wikberg, AL Kirkkola. 1990. Effect of *Lactobacillus* GG yoghurt in prevention of antibiotic associated diarrhoea. *Ann Med* 22:57-59.

Sistek D, R Kelly, K Wickens, T Stanley, P Fitzharris, J Crane. 2006. Is the effect of probiotics on atopic dermatitis confied to food sensitized children? *Clin Exper Allergy* 36:629-633.

Stansbridge EM, V Walker, MA Hall, SL Smith, MR Millar, C Bacon, S Chen. 1993. Effects of feeding premature infants with *Lactobacillus* GG on gut fermentation. *Arch Dis Child* 69:488-492.

Szajewska H, M Kotowska, JZ Mrukowicz, M Armanska, W Mikolajczyk. 2001. Efficacy of *Lactobacillus* GG in prevention of nosocomial diarrhea in infants. *J Pediatr* 138:361-365.

Szymanski H, J Pejcz, M Jawien, A Chmielarczyk, M Strus, PB Heczko. 2006. Treatment of acute infectious diarrhoea in infants and children with a mixture of three *Lactobacillus rhamnosus* strains—a randomized, double-blind, placebo-controlled trial. *Aliment Pharmacol Ther* 23:247-253.

Tannock GW, K Munro, HJM Harmsen, GW Welling, J Smart, PK Gopal. 2000. Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. *Appl Envir Microbiol* 66:2578-2588.

Temmerman R, B Pot, G Huts, J Swings. 2003. Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *Int J Food Microbiol* 81:1-10.

Teuber M, L Meile, F Schwarz. 1999. Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie van Leeuwenhoek* 76:115-137.

Thomas MR, SC Litin, DR Osmon, AP Corr, AL Weaver, CM Lohse. 2001. Lack of effect of *Lactobacillus* GG on antibiotic-associated diarrhea: a randomized, placebo-controlled trial. *Mayo Clin Proc* 76:883-889.

USDA/ARS. 1997. *Data tables: results from USDA's 1994-96 CSFII, Table Set 10.* Available on-line at http://www.ars.usda.gov/sp2userfiles/place/12355000/pdf/csfi3yr.pdf

Uttley AHC, RC George, J Naidoo, N Woodford, AP Johnson, CH Calms, D Morrison, AF Gilfilian, LE Fitch, J Heptonstall. 1989. High-level vancomycin-resistant enterococci causing hospital infections. *Epidemiol Infect* 103:173-181.

Vandenplas Y, S Salvatore, M Viera, T Devreker, B Hauser. 2007. Probiotics in infectious diarrhoea in children: are they indicated? *Eur J Pediatr* 166:1211-1218.

Vanderhoof JA, DB Whitney, DL Antonson, TL Hanner, JV Lupo, RJ Young. 1999. *Lactobacillus* GG in the prevention of antibiotic-associated diarrhea in children. *J Pediatr* 135:564-568.

Vankerckhoven V, G Huys, M Vancanneyt, C Vael, I Klare, M-B Romond, JM Entenze, P Moreillon, RD Wind, J Knol, E Wiertz, B Pot, EE Vaughan, G Kahlmeter, H Goossens. 2007a. Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. *Trends Food Sci Technol* 19:102-114.

Vankerckhoven V, P Moreillon, S Piu, M Giddey, G Huys, M Vancanneyt, H Goossens, JM Entenza. 2007b. Infectivity of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* isolates in a rat model of experimental endocarditis. *J Med Microbiol* 56:1017-1024.

Vendt N, H Grünberg, O Malminiemi, T Tuure, E Wuolijoki, V Tillmann, E Sepp, R Korpela. 2006. Growth during the first 6 months of life in infants using formula enriched with *Lactobacillus rhamnosus* GG: double blind, randomised trial. *J Hum Nutr Dietet* 19:51-58.

Viljanen M, E Savilahti, T Haahtela, K Juntunen-Backman, R Korpela, T Poussa, T Tuure, M Kuitunen. 2005. Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double-blind placebo-controlled trial. *Allergy* 60:494-500.

Vitenskapskomiteen for Mattrygghet (VKM; Norwegian Scientific Committee for Food Safety). 2007. *Risk assessment on use of* Lactobacillus rhamnosus *(LGG) as an ingredient in infant formula and baby foods*. June.

Voravuthikunchai SP, S Bilasoi, O Supamala. 2006. Antagonistic activity against pathogenic bacteria by human vaginal lactobacilli. *Anaerobe*. 2006; 12:221-226.

Wagner RD, T Warner, L Roberts, J Farmer, E Balish. 1997a. Colonization of congenitally immunodeficient mice with probiotic bacteria. *Infect Immun* 65:3345-3351.

Wagner RD, C Pierson, T Warner, M Dohnalek, J Farmer, L Roberts, M Hilty, E Balish. 1997b. Biotherapeutic effects of probiotic bacteria on candidiasis in immunodeficient mice. *Infect Immun* 65:4165-4172.

Wagner RD, M Dohnalek, M Hilty, A Vazquez-Torres, E Balish. 2000. Effects of probiotic bacteria on humoral immunity to *Candida albicans* in immunodeficient *bg/bg-nu/nu* and *bg/bg-nu/+* mice. *Rev Iberoam Micol* 17:55-59.

Wickens K, P Black, T Stanley, E Mitchell, P Fitzharris, GW Tannock, G Purdie, J Crane. 2008. A differential effect of 2 probiotics in the prevention of eczema and atopy: a double-blind, randomized, placebo-controlled trial. *J Allergy Clin Immunol* 122:788-794.

Woods GL and JA Washington. 1995. Antibacterial susceptibility tests: dilution and disk diffusion methods. In Murray PR, EJ Baron, MA Pfaller, FC Tenover, RH Yolken (Eds.), *Manual of clinical microbiology, 6th edition*. Washington, DC: ASM Press, 1327-1341.

Xu J, W Yan, B Yang, Z Li. 2008. Safety assessment of starters culture bacteria in fermented milk in China. J Hygiene Res 37:193-195. [Abstract; article in Chinese]

Zein EF, S Karaa, A Chemaly, I Saidi, W Daou-Chahine, R Rohban. 2008. Lactobacillus rhamnosus septicemia in a diabetic patient associated with probiotic use: a case report. *Ann Biol Clin (Paris)* 66:195-198. [Article in French with English abstract]

Zetterström R, R Bennet, K-E Nord. 1994. Early infant feeding and micro-ecology of the gut. *Acta Paediatr Jap* 36:562-571.

Zhou JS, Q Shu, KJ Rutherfurd, J Prasad, MJ Birtles, PK Gopal, HS Gill. 2000a. Safety assessment of potential probiotic lactic acid bacterial strains *Lactobacillus rhamnosus* HN001, *Lb. acidophilus* HN017, and *Bifidobacterium lactis* HN019 in BALB/c mice. *Int J Food Microbiol* 56:87-96.

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

Zhou JS, PK Gopal, HS Gill. 2001. Porential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin in vitro. *Int J Food Microbiol* 63:81-90.

Zhou JS, CJ Pillidge, PK Gopal, HS Gill. 2005a. Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. *Int J Food Microbiol* 98:211-217.

Zhou JS, KJ Rutherfurd, HS Gill. 2005b. Inability of probiotic bacterial strains *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019 to induce human platelet aggregation in vitro. *J Food Prot* 68:2459-2464.

Zhou JS and HS Gill. 2005. Immunostimulatory probiotic *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019 do not induce pathological inflammation in mouse model of experimental autoimmune thyroiditis. *Int J Food Microbiol* 103:97-104.

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CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF *LACTOBACILLUS RHAMNOSUS* STRAIN HN001 (DR20™) IN CONVENTIONAL FOODS

Prepared for:
Fonterra Co-operative Group
New Zealand

January 2009

CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF *LACTOBACILLUS RHAMNOSUS* STRAIN HN001 (DR20™) IN CONVENTIONAL FOODS

We, the members of the expert panel, have individually and collectively critically evaluated the publicly available information on *Lactobacillus rhamnosus* strain HN001 and other *L. rhamnosus* strains summarized in a monograph prepared by JHEIMBACH LLC, as well as other material deemed appropriate or necessary. Our evaluation included review of the identity and characteristic properties of *L. rhamnosus*, including *L. rhamnosus* strain HN001, the potential exposure resulting from the intended use of *L. rhamnosus* strain HN001, and published research bearing on the safety of *L. rhamnosus* and strain HN001. Our summary and conclusion resulting from this critical evaluation are presented below.

Summary

- The probiotic bacterium that is the subject of this generally recognized as safe (GRAS) determination is *L. rhamnosus* strain HN001, a Gram-positive and non-spore-forming heterofermentative lactic acid bacterium known commercially as DR20™ and as Howaru Rhamnosus™.

- *L. rhamnosus* strain HN001 is intended to be added as a probiotic to foods that can sustain viability of the organism, including but not limited to dairy products (fluid milk and milk drinks, milk-based desserts and meal replacements, dry and powdered milk, yogurt, and cheese); ready-to-eat cereals; fruit juices, nectars, ades, and drinks; confections; chewing gum; and functional/nutritional products at concentrations consistent with cGMP needed to provide up to 10^9 cfu/serving throughout the shelf life of the product. The estimated daily intake of the strain is less than 10^{11} cfu/day.

- *L. rhamnosus* strain HN001 was isolated from cheddar cheese in New Zealand and was deposited with the Australian Government Analytical Laboratories (AGAL) as deposit number NM97/09513.

- *Lactobacillus* is a non-pathogenic genus consisting of over a hundred species with a large variety of phenotypic, biochemical, and physiological properties. *L. rhamnosus* is ubiquitous in the GI tracts of both humans and infra-human mammalian species and has long been ingested during normal food-consumption activities with no apparent adverse effects.

- The whole genome shotgun sequence of *L. rhamnosus* strain HN001 was determined and the resulting sequence was annotated and analysed for genes that may be possible safety concerns. The genome sequence and annotation of the genome and two plasmids were

submitted to the NCBI and published with Refseq accession numbers NZ_ABWJ00000000, NC_011223, and NC_011225.

- No genes encoding for antibiotic resistance, nor any sequences showing significant homology with known antibiotic resistance genes, were identified on either of the plasmids. A range of genes possibly involved in antibiotic resistance were identified in the HN001 genome, but none were closely linked to predicted mobile genetic elements and are thus unlikely to be transferable. This finding is consistent with phenotypic testing that showed that *L. rhamnosus* strain HN001 has no resistance not found in most other members of the species, and thus regarded as intrinsic rather than acquired.

- No genes were identified that are likely to be capable of producing biogenic amines, and none that would indicate a likelihood of virulence or infectivity. These findings are consistent with phenotypic data showing no production of biogenic amines, unusual adherence capability, mucin degradation, adverse metabolic activity, or infectivity.

- Production of *L. rhamnosus* strain HN001 is based on standard fermentation techniques, and all fermentation medium components are food-grade materials. Fonterra has provided food-grade specifications for *L. rhamnosus* strain HN001.

- Several studies in which *L. rhamnosus* strain HN001 was given to BALB/c mice at doses up to 10^{11} cfu/day produced no adverse effects. This finding is consistent with the general absence of adverse effects in studies in which other *L. rhamnosus* strains were administered to mice, hamsters, rats, and rabbits at doses as high as 10^{12} cfu/day.

- The safety of *L. rhamnosus* strain HN001 was also assessed in five studies with human adults, one with children, and one with infants. No adverse effects were observed in any of these studies. The safety of *L. rhamnosus* is supported by a large body of human studies of other *rhamnosus* strains—30 in adults, 24 in children, and 22 in infants—involving over 9000 individuals, with not a single report of an adverse effect other than increased wheezing in one study.

- The European Food Safety Authority (EFSA) classified *L. rhamnosus* as an organism having a Qualified Presumption of Safety (QPS) and thus being "freed from the need for further safety assessment." Similarly, in 2008 the FDA had no questions regarding Mead Johnson's determination that the addition of *L. rhamnosus* strain GG to infant formula is GRAS.

Conclusion

We, the undersigned expert panel members, are qualified by scientific education and experience to evaluate the safety of the addition of probiotic bacteria to conventional foods. We have individually and collectively critically evaluated the materials summarized above.

We recognize that *Lactobacillus* species have a long history of safe use and are appropriately regarded as non-pathogenic and non-toxicogenic. We conclude that *Lactobacillus rhamnosus* strain HN001 has been adequately identified and characterized and that both phenotypic and genotypic research confirm that no concerns exist regarding the safety of ingestion of this probiotic bacterium at levels up to 10^{10} cfu/day. Therefore, we conclude that addition of *L. rhamnosus* strain HN001 to conventional foods as described is safe.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusion. Therefore, the intended use of *L. rhamnosus* strain HN001 is safe, and is GRAS, via scientific procedures.

Joseph F. Borzelleca, Ph.D.
Professor Emeritus
Virginia Commonwealth University School of Medicine
Richmond, Virginia

Signatur _____ Date: 12 January 2009

Walter H. Glinsmann, M.D.
President
Glinsmann Inc.
Arlington, Virginia

Signature: _____ Date: _____

Michael W. Pariza, Ph.D.
Director, Food Research Institute
University of Wisconsin—Madison
Madison, Wisconsin

Signature: _____ Date: _____

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University of Wisconsin—Madison
Madison, Wisconsin

Signature: _____ Date: _____

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Arlington, Virginia

Signature: _____ Date: _____

Michael W. Pariza, Ph.D.
Director, Food Research Institute
University of Wisconsin—Madison
Madison, Wisconsin

Signature: _____ Date: _____

000158

SUBMISSION END

Harry, Molly *

From: Jim Heimbach [jh@jheimbach com]
Sent: Monday, June 22, 2009 4·53 PM
To: Harry, Molly *
Cc: Gaynor, Paulette M
Subject: Re: Questions on GRN 000288
Attachments: FDA QA on GRN 288.20090622.pdf

Molly--

Here are responses to the questions you asked concerning GRN 288. Please let me know if additional questions arise during your review

Regards,
Jim

James T. Heimbach, Ph D., F.A.C.N.
JHeimbach LLC
923 Water Street, P O Box 66
Port Royal VA 22535
tel (+1) 804-742-5548
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e-mail jh@jheimbach com

----- Original Message -----
From: Harry, Molly *
To· Jim Heimbach
Cc: Gaynor, Paulette M
Sent Wednesday, June 03, 2009 4:30 PM
Subject. Questions on GRN 000288

Hi Dr. Heimbach,
The review of GRAS Notice 288 (Lactobacillus rhamnosus strain HN001) is ongoing, and the review team has the following questions:
1. The intended uses are stated to be "those foods which can sustain viable L. rhamnosus for the shelf life of the food, including but not limited to dairy products (fluid milk and milk drinks, milk-based desserts and meal replacements, dry and powdered milk, yogurt, and cheese); ready-to-eat cereals; fruit juices, nectars, ades, and drinks; confections; chewing gum; and functional/nutritional products " (p. 1 of notice)
Please clarify the following:
In your pre-submission meeting, you indicated that ready-to-eat cereals and fruit juices, nectars, ades, and drinks included baby foods (i e infant foods). Please indicate for these foods and for milk products if adult and infant foods are included among the intended uses

b) Please clarify the foods and/or food categories encompassed by "confections" and "functional/nutritional products".
c) Are meat and poultry products included among the intended uses of L. rhamnosus?
d) Please clarify the type of foods which can "sustain" viable L. rhamnosus. For example, are these limited to refrigerated dairy products, refrigerated liquids (e.g. juices), and dried shelf-stable foods or all potential foods listed within the food categories listed above?
Please describe the method and assumptions used to estimate exposure to L. rhamnosus. While we are aware of a brief description provided in the presentation at the pre-submission meeting, the details are not provided in the notice. Further, the wording within GRN 288 suggests a more inclusive list of foods than suggested at the pre-submission meeting. In your description, please indicate if the estimate provided in the notice is based on

potential intake from all conventional foods to which L rhamnosus might be added or to a specific subset

3 The notice describes the stability of the organism in a freeze dried powder stored under nitrogen at room temperature and in infant formula held at 30 degrees Celsius for 1+ year. What is known about the stability of L. rhamnosus in the products listed among your intended uses in GRN 288?

4. After production the notifier reports that the product is sent out to an internationally accredited laboratory before release. The notice reports that the laboratory checked on the purity (other organisms, and metals) physical properties (particle size, color and odor). Can the notifier clearly state how or if the strain they are using has been verified as L rhamnosus HN001.

5. The notifier says that the strain they are using, which is deposited with the Australian Government Analytical Laboratories (deposit # NM97/09513) is a pure culture of L. rhamnosus HN001 According to the World Intellectual Property Organization, deposit # NM97/09513 refers to Bifidobacterium lactis HN019, while deposit # NM97/09514 is biologically pure L. rhamnosus HN001. Please clarify

6. The notifier makes several statements about "strong homology", "significant homology" or "closely linked" genes, but does not explain what these qualifiers mean. Can the notifier describe the criteria that were used to determine "strong" or "significant" homology or "closely linked" genes?

We will forward any additional questions that may arise as the review goes on. Please feel free to contact me if you have any questions.

Sincerely,
Molly Harry, M.S
Division of Biotechnology
and GRAS Notice Review

JHeimbach LLC

June 22, 2009

RE: Questions Concerning GRN 288 (*Lactobacillus rhamnosus* strain HN001)

Molly Harry, M.S.
Division of Biotechnology
and GRAS Notice Review
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Ms. Harry:

Following are responses to the questions you asked about GRAS notice GRN 288 in your e-mail of June 3.

1. The intended uses are stated to be "those foods which can sustain viable L. rhamnosus for the shelf life of the food, including but not limited to dairy products (fluid milk and milk drinks, milk-based desserts and meal replacements, dry and powdered milk, yogurt, and cheese); ready-to-eat cereals; fruit juices, nectars, ades, and drinks; confections; chewing gum; and functional/nutritional products." (p. 1 of notice)

Please clarify the following:

a) In your pre-submission meeting, you indicated that ready-to-eat cereals and fruit juices, nectars, ades, and drinks included baby foods (i.e. infant foods). Please indicate for these foods and for milk products if adult and infant foods are included among the intended uses.

Yes, adult foods, toddler foods, and baby foods (such as fruit juices, strained fruit or vegetables, and infant cereals) are all among the intended uses. Additionally, dairy-based maternal pre- and post-natal nutritional products (for pregnant women and nursing mothers) are included.

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b) Please clarify the foods and/or food categories encompassed by "confections" and "functional/nutritional products".

Confectionery items include sweets, hard candies, lollipops, toffees, candy bars, chocolate, cotton candy, and other sweet items of snack food. The term does not generally apply to cakes, biscuits, or puddings which require cutlery to consume.

Functional/nutritional products has the usual meaning of any fresh or processed food intended or claimed to have a health-promoting property beyond the basic function of supplying nutrients.

c) Are meat and poultry products included among the intended uses of L. rhamnosus?

No.

d) Please clarify the type of foods which can "sustain" viable L. rhamnosus. For example, are these limited to refrigerated dairy products, refrigerated liquids (e.g. juices), and dried shelf-stable foods or all potential foods listed within the food categories listed above?

It is intended that uses may include both foods for which current technology ensures sustainability and also those for which such technology may be developed in the future. *L. rhamnosus* has differing levels of stability in each listed application. It is the notifier's intention both to expand business in areas of good stability and to develop technology to improve stability in areas of current weakness. The strain has excellent stability in milk powders at temperatures as high as 35°C and water activity of 0.2. These values cover a number of applications. Stability of strain HN001 has also been demonstrated in yogurt, liquid yogurt, and cheddar cheese—refrigerated dairy products—and, to a lesser extent, in fruit juice.

2. *Please describe the method and assumptions used to estimate exposure to L. rhamnosus. While we are aware of a brief description provided in the presentation at the pre-submission meeting, the details are not provided in the notice. Further, the wording within GRN 288 suggests a more inclusive list of foods than suggested at the pre-submission meeting. In your description, please indicate if the estimate provided in the notice is based on potential intake from all conventional foods to which L. rhamnosus might be added or to a specific subset.*

The exposure estimate provided in the GRAS notice includes estimated potential intake from all intended uses of the strain. As noted, the intended addition level of *L. rhamnosus* strain HN001 to foods is one that is sufficient to ensure that the bacterium remains present at a level of 10^9 cfu/serving of the food throughout its shelf life. What that addition level is will vary by food category, since the stability of the organism is different in different foods and because different categories of foods have different shelf lives. The foods that are most widely consumed for their probiotic benefits are dairy products such as yogurt, and these are also the foods in which *L. rhamnosus* is most stable. Consequently, these are foods in which less overage in probiotic addition is needed than some other foods.

The estimated daily intake of *L. rhamnosus* strain HN001 from the intended use is up to 10^{11} cfu/day. If no overage were needed in adding the probiotic to foods, or if all foods were consumed late in their shelf life when bacterial count had declined, this level of intake would be achieved only by those who consumed 100 servings/day of foods supplemented with *L. rhamnosus*. A more reasonable estimate of the likely average level of *L. rhamnosus* in supplemented foods is between 5×10^9 and 10^{10} cfu/serving. At these concentrations, between 10 and 20 servings of foods containing the strain are required to ingest 10^{11} cfu. Since the average individual eats only about 20 servings per day of all food combined*, and it is most unlikely that half or more of them will include *L. rhamnosus* strain HN001, 10^{11} cfu/day is a very conservative estimate of potential daily intake.

*For example, Millen et al. (2006) found that women in their study consumed an average of 15.3 servings of grain, vegetables, fruit, dairy, and protein sources combined, while the average man consumed 23.4 servings.

Millen AE, D Midthune, FE Thompson, V Kipnis, AF Subar. 2006. The National Cancer Institute Diet History Questionnaire: Validation of pyramid food servings. *Am J Epidemiol* 163:279-288.

3. *The notice describes the stability of the organism in a freeze dried powder stored under nitrogen at room temperature and in infant formula held at 30 degrees Celsius for 1+ year. What is known about the stability of L. rhamnosus in the products listed among your intended uses in GRN 288?*

In all applications, the stability of the probiotic is tested at inoculation and over the entire shelf life to show viable count throughout the life of the finished product. Depending upon the product, the stability varies and so do the shelf-life requirements (temperature, time, etc). As noted earlier, stability has been demonstrated in milk-based powders as well as such refrigerated dairy products as yogurt, liquid yogurt, and cheddar cheese. Limited data indicate satisfactory stability in fruit juices.

4. *After production the notifier reports that the product is sent out to an internationally accredited laboratory before release. The notice reports that the laboratory checked on the purity (other organisms, and metals) physical properties (particle size, color and odor). Can the notifier clearly state how or if the strain they are using has been verified as L. rhamnosus HN001.*

   Fonterra ensures (as shown in the flow chart covering manufacture of strain HN001 on page 13 of the GRAS notice) that the fermenter is inoculated with a pure culture of *L. rhamnosus* strain HN001 withdrawn from the cryobank. At the end of the ,manufacturing process, Q/A on each batch is performed for foreign matter, microbial contamination, color, odor, and lactobacillus count and activity (using method NZTM2 132.72, a copy of which is attached). Although this test method determines colony identity based on cell morphology and consequently cannot distinguish specific strains, it does ensure that the bacteria present are *Lactobacillus*. Since fermentation is started with a pure culture of *L. rhamnosus* strain HN001, there is no reason to doubt that the lactobacilli present in the final product would be anything but strain HN001.

5. *The notifier says that the strain they are using, which is deposited with the Australian Government Analytical Laboratories (deposit # NM97/09513) is a pure culture of L. rhamnosus HN001. According to the World Intellectual Property Organization, deposit # NM97/09513 refers to Bifidobacterium lactis HN019, while deposit # NM97/09514 is biologically pure L. rhamnosus HN001. Please clarify.*

   I apologize. As I noted in my response to FDA's questions concerning GRN 281, this is a simple typographical error. The deposit number for *L. rhamnosus* strain HN001 is indeed #NM97/09514 rather than 09513.

6. *The notifier makes several statements about "strong homology", "significant homology" or "closely linked" genes, but does not explain what these qualifiers mean. Can the notifier describe the criteria that were used to determine "strong" or "significant" homology or "closely linked" genes?*

   In Section 2.4.4.1, we stated: "No genes encoding for antibiotic resistance, nor any sequences showing significant homology with known antibiotic resistance genes, were identified on either of the plasmids."

   For the sequences in question, the output of the NCBI's PGAAP and Integrated Genomics annotation process (explained in Section 2.4.2) were examined for any genes, protein domains, or clusters of orthologous groups associated with antibiotic resistance, none were identified. The PGAAP output included:

   - BLAST searching of the NCBI Bactprot database (containing all proteins from complete prokaryotic chromosomes and plasmids from RefSeq records) with an expect threshold of 1e-06 and the top five BLAST hits returned;

- RPS-BLAST searches with an expect threshold of 1e-02 of the NCBI Conserved Domain Database for domain assignment; and

- COGnitor searching of the NCBI COG database.

The expect threshold specifies the statistical significance threshold for reporting matches against database sequences.

In 2.4.4.1, we also stated: "A range of other genes possibly involved in antibiotic resistance were identified in the HN001 genome, but none were closely linked to predicted mobile genetic elements and are thus unlikely to be transferable."

Of the genes identified that could possibly be involved in antibiotic resistance, those where any transferrable elements were identified on the same contig sequence are described below:

- A gene annotated as encoding a "multidrug resistance protein B" is at one end of an 8.5 kb contig, with a single transposase at the other end of this contig. The presence of a single transposon is expected to be insufficient to enable transfer of over 8 kb of DNA.

- A 141 kb contig contains several putative antibiotic/drug export proteins. This contig has at its 5'-extremity an integrase/transposase-like protein. 70 kb from this is a protein annotated as phage-infection protein, and a further 15 kb from this is a putative plasmid-related RepB family protein (although its annotation as a RepB protein by Integrated Genomics is difficult to understand, as BLAST searching of this sequence against the NR database at NCBI with an expect threshold of 0.1 identifies no similarity to any Rep proteins). Another 45 kb from this is a second putative phage-infection protein. The 141 kb contig carries two genes annotated as encoding "multidrug resistance protein B." One of these genes is about 9 kb upstream from the integrase/transposase-like protein and 60 kb downstream from one of the possible phage-infection proteins, while the other "multidrug resistance protein B" gene is about 13 kb upstream from the putative repB protein and 30 kb downstream from the second phage infectivity protein. Finally, a putative β-lactamase family protein is 10 kb from the RepB and 35 kb from the second prolactin-induced protein.

- One 165 kb contig carries a putative ABC-type multidrug resistance transporter; the genes for this are 90 kb from a transposase sequence.

- A 71 kb contig carries a possible tetronasin resistance operon, this is 30 kb away from a possible transposon.

- Finally, an 83 kb contig carries a possible efflux pump antibiotic resistance protein, this is 62 kb from a possible transposase.

The arrangement of these possible antibiotic resistance genes and genes associated with mobile genetic elements seem very unlikely to lead to horizontal gene transfer of the sequences in question, which may or may not encode true antibiotic resistance proteins.

In Section 2.4.4.4, we stated: "... one sequence shows strong homology to a putative hemolysin..."

The strong homology here was a BLAST match in the top five BLAST matches returned by the PGAAP annotation procedure, with an expect threshold of 2e-122 to the sequence in question.

NZTM2 132.72

Pro-biotic Culture DR 20
Method for Counting Freeze Dried Powder or Milk Powders containing DR20

1.1 Scope

This method is for the enumeration of freeze-dried DR20 (*Lactobacillus rhamnosus*) probiotic culture and dry blended milk powders containing DR20.

1.2 Validation Status

This method is sourced from:
"The survival of freeze-dried bacterial cultures after blending in milk powders". J T Harnett. 1997. Report NZDRI-1997-111. New Zealand Dairy Research Institute, Palmerston North.
This method is not validated.

1.3 Principle

A defined test portion or a series of decimal dilutions of the sample is mixed with MRS Agar (MRSA) and incubated anaerobically at 37 °C for 72 hours.
The number of microorganisms per gram of the original sample is calculated from the number of colonies on selected plates.

1.4 Apparatus

- A balance weighing to 0.1g
- A stomacher e.g. Stomacher 400 (Seaward Medic Lab), or an equivalent
- A vortex mixer
- A waterbath controlled to 45 ±□1 °C
- The GasPak system or equivalent
- An incubator controlled to 37 ±1 °C

1.5 Diluent and Medium

0.1% peptone

MRS Agar

Beef Extract	10.0 g
Protease peptone No.3	10.0 g
Yeast Extract	5.0 g
Dextrose	20.0 g
Polysorbate 80	1.0 g
Ammonium citrate	2.0 g
Sodium acetate	5.0 g
Magnesium sulphate	0.1 g
Managanese sulphate	0.05 g
Dipotassium phosphate	2.0g
Agar	15 g
Distilled or deionised water	1000 mL

pH 6.8 ±□0.2 at 25℃.

Add the ingredients to the water. Mix thoroughly and heat with frequent agitation just sufficiently to completely dissolve the ingredients. Adjust the pH so that after sterilisation it is 6.8 ±□0.2 at 25℃. Sterilise at a maximum temperature of 121℃ for 15 mins.

1.6 Procedure

The performance of new batches of MRSA should be checked using a laboratory culture of *Lactobacillus rhamnosus*.
A culture of *Lactobacillus rhamnosus* should be used as a positive control during testing

Preparation of Sample

1 Weigh out 10 g of powder sample into a sterile stomacher bag
2 Add 90 mL of 'room temperature' (20 ±□1℃) 0.1% peptone to the powder sample and stomach on high for 2 minutes to create an homogenised mixture.
3 Allow the stomached sample to stand at room temperature for 30 minutes.
4 Stomach on high for 1 minute.

Dilutions

1 Prepare a dilution by transferring 1 mL of prepared sample to 9 mL of sterile 0.1% diluent. Mix by vortexing for 5-10 seconds.
2 Transfer 1 mL of this diluted sample to a second 9 mL of 0.1% peptone.
3 Repeat step 2 for the number of dilutions required, each time adding 1 mL of the previous dilution to 9 mL of 0.1% peptone.

Plating

1 Transfer 1 mL of the selected dilutions to each of four replicate Petri dishes.
2 Pour about 15 mL of molten MRSA agar tempered to 45 ±1 °C into each Petri dish.
3 Mix immediately after pouring into each Petri dish sufficiently to obtain evenly dispersed colonies after incubation. Allow agar to solidify at room temperature.
   Prepare the following test control plates:
   - *Blank Control Plates*:
     Pour at least one plate from each batch of MRSA used. Leave uninoculated.
   - *Positive Control Plate*:
     Inoculate a diluted sample of the positive control organism into a sterile Petri dish. Add 15 – 18 mL of MRSA tempered to 45 ± 1 °C. Mix well.
4 Incubate all plates anaerobically at 37 ±1 °C for 72 ± 3 hours.

Interpretation

1 Examine the plates for the presence of typical colonies using a colony counter in subdued light.
   Note: typical DR20 colonies are cream coloured with a shiny convex surface.
   Colonies are 2 – 4 mm in diameter.
2 Select all replicate sample plates, at two successive dilutions if applicable containing between 10 and 300 colonies.

1.7 Calculation and Report

Count and record the number of colonies on each selected plate.
Average the count obtained from each set of four replicate plates.
Express the result as colony forming units per gram.

1.8 Precision

Precision values are not available for this method.

Harry, Molly *

From: Jim Heimbach [jh@jheimbach.com]
Sent: Friday, August 07, 2009 3:51 PM
To: Harry, Molly *
Cc: Gaynor, Paulette M
Subject: Re: Questions on GRN 000288
Attachments: FDA 2nd QA on GRN 288 20090807 pdf; Ascorbic Acid Specification.pdf; COA - Yeast Extract Gistex LS.pdf; COA Ascorbic Acid.pdf; COA Dextrose.tif; COA Magnesium Sulfate.pdf; COA Maltodextrin.pdf, COA Manganese Sulfate.pdf, COA Potassium Diphosphate.pdf; COA Sodium Acetate.pdf, COA Sorbitol P pdf; COA Sucrose.pdf, COA Trisodium Citrate.pdf; NZTM1 D7.pdf, NZTM2 43.1 pdf; NZTM2 43.2 pdf, NZTM2 44.1.pdf; NZTM2 45.2.pdf; NZTM2 46.1.pdf; NZTM2 47 2 pdf; NZTM2 48 5.pdf, NZTM2 50 1 pdf; NZTM2 53.2.pdf; NZTM2 58.2.pdf; NZTM2 59 1 pdf; NZTM2 59.3 pdf; NZTM2 60 1 pdf; NZTM2 61.1.pdf, NZTM2 132 72 pdf

Dear Molly--

Attached are responses to your questions, copies of specification or analytical-results sheets for substances used in the growth medium and cryoprotectant, and descriptions of New Zealand technical analytical methods.

I'm looking forward to our telephone call on Monday

Regards,
Jim

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----- Original Message -----
From: Harry, Molly *
To: Jim Heimbach
Cc: Gaynor, Paulette M
Sent: Thursday, July 30, 2009 4:34 PM
Subject: Questions on GRN 000288

Dear Dr. Heimbach,

We have identified the following additional questions regarding this notice:

1. The notifier provides information from studies done using *L. rhamnosus* GG, however, on page 7, the notifier states that "It is clear from the PFGE patterns that strain HN001 is not very closely related to strain GG---". Please provide a more thorough analysis in which *L rhamnosus* GG and *L. rhamnosus* HN001 are more closely compared, especially for traits involved in virulence.

2. In Section 2.5 "Production Process" on page 11 of the notice polysorbate is mentioned as a component of the growth medium. Please provide a brief update of the method of manufacture, including any changes in ingredients used therein.

3. The notifier states that growth medium components are "food grade", however, it is not clear if the production process complies with current good manufacturing practices (cGMP). Please provide a description of the ingredients used in the method of manufacture, including specifications (or reference to FCC specifications where applicable), statement of use in compliance with FDA regulations, and a positive statement of compliance with cGMPs

4. The specifications on Table 2 (page 14) and tests on Table 1 (page 12) of the submission cite methods from the New Zealand Technical Association. Please provide copies of these methods or a description of each of the methods cited.

5. Section 2.6 (page 14) Specifications for *L. rhamnosus* HN001 include protein 60-70 g/100g. Please clarify how the protein content was determined in the final ingredient For completeness, please provide an updated specifications table

6. GRN 000288 specifically states that the intended uses include infant foods. 21CFR105.3(e) defines the term infant as persons not more than 12 months old. Previous GRAS notices for use of similar ingredients in infant formula have excluded immunocompromised infants from the population intended to consume the ingredient. Please discuss the inclusion/exclusion of immunocompromised individuals in the list of potential users.

Please feel free to contact us if you have any questions

Sincerely,

Molly Harry, M.S.
Division of Biotechnology
and GRAS Notice Review

JHeimbach LLC

RECEIVED
AUG - 7 [redacted]
Division of Biotechnology and GRAS Notice Review

August 7, 2009

RE: Additional Questions Concerning GRN 288 (*Lactobacillus rhamnosus* strain HN001)

Molly Harry, M.S.
Division of Biotechnology
and GRAS Notice Review
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Ms. Harry:

Following are responses to the additional questions you asked about GRAS notice GRN 000288 in your e-mail of July 30.

Sincerely,

(b)(6)

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

923 Water Street, P.O. Box 66, Port Royal Virginia 22535, USA
tel. (+1) 804-742-5548 fax (+1) 202-478-0986 jh@jheimbach.com

1. The notifier provides information from studies done using L. rhamnosus GG, however, on page 7, the notifier states that "It is clear from the PFGE patterns that strain HN001 is not very closely related to strain GG---". Please provide a more thorough analysis in which L. rhamnosus GG and L. rhamnosus HN001 are more closely compared, especially for traits involved in virulence.

PFGE is a technique to differentiate microorganisms at the strain level. The comparison is from a taxonomic perspective and is not necessarily indicative of phenotypic similarity or difference; thus, it tells us nothing regarding virulence or other traits. It is possible for two strains of *Lactobacillus rhamnosus* to be identical in all phenotypic characteristics and indistinguishable by other methods, but yet be differentiated by PFGE. The purpose of Figure 2 on page 7 is to demonstrate that *L. rhamnosus* HN001 is not *L. rhamnosus* GG and is not a re-isolate or a "twin" of that strain, but rather a different strain.

The discussion of safety and virulence is more appropriate at the species level, and at that level both strains, HN001 and GG, belong to species *rhamnosus.* Therefore it is valid to draw parallels regarding safety characteristics at the species level, and it is for that reason that research studies on LGG were included in the HN001 monograph. Genomic analyses of both strains have been completed and published; that of HN001 is discussed in the GRAS monograph and that of LGG has been discussed in other places (e.g., in GRN 000231). Neither strain harbors genes that are associated with virulence characteristics. Indeed, *Lactobacillus* species are generally regarded as non-pathogenic, and thus there is no basis on which to compare the potential for virulence between the two strains.

2. In Section 2.5 "Production Process" on page 11 of the notice polysorbate is mentioned as a component of the growth medium. Please provide a brief update of the method of manufacture, including any changes in ingredients used therein.

The listing of polysorbate is an error—the use of polysorbate was discontinued some time ago, while sorbitol is used. Other than that the description as provided in the GRAS notice is correct. As requested, the method of manufacture is summarized below, unchanged (except for the replacement of polysorbate by sorbitol) from the GRAS notice:

Production of *L. rhamnosus* strain HN001 begins with preparation directly from the production master stock of approximately 30 batches of production working stock, which are small aliquots in cryovials held at -80°C. Renewal of the production working stock occurs approximately once every 5 years. The Fonterra Microbial Fermentation Unit at Palmerston North is certified as compliant with all requirements of standard ISO 9001:2000 for the manufacture of starters including probiotics. The culturing of *L. rhamnosus* strain HN001 is carried out through the following steps, described schematically in Figure 3. All culturing steps take place in a defined growth medium based on reconstituted skim milk (RSM), which additionally includes yeast extract, dextrose, magnesium sulfate, sodium acetate, manganese sulfate, dibasic potassium phosphate, trisodium citrate, and sorbitol. The cryoprotectant (added at step 4 below) consists of maltodextrin, sucrose, and ascorbic acid. All components of the growth medium and the cryoprotectant are food grade.

1. Working seed vials are prepared containing 1-2 ml aliquots of viable cells and held at -80°C.
2. Each seed vial is used to inoculate a small flask which is incubated at 37°C overnight in a small inoculum fermenter until the culture reaches stationary phase. Since *L. rhamnosus* produces lactic acid, ammonium hydroxide is added as needed to maintain the pH of the broth at approximately 5.8.
3. This broth culture is added to the main production fermenter and incubated at 37°C overnight until it reaches stationary phase. Again, ammonium hydroxide is used as needed to maintain the pH at approximately 5.8.
4. The fermenter broth is chilled and concentrated approximately 15-fold through a cell-concentrating centrifuge, and the cyroprotectant is added.
5. The concentrated slurry is poured into sterilized trays which are placed in a lyophilizer at -30°C and under negative pressure.
6. After lyophilization is complete, the freeze-dried powder is milled to specification and vacuum packed under nitrogen; it is maintained at -30°C until quality-assurance is completed.
7. Finally, the frozen and freeze-dried product is tested in an internationally accredited laboratory before release.

3. The notifier states that growth medium components are "food grade", however, it is not clear if the production process complies with current good manufacturing practices (cGMP). Please provide a description of the ingredients used in the method of manufacture, including specifications (or reference to FCC specifications where applicable), statement of use in compliance with FDA regulations, and a positive statement of compliance with cGMPs .

Specifications for all components of the growth medium and the cryoprotectant are attached to this e-mail. Fonterra Cooperative Group, Ltd., certifies that all production steps for *L. rhamnosus* strain HN001 are performed in full compliance with current good manufacturing practice.

4. The specifications on Table 2 (page 14) and tests on Table 1 (page 12) of the submission cite methods from the New Zealand Technical Association. Please provide copies of these methods or a description of each of the methods cited.

The New Zealand Technical Association (NZTA) methods cited in Table 2 on page 14 include the following:

- NZTM2 43.1: Aerobic Plate Count [N.B. Table 2 erroneously cites this analysis as NZTM 43.2]
- NZTM2 44.1: *Bacillus cereus* Count
- NZTM2 46.1: *Clostridium perfringens* Count
- NZTM2 47.2: Coagulase-Positive *Staphylococci* Detection
- NZTM2 48.5: *E. coli* and Coliforms Detection
- NZTM2 50.1: *Enterococci* Count
- NZTM2 53.2: *Listeria* Detection
- NZTM2 58.2: *Salmonella* Detection
- NZTM2 61.1: Yeasts and Molds Count
- NZTM2 132.72: *L. rhamnosus* activity

Table 1 on page 12 cites these same methods along with the following:

- NZTM1 D7 2.3: Stains and Staining Procedures
- NZTM2 45.2: β-Hemolytic *Streptococci* Detection
- NZTM2 59.1: Mesophilic Aerobic Spore Count
- NZTM2 59.3: Sulfite-Reducing Clostridial Spore Count
- NZTM2 60.1: Thermophilic Bacteria Count

Copies of these methods are attached to this e-mail.

5. Section 2.6 (page 14) Specifications for L. rhamnosus HN001 include protein 60-70 g/100g. Please clarify how the protein content was determined in the final ingredient. For completeness, please provide an updated specifications table.

The protein figure was an error, resulting from Fonterra using a template and failing to correct one of the cells. The actual "protein" content, based on the Kjeldahl method, is about 7%. However, as you know, this method simply measures nitrogen. No intact milk proteins are used either as part of matrix material or as cryoprotectant. The only milk protein-related material that is used in the manufacturing process is casein peptone, generated by extensive hydrolysis of casein, in the growth medium. Note that we are not making any claim that the ingredient is free of milk protein or is hypoallergenic.

A corrected/i[dated specifications table follows.

Parameter	Unit	Specification	Method
Description		Freeze-dried powder	Inspection
Particle size	µm	NMT[2] 300	Sieving
Color		Light tan	Inspection
Odor		Sweet	Inspection
L. rhamnosus activity	cfu[1]/g	NLT[3] 10^{11}	NZTM2[4] No. 132.72
Bulk density	g/mL	0.75	
pH (10% solution)		4.5 – 7.2	
Ignition temperature	°C	460	
Proximates			
Carbohydrate	g/100g	30 – 40	
Protein	g/100g	6 – 8	Kjeldahl
Moisture	g/100g	2 – 3	
Heavy metals			
Lead	mg/kg	NMT 0.1	ICP-MS[5]
Arsenic	mg/kg	NMT 0.2	ICP-MS
Cadmium	mg/kg	NMT 0.1	ICP-MS
Mercury	mg/kg	NMT 0.1	ICP-MS
Microbiological purity			
Total aerobic bacteria	cfu/g	NMT 1	NZTM2 No. 43.2
Enterococci	cfu/g	NMT 1	NZTM2 No. 50.1
Yeast and molds	cru/g	NMT 1	NZTM2 No. 61.1
Clostridium perfringens	cfu/g	NMT 1	NZTM2 No. 46.1
Bacillus cereus	cfu/g	NMT 1	NZTM2 No. 44.1
Coliforms	cfu/g	NMT 1	NZTM2 No. 48.5
Staphylococcus aureus	cfu/g	NMT 1	NZTM2 No. 47.2
Escherichia coli		Not detected in 1 g	NZTM2 No. 48.5
Listeria		Absent in 25 g	NZTM2 No. 53.2
Salmonellae		Absent in 25 g	NZTM2 No. 58.2

1. cfu = colony forming units
2. NMT = not more than
3. NLT = not less than
4. NZTM = New Zealand Technical Manual
5. ICP-MS = inductively coupled plasma mass spectrometry

6. GRN 000288 specifically states that the intended uses include infant foods. 21CFR105.3(e) defines the term infant as persons not more than 12 months old. Previous GRAS notices for use of similar ingredients in infant formula have excluded immunocompromised infants from the population intended to consume the ingredient. Please discuss the inclusion/exclusion of immunocompromised individuals in the list of potential users.

Lactobacillus rhamnosus strain HN001 is intended for use only in conventional foods and foods that are intended for consumption by healthy infants and toddlers. This does not include infant formula, which is addressed in GRN 000281, and the intended use of HN001 does not include foods intended for consumption by immunocompromised or other non-healthy individuals.

INTERCHEM
Agencies Limited

Ascorbic Acid
BP2003/USP27

Manufactured by Jiangsu Jiangshan Pharmaceutical Co Ltd, China

Product Specification

Appearance	:	Powder
Identification	:	Positive
Melting point	:	Approx. 190°C
pH (5% W/V)	:	2.1 – 2.6
Specific optical rotation	:	+20.5° - +21.5°
Clarity and colour of solution	:	Clear, ≤BY7
Copper	:	≤0.0003%
Heavy Metals	:	≤0.001%
Iron	:	≤0.0002%
Mercury	:	≤0.0001%
Zinc	:	≤0.0025%
Arsenic	:	≤0.0003%
Lead	:	≤0.0002%
Oxalic Acid	:	≤0.2%
Loss on drying	:	≤0.1%
Sulphated Ash	:	≤0.1%
Assay	:	99.0 – 100.5
Organic Vol. Impurities	:	Meets USP requirements

Confirms to USP27/BP2003/PH EURIII/DAB98/FCCV/
European Directive 96/77/CE

[illegible] ROAD
[illegible]
PRIVATE BAG [illegible]
[illegible]
NEW ZEALAND
PHONE [illegible]
FAX [illegible]

DSM Food Specialties

DSM

Fonterra Co-operative Group Ltd
PO BOX 459
HAMILTON WAI 2001
NIEUW-ZEELAND

Date	: 17.09.2008
Your purchase order	: 8000047474
Our order number	: 203116

Certificate of Analysis

Material Our / Your reference

2426 - GISTEX LS FERM POWDER, 25 kg bag

Batch LFGRD19 / Best Before Date 14.12.2009

Characteristic	Unit	Value
Dry matter	%	96.3
[illegible]		22.2
pH		6.8
[illegible]	%	0.2
[illegible]	%	10.4
Total Plate Count	/g	60
B. cereus	/g	< 0.0
Salmonellae		Negative
Moulds	/g	< 10
Yeasts	/g	< 10
Enterobacteriaceae	/g	< 0.3
E.coli	/g	< 0.3

In addition to the results listed on the certificate of analysis we confirm that each individual batch meets the specification limits as stated in our compliance product data sheets.

This document is a non-signed computer form generated after release by the QA manager H Overweg

[illegible]

Registered trademark of DSM

COPY

A200506219

CERTIFICATE OF ANALYSIS

CPL 108548 **ASCORBIC ACID BP2003/USP27**

DRAWN UNDER DOCUMENTARY CREDIT NO. LMP37718/1107 DATED MAY 30, 2005 ISSUED BY ANZ BANK, CHRISTCHURCH NEW ZEALAND DECEMBER 12, 2006 RE-VALIDATE EXPIRY DATE

CERTIFICATE No. A1601 MANUFACTURE DATE 2005.6.18

BATCH No. [redacted] QUANTITY 1000KG

Test Items	Test Parameters	Test Results
APPEARANCE	POWDER	PASS
IDENTIFICATION	POSITIVE	POSITIVE
MELTING POINT	ABOUT 190°C	191°C
PH(5%, W/V)	2.1 – 2.6	2.3
SPECIFIC OPTICAL ROTATION	+20.5° – +21.5°	+20.9°
CLARITY AND COLOR OF SOLUTION	CLEAR, ≤ BY7	CLEAR, <BY7
COPPER	≤ 0.0003%	< 0.0003%
HEAVY METALS	≤ 0.001%	< 0.0003%
IRON	≤ 0.0002%	< 0.0002%
MERCURY	≤ 0.0001%	< 0.0001%
ZINC	≤ 0.0025%	< 0.0025%
ARSENIC	≤ 0.0003%	< 0.0003%
LEAD	≤ 0.0002%	< 0.0002%
OXALIC ACID	≤ 0.2%	< 0.2%
LOSS ON DRYING	≤ 0.1%	< 0.05%
SULPHATED ASH	≤ 0.1%	0.02%
ASSAY	99.0 – 100.5%	99.8%
ORGANIC VOL. IMPURITIES		MEETS USP REQUIREMENT

CONCLUSION THE PRODUCT CONFORMS TO USP27/BP2003/PH.EUR.III/DAB98/FCC/EUROPEAN DIRECTIVE 96/77/CE

SIGNED (b)(6) QUALITY DEPT. MANAGER (b)(6) ANALYST

NEW EXPIRE DATE 2008.8.17

江苏江山制药有限公司 JIANGSU JIANGSHAN PHARMACEUTICAL CO., LTD.

WE HEREBY CERTIFY THAT WE ARE THE MANUFACTURER OF THE ANALYZED PRODUCT.
WE HEREBY CERTIFY THAT THE ABOVE MENTIONED PRODUCT IS NOT MORE THAN SIX MONTHS OLD AT [redacted] OF SHIPMENT.

(b)(6)

Authorized Signature(s)

JIANGSU JIANGSHAN PHARMACEUTICAL CO., LTD.

JIANGSHAN ROAD,JINGJIANG,JIANGSU(214500) P.R.CHINA TEL:86-523-4839183 FAX:86-523-4831013

PENFORD
NEW ZEALAND LIMITED
319 Church Street
Onehunga Auckland New Zealand
Telephone: 64 9 634 2119
Facsimile: 64 9 636 7519

Date of Issue: 05/07/07
Product Name: DEXTROSE MONOHYDRATE
Batch Number: 3536XM
Date Of Manufacture: 20/12/06
Best Before: 20/12/08

Certificate Of Analysis

Test	Test Result	Units	Specification
Moisture	8.89	%	<=10
Dextrose Equivalent	99.69	%	>=99
Specific Rotation	53.35		52.5|54
NSR	PASS		*
Sulphur Dioxide	1.28	ppm	<=10
Taste & Odour	PASS		*
Total Plate Count	0	/g	<=1000
Coliforms	ABSENT	/10g	Absent
E. Coli	ABSENT	/g	Absent
Yeast	0	/g	<=50
Mould	0	/g	<=50
Salmonella	ABSENT	/10g	Absent

Does not contain wheat gluten. Does not require labelling as a genetically modified food, in accordance with food standard A18.
Microbiological results below the 'limit of detection' are quoted as '0'.
Results quoted have been transferred from the manufacturer's or supplier's CoA.

Name: Jeet
Prasad

Certificate of Analysis

http://certificates.merck.de

Date of print: 12.11.2007

1.05882.2500 Magnesium sulfate heptahydrate extra pure Ph Eur,BP,USP,JP

Batch K33862182

	Batch Values	
Assay (complexometric, calculated on dried substance)	99 7	%
Identity	passes test	
Appearance of solution	passes test	
pH-value (5 %; water)	6.2	
Acidity or alkalinity	conforms	
Chloride (Cl)	≤ 0.014	%
Heavy metals (as Pb)	≤ 0 0005	%
As (Arsenic)	≤ 0.0002	%
Ca (Calcium)	< 0.005	%
Fe (Iron)	≤ 0.0010	%
Se (Selenic)	≤ 0 001	%
Zn (Zinc)	passes test	
Loss on drying (450 °C)	51.1	%
Organic volatile impurities (according to USP)	conforms	
Residual solvents (Ph Eur /ICH)	Excluded by production process	

Certificate of Analysis

1.05882.2500	Magnesium sulfate heptahydrate extra pure Ph Eur,BP,USP,JP
Batch	K33862182

Date of examination (DD MM YYYY)	*27 09 2004*
Minimum shelf life (DD MM YYYY)	*30 09 2009*

Corresponds to Ph.Eur.,BP,USP,JP

Dr. Thorsten Clajus

responsible laboratory manager quality control

This document has been produced electronically and is valid without a signature

Where Innovation Comes Naturally
GPC
Grain Processing Corporation
1600 Oregon Street
P.O. Box 349 Muscatine, Iowa 52761-1494 USA
Phone: 563-264-4265 Fax: 563-264-4289

Date: 11/21/07

Salkat New Zealand LTD
Super Freight Ltd
Manu Tapu Dr, Airport Oaks
Auckland
New Zealand

Certificate of Analysis

The following material manufactured by Grain Processing Corporation, Muscatine, Iowa, was tested and approved for shipment.

Customer Code Number. . . .

Customer Order Number . . . 2754A

GPC Confirm Number. 0663502

Product . . . . . MALTRIN M040 Maltodextrin

Car or Truck Number . . . .

Lot Number. M0729801

Number of Bags 00320

Date of Expiration. 10/25/2009

Date of Manufacture 10/25/2007

Test	Result
Dextrose Equivalent	6.5
% Moisture	4.3
% Ash	.24
pH (20% Solution)	4.4
Standard Plate Count (CFU/g)	<10
Enterobacteriaceae (CFU/g)	<10
E.Coli (10g)	Neg
Yeast (CFU/g)	<10
Mold (CFU/g)	<10
Salmonella (25g)	Neg
Staphylococcus aureus (/g)	<2 /g
L. Monocytogenes (25g)	Neg

No genetically modified plant DNA was detected.

(b)(6)

Debra L. Doehrmann
Quality Assurance Coordinator

Certificate of Analysis

http://certificates.merck.de

Date of print: 12.11.2007

1.05999.1000 Manganese(II) sulfate monohydrate spray dried EMPROVE® Ph Eur,USP,FCC

Batch F1427999

	Spec. Values		Batch Values	
Assay				
komplexometric, $MnSO_4$ H_2O	98 0 - 102 0	%	98.8	%
complexometric, calc on the ignited basis	99 0 - 101 0	%	99 6	%
Identity	passes test		passes test	
Appearance of solution	passes test		passes test	
Chloride (Cl)	≤ 0 005	%	≤ 0.005	%
Heavy metals (as Pb)	≤ 0 002	%	≤ 0.002	%
As (Arsenic)	≤ 0 0003	%	≤ 0 0003	%
Ca (Calcium)	≤ 0 01	%	0 0001	%
Fe (Iron)	≤ 0 001	%	≤ 0 001	%
Pb (Lead)	≤ 0.0004	%	≤ 0 0004	%
Se (Selenic)	≤ 0.003	%	≤ 0 003	%
Zn (Zinc)	≤ 0 005	%	≤ 0.005	%
Substances not precipitated by ammonium sulfide	≤ 0.5	%	0.1	%
Residual solvents (Ph. Eur./USP/ICH)	excluded by productions process		excluded by productions process	
Organic volatile impurities (according to USP)	conforms		conforms	
Loss on ignition (500 °C)	10 5 - 12.0	%	11.4	%

Certificate of Analysis

1.05999.1000	Manganese(II) sulfate monohydrate spray dried EMPROVE® Ph Eur,USP,FCC
Batch	F1427999

Date of examination (DD MM YYYY):	*07 03 2006*
Minimum shelf life (DD MM.YYYY).	*31 03 2011*

Dr. Andreas Lang

responsible laboratory manager quality control

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JIANGYIN CHENGXING HOUSEHOLD CHEMICALS CO.,LTD.

SOUTH CHENGNAN BRIDGE, RENMIN ROAD(E), JIANGYIN CITY, JIANGSU, CHINA

EL:0086-510-6281321 FAX:0086-510-6281884

Certificate of Analysis

Product name: Potassium Phosphate Dibasic -food grade

Test Basis: FCC-IV

Lot No.:051230

Manufacturing Date: 060227

Expiry Date: 070227

Index Name	Specification	Actual Test Result
Assay:	min. 98%	98.8%
Loss on drying:	max. 2%	0.25%
Insoluble matter:	max. 0.20%	0.02%
Fluoride:	max 10mg/kg	<10 mg/kg
Heavy mental:	max 15mg/kg	<10 mg/kg
Lead:	max. 2mg/kg	< 1mg/kg
Arsenic :	max. 3mg/kg	< 1mg/kg

Conclusion: Pass Test Quantity: 7mts

Laboratory Test:QuMinYan Person In Charge:ShenJiangLi

YONG ZE 天津永泽国际贸易有限公司
TIANJIN YONGZE INTERNATIONAL TRADE CO., LTD.
地址：天津市河东区十二经路与六纬路交口润泉公寓 9-2-1803
ADD: [illegible] NO.2-2-1803 RUNQUAN MANSION, THE CROSS [illegible]
[illegible] 6TH WEI ROAD, HEDONG DISTRICT, TIANJIN, CH[illegible]
TEL:+86-22-24[illegible]151530 FAX:+86-22-24[illegible]

CERTIFICATE OF ANALYSIS

PURCHASE ORDER NO.[illegible]
PROFORMA INVOICE [illegible]
INVOICE NO.07YZSI[illegible]
BATCH NO.070412
DATE:APR.10,2007

SODIUM ACETATE ANHYDROUS EDIBLE GRADE

INDEX NAME	SPECIFICATION	RESULT
ASSAY %	99-101	[illegible]
CHLORIDE (Cl) %	≤0.005	0.0[illegible]
ARSENIC (As) %	≤0.0004	0.00[illegible]
HEAVY METAL (AS PB) %	≤0.0006	0.00[illegible]
PH VALUE	7.5-9.0	8.5

天津永泽国际贸易有限公司
TIANJIN YONGZE INTERNATIONAL TRADE CO.,LTD.

14/11/07

ROQUETTE

LC 1 EEHZ

CERTIFICATE OF ANALYSIS/QUALITY

MEDCHEM INGREDIENTS LTD

118 ASQUITH AVENUE

ST LUKES

AUCKLAND / NEW ZEALAND

NEOSORB P 20/60 DC - SORBITOL E420I

CUSTOMER. . MED CHEM/N.ZELANDE

423316 L

INVOICE..... EE776T1
TONNAGE..... 9.500 KG
CONTRACT.... E36550L
ORDER..... 02255
BATCH..... E617G
MANUF&TESTED 18 NOVEMBER 2007

EXPIRY DATE. 01 DEC 2012

DESCRIPTION WHITE CRISTALLINE POWDER.
SORBITOL, SWEET AND COOL TASTE.

TOTAL SUGARS	%	0,16
REDUCING SUGARS	%	< 0,10
LOSS ON DRYING	%	0,15
CHLORIDE	PPM	< 0,4
SULPHATE	PPM	< 3
LEAD	PPM	< 0,500
SULPHATED ASH	%	< 0,02
ARSENIC	PPM	< 1,000
NICKEL	PPM	< 1,00
PH IN SOLUTION		5,8
D-SORBITOL	%	98,9

(b)(6)

FACTORY LESTREM/MERCIER FREDERIC , 25 FEBRUARY 2008

QUALITY ASSURANCE MANAGER

Certificate has an electronic signature.

Certificate of Analysis

To: Fax No: 063504624

Fonterra Research Centre

Dairy farm Rd, Palmerston

From NZ Sugar Co Date: 17/08/2007

These analyses represent sugar products delivered to you under the following datecodes .

Product Date: 8/08/2007

Datecode: 22071-3

Order number SO: 167914

Customer Order No: 8000038801

Extra Fine White Sugar

	Results	Specifications Max	Min	Units	Method
Cane Sugar	99.93	-	99.9	%	Difference
Reducing Sugar	0.014	0.04	-	%	ICUMSA GS2/3-5
Ash	0.01	0.02	-	%	ICUMSA GS2/3-17
Water	0.03	0.04	-	%	HMA
Colour	30	50	-	CI	Spectro
Turbidity	0	5	-		
Insoluble matter	0.09	5	-	ppm	Filtration
C425µ	1.2	4	-	%	ICUMSA GS[illegible]
Thermophilic Spore Count	10	150	-	/10g	ICUMSA GS2/3-49
Yeasts	1	10	-	/10g	ICUMSA GS2/3-47
Moulds	<1	10	-		

Pathogens tested annually (Salmonella, Coliforms, Listeria): results negativ

Appearance	Fine granular white crystal
Flavour/Aroma	Typical of white sugars
Allergens	absent
Shelf life	greater than 2 years
GM Status	Contains no GM ingredients processing aids or additives during production

(b)(6) 20/8/07

Q C Analyst
Chelsea Refinery ..... (b)(6) ...

Jungbunzlauer Austria AG
Schwarzenbergplatz 16
A-1011 Wien
Phone: +43 1/50200-0
www.jungbunzlauer.com

Interchem Agencies Ltd. - c/o Nestle
7 Gladstone Road, Northcote
1310 AUCKLAND
NEUSEELAND

Jungbunzlauer

Certificate of Analysis

Creation date	Your order no. / Date	Delivery note / Date of dispatch
14.08.2007	A20331/A / 26.04.2007	80195505 000030 / 14.08.2007
Our reference / Date	**Client number**	**Date of arrival**
173109 000030 / 26.04.2007	208749	28.09.2007

Material / Product / Description: 101650 / / Trisodium Citrate fine F6000 in 25 kg bags

Country of origin: AT

Shipping point: Jungbunzlauer Austria AG Werk Pernhofen 2064 Wulzeshofen

Batch	Quantity	Date of production	Date of expiry	Production plant
1102978	3,500 KG	03.08.2007	08.2010	Pernhofen/Wulzeshofen AT

Parameter	Unit	Specification		Value
Characteristics				conforms
white, granular crystals or a white, crystalline powder,				
freely soluble in water, practically insoluble in ethanol (96%)				
Appearance of crystals				conforms*
Identification				conforms*
Assay	%	99.00	- 100.50	99.95
Water (Loss on drying)	%	11.00	- 13.00	12.11
pH in 1% solution		7.50	- 9.00	conforms*
pH in 5% solution		7.50	- 9.00	8.39
Readily carbonis. subst. (Ph.Eur./EC/JP)				conforms
Odour				conforms*
Appearance of solution				conforms*
Acidity				conforms*
Alkalinity				conforms*
Heavy metals as lead	mg/kg	0.0	- 5.0	conforms*
Chloride	mg/kg	0.0	- 50.0	conforms
Arsenic	mg/kg	0.0	- 1.0	conforms*
Lead	mg/kg	0.0	- 0.5	conforms*
Mercury	mg/kg	0.0	- 0.5	conforms*
Oxalic acid/Oxalate	mg/kg	0	- 100	conforms*
Sulphate (< 100 mg/kg)				conforms*
Tartrate (USP/JP)				conforms*
Bulk density	g/l	850	- 1100	conforms*
Particles on Mesh 25 (0.71mm)	%	0.00	- 10.00	conforms*
Particles on 0,63 mm	%	0.00	- 10.00	conforms*
Particles through 0,20mm	%	0.00	- 15.00	conforms*
Particles < Mesh 100 (0,150mm)	%	0.00	- 15.00	conforms*

We herewith confirm that this product meets the requirements of the latest edition of the European Pharmacopoeia (Ph. Eur.), the United States Pharmacopoeia (USP), the Food Chemical Codex (FCC) and of Commission Directive 96/77/EC. All analytical methods are in accordance with the latest requirements of the European Pharmacopoeia (Ph. Eur.), the United States Pharmacopoeia (USP), the Food Chemical Codex (FCC) or equivalent methods. Test methods are available on request.

*) analysis is confirmed based on In-Process-Control or by random testing.

QUALITY CONTROL MANAGER
Dr. Otto Bollmann
ISO 9001 certified by Lloyd's RQA - Certificate N° 200628
This computer generated certificate is valid without signature.

D7 Stains and Staining Procedures

D7.1 Stains

Stains for Simple Staining

Crystal violet stain

Crystal violet	10.0 g
Distilled or deionised water	1000 ml

Completely dissolve the crystal violet in the water. Store in well-stoppered bottles at room temperature.

Loeffler's methylene blue stain

Methylene blue, 0.8% (w/v) in 95% ethanol	300 ml
Potassium hydroxide, 1% (w/v)	10 ml
Distilled or deionised water	900 ml

Mix the methylene blue solution with the water. Add the potassium hydroxide solution and mix. Store in well-stoppered dark glass bottles to protect from light.

Solutions for Gram Staining

Ammonium oxalate crystal violet stain (primary stain)

Crystal violet (or methyl violet 6B)	20.0 g
Ethanol (95%)	200 ml
Ammonium oxalate	8.0 g
Distilled or deionised water	800 ml

Completely dissolve the crystal violet in the ethanol. Dissolve the ammonium oxalate in the water. Mix together these two solutions. Store in well-stoppered glass bottles at room temperature.

Lugol's iodine solution (mordant or fixative)

Iodine	3.3 g
Potassium iodide	6.7 g
Distilled or deionised water	1000 ml

Completely dissolve the potassium iodide in a minimum volume of the water. Add the iodine and dissolve. Make up to volume with distilled water. Store in well-stoppered dark glass bottles to protect from air and light.

Iodine–acetone solution (decoloriser)

Stock iodine solution

Iodine	10.0 g
Potassium iodide	6.0 g
Ethanol (95%)	90 ml
Distilled or deionised water	10 ml

Completely dissolve the potassium iodide in a minimum volume of distilled water. Add the iodine and dissolve. Add the remainder of the distilled water. Make up to volume with ethanol. Store in a well-stoppered dark glass bottle to protect from air and light.

Working solution (decoloriser)

Stock iodine solution	35 ml
Acetone	965 ml

Mix together the stock iodine solution and acetone. Store in well-stoppered dark glass bottles to protect from evaporation and light.

Safranin stain (counterstain)

Safranin	5.0 g
Distilled or deionised water	1000 ml

Completely dissolve the safranin in the water. Store in well-stoppered bottles at room temperature.

Note: Neutral red or basic fuchsin are also suitable Gram counterstains.

Solutions for Spore Staining (Fleming's Method)

Ziehl-Neelsen strong carbol fuchsin stain (primary stain)

Fuchsin	10.0 g
Phenol crystals	50.0 g
Ethanol (95%)	100 ml
Distilled or deionised water	1000 ml

Add the fuchsin to the phenol and dissolve by gently shaking while heating over a boiling water bath. Add the ethanol and mix. Add the water and mix. Filter through coarse filter paper. Store in well-stoppered bottles at room temperature.

Decoloriser

Aqueous sodium sulphite (5%), or
Hydrochloric acid (0.5%), or
Ethanol (95%)

Methylene blue stain (counterstain)

Methylene blue	10.0 g
Distilled or deionised water	1000 ml

Completely dissolve the methylene blue in the water. Store in well-stoppered bottles at room temperature.

Carbol Thionin Stain

Thionin	0.2 g
5% phenol solution	100 ml

Completely dissolve the thionin in the phenol solution. Store in well-stoppered bottles at room temperature.

***Methylene Blue Stain (Levowitz-Weber Modification of Newman*-Lampert)**

Glacial acetic acid	4.0 ml
Methylene blue chloride	0.6 g
Ethanol (95%)	52.0 ml
Xylene	44.0 ml

Completely dissolve the methylene blue chloride gradually into a mixture of the ethanol and xylene in a stoppered 200 ml flask. Mix by swirling and allow to stand overnight in the refrigerator at 2–5 °C. Filter through fine paper (e.g. No. 42 Whatman) and add 4 ml of glacial acetic acid to the filtrate. Store in a stoppered bottle in a cool dark place.

D7.2 Staining Procedures

Preparation of Smears

1. Place a loopful of the culture on to a clean glass microscope slide. Cultures from solid media should be emulsified in sterile 0.85% (w/v) saline on the microscope slide, using only sufficient culture to produce a light smear.

2. Leave this smear to air dry, and then heat fix by passing rapidly through a low gas flame. Do not overheat. Allow to cool.

Simple Stains

1. Place the prepared slide, smear facing up, on a staining rack over the sink.

2. Cover the smear with a few drops of stain, and leave for 15–20 seconds.

3. Rinse off the stain with tap water, and blot the slide dry. Take care to avoid rubbing the smear. Alternatively, allow to air dry or pass rapidly through a low gas flame to speed the drying process. Do not overheat.

4. Examine under a microscope using the 100 x oil immersion objective lens.

Gram Stain

1. Place the prepared slide, smear facing up, on a staining rack over the sink.

2. Cover the smear with a few drops of ammonium oxalate crystal violet stain, and leave for 30 seconds.

3. Pour off this stain.

Note: Do not rinse with tap water at this stage.

Cover the smear with a few drops of Lugol's iodine, and leave for 30 seconds.

4. Pour off the Lugol's iodine, and rinse the smear with iodine–acetone solution until the colour just fades.

Note: Do not over-decolorise.

5. Rinse immediately with tap water. Pour off the excess water, and cover the smear with a few drops of safranin. Leave for 30 seconds, and rinse with tap water.

6. Blot the slide dry. Take care to avoid rubbing the smear. Alternatively, allow to air dry or pass rapidly through a low gas flame to speed the drying process. Do not overheat.

7. Examine under a microscope using the 100 x oil immersion objective lens.

   Gram-positive bacteria stain purple.

   Gram-negative bacteria stain red.

Spore Stain (Fleming's Method)

1. Place the prepared slide, smear facing up, on a staining rack over the sink.

2. Cover the entire slide with the Ziehl-Neelsen stain, and heat to steaming for 3–5 minutes, by passing a flame under the slide.

Note: Do not allow to boil.

3. Rinse with tap water. Apply decoloriser to the slide and leave for 30 seconds.

4. Rinse with tap water. Apply the methylene blue and leave for 60 seconds. Rinse with tap water.

5. Blot the slide dry. Take care to avoid rubbing the smear. Alternatively, allow to air dry or pass rapidly through a low gas flame to speed the drying process. Do not overheat.

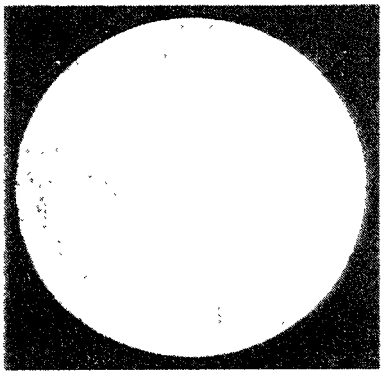
6. Examine under a microscope using the 100 x oil immersion objective lens.

Vegetative cells stain blue.

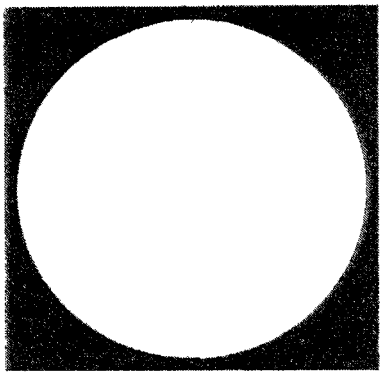
Spores stain purple.

D7.3 Gram Stains

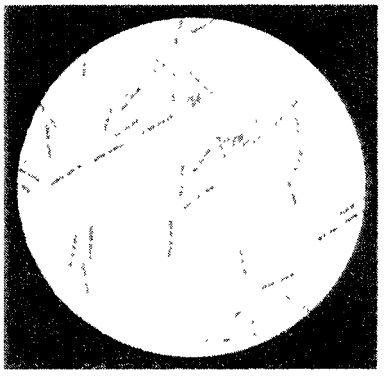
The following photographs show examples of bacteria prepared using Gram stains. (Reprinted with thanks to Becton Dickinson & Co. and Biolab Scientific.)



Gram-Positive Cocci
Staphylococcus aureus



Gram-Negative Rods
Escherichia coli

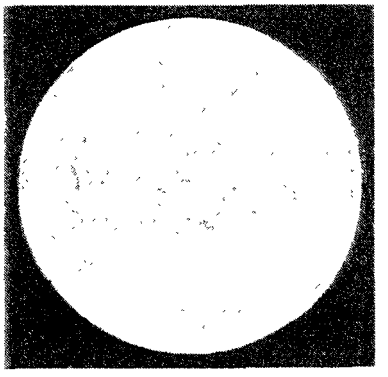


Gram-Positive Rods
Clostridium perfringens

D7.4 Yeast and Mould Stains

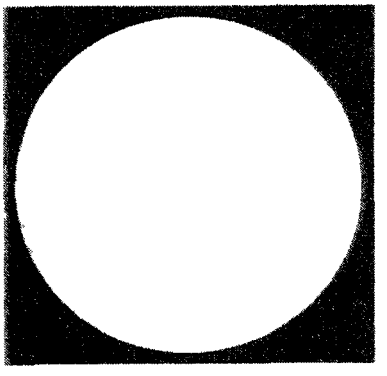
The following photographs depict examples of yeasts and moulds. (Reprinted with thanks to Becton Dickinson & Co. and Biolab Scientific.)

Yeast

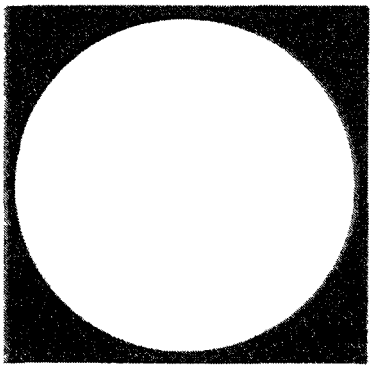


Saccharomyces sp

Moulds



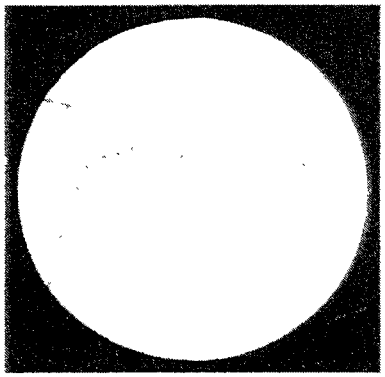
Aspergillus sp
(x 125)



Penicillium sp
(x 125)

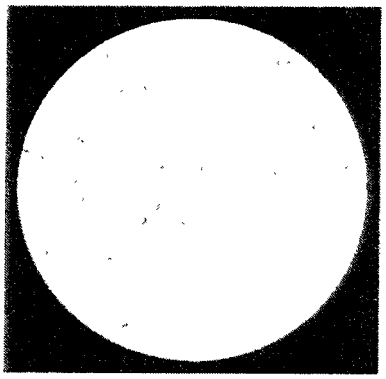
D7.5 Spore Stains

The following photographs show examples of fuschin-methylene-blue-stained spores. (Reprinted with thanks to Becton Dickinson & Co. and Biolab Scientific.)



Bacillus anthracis

The photograph shows blue chains of vegetative cells and red chains of spores. Faint blue cell material is just visible around some spores, which are centrally located within the cells.



Clostridium tetani

The photograph shows blue vegetative cells with red, distally located spores.

43.1 Aerobic Plate Count at 30 °C

43.1.1 Scope

This method is used to count micro-organisms in milk and milk products using a colony count technique at 30 °C.

This is regarded as the reference method. In cases where use of an alternative method is allowed, equivalence to this method must be demonstrated by a suitable means (eg NZCP13, AS/NZS 4659 etc). It must be ensured that equivalence rules stipulated by controlling bodies such as MAF are fully met.

43.1.2 Validation Status

This method is based on:

- IDF Standard 100B: 1991. *Milk and Milk Products. Enumeration of Micro-organisms. Colony Count Technique at 30°C,* and ISO 4833:2003. *Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of micro-organisms – Colony Count Technique at 30 °C.*

The validation status of these methods is not known.

43.1.3 Principle

A defined test portion or a series of decimal dilutions of the sample is mixed with milk plate count agar, and incubated aerobically at 30 °C for 72 hours. Agar plates are overlaid if necessary.

The number of micro-organisms per gram or per millilitre of the original sample is calculated from the number of colonies counted on selected plates.

43.1.4 Diluent

- Quarter-strength Ringers solution
- Phosphate buffer solution (KH_2PO_4)
- 0.1% peptone
- Peptone-Saline solution.

43.1.5 Medium

Milk Plate Count Agar (MPCA)

Tryptone	5.0 g
Yeast extract	2.5 g
Glucose monohydrate	1.0 g
Skimmed milk powder	1.0 g
Agar	15.0 g*
Distilled or deionised water	1000 mL

pH 7.0 ± 0.2 at 25 °C.

* The exact agar content may vary, depending on the manufacturer's specifications.

Add all ingredients to the water. Skimmed milk powder must be added to a suitable commercial preparation if it is not already included in the dehydrated medium (refer to the note below). The agar-skimmed milk powder mix should be allowed to stand for about 15 minutes for rehydration to occur.

Dissolve with frequent agitation and gentle heating. Adjust the pH, if necessary, so that after sterilisation, it is 7.0 ± 0.2 at 25 °C. Sterilise at a maximum temperature of 121 °C for 15 minutes. Cool to 45 ± 1 °C before use. If the agar is to be stored, the bottles should be placed in the dark at 2-5 °C, for no longer than 1 month.

Note: Alternatively, plate count agar or standard methods agar which meet the above composition may be used, with the addition of 1.0 g of medium heat, standard specification skimmed milk powder. This powder shall be proven to be free of inhibitory substances by comparative tests using skimmed milk powder known to be free of such substances. Such testing may only be performed by laboratories holding suitable accreditation (e.g. from Telarc) for the inhibitory substances test (NZTM 2, Method 51.1). Powder from a suitable batch may be used for an entire dairy season provided that this powder is repackaged into sealed, airtight containers and protected from light.

43.1.6 Quality Control

When checking the performance of new batches of MPCA, actual product from the manufacturing plant should be used, rather than control cultures. This is because slight changes in media composition or processing conditions may alter the sensitivity of media for detecting bacteria present in the product.

43.1.7 Plating

1. Prepare a sample of the product as described in Section 41. Agitate the sample just prior to taking test portions, to ensure sample homogeneity. Where possible, use reconstitution methods sourced from ISO 8261/IDF 122: 2001 (E).
2. Take a sterile Petri dish. Using a sterile pipette, transfer 1 mL of the sample into the dish. Repeat using a further dish if a duplicate is required.

   Note: This represents a 10^0 dilution for liquid products, and a 10^{-1} dilution for most prepared products. Refer to Section 41 for exceptions.

   Note: Refer to Section 10 for recommendations on the frequency of duplicate plates and dilutions.

3. If a further dilution is required, transfer 1 mL of the sample into 9 mL of sterile 0.1% peptone diluent. Mix by aspirating 10 times with a fresh pipette, or vortexing for 5-10 seconds. Transfer 1 mL of this dilution into a Petri dish. Transfer a further 1 mL of this into another dish if a duplicate is required.

   Note: This represents a 10^{-1} dilution for liquid products, and a 10^{-2} dilution for most prepared products.

4. Repeat Step 3 for the number of dilutions required, each time adding 1 mL of the previous dilution to 9 mL of 0.1% peptone, and transferring 1 mL of this into each dish.

5. Pour about 15 mL of molten MPCA agar tempered to 45 ± 1 °C into each Petri dish.

   Note: The time elapsing between the preparation of the sample and the addition of the agar must not exceed 15 minutes.

6. Mix immediately after pouring into each Petri dish by rotating the dish sufficiently to obtain evenly dispersed colonies after incubation. Allow the agar to solidify at room temperature.

7. If spreading colonies are expected, overlay the solidified plates with a further 5 mL of MPCA agar, and allow to solidify at room temperature.

8. Prepare blank control plates, by pouring about 15 mL of molten MPCA agar into a Petri dish.

9. Incubate all plates aerobically at 30 ± 1 °C for 72 ± 3 hours.

Note: Incubate the plates in an inverted position. It is recommended that stacks of plates be no more than six high. Stacks of plates should be separated from one another and from the walls and top of the incubator.

43.1.8 Interpretation

1. Examine the plates for the presence of colonies, preferably using a colony counter in subdued light.

2. Examine the blank control plates. There should be no colonies present. Otherwise, investigate the source of contamination.

3. Select all sample plates, at two successive dilutions if applicable, containing between 10 and 300 colonies.

43.1.9 Calculation and Report

1. Count and record the number of colonies on each selected plate.

   Note: Count the colonies promptly after incubation.

   Note: Avoid counting particles of extraneous matter as colonies. Examine under higher magnification if necessary.

   Note: Do not overlook pin-point colonies, which must be included in the count. Examine under higher magnification if necessary.

   Note: A quick daily inspection of the plates may be made, as it is usually possible to detect Second Grade milk within 24 hours and certainly by 48 hours. Early detection of such milk is important both for the supplier and the processor.

2. Calculate the result as described in NZTM 1 Calculation of Counts section.

3. Express the result as "Aerobic plate count at 30 °C, colony forming units per gram (or per mL)".

43.1.10 Precision

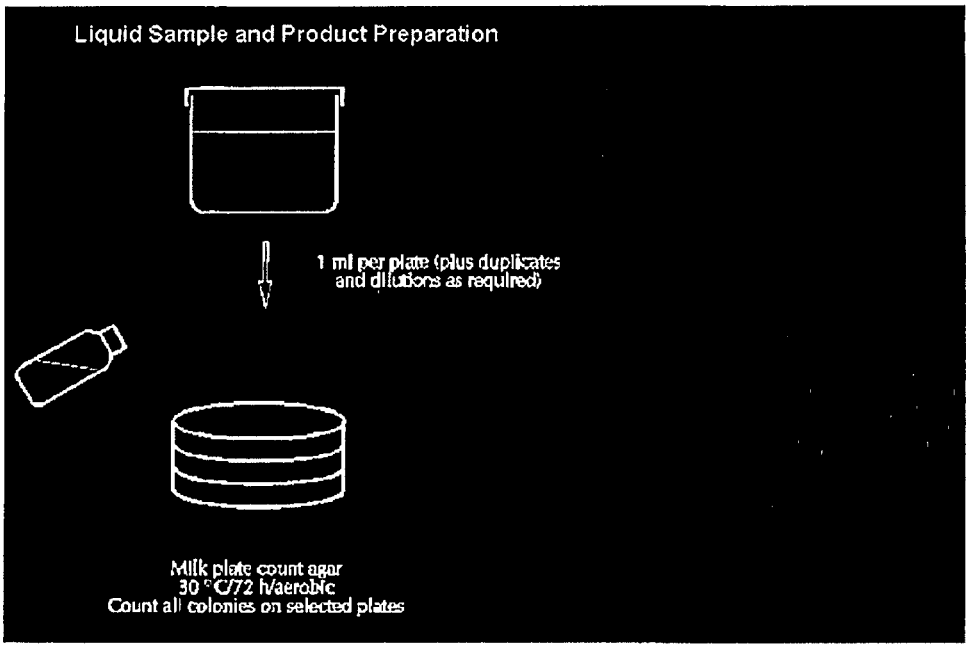
Precision values for this method are not available.

However, IDF Standard 100B states some indicative figures based on a random variation of colony counts, alone, but notes that greater variation may be found in practice.

43.1.11 Notes

1. Some colonies may be very small. These pin point colonies must be included in the counts.

Flow Chart 43.1.1: Aerobic Plate Count at 30 °C



43.2 Aerobic Plate Count for Probiotic Dairy Goat Milk Powders

43.2.1 Scope

This method is used to determine the levels of general aerobic plate count (APC) organisms in dairy goat milk powders supplemented with Lactic Acid bacteria (LAB) such as Bifidobacteria and Lactobacilli.

43.2.2 Validation Status

This test method is based on:

- IDF Standard 100B: 1991. Milk and Milk Products. Enumeration of Micro-organisms. Colony Count Technique at 30°C.

According to IDF, this method has not been validated for any product.

The following modifications have been made to the method:

- Instructions for reading of MPCA plates have been modified to distinguish LAB lawn growth from general growth.
- Instructions for the confirmation of identity of LAB bacteria have been added.

The modifications have not been validated.

43.2.3 Principle

This method is used to enumerate general Aerobic Plate Count bacteria at 30°C.

In dairy goat milk powders supplemented with LAB at high concentrations (10^6 cfu/g), many non-selective assays cannot distinguish between general aerobic plate count (APC) flora, and the specific added LAB flora. This can give rise to APC counts far in excess of specification requirements if differentiation of LAB bacteria from the APC is not carried out.

Identification/differentiation of the LAB colonies on incubated MPCA agar plates allows differentiation and reporting of only the general APC organisms.

43.2.4 Apparatus

- An incubator controlled at 30 ± 1 °C.
- A timer.
- A waterbath controlled at 45 °C for agar.
- A 1 mL pipette.

43.2.5 Media and Reagents

- Milk Plate Count Agar (MPCA)

Tryptone	5.0 g
Yeast extract	2.5 g
Glucose monohydrate	1.0 g
Skimmed milk powder	1.0 g
Agar	15.0 g*
Distilled or deionised water	1000 mL

pH 7.0 ± 0.2 at 25°C.

*The exact agar content may vary, depending on the manufacturers specifications.

Add all ingredients to the water. Skimmed milk powder must be added to a suitable commercial preparation if it is not already included in the dehydrated medium (refer to the note below). The agar-skimmed milk powder mix should be allowed to stand for about 15 minutes for rehydration to occur.

Dissolve with frequent agitation and gentle heating. Adjust the pH, if necessary so that after sterilisation it is 7.0 ± 0.2 at 25°C. Sterilise at a maximum temperature of 121°C for 15 minutes. Cool to 45 ± 1°C before use. If the agar is to be stored, the bottles should be placed in the dark at 2 – 5°C, for no longer than 1 month.

Note: Alternatively, plate count agar or standard methods agar which meet the above composition may be used, with the addition of 1.0 g of medium heat, standard specification skimmed milk powder. This powder shall be proven to be free of inhibitory substances by comparative tests using skimmed milk powder known to be free of such substances. Such testing may only be performed by laboratories holding suitable accreditation (eg from IANZ) for the inhibitory substances test (NZTM2, Method 51.1). Powder from a suitable batch may be used for an entire dairy season provided that this powder is repackaged into sealed, airtight containers and protected from light.

43.2.6 Procedure

Plating

1. Prepare a sample of product as described in Section 41. Agitate the sample just prior to taking test portions, to ensure sample homogeneity.

2. Take a sterile petri dish. Using a sterile pipette transfer 1 mL of the sample into the dish.

   Note: This represents a 10^{-1} dilution for dairy goat milk powder.

3. Pipette 1 mL each of any further dilutions into separate petri dishes.

4. Add 15 - 20 mL of MPCA agar tempered to 45 ± 1 °C.

Note: The time elapsing between the preparation of the sample and the addition of the agar must not exceed 15 minutes.

5. Mix well and allow to solidify at room temperature. If necessary, overlay solidified agar plates10^{-1} with a further 5 mL of MPCA (to prevent growth of spreaders) and allow to solidify at room temperature.

6. Incubate inverted plates aerobically at 30 ± 1 °C for 72 ± 3 hours.

Reading

1. Count all colonies and record total count.

   Note: Distinguish between undissolved particles and actual colonies under the microscope.

2. Distinguish between suspected LAB and other general (APC) bacteria.

   Note: The presence of LAB will be observed as a lawn of pinpoint colonies in the agar plate that is better identified with microscope than the naked eye. The growth of other APC organisms is more likely to be represented by large colonies, easily differentiated by the eye.

3. If necessary, carry out further tests to confirm that the pinpoint lawn on incubated MPCA plates is composed of LAB.

Confirmation

1. Work through the following confirmation methods on any plates that have suspect LAB colonies.

2. Sensory

   The fermentation process of the LAB produces a buttery odour of diactyl. This aroma will be pronounced on plates with high numbers of LAB.

3. Gram Stain and Morphology

   Lactobacilli are Gram positive, non-spore forming rods or cocci.

   Bifidobacteria are Gram positive, non-motile, non-sporulating Gram-positive rods with varying appearance.

4. Catalase Test

   LAB are catalase negative bacteria.

43.2.7 Results

Report only non-LAB bacteria ie large colonies deemed to be part of the general APC on the MPCA agar plate.

Calculate the result as described in NZTM1 Calculation of Counts section.

Express the result as "aerobic plate count at 30°C colony forming units per gram".

43.2.8 Precision

No precision values are available for this method.

43.2.9 References

- Orrhage K., Nord C.E. "Bifidobacteria and Lactobacilli in human health". Drugs Exptl. Clin. Res. 2000; XXVI (3): pages 95-111.

44.1 *Bacillus cereus*; Count

44.1.1 Scope

This method is used to count *Bacillus cereus* in products intended for human consumption or animal feeding stuffs and environmental samples using a plating and confirmatory testing technique.

44.1.2 Validation Status

This method is based on:

- ISO 7932 : 2004(E). *Microbiology of food and animal feeding stuffs – horizontal method for the enumeration of presumptive Bacillus cereus, – colony count technique at 30°C.*

According to ISO, this method has been validated.

The following modifications have been made to the method:

- Suspicious colonies showing a typical haemolysis reaction in confirmatory testing are reported as Bacillus cereus colony forming units per gram (or per mL).
- Additional notes on interpretation of haemolysis have been sourced from FDA - BAM.

This modified method is not validated.

44.1.3 Principle

A defined test portion or a series of decimal dilutions of the sample is spread on to the surface of mannitol egg yolk polymyxin agar. The plates are incubated aerobically at 30°C for 18 - 48 hours. Formation of clearly visible colonies enables testing to proceed after 18 hours of incubation. The plates are examined for suspicious colonies and these colonies are counted. Suspicious colonies are large, pink (indicating that mannitol has not been fermented and the phenol red indicator has therefore remained red), and generally have a surrounding zone of precipitation (indicating the production of the enzyme lecithinase, which hydrolyses the lecithin in egg yolk).

Five presumptive colonies from each plate are confirmed by assessment of haemolysis on sheep blood agar.

It is important to note that this method is not capable of differentiating between *B cereus* and *B cereus* var. *mycoides, B anthracis* and *B thuringiensis.*

Differentiation is not really necessary because:

- it is extremely unlikely that *B anthracis* and *B thuringiensis* will be recovered from any dairy products
- some strains of *B cereus* var. *mycoides* may also act as food pathogens.

44.1.4 Diluent

- 0.1% peptone.

44.1.5 Media and Reagents

Mannitol Egg Yolk Polymyxin (MYP) Agar

Base medium

Beef extract	1.0g
Peptone	10.0g
D-mannitol	10.0g
Sodium chloride (NaCl)	10.0g
Phenol red	0.025g
Agar	12-18g*
Distilled or deionised water	900mL

pH 7.2 ± 0.2

* According to the manufacturer's specifications.

Add the ingredients to the water. Mix thoroughly and heat, if necessary, to completely dissolve the ingredients. Adjust the pH so that after sterilisation it is 7.2 ± 0.2 at 25°C. Dispense 90mL volumes into bottles. Sterilise at 121°C for 15 minutes.

Polymyxin B sulphate -10^4 IU/mL

Polymyxin B sulphate	1×10^6 IU
Distilled or deionised water	100mL

Add the polymyxin B sulphate powder to the water.
Mix thoroughly to completely dissolve.

Filter sterilise into a sterile dark bottle.

Note: The weight of polymyxin B sulphate required to obtain a concentration of 1×10^6 IU/mL is calculated as follows:

$$\text{Weight (mg)} = \frac{1 \times 10^6 \text{IU}}{\text{Activity (IU/mg)}}$$

Egg yolk emulsion - 20%

Wash fresh and intact hens' eggs using a brush and liquid detergent. Rinse well under running water. Immerse in 70% (v/v) ethanol for 10 minutes and air dry. Aseptically break each egg and separate the yolk. Put the yolks into a sterile measuring cylinder and add four parts by volume of sterile water. Heat the mixture for 2 hours in a water bath at 45°C. Leave for 18 - 24 hours at 0-5°C to allow a precipitate to form. Collect the supernatant aseptically. The emulsion may be stored at 5 ± 3°C for up to 72 hours.

Complete medium (MYP agar)

Base medium	90.0mL
Polymyxin B sulphate -10^4 IU/mL	1.0mL
Egg yolk emulsion	10.0mL

Melt the base medium. Cool to 45 ± 1°C in a water bath. Add the other components, mixing well after each addition. Pour 15 - 20 mL of complete medium into each Petri dish. Allow to solidify at room temperature. Plates may be stored prior to drying at 5 ± 3°C for up to 4 days.

Sheep Blood Agar

Base Medium

Proteose peptone or equivalent peptone	15.0g
Liver Hydrolysate	2.5g
Yeast Extract	5.0g
Sodium chloride (NaCl)	5.0g
Agar	12-18g*
Distilled or deionised water	1000mL

pH 7.0 ± 0.2

* According to the manufacturer's specifications.

Dissolve the components in water by boiling.

Adjust the pH if necessary, so that after sterilisation it is 7.0 ± 0.2 at 25°C.

Dispense and sterilise at a maximum temperature of 121°C for 15 minutes.

Defibrinated Sheep Blood

Complete Medium

Base Medium	100mL
Defibrinated Sheep Blood	5 – 7mL

After cooling to 44 – 47 °C, add defibrinated sheep blood to the base medium. Mix.

Pour at least 12mL portions of the complete medium into sterile Petri dishes and allow to solidify.

44.1.6 Quality Control Organisms

Positive Control:
- *Bacillus cereus* NZRM 5.

MYP Negative Control (**Note:** optional to the method):
- *Bacillus subtilis* NCTC 3610.

Haemolysis Negative Control (**Note:** optional to the method):
- *Enterococcus faecalis NCTC 5957.*

44.1.7 Plating

1. Prepare a sample of the product as described in Section 41. Agitate the sample to ensure homogeneity and take test portions as soon as possible. Where possible use reconstitution methods sourced from ISO 8261/ IDF 122:2001 (E).

2. Take three dried MYP agar plates. Using a sterile pipette, transfer a **total** of 1.0mL of the sample equally on to the surface of the three plates. Repeat using a further three plates if a duplicate is required. Treat each set of three plates as a single plate for purposes of counting and confirmation, i.e. each set of three plates represents 0.1g of product for purposes of counting and confirmation, where a 10^{-1} dilution of the sample has been plated.

   Note: This represents a 10^{0} dilution for liquid products, and a 10^{-1} dilution for most prepared products, **over the three plates**. Refer to Section 41 for exceptions.

   Note: Refer to NZTM 1 Frequency of Duplicate Plating and Decimal Dilutions for Counts section for recommendations on the frequency of duplicate plates and dilutions.

   Note: Dry the plates prior to use by placing them in a drying cabinet, agar surface down and without lids, at about 50°C for about 30 minutes, or 35 - 37°C for about 60 minutes.

3. If a further dilution is required, take another dried MYP plate, and transfer 0.1mL of the sample on to the plate. Repeat if a duplicate is required.

   Note: This represents a 10^{-1} dilution for liquid products, and a 10^{-2} dilution for most prepared products.

4. If a further dilution is required, transfer 1mL of the sample into 9mL of sterile 0.1% peptone diluent. Mix by aspirating 10 times with a fresh pipette, or vortexing for 5 - 10 seconds. Transfer 0.1mL of this dilution on to a dried MYP plate. Transfer a further 0.1mL of the dilution on to another MYP plate if a duplicate is required.

5. Repeat Step 4 for the number of dilutions required, each time adding 1mL of the previous dilution to 9mL of 0.1% peptone, and transferring 0.1mL of this on to each plate.

6. Using a sterile glass spreader for each plate, carefully spread the inoculum over the entire surface of the agar. Avoid touching the sides of the dish with the spreader.

   Note: As *B. cereus* spores may survive flame sterilising, it is advisable to use a fresh sterile spreader for each sample.

   Note: The time elapsing between the preparation of the sample and the spreading of the inoculum must not exceed 15 minutes.

7. Prepare the following test control plates:

   - Blank Control Plate:
     Leave uninoculated.

   - Positive Control Plate:
     Streak with the positive control organism.

   - Negative Control Plate:
     Streak with the negative control organism.

8. Leave the plates for 15 minutes at room temperature to allow the absorption of the inoculum into the agar medium.

9. Incubate the inoculated plates aerobically at 30 ± 1°C for 18 - 24 hours.
   If colonies are not clearly visible, incubate the plates for a further 24 hours.

   Note: Incubate the plates in an inverted position. It is recommended that stacks of plates be no more than six high. Stacks of plates should be separated from one another and from the walls and top of the incubator.

44.1.8 Interpretation

1. Examine the plates for the presence of suspicious (presumptive) colonies:

   - pink (indicating that mannitol has not been fermented),

   - generally surrounded by a zone of precipitation (indicating the production of lecithinase).

Note: If numerous mannitol fermenting colonies are present, the acid produced by them may reduce or obscure the pink of the *B. cereus* colonies.

Note: Some *B. cereus* strains produce little or no lecithinase, so the precipitation zone will be reduced or absent. These colonies should be subject to confirmation tests.

Note: The colonies or MYP agar that give typical reactions may vary in morphology from a 'ground glass' appearance, to large, amorphous and highly irregular colonies. In the case of *B cereus* var. *mycoides,* the colonies will have a *rhizoid* appearance. All colony types (including the *rhizoid* colonies) should be included in the presumptive count.

2. Examine the test control plates:

   Blank control plate
   There should be no colonies present. Otherwise, investigate the source of contamination.

   Positive control plate
   It should contain typical *B. cereus* colonies. Otherwise, investigate the cause of the problem.

   Negative control plate
   It should contain colonies which are lecithinase negative. Otherwise, investigate the cause of the problem.

3. Select all sample plates, at two successive dilutions if applicable, containing between 15 and 150 presumptive colonies.

4. Count and record the number of presumptive colonies on each of these plates. (Note that the three plates inoculated with a total of 1mL of sample are treated as a single plate. However, it will be necessary to record the result for each individual plate, as well as the total count.)

44.1.9 Confirmation

1. Choose colonies from the plates as follows:

   - Positive Control Plate:
     Choose a well-isolated colony.

   - Negative Control Plate:
     Choose a well-isolated colony. (Unless an alternative is specified).

   - Sample Plates:
     Choose at least five well-isolated presumptive colonies from each set of selected sample plates. If there are less than five presumptive colonies, choose all of them.

2. If it is not possible to choose well-isolated colonies, streak at least five presumptive colonies on to dried MYP plates, incubate as before, and choose one well isolated presumptive colony from each plate.

3. Confirm each colony as follows:

   Haemolysis test on Sheep Blood Agar

   Streak, stab or spot the selected colonies onto the surface of sheep blood agar to allow clear interpretation of the haemolysis reaction.

   Incubate at 30°C for 24 ± 2 hours.

   Interpretation
   Examine plates for haemolytic activity.
   B. cereus cultures are usually strongly haemolytic and produce a 2 – 4 mm clear zone of haemolysis (*B. haemolysis*) surrounding growth.

4. A summary of tests is shown in Table 44.2.2.

Table 44.2.2 Test Summary

Test	Result confirming *B. cereus*
MPY Agar	Formation of pink colonies surrounded by precipitate (see section 44.2.8)
Haemolysis	Positive Reaction (note the width of the haemolysis zone may vary)

44.1.10 Expression of Results

Calculate the result as described in NZTM 1 Calculation of Counts section.

Express the result as "Bacillus cereus, colony forming units per gram (or per mL)"

44.1.11 Precision

According to data collected by ISO in an interlaboratory trial examining fresh cheese samples:

- The repeatability standard deviation S_r ($\log_{10}$ cfu/g) was in the range 0.05 – 0.12.

- The reproducibility standard deviation S_R ($\log_{10}$ cfu/g) was in the range 0.08 – 0.17.

  See Annex B of ISO 7932 : 2004(E) for further details.

44.1.12 Note

1. This method is not adequate to differentiate between B. cereus and the closely related members of this group (B. anthracis, B.thuringiensis and B. cereus var. mycoides). In the event that full differentiation is required, refer to FDA BAM, Online : 2001. Chapter 14.

44.1.13 44.1.13 References

- Fleiss, J.L. Statistical Methods for Rates and Proportions. 2nd Edition. John Wiley & Sons, New York, 1981.
- FDA. Bacteriological Analytical Manual. Online : 2001. Chapter 14. *Bacillus cereus.*

45.2 β-Haemolytic Streptococci; Detection

45.2.1 Scope

This method is used to detect the presence of β-haemolytic streptococci (including *S. pyogenes*) in milk and milk products using an enrichment, plating and confirmatory testing technique.

45.2.2 Validation Status

This method is based on:

- An interim method which has been used by the New Zealand dairy industry for several years. No suitable international or national reference method is currently available for testing this organism in dairy products.

This method is not validated.

45.2.3 Principle

A defined test portion of the sample is added to triplicate tubes of streptosel broth (streptococcus enrichment broth). The azide and sulphide in the medium inhibit Gram-negative organisms, and the low level of crystal violet suppresses many Gram- positive organisms, but not streptococci. The tubes are incubated aerobically at 37 °C for 24 hours.

A loopful of each broth is streaked on to blood agar. The plates are incubated anaerobically at 37 °C for 24 hours. Anaerobic conditions prevent oxidation of the oxygen-labile streptolysin, the substances produced by the bacteria which lyse red blood cells. The plates are examined for suspicious colonies. Suspicious colonies are surrounded by a clear zone (indicating complete haemolysis or β-haemolysis).

Selected colonies are confirmed by Gram stains and catalase tests. If a presumptive *Streptococcus pyogenes* (Lancefield Group A) result is required, a further confirmatory bacitracin sensitivity test is carried out.

45.2.4 Media and Reagents

Streptosel Broth (Streptococcus Enrichment Broth)

	Single Strength	Double Strength
Peptone from casein	14.4 g	28.8 g
Peptone from soybean meal	5.0 g	10.0 g
Sodium chloride	4.0 g	8.0 g
Sodium citrate	1.0 g	2.0 g
L-Cystine	0.2 g	0.4 g
Sodium sulphite	0.2 g	0.4 g
D-Glucose	5.0 g	10.0 g
Sodium azide	0.2 g	0.4 g
Crystal violet	0.0002 g	0.0004 g

Distilled or deionised water	1000 mL	1000 mL

pH 7.4 ± 0.2

Completely dissolve the ingredients in the water. Adjust the pH so that after sterilisation it is 7.4 ± 0.2 at 25 °C. Dispense 10 mL volumes into test tubes, and larger volumes of double strength into suitable containers. Sterilise at a maximum temperature of 121 °C for 15 minutes.

Blood Agar

Columbia blood agar base

Pancreatic digest of casein	12.0 g
Peptic digest of animal tissue	5.0 g
Yeast extract	3.0 g
Beef extract	3.0 g
Starch	1.0 g
Sodium chloride	5.0 g
Agar	12-18 g*
Distilled or deionised water	1000 mL

pH 7.3 ± 0.2

* According to the manufacturer's specifications.

Add the ingredients to the water. Mix thoroughly and boil with frequent agitation just sufficiently to completely dissolve the ingredients. Adjust the pH so that after sterilisation it is 7.3 ± 0.2 at 25 °C. Dispense 200 mL volumes into bottles. Sterilise at a maximum temperature of 121 °C for 15 minutes. Cool to 45-50 °C.

Defibrinated sheep blood

Complete medium

Columbia agar base	200 mL
Defibrinated sheep blood	10 mL

Melt the Columbia agar base. Cool to 45-50 °C. Add the blood, and mix thoroughly. Pour 12-15 mL volumes into Petri dishes. Allow to solidify at room temperature. Store at 2-5 °C for up to 1 week.

Catalase Reagent (3% H_2O_2)

Hydrogen peroxide, 30%	10 mL
Distilled or deionised water	90 mL

Mix. Store in a dark bottle at 2-5 °C. Discard when the solution no longer produces bubbles with the positive control organism.

Caution: Hydrogen peroxide is an oxidising agent. Avoid contact with eyes and skin.

Brain Heart Infusion (BHI) Broth

Calf brain infusion	12.5 g
Beef heart infusion	5.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Glucose	2.0 g
Disodium hydrogen phosphate	2.5 g
Distilled or deionised water	1000 mL

pH 7.4 ± 0.2

Completely dissolve the ingredients in the water.

Adjust the pH so that after sterilisation it is 7.4 ± 0.2 at 25 °C. Dispense 5 mL volumes into test tubes. Sterilise at a maximum temperature of 121 °C for 15 minutes. The medium may be stored at 2-5 °C for up to 3 months.

Bacitracin Disks

Paper disks containing 0.04-0.05 units of bacitracin. Store at 2-5 °C.

Gram Stain Solutions

See NZTM 1 Stain and Staining Procedures section.

45.2.5 Quality Control Organisms

Positive Control:
- *Streptococcus pyogenes* NZRM 2264.

Negative Control (**Note:** optional to the method):
- *Streptococcus lactis* NZRM 2274.

Catalase-Positive Control (**Note**: optional to the method):
- *Staphylococcus aureus* NZRM 87.

45.2.6 Enrichment

1. Prepare a sample of the product as described in Section 41 of this manual. Agitate the sample just prior to taking test portions, to ensure sample homogeneity. Where possible, use reconstitution methods from IDF Standard 122C: 1996.

2. Determine the volume of prepared sample to be added to each streptosel broth based on:

   - the dilution factor of the prepared sample,
   - the weight or volume of the product required to determine compliance with the specification.

   See Table 45.2.1 for examples.

Table 45.2.1: Examples of enrichment regimes (for a reconstituted product at 10^{-1} dilution)

Product specification	Product weight	Reconstituted sample volume	Streptosel broth volume and strength
per 0.1 g	0.1 g	1.0 mL	10 mL of single strength
per 1.0 g	1.0 g	10.0 mL	10 mL of double strength
per 10.0 g	10.0 g	100.0 mL	100 mL of double strength

Note: Do not add more than 1 mL of sample to 10 mL of single strength broth. When inoculating double strength broths, the final concentration of the broth ingredients must be equivalent to that of single strength broths.

3. Take three test tubes (or other containers as appropriate) of streptosel broth at the appropriate strength and volume. Using a sterile pipette transfer the amount of sample determined above into each broth.

   Note: The time elapsing between the preparation of the sample and the inoculation of the broth must not exceed 15 minutes.

4. Prepare further streptosel broths as follows:

   - Blank Control Broth:
     Leave this broth uninoculated.

   - Positive Control Broth:
     Inoculate this broth with the positive control organism.

   - Negative Control Broth:
     Inoculate this broth with the negative control organism.

     Note: If double strength broths were used for sample testing, the controls should also use double strength broths. Dilute these to single strength using an equal volume of sterile water. For the positive and negative controls, do this **before** inoculating the control organisms.

5. Incubate all broths aerobically at 37 ± 1 °C for 24 ± 2 hours.

45.2.7 Plating

1. Take three dried blood agar plates. Using a sterile loop for each broth, streak a loopful of broth on to the surface of each plate, so as to obtain well isolated colonies after incubation.

   Note: Dry the plates prior to use by placing them in a drying cabinet, agar surface down and without lids, at about 50 °C for about 30 minutes, or 35-37 °C for about 60 minutes.

2. Similarly streak a dried blood agar plate from the blank control broth, one from the positive control broth and one from the negative control broth.

3. Incubate all plates anaerobically at 37 ± 1 °C for 24 ± 2 hours.

   Note: See NZTM 1 Anaerobic and Co_2 - Enriched Incubation section for information regarding anaerobic incubation.

   Note: Incubate the plates in an inverted position.

45.2.8 Interpretation

1. Examine the plates for the presence of suspicious (presumptive) colonies.

   Presumptive colonies of β-haemolytic streptococci are:

   - about 0.5 mm in diameter, transparent or translucent, domed, with a shiny or semi-matt surface and an entire edge;
   - surrounded by a clear zone (indicating complete lysis of the red blood cells or β-haemolysis).

2. Examine the test control plates:

   Blank control plate

   There should be no colonies present. Otherwise, investigate the source of contamination.

   Positive control plate

   It should contain typical β-haemolytic colonies. Otherwise, investigate the cause of the problem.

   Negative control plate

   It should contain colonies that are α-haemolytic (partially haemolytic). Otherwise, investigate the cause of the problem.

3. Examine the sample plates. Select all plates with presumptive colonies.

4. Choose colonies from the plates for confirmation as follows:

   - Negative Control Plate:
     Choose a well-isolated colony. (Unless an alternative is specified).

   - Sample Plates:
     Choose at least two well-isolated presumptive colonies from each selected plate. If there is only one presumptive colony, choose it.

5. If it is not possible to choose well-isolated colonies, streak at least two presumptive colonies on to dried blood agar plates, incubate at as before, and choose one well-isolated presumptive colony from each plate.

45.2.9 Confirmation

1. Confirm each colony as follows:

   Gram stain

   Inoculate a portion of the colony into a tube of BHI. Incubate aerobically at 37 ± 1 °C for 18-24 hours. Carry out a Gram stain from the broth (see NZTM 1 Stain and Staining Procedures section).

   Result

   β-Haemolytic streptococci are Gram-positive, ovoid to spherical, and usually in moderate to long chains.

   Catalase production

   Note: Use *Staphylococcus aureus* NZRM 87 as the catalase-positive control.

   Add a drop of catalase reagent to a smear of a portion of one well-isolated colony on a clean glass slide. If the result is positive (i.e. if bubbles of oxygen are released) then another well-isolated colony **must** be streaked on to a non-selective medium such as tryptic soy agar and incubated aerobically at 37 ± 1°C for 18-24 hours.

   This step is mandatory if a positive catalase test is obtained since the blood from the blood agar plates may give false positive catalase results.

   Interpretation

   As stated above, catalase-positive bacteria produce bubbles of oxygen gas in the reagent within 10 seconds.

   Result

   β-Haemolytic streptococci are catalase-**negative**. (*S. aureus* is catalase-positive.)

2. Proceed as follows:

   - If the product specification is for β-haemolytic streptococci, go to Section 45.2.10.

   - If the product specification is for presumptive *S. pyogenes* (Lancefield Group A) go to Step 3 below.

3. **Bacitracin sensitivity**

   For each colony that tested Gram-positive and catalase-negative, streak a loopful of the BHI used for Gram staining over the surface of a blood agar plate to obtain a confluent growth after incubation. Aseptically place a 0.05 units bacitracin disk on to the surface of the plate and incubate aerobically at 37 ± 1 °C for 18-24 hours.

   Interpretation

   Bacteria that are sensitive to bacitracin produce a zone of growth inhibition around the disk.

   Result

   S. pyogenes is highly sensitive to bacitracin and produces a broad zone. (*S. lactis* is bacitracin-resistant.)

4. A summary of the tests is shown in Table 45.2.2.

   Table 45.2.2: Test summary

Test	Result
β-haemolytic streptococci	
Blood agar	Colonies surrounded by zones of complete clearing (β-haemolysis).
Gram stain	Gram-positive, oval to spherical cocci, in moderate to long chains.
Catalase test	Negative; does not produce gas.
S. pyogenes	
Bacitracin sensitivity	Sensitive; produces a broad zone of growth inhibition.

45.2.10 Expression of Results and Report

β-haemolytic streptococci

β-haemolytic streptococci are reported as being **"Detected"** in the sample for the amount of product tested in each tube, when two or three of the enrichment tubes are confirmed as positive.

β-haemolytic streptococci are reported as being **"Not detected"** in the sample, when none or only one of the enrichment tubes is confirmed as positive.

Example

If 1 mL of a 10^{-1} dilution of the sample was added to each of three streptosel broth tubes, and two or three tubes were confirmed as containing β-haemolytic streptococci, the result is expressed as **"Detected per 0.1 g (or 0.1 mL)".**

If none or only 1 tube was confirmed positive, the result is expressed as **"Not detected per 0.1g (or 0.1 mL)"**.

Example

If 10 mL of a 10^{-1} dilution of the sample was added to each of three streptosel broth tubes, and two or three tubes were confirmed as containing β-haemolytic streptococci, the result is expressed as **"Detected per gram (or per mL)".**

If none or only one result is expressed as **"Not detected per gram (or per mL)"**.

Streptococcus pyogenes

Report as presumptive *Streptococcus pyogenes* **"Detected"** or **"Not detected"** according to the criteria described above.

45.2.11 Precision

Precision values are not applicable to detection methods.

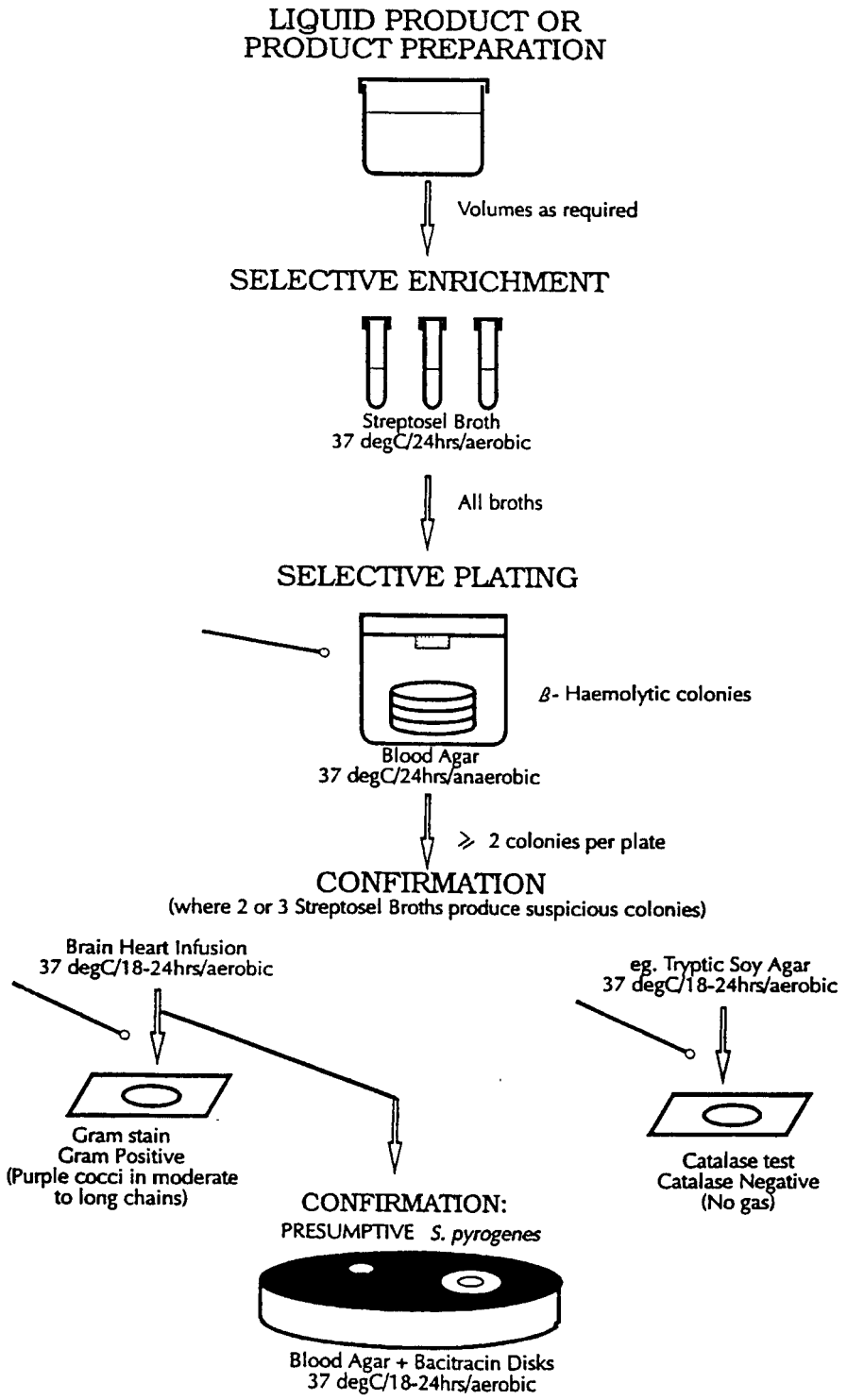
45.2.12 Notes

1 A few strains of β-haemolytic streptococci (including group A) do not produce streptolysin S, but streptolysin O will be detected when they are incubated anaerobically as in this method.

2 Bacitracin sensitivity identifies *S. pyogenes* with over 90% accuracy, but a few strains of *S. pyogenes* do not show growth inhibition in the bacitracin test.

Note: At any step in the test where β-haemolytic streptococci are shown to be **absent** in two or three of the streptosel broth enrichment tubes further testing is not required, and β-haemolytic streptococci are reported as being not detected in the sample (see Section 45.2.10). However, for purposes of product quality control, it is recommended that all presumptive positive tubes be confirmed.

Flow Chart 45.2.1: β-Haemolytic Streptococci; Detection



46.1 *Clostridium perfringens*; Count

46.1.1 Scope

This method is used to count *Clostridium perfringens* in milk and milk products using a plating and confirmatory testing technique.

46.1.2 Validation Status

This method is based on:

- ISO 7937:2004(E). *Microbiology of Food and Animal Feeding Stuffs. Horizontal method for enumeration of Clostridium perfringens. Colony count technique.*

The validation status of this method is not known.

46.1.3 Principle of Method

A defined test portion or a series of decimal dilutions of the sample is mixed with sulphite cycloserine agar in Petri dishes, and overlaid with the same medium. The plates are incubated anaerobically at 37 °C for 18-22 hours, and examined for suspicious black colonies (indicating production of H_2S).

Suspicious colonies are confirmed by a series of biochemical tests. The ratio of suspicious to confirmed *C. perfringens* colonies on the selected plates enables the determination of a *C. perfringens* count in the sample.

46.1.4 Diluent

- 0.1% peptone.

46.1.5 Media and Reagents

Sulphite Cycloserine (SC) Agar

Base medium	
Tryptose	15.0 g
Soytone	5.0 g
Yeast extract	5.0 g
Ammonium iron (III) citrate	1.0 g
Disodium disulphite, anhydrous	1.0 g
Agar	12-18 g*
Distilled or deionised water	1000 mL

pH 7.6 ± 0.2

* According to the manufacturer's specifications.

Add the ingredients to the water. Mix thoroughly and boil with frequent agitation just sufficiently to completely dissolve the ingredients. Adjust the pH so that after sterilisation it is 7.6 ± 0.2 at 25 °C. Sterilise at a maximum temperature of 121 °C for 15 minutes.

Store base medium at 2 – 8 °C for up to two weeks. If SC agar plates are required for confirmation with Nitrate Motility and Lactose Gelatin, transfer about 15 mL of the base medium (cooled to 44 – 47 °C) into Petri dishes and allow to solidify. Immediately before use, dry the plates.

D-cycloserine solution — 4%

D-cycloserine (use white crystalline powder only)	4.0 g
Distilled or deionised water	100 mL

Add the D-cycloserine to the water. Mix thoroughly to completely dissolve. Filter sterilise into a sterile bottle. Store at 0-5 °C. Discard after 4 weeks storage.

Complete medium (SC Agar)

Base medium	1000 mL
D-cycloserine solution - 4%	10 mL

Melt the base medium. Cool to 50 °C. Add the D-cycloserine and mix thoroughly. For spread plates, pour about 15 mL of complete medium into each Petri dish. Allow to solidify at room temperature. Plates may be stored prior to drying at 4 °C for up to 24 hours, or at room temperature for up to 4 hours. For pour plates, temper to 45 ± 1 °C and use immediately.

Fluid Thioglycollate Medium

Pancreatic digest of casein	15.0 g
L-cystine	0.5 g
Dextrose (D-glucose)	5.5 g
Yeast extract	5.0 g
Sodium chloride	2.5 g
Sodium thioglycollate (mercaptoacetate)	0.5 g
Agar	0.5-0.8 g
Resazurin	0.001 g
Distilled or deionised water	1000 mL

pH 7.1 ± 0.2

Add the ingredients to the water. Mix thoroughly and boil with frequent agitation just sufficiently to completely dissolve the ingredients. Adjust the pH so that after sterilisation it is 7.1 ± 0.2 at 25 °C. Dispense 10 mL volumes into test tubes. Sterilise at a maximum temperature of 121 °C for 15 minutes Just prior to use, heat in a boiling water bath for 15 minutes to de-aerate the medium and cool rapidly to 37 °C.

Lactose Sulphite (LS) Medium

Base medium

Enzymatic digest of casein	5.0 g
Yeast extract	2.5 g
Sodium chloride	2.5 g
Lactose	10.0 g
L-cysteine hydrochloride	0.3 g
Water	1000 mL

Dissolve the components in the water (by boiling if necessary). Adjust the pH so that after sterilisation it is 7.1 ± 0.2 at 25 °C. Dispense 8 mL portions into test tubes or bottles with inverted Durham tubes and sterilise at 121 °C for 15 minutes. The medium may be stored at 1-5 °C for up to 4 weeks.

Disodium disulphite, anhydrous solution

Anhydrous disodium disulphite ($Na_2S_2O_5$)	1.2 g
Water	100 mL

Dissolve the disodium disulphite in the water and sterilise by filtration. Use the solution within 24 hours.

Ammonium iron (III) citrate solution

Ammonium iron (III) citrate	1.0 g
Water	100 mL

Dissolve the ammonium iron (III) citrate in water and sterilise by filtration. Use the solution with 24 hours.

Complete medium

Just prior to use, de-aerate the base medium by placing in a boiling water and flowing steam bath for 15 minutes. Cool rapidly, then add:

Disodium disulphite solution	0.5 mL
Ammonium iron (III) citrate	0.5 mL

to each 8 mL tube of LS base medium.

Use the complete medium within 24 hours.

Nitrate Motility Medium

Enzymatic digest of casein	5.0 g
Meat extract	3.0 g
Galactose	5.0 g
Glycerol	5.0 g
Potassium nitrate (KNO_3)	1 0 g
Disodium hydrogen orthophosphate (Na_2HPO_4)	2.5 g
Agar	1.0 – 5.0 g
Water	1000 mL

Dissolve the components in the water (by boiling if necessary). Adjust the pH so that it will be 7.3 ± 0.2 at 25 °C after sterilisation.

Transfer the medium to 10 mL volumes in tubes, and sterilise at 121 °C for 15 minutes. If not used the same day, store at 2 – 8 °C.

Just prior to use, heat in a boiling water or steam bath for 15 minutes and then cool rapidly to the incubation temperature.

Discard unused medium 4 weeks after preparation.

Nitrite Detection Reagent

5-Amino-2-napthalenesulfonic acid (5-2-ANSA) solution

Dissolve 0.1 g of 5-2-ANSA in 100 mL of 15% (volume fraction) acetic acid solution. Filter through a filter paper.

Store in a well-stoppered brown bottle at 2 – 8 °C.

Sulfanilic acid solution

Dissolve 0.4 g of sulfanilic acid in 100 mL of 15% (volume fraction) acetic acid solution. Filter through a filter paper.

Store in a well-stoppered brown bottle at 2 – 8 °C.

Complete reagent

Mix equal amounts of the two solutions (ANSA and sulfanilic acid) just before use. Discard unused reagent immediately.

Zinc Dust

Lactose – Gelatin Medium

Enzymatic digest of casein	15.0 g
Yeast extract	10.0 g
Lactose	10.0 g
Gelatin	120.0 g
Phenol red	0 05 g
Water	1000 mL

Dissolve the components, except the lactose and phenol red, in the water. Adjust the pH so that after sterilisation it will be 7.5 ± 0.2 at 25 °C.

Add the lactose and phenol red, dispense 10 mL portions into test tubes and sterilise at 121 °C for 15 minutes. If not used on the same day, store at 2 – 8 °C.

Just prior to use, heat in a boiling water or flowing steam for 15 minutes, then cool rapidly to the incubation temperature.

Discard unused medium 3 weeks after preparation.

46.1.6 Quality Control Organisms

Positive Control:
- *Clostridium perfringens* NZRM 20

Negative Control (**Note:** optional to the method):
- *Clostridium sporogenes* NZRM 1097

46.1.7 Plating

1. Prepare a sample of the product as described in Section 41. Agitate the sample to ensure homogeneity and take test portions as soon as possible. Where possible, use reconstitution methods sourced from ISO 8261/ IDF 122:2001.

2. Take a sterile Petri dish. Using a sterile pipette, transfer 1 mL of the sample into each dish. Repeat if a duplicate plate is required.

   Note: This represents a 10^0 dilution for liquid products, and a 10^{-1} dilution for most prepared products. Refer to Section 41 for exceptions.

   Note: Refer to NZTM 1 Frequency of Duplicate Plating and Decimal Dilutions section for recommendations on the frequency of duplicate plates and dilutions.

3. If a further dilution is required, transfer 1 mL of the sample into 9 mL of sterile 0.1% peptone diluent. Mix by aspirating 10 times with a fresh pipette, or vortexing for 5-10 seconds. Transfer 1 mL of this dilution into a Petri dish. If a duplicate plate is required, transfer a further 1 mL into another Petri dish.

   Note: This represents a 10^{-1} dilution for liquid products, and a 10^{-2} dilution for most prepared products.

4. Repeat Step 3 for the number of dilutions required, each time adding 1 mL of the previous dilution to 9 mL of 0.1% peptone.

5. Pour 15-20 mL of molten SC medium, tempered to 45 ± 1 °C into each Petri dish.

6. Mix immediately after pouring into each Petri dish by rotating the dish sufficiently to obtain evenly dispersed colonies after incubation. Allow the agar to solidify at room temperature.

   Note: The time elapsing between the preparation of the sample and the addition of the agar must not exceed 15 minutes.

7. Prepare the following test controls, using pre-poured and dried SC plates.

   - Blank Control Plate:
     Leave uninoculated.

   - Positive Control Plate:
     Inoculate with the positive control organism.

   - Negative Control Plate:
     Inoculate with the negative control organism.

   Note: Dry the plates prior to use by placing them in a drying cabinet, agar surface down and without lids, at about 50 °C for 30 minutes, or at 35-37 °C for 1 hour.

8. Add a 10 mL overlay of SC medium, tempered to 45 ± 1 °C, to each dish. Allow the agar to solidify at room temperature.

9. Incubate the plates anaerobically at 37 ± 1 °C for 18-22 hours. Longer incubation may lead to excess blackening around the edges of the plates.

   Note: See NZTM 1 Anaerobic and CO_2 — Enriched Incubation section for information regarding anaerobic incubation.

46.1.8 Interpretation

1. Examine the plates for the presence of suspicious (presumptive) colonies.

   Presumptive *C. perfringens* colonies are black.

2. Examine the test control plates:

   Blank Control Plate

   There should be no colonies present. Otherwise, investigate the source of contamination.

Positive Control Plate

It should contain typical black *C. perfringens* colonies. Otherwise, investigate the cause of the problem.

Negative Control Plate

It should contain presumptive black colonies. Otherwise, investigate the cause of the problem.

3. Select sample plates, at two successive dilutions if applicable, containing between 15 and 150 presumptive colonies.

4. Count and record the number of presumptive colonies on each of these plates.

5. Choose colonies from the plates for confirmation as follows:

   - Positive Control Plate:
     Choose a well-isolated colony.

   - Negative Control Plate:
     Choose a well-isolated colony (unless an alternative is specified).

   - Choose a total of 5 well-isolated presumptive colonies from the selected plates representing the sample. If there are less than 5 presumptive colonies, choose all of them.

46.1.9 Confirmation

Confirm each colony using one of two confirmation regimes described below:

Fluid thioglycollate and LS medium

Inoculate each selected colony into fluid thioglycollate medium. Incubate anaerobically at 37 ± 1 °C for 18-24 hours.

After incubation, transfer with no delay 5 drops of the incubated thioglycollate culture to lactose sulphite medium with a sterile pipette. Incubate at 46 ± 1 °C for 18-24 hours in a waterbath.

Note: The reaction obtained at 46 °C in LS medium is very specific for *Clostridium perfringens* and some strains of *Clostridium paraperfringens* and *Clostridium absonum.* It is, therefore, not necessary to ensure that colonies picked from the TSC agar plates are pure before inoculation into thioglycollate broth and subsequently into the lactose sulphite medium.

Interpretation
Examine the tubes of LS medium for gas production and black precipitate (iron sulphite precipitate). Durham tubes more than one-quarter full of gas and tubes having a black precipitate are considered positive.

If the Durham tube's LS medium is less than one-quarter full of gas, transfer 5 drops of incubated LS medium to another tube of LS medium. Incubate at 46 ± 1 °C for 18-24 hours.

Examine the tube as described above.

Nitrate motility medium and lactose gelatin medium

Note: this technique requires well isolated colonies. If it is not possible to select well isolated colonies (e.g. due to overgrowth of SC Agar), inoculate 5characteristic colonies into pre-deaerated fluid thioglycollate medium. Incubate under anaerobic conditions at 37 °C for 18 – 24 hours. Streak growth from incubated tubes to SC base agar plates and overlay with 10 mL of SC base agar. Incubate SC agar plates anaerobically at 37 °C for 18 – 24 hours. Select at least one characteristic and well seperated colony from each incubated SC agar plate.

If necessary, repeat the streaking and inoculation on SC base agar until well isolated, characteristic black colonies are obtained.

Nitrate motilitymedium

Stab inoculate each selected colony into the freshly de-aerated nitrate motility medium.

Incubate under anaerobic conditions at 37 °C for 24 hours. Examine the incubated tube of motility medium for the type of growth along the stab line. Motility is evident from diffuse growth out into the medium away from the stab line.

Test for the presence of nitrite by adding, wth the graduated pipette, 0.2 mL – 0.5 mL of nitrate detection reagent to each tube of nitrate motility medium.

The formation of a red colour confirms the reduction of nitrate to nitrite. If no red colour is formed within 15 minutes add a small amount of zinc dust and allow to stand for 10 minutes. If a red colour is formed after addition of zinc dust, no reduction of nitrate has taken place.

Lactose – gelatin medium

Inocuate each selected colony into freshly deaerated lactose – gelatin medium. Incubate under anaerobic conditions at 37 °C for 24 hours.

Examine the tubes of incubated lactose – gelatin medium for the presence of gas and a yellow colour (due to acid formation) indicating fermentation of lactose.

Chill the tubes for 1 hour at 5 °C and check for gelatin liquefaction. If the medium has solidified re-incubate for an additional 24 hours and again check for gelatin liquefaction.

Table 46.1.1: Test Summary

Test	Result for *Clostridium perfringens*
SC agar	Black colonies
LS medium	Gas production and black precipitate
Nitrate motility	Non – motile, reduce nitrate to nitrite
Lactose gelatin	Produce acid and gas from lactose and liquefy gelatin in 48 hours. (Note: cultures that show a faint reaction for nitrite (i.e. faint colour pink) shall be eliminated as *Clostridium perfringens* consistently gives an intense and immediate reaction).

46.1.10 Expression of Results

1. Calculate the result as described in NZTM 1 Calculation of Counts section.

2. Express the result as "*Clostridium perfringens*, colony forming units per gram (or per mL)".

46.1.11 Precision

Precision values are as discussed in ISO 7937 2004 (E).

46.1.12 Notes

1. Samples should be analysed as soon as possible after collection, preferably within 10 hours. They should not be refrigerated or frozen for purposes of storing prior to testing, nor should they be subjected to sudden cooling.

2. *Clostridium perfringens* are oxygen-sensitive, so reconstitution, inoculation of tests and anaerobic incubation should be carried out promptly.

Harry, Molly *

From: Jim Heimbach [jh@jheimbach.com]
Sent: Thursday, September 03, 2009 10:16 AM
To: Harry, Molly *
Subject: Re: Clarification needed for GRN 288

Dear Molly--

A response to your question follows the copy of your e-mail. Please contact me if you need additional information.

Jim H.

James T. Heimbach, Ph.D., F.A C.N.
JHeimbach LLC
923 Water Street, P.O. Box 66
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cell (+1) 202-320-3063
e-mail jh@jheimbach.com
----- Original Message -----
From: Harry, Molly *
To: Jim Heimbach
Sent: Thursday, September 03, 2009 9:47 AM
Subject: Clarification needed for GRN 288

Hi Dr. Heimbach,

You may have already provided a response to this question for GRN 281, but the team would like you to provide a response for GRN 288 as well since it is a separate document.

The notifier has provided certificates of analysis and information regarding components of the media used to produce L. rhamnosus strain HN001. However, the information provided regarding the casein hydrolysate was limited. Please provide a brief description of the casein hydrolysate (including specifications if available), including a statement regarding the safety and suitability of the enzyme used to produce this ingredient.

Thanks.

Molly Harry

The identity of the protease used to hydrolyze casein protein to provide the peptone product used in the growth medium is proprietary, but we canconfirm that it is a food-grade enzyme permitted for this use in the United States (Pariza and Johnson 2001). The amino-acid composition and molecular-weight distribution of the hydrolysate is shown below.

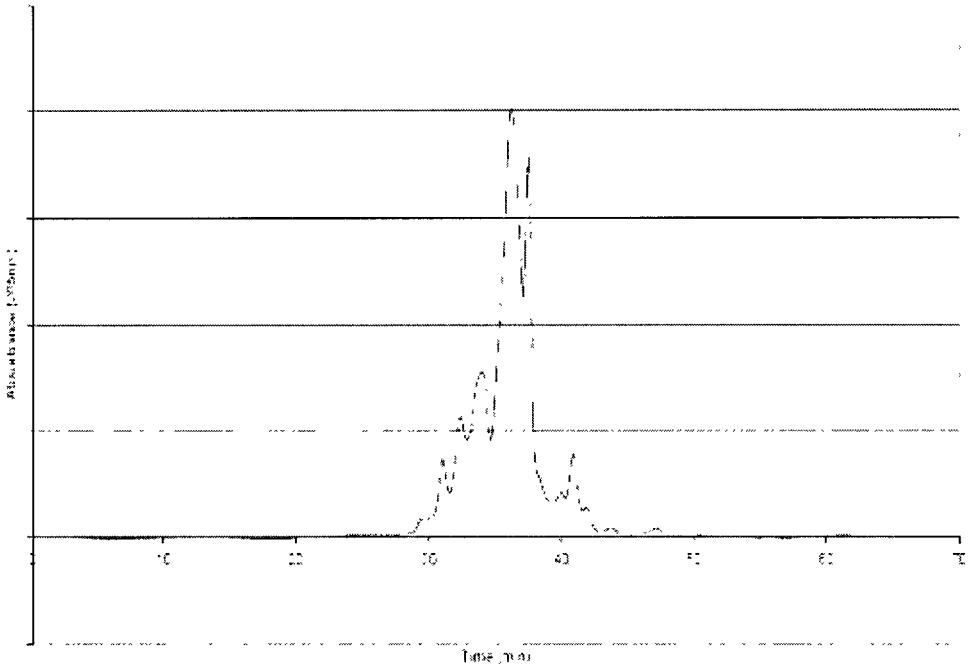
Reference

Pariza MW and EA Johnson. 2001. Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. *Reg Toxicol Pharmacol* 33:173-186.

Amino Acid Composition (mg/g of sample)

Amino Acid	Total
Alanine	2.75
Arginine	5.46
Asparagine or aspartic acid	61.0
Cystine	4.8
Glutamine or glutamic acid	202.0
Glycine	15.8
Histidine	22.3
Isoleucine	42.0
Leucine	87.5
Lysine	55.3
Methionine	23.2
Phenylalanine	48.6
Proline	101.0
Serine	48.0
Threonine	37.4
Tryptophan	14.8
Tyrosine	40.2
Valine	52.6

Molecular Weight Profile



Harry, Molly *

From: Jim Heimbach [jh@jheimbach.com]
Sent: Thursday, October 01, 2009 12:12 PM
To: Harry, Molly *
Cc: Gaynor, Paulette M
Subject: Re: Clarification needed for GRN 288

Dear Molly and Paulette--

Thank you for your telephone call this morning. Following are my responses to the clarification questions posed in your e-mail to me on September 11, 2009.

1. In place of "functional/nutritional food products," please list the specific food categories [from 21 CFR 170.3(n)] that are among the intended uses of the ingredient.

The specific food categories, as listed in 21 CFR 170.3(n), to which the subject probiotic strain may be added include:

(3) Beverages, such as sports drinks, energy drinks, and similar nutritional beverages

(23) Grain products, such as energy bars

(25) Hard candies, such as lozenges

(33) Plant protein products, including soy-based bars and beverages

2. Clarify intended use level and consumer exposure information.

I agree that this information is stated inconsistently and unclearly and I apologize. The intent is for the maximum addition level to be that, consistent with cGMP, needed to provide 10^9 cfu/serving throughout the shelf life of the product. This was occasionally stated as "up to 10^9 cfu/serving," which was simply intended as recognition that some products may have lower targeted probiotic levels than others. However, the intended use level should have been simply stated as "the concentration needed, consistent with cGMP, to provide 10^9 cfu/serving throughout the shelf life of the product."

Survival of probiotic bacteria in the food matrix is a complex issue, but for all foods some loss may be expected in the numbers of viable organisms over time. Thus, if a minimum concentration is to be available over the shelf life of the food, the addition of some level of overage is required. Variables that affect the amount of overage needed include the intended shelf life; the moisture level and water activity of the food; temperatures during final processing, shipping, and storage; pH; and exposure to oxygen.

Probiotic bacteria added to foods in dried form are metabolically quiescent, but their survival is still sensitive to these variables. Generally, survival is lessened, and therefore the overage must be greater, in foods with the following characteristics:

- longer intended shelf life
- greater level of water activity
- storage at higher temperature (e.g., ambient v. refrigerator)
- more acidic pH (e.g., in fruit juices)
- greater exposure to free oxygen

For foods that will be refrigerated and those with low water activity (which is most of the foods for which use is intended), a half-log increase in the concentration (i.e., to $5x10^9$ cfu/serving) is adequate to ensure survival of 10^9 cfu/serving through the shelf life. For a small number of foods, this level of overage may not be adequate and cGMP will allow addition of a full log (to 10^{10} cfu/serving) of overage.

For an extended discussion of the interaction between food-matrix characteristics and probiotic survival, see Lee YK and S Salminen. 2009. *Handbook of probiotics and prebiotics*, 2nd ed. Hoboken NJ: Wiley.

With regard to exposure estimation, a truly "worst case" scenario envisions a consumer puchasing foods early in their shelf lives that contain the highest expected overage levels, thus containing as high as 10^{10} cfu/serving, and consuming 10 servings of these foods in a day. This extreme case could potentially result in exposure as high as 10^{11} cfu/day. (Note that this scenario reveals one of the limitations of estimating exposure based on existing data on food consumption. No data, for example, are available on consumption of fruit juices containing probiotics, and so estimated intake must be based on total fruit juice consumption, which greatly overestimates the likely consumption of fruit juice with probiotics.)

A more realistic estimate of exposure is that consumers may consume as many as three or four servings per day of products containing between 10^9 and $5x10^9$ cfu/serving, and thus having an exposure in the range of 10^{10} cfu/day. This is an order of magnitude less than the 10^{11} cfu/day that the Expert Panel determined to be both safe and GRAS.

I hope that these responses are satisfactory in resolving your concerns.

Regards,
Jim

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fax (+1) 202-478-0986
cell (+1) 202-320-3063
e-mail jh@jheimbach.com

----- Original Message -----
From: Harry, Molly *
To: Jim Heimbach
Cc: Gaynor, Paulette M
Sent: Friday, September 11, 2009 11:54 AM
Subject: Clarification needed for GRN 288

Hi Dr. Heimbach,

We have a couple of areas in GRN 000288 that we will like further clarifications on.

1. In your June 22, 2009 response to our earlier questions, one of which was about "Functional/nutritional" products, you explained in part: "Functional/nutritional products has the usual meaning of any fresh or processed food intended or claimed to have a health-promoting property beyond the basic function of supplying nutrients".

In place of "functional/nutritional food products", please list the specific food categories (from 21 CFR 170.3(n)) that are among the intended uses of the ingredient. If the food categories are too broad to describe the intended uses, please provide a list of foods (e.g. energy bars, sports drinks, medical foods).

2. The intended use level and consumer exposure information appear to be stated inconsistently in different sections of the notice. For example:

i. Page 1, section 1.3 the notice states in part: "...concentration needed, consistent with cGMP, to provide up to 109 colony forming units (cfu) L. rhamnosus per serving of the food." "Anticipated consumer exposure from these intended uses is less than 1011 cfu/day, well within levels that have been shown to be safe."

ii. Page 16, section 3 in part states: "... L. rhamnosus strain HN001 is intended to be added to conventional foods at concentrations consistent with cGMP needed to provide at least 109 cfu/serving..."

iii. Section 3.2. of page 16 states in part: "L. rhamnosus is expected to be present in a limited number of foods at between 109 and 1011cfu/serving, usually at less than 1010 cfu/serving." "Its likely maximum ingestion is thus less than 1011 cfu/day, well within levels that have been shown to be safe."

iv. Page 1, second bullet of the Conclusion of the Expert Panel in part states: "...at concentrations consistent with cGMP needed to provide up to 109cfu/serving throughout the shelf life of the product. The estimated daily intake of the strain is less than 1011 cfu/day."

v. Page 3, second paragraph of the Conclusion of the Expert Panel states in part: "...confirm that no concerns exist regarding the safety of ingestion of this probiotic bacterium at levels up to 1010cfu/day."

Please clarify the intended use level and the exposure per day. We recommend that the intended use level and exposure be consistent with what the Expert Panel based their conclusions on.

Thanks.

Molly Harry

Harry, Molly *

From: Jim Heimbach [jh@jheimbach.com]
Sent: Wednesday, October 07, 2009 11:59 AM
To: Harry, Molly *
Cc: Gaynor, Paulette M
Subject: Re: Clarification needed for GRN 288

Dear Molly and Paulette--

I think probably what I will need to do is get new signature pages from the three members of the Expert Panel with the exposure being up to 10E11 cfu/day. However, before I do that let me give you an e-mail string that shows what happened and perhaps you will find it satisfactory as an explanation. As you'll see, at the very end of the exchange I made a mistake in not noticing the new inconsistency in the final conclusion of the panel.

Let me know if this explanation (along with the copies of the e-mails from the members of the Panel) is sufficient, or whether I need to get new signature pages.

Regards,
Jim

James T. Heimbach, Ph.D., F.A.C.N.
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March 12, 2009

Dear All--

Late last year we determined that Fonterra's *L. rhamnosus* strain is GRAS for addition to a specific list of conventional foods. Much to my frustration, Fonterra changed the intended use at the last minute. (Literally--I had 3 copies of the GRAS notice all bound and in the box, ready to send to FDA.)

They wanted to change the intended use to state that the probiotic could be added to any food that would sustain viable *L. rhamnosus* through its shelf life. Given the large range of foods already listed, this really didn't change much. (The old categories are listed now as "including but not limited to") Nevertheless, it took them two months to be happy with my suggested rewording.

So . . .

Here is a new copy of the monograph. The only change is in section 3, Intended Use and Consumer Exposure, which now reads as shown at the end of this e-mail. Please review and let me know if you agree that this amended use is GRAS. If so, I will send you a new Conclusion Statement to sign.

Thanks--
Jim

3. Intended Use and Exposure

L. rhamnosus strain HN001 is intended to be added to conventional foods at concentrations

consistent with cGMP needed to provide at least 10^9 cfu/serving throughout the shelf life of the product. This addition level will usually be between 5 x 10^9 and 10^{11} cfu/serving, which provides for the loss of viability of from 80% to 99% of the bacteria added. The strain's function is to serve as a probiotic microorganism.

3.1. Food Categories for Addition of *L. rhamnosus*

The foods to which *L. rhamnosus* is intended to be added are those foods that can sustain viable *L. rhamnosus* for the shelf life of the food, including but not limited to dairy products (fluid milk and milk drinks, milk-based desserts and meal replacements, dry and powdered milk, yogurt, and cheese); ready-to-eat cereals; fruit juices, nectars, ades, and drinks; confections; chewing gum; and functional/nutritional products.

3.2. Estimated Daily Intake of *L. rhamnosus*

L. rhamnosus is expected to be present in a limited number of foods at between 10^9 and 10^{11} cfu/serving, usually at less than 10^{10} cfu/serving. It will not proliferate in the foods and beverages to which it is added, but instead will decline over the shelf-life of the food. Its likely maximum ingestion is thus less than 10^{11} cfu/day, well within levels that have been shown to be safe.

March 14, 2009

Jim,

I have not received a new copy of the monograph but the changes you have detailed below are fully acceptable to me and I am ready to sign-off on a new Conclusion Statement.

Best regards as always,

Walter [Glinsmann]

March 16, 2009

Dear Joe and Mike--
I sent the following note last Thursday. Walter has indicated that he agrees that the intended use of the strain, as revised, is still GRAS. Please review and give me your opinions.

In the original note I said that I would send out a new Conclusion statement for your signatures once we all agree on the GRAS status, but that won't actually be necessary--the signature page that I already have won't change, so I can just use it once you all certify that you accept the GRAS status. The only change in the statement of the Conclusion of the Expert Panel is this one bullet:

- *L. rhamnosus* strain HN001 is intended to be added as a probiotic to foods that can sustain viability of the organism, including but not limited to dairy products (fluid milk and milk drinks, milk-based desserts and meal replacements, dry and powdered milk, yogurt, and cheese); ready-to-eat cereals; fruit juices, nectars, ades and drinks; confections; chewing gum; and functional/nutritional products at concentrations consistent with cGMP needed to provide up to 10^9 cfu/serving throughout the shelf life of the product. The estimated daily intake of the strain is less than 10^{11} cfu/day.

Regards,

Jim

March 17, 2009

Greetings, Jim, Mike, and Walter.
I agree with Walter that the intended uses are also GRAS.
It is always a pleasure working with each of you.
Fondest personal regards.
Joe [Borzelleca]

March 17, 2009

I concur-- Mike Pariza

RECEIVED
OCT 1 2 [redacted]
Division of
Biotechnology and
GRAS Notice Review

CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF *LACTOBACILLUS RHAMNOSUS* STRAIN HN001 (DR20™) IN CONVENTIONAL FOODS

Prepared for:
Fonterra Co-operative Group
New Zealand

Revised
October 2009

CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF *LACTOBACILLUS RHAMNOSUS* STRAIN HN001 (DR20™) IN CONVENTIONAL FOODS

We, the members of the expert panel, have individually and collectively critically evaluated the publicly available information on *Lactobacillus rhamnosus* strain HN001 and other *L. rhamnosus* strains summarized in a monograph prepared by JHEIMBACH LLC, as well as other material deemed appropriate or necessary. Our evaluation included review of the identity and characteristic properties of *L. rhamnosus*, including *L. rhamnosus* strain HN001, the potential exposure resulting from the intended use of *L. rhamnosus* strain HN001, and published research bearing on the safety of *L. rhamnosus* and strain HN001. Our summary and conclusion resulting from this critical evaluation are presented below.

Summary

- The probiotic bacterium that is the subject of this generally recognized as safe (GRAS) determination is *L. rhamnosus* strain HN001, a Gram-positive and non-spore-forming heterofermentative lactic acid bacterium known commercially as DR20™ and as Howaru Rhamnosus™.

- *L. rhamnosus* strain HN001 is intended to be added as a probiotic to foods that can sustain viability of the organism, including but not limited to dairy products (fluid milk and milk drinks, milk-based desserts and meal replacements, dry and powdered milk, yogurt, and cheese); ready-to-eat cereals; fruit juices, nectars, ades, and drinks; confections; chewing gum; and functional/nutritional products at concentrations consistent with cGMP needed to provide up to 10^9 cfu/serving throughout the shelf life of the product. The estimated daily intake of the strain is less than 10^{11} cfu/day.

- *L. rhamnosus* strain HN001 was isolated from cheddar cheese in New Zealand and was deposited with the Australian Government Analytical Laboratories (AGAL) as deposit number NM97/09513.

- *Lactobacillus* is a non-pathogenic genus consisting of over a hundred species with a large variety of phenotypic, biochemical, and physiological properties. *L. rhamnosus* is ubiquitous in the GI tracts of both humans and infra-human mammalian species and has long been ingested during normal food-consumption activities with no apparent adverse effects.

- The whole genome shotgun sequence of *L. rhamnosus* strain HN001 was determined and the resulting sequence was annotated and analysed for genes that may be possible safety concerns. The genome sequence and annotation of the genome and two plasmids were

submitted to the NCBI and published with Refseq accession numbers NZ_ABWJ00000000, NC_011223, and NC_011225.

- No genes encoding for antibiotic resistance, nor any sequences showing significant homology with known antibiotic resistance genes, were identified on either of the plasmids. A range of genes possibly involved in antibiotic resistance were identified in the HN001 genome, but none were closely linked to predicted mobile genetic elements and are thus unlikely to be transferable. This finding is consistent with phenotypic testing that showed that *L. rhamnosus* strain HN001 has no resistance not found in most other members of the species, and thus regarded as intrinsic rather than acquired.

- No genes were identified that are likely to be capable of producing biogenic amines, and none that would indicate a likelihood of virulence or infectivity. These findings are consistent with phenotypic data showing no production of biogenic amines, unusual adherence capability, mucin degradation, adverse metabolic activity, or infectivity.

- Production of *L. rhamnosus* strain HN001 is based on standard fermentation techniques, and all fermentation medium components are food-grade materials. Fonterra has provided food-grade specifications for *L. rhamnosus* strain HN001.

- Several studies in which *L. rhamnosus* strain HN001 was given to BALB/c mice at doses up to 10^{11} cfu/day produced no adverse effects. This finding is consistent with the general absence of adverse effects in studies in which other *L. rhamnosus* strains were administered to mice, hamsters, rats, and rabbits at doses as high as 10^{12} cfu/day.

- The safety of *L. rhamnosus* strain HN001 was also assessed in five studies with human adults, one with children, and one with infants. No adverse effects were observed in any of these studies. The safety of *L. rhamnosus* is supported by a large body of human studies of other *rhamnosus* strains—30 in adults, 24 in children, and 22 in infants—involving over 9000 individuals, with not a single report of an adverse effect other than increased wheezing in one study.

- The European Food Safety Authority (EFSA) classified *L. rhamnosus* as an organism having a Qualified Presumption of Safety (QPS) and thus being "freed from the need for further safety assessment." Similarly, in 2008 the FDA had no questions regarding Mead Johnson's determination that the addition of *L. rhamnosus* strain GG to infant formula is GRAS.

Conclusion

We, the undersigned expert panel members, are qualified by scientific education and experience to evaluate the safety of the addition of probiotic bacteria to conventional foods. We have individually and collectively critically evaluated the materials summarized above.

We recognize that *Lactobacillus* species have a long history of safe use and are appropriately regarded as non-pathogenic and non-toxicogenic. We conclude that *Lactobacillus rhamnosus* strain HN001 has been adequately identified and characterized and that both phenotypic and genotypic research confirm that no concerns exist regarding the safety of ingestion of this probiotic bacterium at levels up to 10^{11} cfu/day. Therefore, we conclude that addition of *L. rhamnosus* strain HN001 to conventional foods as described is safe.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusion. Therefore, the intended use of *L. rhamnosus* strain HN001 is safe, and is GRAS, via scientific procedures.

Joseph F. Borzelleca, Ph.D.
Professor Emeritus
Virginia Commonwealth University School of Medicine
Richmond, Virginia

Signature (b)(6) Date: 12 October 2009

Walter H. Glinsmann, M.D.
President
Glinsmann Inc.
Arlington, Virginia

Signature: _____ Date: _____

Michael W. Pariza, Ph.D.
Professor of Food Science
University of Wisconsin—Madison
Madison, Wisconsin

Signature: _____ Date: _____

Conclusion

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Joseph F. Borzelleca, Ph.D.
Professor Emeritus
Virginia Commonwealth University School of Medicine
Richmond, Virginia

Signature: _____ Date: _____

Walter H. Glinsmann, M.D.
President
Glinsmann Inc.
Arlington, Virginia

Signature: (b)(6) Date: Oct. 9, 2009

Michael W. Pariza, Ph.D.
Professor of Food Science
University of Wisconsin—Madison
Madison, Wisconsin

Signature: _____ Date: _____

Conclusion

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It is also our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusion. Therefore, the intended use of *L. rhamnosus* strain HN001 is safe, and is GRAS, via scientific procedures.

Joseph F. Borzelleca, Ph.D.
Professor Emeritus
Virginia Commonwealth University School of Medicine
Richmond, Virginia

Signature: _____ Date: _____

Walter H. Glinsmann, M.D.
President
Glinsmann Inc.
Arlington, Virginia

Signature: _____ Date: _____

Michael W. Pariza, Ph.D.
Professor of Food Science
University of Wisconsin—Madison
Madison, Wisconsin

Signature: [redacted] (b)(6) Date: October 9, 2009

Harry, Molly *

From: Jim Heimbach [jh@jheimbach.com]
Sent: Tuesday, October 20, 2009 1:46 PM
To: Harry, Molly *
Cc: Gaynor, Paulette M
Subject: Re: Request For Clarifications on GRN 000288
Attachments: Panel Conclusion for HN001 in Conventional Foods.20091020.pdf

Dear Molly--
I apologize for not catching the error in the deposit number in the Conclusion of the Expert Panel. Attached is a version with that corrected.

With regard to intended uses, I actually think that the best statement was that in the original GRAS notice before we started tinkering with it--"The foods to which *L. rhamnosus* is intended to be added are those foods that can sustain viable *L. rhamnosus* for the shelf life of the food..." The exposure level signed off on by the Expert Panel is sufficiently high to cover this broad statement of use. This statement clearly excluded retorted foods, since such a food can obviously not sustain viable bacteria. It also avoided getting into such details as whether "energy bars" (a food category that did not exist at the time section 170.3(n) was written) includes only grain-based bars or also soy-based bars (or, I suppose someday, pea-flour based bars, bean-flour based bars, chick-pea flour based bars, etc.) In the case of probiotics, where exposure and safety are estimated based on orders of magnitude, the sort of precision appropriate for chemical ingredients is simply not needed.

Nevertheless...

No, we do not plan to add probiotic to retorted foods. They actually got included more or less by accident--we were indicating that we did wish to include toddler foods as well as adult foods and had at one point specifically listed cereals and fruit juices; strained fruits and vegetables got added in defining the category of toddler foods.

Yes, soy-based meal-replacement or energy bars would be included. (Actually, your suggested wording specifically includes "soy-based drinks and meal-replacements.")

Yes, we wish to include the entirety of 170.3(n)(3) excluding soft drinks.

No, we do not wish to include 170.3(n)(10) dairy analogs.

Yes, we do wish to include 170.3(n)(5) and 170.3(n)(31) cheeses and other milk products.

Regards,
Jim

James T. Heimbach, Ph.D., F.A.C.N.
JHeimbach LLC
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tel (+1) 804-742-5548
fax (+1) 202-478-0986
cell (+1) 202-320-3063
e-mail jh@jheimbach.com

----- Original Message -----
From: Harry, Molly *
To: Jim Heimbach
Cc: Gaynor, Paulette M
Sent: Tuesday, October 20, 2009 12:08 PM

Subject: Request For Clarifications on GRN 000288

Dear Dr. Heimbach,
Below is the suggested text regarding the food categories to which Fonterra intends to add the L. rhamnosis HN001 ingredient.
"The notice informs FDA of the view of Fonterra that L. rhamnosus strain HN001 is GRAS, through scientific procedures, for use as an ingredient in dairy products (fluid milk and milk drinks, milk-based desserts and meal replacements, dry and powdered milk, yogurt, and cheese), soy-based drinks and meal-replacements, ready-to-eat cereals, fruit juices, nectars, ades, and drinks, confections, chewing gum, sports drinks, energy drinks and bars, hard candies, and infant and toddler foods (cereals, strained fruits and vegetables) at a level to provide up to 109 colony forming units (cfu) per standard serving".
Please confirm (or otherwise provide clarification) that the intended uses in this paragraph cover the proposed uses listed in the notice, as well as in the additional correspondence sent on June 22, 2009 and Oct 1, 2009.
We note that, as written, the proposed uses appear to include foods subjected to retort (e.g. strained vegetables for toddlers). We believe that retort would kill the probiotic. Is this correct?
We note that you indicated the food category grain products (e.g. energy bars) among the proposed uses. Does this include soy-based meal replacement bars? What other foods are included in this category, if any?
We note that you indicated the food category beverages among the intended uses. Do the intended categories include all beverages and beverage bases listed therein (21 CFR 170.3(n)(3)) excluding soft drinks?
You have listed certain dairy products as examples of intended uses of the HN001 ingredient. Do the proposed uses include items listed within the broader categories of 21 CFR 170.3(n)(10) dairy product analogs; 21 CFR 170.3(n)(5) cheeses; 21 CFR 170.3(n)(31) milk products?.
Additionally, the new Expert Panel conclusion page sent to us on October 12, 2009 still contains the wrong deposit number NM97/09513 (page 1 3rd bullet), it was corrected in the June 22,2009 amendments to NM97/09514. Please clarify this.
Thanks.
Molly Harry

RECEIVED
OCT 20 2009
BY: (b)(6)

CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF *LACTOBACILLUS RHAMNOSUS* STRAIN HN001 (DR20™) IN CONVENTIONAL FOODS

Prepared for:
Fonterra Co-operative Group
New Zealand

January 2009

CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF *LACTOBACILLUS RHAMNOSUS* STRAIN HN001 (DR20™) IN CONVENTIONAL FOODS

We, the members of the expert panel, have individually and collectively critically evaluated the publicly available information on *Lactobacillus rhamnosus* strain HN001 and other *L. rhamnosus* strains summarized in a monograph prepared by JHEIMBACH LLC, as well as other material deemed appropriate or necessary. Our evaluation included review of the identity and characteristic properties of *L. rhamnosus*, including *L. rhamnosus* strain HN001, the potential exposure resulting from the intended use of *L. rhamnosus* strain HN001, and published research bearing on the safety of *L. rhamnosus* and strain HN001. Our summary and conclusion resulting from this critical evaluation are presented below.

Summary

- The probiotic bacterium that is the subject of this generally recognized as safe (GRAS) determination is *L. rhamnosus* strain HN001, a Gram-positive and non-spore-forming heterofermentative lactic acid bacterium known commercially as DR20™ and as Howaru Rhamnosus™.

- *L. rhamnosus* strain HN001 is intended to be added as a probiotic to foods that can sustain viability of the organism, including but not limited to dairy products (fluid milk and milk drinks, milk-based desserts and meal replacements, dry and powdered milk, yogurt, and cheese); ready-to-eat cereals; fruit juices, nectars, ades, and drinks; confections; chewing gum; and functional/nutritional products at concentrations consistent with cGMP needed to provide up to 10^9 cfu/serving throughout the shelf life of the product. The estimated daily intake of the strain is less than 10^{11} cfu/day.

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- Several studies in which *L. rhamnosus* strain HN001 was given to BALB/c mice at doses up to 10^{11} cfu/day produced no adverse effects. This finding is consistent with the general absence of adverse effects in studies in which other *L. rhamnosus* strains were administered to mice, hamsters, rats, and rabbits at doses as high as 10^{12} cfu/day.

- The safety of *L. rhamnosus* strain HN001 was also assessed in five studies with human adults, one with children, and one with infants. No adverse effects were observed in any of these studies. The safety of *L. rhamnosus* is supported by a large body of human studies of other *rhamnosus* strains—30 in adults, 24 in children, and 22 in infants—involving over 9000 individuals, with not a single report of an adverse effect other than increased wheezing in one study.

- The European Food Safety Authority (EFSA) classified *L. rhamnosus* as an organism having a Qualified Presumption of Safety (QPS) and thus being "freed from the need for further safety assessment." Similarly, in 2008 the FDA had no questions regarding Mead Johnson's determination that the addition of *L. rhamnosus* strain GG to infant formula is GRAS.

Conclusion

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It is also our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusion. Therefore, the intended use of *L. rhamnosus* strain HN001 is safe, and is GRAS, via scientific procedures.

Joseph F. Borzelleca, Ph.D.
Professor Emeritus
Virginia Commonwealth University School of Medicine
Richmond, Virginia

Signature (b)(6) Date: 12 October 2009

Walter H. Glinsmann, M.D.
President
Glinsmann Inc.
Arlington, Virginia

Signature: _____ Date: _____

Michael W. Pariza, Ph.D.
Professor of Food Science
University of Wisconsin—Madison
Madison, Wisconsin

Signature: _____ Date: _____

Conclusion

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Joseph F. Borzelleca, Ph.D.
Professor Emeritus
Virginia Commonwealth University School of Medicine
Richmond, Virginia

Signature: _____ Date: _____

Walter H. Glinsmann, M.D.
President
Glinsmann Inc.
Arlington, Virginia

Signature: (b)(6) Date: Oct. 9, 2009

Michael W. Pariza, Ph.D.
Professor of Food Science
University of Wisconsin—Madison
Madison, Wisconsin

Signature: _____ Date: _____

Conclusion

We, the undersigned expert panel members, are qualified by scientific education and experience to evaluate the safety of the addition of probiotic bacteria to conventional foods. We have individually and collectively critically evaluated the materials summarized above.

We recognize that *Lactobacillus* species have a long history of safe use and are appropriately regarded as non-pathogenic and non-toxicogenic. We conclude that *Lactobacillus rhamnosus* strain HN001 has been adequately identified and characterized and that both phenotypic and genotypic research confirm that no concerns exist regarding the safety of ingestion of this probiotic bacterium at levels up to 10^{11} cfu/day. Therefore, we conclude that addition of *L. rhamnosus* strain HN001 to conventional foods as described is safe.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusion. Therefore, the intended use of *L. rhamnosus* strain HN001 is safe, and is GRAS, via scientific procedures.

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