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JHeimbach LLC

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May 27, 2008

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), BioGaia AB of Stockholm, Sweden, through me as its agent, herby provides notice of a claim that the use of the probiotic bacterium described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because BioGaia AB 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. Please note that the signatures of the four members of the Expert Panel appear on page 92 of the document.

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 of *Lactobacillus reuteri* Strain DSM 17938

Prepared for
BioGaia AB
Stockholm, Sweden

Prepared by
JHeimbach LLC
Port Royal VA

May 2008

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1. GRAS Exemption Claim

1.1. Name and Address of Notifier

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1.2. Name of GRAS Organism

The subject of this Generally Recognized as Safe (GRAS) determination is a strain of the probiotic bacterium *Lactobacillus reuteri*. The strain is known commercially as *L. reuteri Protectis*. It has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and referenced as DSM 17938. This strain is derived by the deletion of two plasmids, using natural methods, from a strain deposited in the American Type Culture Collection (ATCC) in 1995 and referenced as ATCC 55730. Strain ATCC 55730 is also referred to in the scientific literature by the designations SD 2112, ING 1, and MM 53, but the designation ATCC 55730 is used throughout this document. An organism derived from strain ATCC 55730 by the deletion of one plasmid was deposited by BioGaia in the DSMZ as DSM 17686. This strain was then used as the starting point for the deletion of the second plasmid to produce strain DSM 17938. Both cured strains, DSM 17686 and DSM 17938, are substantially equivalent to their parent strain in all respects other than possession of these plasmids (which contain genes encoding for antibiotic resistance) and thus share its safety profile. Most of the research on this strain of *L. reuteri* was performed using parent strain ATCC 55730.

1.3. Intended Use and Consumer Exposure

L. reuteri strain DSM 17938 is intended to be added to a variety of foods at the concentration needed to provide 10^8 colony forming units (cfu) *L. reuteri* per serving of the food, which may be as high as 10^9 cfu at the time of manufacture. The foods to which *L. reuteri* is intended to be added are processed cheeses, yogurt, ice cream, fruit juices and drinks, beverage bases, energy bars and drinks, and chewing gum. Some of these conventional foods may be consumed by infants and children, and others may be specifically targeted to infants and children. It is also planned to incorporate *L. reuteri* in a drinking straw at the same level, 10^8 cfu, such that the probiotic bacteria can be ingested while consuming any beverage. The total estimated consumer exposure from these intended uses is less than 10^9 to 10^{10} cfu/day.

1.4. Basis for GRAS Determination

BioGaia's GRAS determination for the intended use of *L. reuteri* strain DSM 17938 is based on scientific procedures as described under 21 CFR §170.30(b).

Determination of the safety and GRAS status of the intended addition to food of *L. reuteri* strain DSM 17938 was made through the deliberations of an Expert Panel consisting of Berthold V. Koletzko, M.D., Daniel J. O'Sullivan , Ph.D., Mary Ellen Sanders, Ph.D., and John A. Thomas, 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. reuteri* DSM 17938 resulting from its intended use, and individually and collectively concluded that no evidence exists in the available information on *L. reuteri* strain DSM 17938, its parent strain ATCC 55730, or other *L. reuteri* strains, that demonstrates or suggests reasonable grounds to suspect a hazard to adults or to infants or children under the intended conditions of use of *L. reuteri* strain DSM 17938.

It is the Expert Panel's opinion that other qualified scientists reviewing the same publicly available data would reach the same conclusion. Therefore, *Lactobacillus reuteri* strain DSM 17938 is GRAS by scientific procedures under the conditions of use described.

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 reuteri*. The strain is known commercially as *L. reuteri Protectis*. It was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) on 30 January 2006 and referenced as DSM 17938. This strain is derived by the deletion of two plasmids from the original strain deposited in the American Type Culture Collection (ATCC) on 6 December 1995 and referenced as ATCC 55730. Strain ATCC 55730 is also referred to in the scientific literature by the designations SD 2112[1], ING 1, and MM 53[2], but the designation ATCC 55730 is used throughout this document. An organism derived from strain ATCC 55730 by the deletion of one plasmid was deposited by BioGaia in the DSMZ as DSM 17686. This strain was then used as the starting point for the deletion of the second plasmid to produce strain DSM 17938. Both cured strains, DSM 17686 and DSM 17938, are substantially equivalent to their parent strain in all respects other than possession of these plasmids (which contain genes encoding for antibiotic resistance) and thus share its safety profile. Most of the research on this strain of *L. reuteri* was performed using parent strain ATCC 55730.

2.2. Description of the GRAS Organism

Lactobacillus reuteri is a Gram-positive bacterium that is a member of the broad classification of lactic acid bacteria (LAB). LAB are 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, nonsporing, catalase-negative and 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 possible exception of 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 food borne 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

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1 The original ATCC deposit of the bacterium was into the "Safe Deposit" (or SD) collection on 29 November 1994 and it thus received the designation SD 2112; the designation was changed when it was moved to the public collection.

2 At one time the laboratory assigned strain names based on the origin of the strain and the prefix MM was used for strains derived from "mothers milk."

are safe for use in conventional foods that may be consumed by all members of the general population.

Lactobacillus is a non-pathogenic genus of LAB that consists of over 112 recognized species as of November 2007 (EFSA 2007). Lactobacilli grow under reduced oxygen conditions in habitats where ample nutrients exist. They are used in commercial applications for the fermentation of dairy products, fruits, vegetables, and meats (Aguirre and Collins 1993; Gasser 1994). Some *Lactobacillus* species 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). Like other *Lactobacillus* species, *L. reuteri* is rod-shaped, measuring about 0.7 to 1.0 μm in diameter and 2.0 to 5.0 μm in length (Cogan 1996). It occurs in clusters, singly, or in pairs.

Lactobacilli 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. reuteri* is an obligate heterofermenter (Cogan 1996; Kandler et al. 1980). Recent studies of *L. reuteri* ATCC 55730 have determined that both the Embden-Meyerhof pathway and the phosphoketolase pathway are employed by this strain to ferment glucose or sucrose (Arskold et al. 2008). The main flux is through the latter pathway, while the former pathway is used as a shunt. During exponential growth phase, over 70% of the flux goes through the phosphoketolase pathway; this rises to 84% if the substrate is composed solely of sucrose. Genomic analysis of ATCC 55730 confirmed the presence of genes for both glycolytic pathways (Arskold et al. 2008).

L. reuteri is found in the gastrointestinal tract of a number of species, including humans (Kandler and Weiss 1986). Indeed, *L. reuteri* is the only *Lactobacillus* species reported to inhabit the gastrointestinal tract of all vertebrates and mammals, ranging from birds to humans (Casas and Dobrogosz 2000). *L. reuteri* has been isolated in living form from every part of the digestive tract—the oral cavity, stomach, small intestine, and colon, as well as from stool samples and from the vagina (Reuter 2001). *L. reuteri* is also found in human breast milk; as discussed below, strain ATCC 55730, the parent of strain DSM 17938, was isolated from this source. Sinkiewicz and Nordstrom (2005; abstract only) studied the occurrence of *L. reuteri* (as well as other lactobacilli and bifidobacteria) in milk from lactating mothers in urban and rural areas of Sweden, Denmark, Israel, South Africa, South Korea, Japan, and Peru. Overall, 12% of the mothers had detectable counts of *L. reuteri* in their milk, with a higher rate of incidence in rural than urban areas.

The type strain of what was later designated as *L. reuteri* was isolated from the intestine of a human adult by Lerche and Reuter (1962) and designated as the reference strain of *L. fermentum* biotype IIb (Hansen 1968). The strain was deposited by Reuter in 1964 at the ATCC as *L. fermentum* Beijerinck and assigned the number ATCC 23272; it was later transferred to the DSMZ with the number DSM 20016. Kandler et al. (1980) described this biotype as *L. reuteri*, a newly designated species of heterofermentative *Lactobacillus* according to DNA-DNA homology. While *L. reuteri* and *L. fermentum* are phenotypically similar, they differ in molecular phenotypology in possessing different peptidoglycan types (Klein et al. 1998). The parent of the cured strain that is the subject of this GRAS determination, ATCC 55730, was isolated from the

milk of a lactating woman in Huancayo, Peru, at the Agrarian University "La Molina" in Lima (Johnson et al. 2006); it was identified and characterized by Casas and Dobrogosz (1996).

Bath et al. (2005) subjected DNA from *L. reuteri* ATCC 55730 to 4x shotgun genome sequencing in order to identify and characterize the extracellular proteins, i.e., cell-surface-associated proteins that mediate nutrient uptake, environmental sensing, adhesion to intestinal surfaces, and other host interactions. Bioinformatic analysis exposed 126 genes encoding putative extracellular proteins in the genome. The largest class of proteins with a predicted function was enzymes, which were encoded by 44 genes, followed by transport proteins (13 genes), proteins of miscellaneous function (6 genes), regulators or signal transduction components (4 genes), conserved proteins of unknown function (35 genes), and unconserved proteins of unknown function (24 genes). Most of these proteins are widely distributed (50 universally, 33 in all Gram-positive bacteria, 13 in all LAB, and 7 in all lactobacilli), but 24 have not been identified in other bacteria.

The ability of *L. reuteri* to transit the harsh acidic conditions of the stomach (with fasting pH of about 1.5 and feeding pH between 3.0 and 5.0) successfully after oral ingestion (Wall et al. 2007) likely contributes to its ability to influence human physiology. In studies of the effect of acid shock on *L. reuteri* strain ATCC 55730, Wall et al. (2007) found that a number of gene expressions are up-regulated to provide protection. Paramount among these are *clpL*, encoding an ATPase with chaperone activity, lr1516, encoding a putative esterase, and lr1797, putatively encoding a phosphatidylglycerophosphatase. Mutation studies showed that damage to these genes resulted in a variant with severely lowered ability to survive low pH conditions.

As is discussed in more detail in Section 4.3.4, a 21x shotgun sequencing of *L. reuteri* DSM 17938 predicted a total of 2,299 potential genes, likely representing over 98% of the gene content of the strain (O'Sullivan 2008). An analysis of the genome annotation did not reveal any gene or gene cluster known to be involved in virulence or antibiotic resistance.

2.3. Characterization of Strains DSM 17686 and DSM 17938

2.3.1. Curing of the Plasmid pLR581

2.3.1.1. Identification of Plasmids in *L. reuteri* ATCC 55730

L. reuteri ATCC 55730 exhibits resistance to the antibiotic tetracycline. Roos and Rosander (2005) performed a whole-genome shotgun sequencing of *L. reuteri* ATCC 55730. After annotation of all open reading frames in the genome sequence, 4 contigs were found to harbor plasmid-related genes. These contigs were built up of a higher number of sequencing runs, indicating an elevated copy number compared with genes located on the chromosome. The 4 plasmids have the sizes 8.1 kb (pLR580), 12.9 kb (pLR581), 18.3 kb (pLR585), and 24.9 kb (pLR584).

2.3.1.2. Isolation of Tetracycline Resistance

Roos and Rosander (2005) then subjected *L. reuteri* ATCC 55730 to protoplast formation, similar to an earlier report on curing of *L. reuteri* (Vescovo et al. 1984), in order to obtain a strain cured from the tetracycline resistance. This method of curing is a natural process rather

than a biotechnological approach, in which the bacteria are starved of nutrients and temporarily stressed, inducing the spontaneous loss of unnecessary plasmids. After incubation in the protoplast buffer, a hundred-fold more colonies were obtained on the MRS plates with sucrose than on those without sucrose, indicating an efficient formation of protoplasts. Roos and Rosander (2005) examined 200 colonies from the sucrose plates for growth or non-growth on MRS agar with and without tetracycline. Seven non-growing colonies were re-plated and grew on MRS with tetracycline, indicating that these colonies were false candidates. One colony, however, grew well on the control MRS plate but not at all on the MRS plate with tetracycline. This colony was re-plated and assumed to be a true candidate; i.e., one that did not exhibit tetracycline resistance.

The tetracycline sensitive candidate was analyzed using polymerase chain reaction (PCR; Roos and Rosander 2005). Both lr2004 (a gene encoding a replication protein in pLR581) and lr1996 (*tetW*) were lacking, whereas the genes representing the other 3 plasmids were still found in the candidate, indicating that it was cured only from the *tetW*-containing plasmid.

2.3.1.3. Substantial Equivalence of the Parent and Daughter Strains

Roos and Rosander (2005) found that the plasmid pLR581 harbored 13 other genes besides *tetW*. These genes and their putative functions were identified, and none of the genes was thought to be of importance for the probiotic effect of the strain. The repetitive extragenic palindromic-PCRs (rep-PCRs) comparing the parent and daughter strains were identical with regard to the selected primers, showing both that the daughter strain was a true variant of *L. reuteri* ATCC 55730 and not a contaminant, and that removal of pLR581 did not affect the rep-PCR. Roos and Rosander (2005) compared the colony and cell morphology of the parent and daughter strains. No differences in either the colony or cell morphology were detected. Additionally, the fermentation patterns of the 2 strains were compared using api 50 CHL medium and no differences could be detected (Roos and Rosander 2005). Finally, the production of reuterin was of the same magnitude for the 2 strains.

As a final check on the deletion of tetracycline resistance, Roos and Rosander (2005) compared the minimum inhibitory concentrations (MIC) of tetracycline for the parent and daughter strains. The MIC were found to be 256 and 6 µg/ml, respectively, demonstrating that *tetW* alone is responsible for the tetracycline resistance of *L. reuteri* ATCC 55730 and that the curing was successful.

The cured strain, identical with the parent strain except for the deletion of plasmid pLR581, was deposited at DSMZ and given the designation *Lactobacillus reuteri* DSM 17686. As was confirmed by the testing performed by Roos and Rosander (2005), it is substantially equivalent to its parent strain and the evidence demonstrating the safety of *L. reuteri* ATCC 55730 is equally applicable to strain DSM 17686.

2.3.2. Curing of the Plasmid pLR585

2.3.2.1. Identification of Lincomycin Resistance Gene

During a screen of lactic acid bacteria in which over 200 isolates from 90 different sources were tested, including 74 samples of *Lactobacillus* strains, Kastner et al. (2006) found

that *L. reuteri* ATCC 55730 appeared to exhibit resistance to lincomycin and clindamycin and to contain the resistance gene *lnuA*. Roos and Rosander (2006) determined the MIC for lincomycin and clindamycin for a variety of *L. reuteri* strains, including ATCC 55730, DSM 17686 (SD2112s), DSM 20016, ATCC 55148 (11284), ATCC PTA 4659 (MM2-3), ATCC PTA 5289 (FJ1), and ATCC PTA 6475 (MM4-1A). Strains ATCC 55730 and its daughter strain DSM 17686 (the evolution of which was described above in Section 2.3.1) were both found to be resistant to lincomycin but not to clindamycin.

Using the genome sequence information described in Section 2.3.1.1, above, Roos and Rosander (2006) searched for the lincomycin-resistance gene *lnuA* and identified it as the open reading frame lr2105 on plasmid pLR585, the previously identified 18.3 kb plasmid. They found that this plasmid also harbored genes encoding a multidrug resistance protein (lr2089) and a polyketide antibiotics exporter (lr2096 and lr2097). Roos and Rosander (2006) also determined that none of the genes on the plasmid had any evident connection or relevance of importance for the known probiotic characteristics of *L. reuteri* ATCC 55730.

2.3.2.2. Isolation of Lincomycin Resistance

Roos and Rosander (2006) began with the daughter strain (DSM 17686) that had already been cured from pLR581 and subjected it to protoplast formation in order to obtain a strain cured from pLR585, using a protocol similar to that used to cure *L. reuteri* ATCC 55730 from the *tetW*–containing plasmid pLR581 as described above. After the protoplast procedure, one colony was found to grow well on the MRS plate but not at all on the MRS plate with lincomycin.

The plasmids were analysed with PCR (Roos and Rosander 2006). Both pLR585 and pLR581 were absent, whereas the other two plasmids were still found. This result confirmed that the new strain was cured from both the *tetW*-plasmid pLR581 and the *lnuA*-plasmid pLR585. The absence of *lnuA* was also confirmed by PCR.

2.3.2.3. Substantial Equivalence of the Parent and Both Daughter Strains

The rep-PCR patterns comparing the parent strain (ATCC 55730) and both daughter strains were identical with regard to the selected primers (Figure 1), showing that both daughter strains are true variants of *L. reuteri* ATCC 55730 and not contaminants, and that removal either of pLR581 alone or of pLR581 and pLR585 together does not affect the rep-PCR (Roos and Rosander 2006).

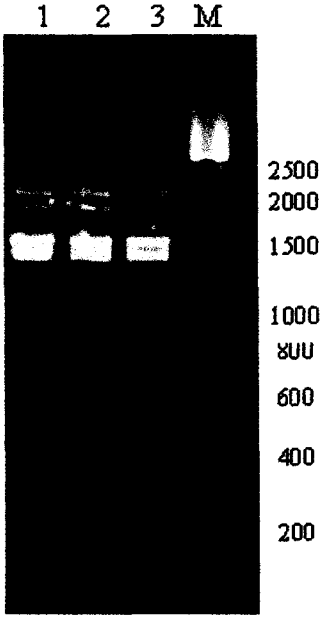

Figure 1. Rep-PCRs of Parent and Daughter Strains
(1: DSM17938; 2: DSM 17686; 3: ATCC 55730; M: Mw marker [sizes in bp])

Roos and Rosander (2006) compared the colony and cell morphology of the parent strain and both daughter strains. No differences in either the colony or cell morphology were detected among the 3 strains. Additionally, the fermentation patterns of the 3 strains were compared using api 50 CHL and no differences could be detected. Finally, the production of reuterin was of the same magnitude for all 3 strains.

The growth of the 2 daughter strains was compared with that of ATCC 55730 (Roos and Rosander 2006). No difference in generation time could be detected; however, both cured strains grew to a significantly higher density than ATCC 55730. The 3 strains were also co-cultured in MRS broth for approximately 30 generations. The results revealed that the cured strains had an advantage when growing *in vitro*. Equal amounts of the strains were inoculated together, but at the end of the experiment the mixture consisted of only 6% ATCC 55730 (with all 4 plasmids), 38% DSM 17686 (with 3 plasmids), and 56% of the new daughter strain with only 2 plasmids. Roos and Rosander (2006) speculated that the cured strains may survive better in stationary phase or be more competitive, possibly because the loss of two plasmids might have resulted in a decreased reproductive burden (less DNA to replicate) and thereby higher competitive ability.

Roos and Rosander (2006) compared the mucus binding ability of the 3 strains, finding no difference, and the acid tolerance, finding that the 2 daughter strains were somewhat more likely to survive at pH 2.0 than was the parent strain, ATCC 55730; the authors offered no explanation for this difference. There were no differences among the strains in tolerance to bovine bile in either stationary or exponential growth phase.

As a final check on the deletion of lincomycin resistance, Roos and Rosander (2006) compared the minimum inhibitory concentrations (MIC) of lincomycin (as well as tetracycline)

for the parent and both daughter strains. The MIC for tetracycline was found to be 256 µg/ml for the parent strain and 12-16 µg/ml for the 2 daughter strains, demonstrating that tetracycline resistance was not affected by deletion of the second plasmid. With regard to lincomycin, the MIC for both ATCC 55730 and DSM 17686 were 19 µg/ml, while that for the new strain with plasmid pLR585 deleted was only 0.5 µg/ml, showing complete removal of lincomycin resistance.

This strain, identical with the parent strain ATCC 55730 except for deletion of plasmids pLR581 and pLR585, was deposited at DSMZ and given the designation *Lactobacillus reuteri* DSM 17938. As was confirmed by the testing performed by Roos and Rosander (2005 and 2006), it is substantially equivalent to its parent strain and the evidence demonstrating the safety of *L. reuteri* ATCC 55730 is equally applicable to strain DSM 17938.

2.4. Production Process

Production of *L. reuteri* strain DSM 17938 is carried out by independent suppliers under contract with BioGaia. At the current time these suppliers, who are responsible for fermentation and freeze-drying, include Chr Hansen, Danisco, and Medipharm; other suppliers may be contracted in the future, but all lots of *L. reuteri* strain DSM 17938 entering the market will meet the established food-grade specifications described in the following section.

Production of *L. reuteri* strain 17938 begins with preparation directly from the deposit at Microbial Developments Ltd. of working cell banks (WCB), which are frozen 1-2 ml aliquots in cryovials held at -80°C. The growth medium used to prepare the WCB is deMan Rogosa Sharpe (MRS) broth, which is widely available commercially. The current supplier of medium is Merck KGaA, but other suppliers may be used in the future. All components of the growth medium are food-grade materials approved for this application. After confirming the bacterial identity through DNA fingerprinting using repetitive sequence based polymerase chain reaction (rep-PCR), the cryovials are delivered to the suppliers on dry ice to prevent thawing, usually in batches of 20 to 30 vials per shipment.

While there is some variation in the production methods used by the various suppliers, they do not differ in their essentials. All growth media are based on skim milk or hydrolyzed milk protein, but the precise compositions may vary. Similarly, all suppliers include cryoprotective coatings that differ in minor details. All components of the fermentation media and the cryoprotection are food grade and are permitted for these applications; all suppliers maintain quality-assurance programs that include verifying the purity and freedom from microbial contamination of incoming materials. All equipment is steam cleaned and sterilized prior to each use.

At the supplier, each cryovial is used to inoculate a small laboratory-scale flask containing a culture medium, which then undergoes a 10-14-hour temperature-controlled incubation until the culture reaches stationary phase. This broth culture is used to inoculate a starter fermenter in a 100-200-liter vessel, which in turn is allowed to grow for 10-14 hours until it reaches early stationary phase. Finally, it is transferred to a large production fermentation vessel (about 5-20 cubic meters). The media used in the fermenter are batch sterilized except for heat sensitive components, which are sterilized separately prior to introduction of the culture.

The sterilization is achieved by heating to a temperature of 121°C under pressure and maintaining this temperature for 30 minutes. The fermentation batch is cooled by circulating cold water through the jacket of the fermentation vessel.

Since *L. reuteri* produces lactic acid, NaOH is added as needed to maintain the pH of the broth at approximately 6.0. Bacterial growth is monitored by measuring acid production, which diminishes significantly as stationary phase is neared, approximately 8-12 hours after the broth is transferred to the fermentation vessel. Approximately 30 minutes after reaching stationary phase, the broth culture is chilled in a plate-and-frame heat exchanger and then centrifuged. During centrifugation the bacterial cells are washed twice with distilled water and the cryoprotective substances are added. (For those cryoprotective formulations that include gelatin, the supplier provides certification that it of porcine origin or of bovine origin from certified BSE-free sources, from animals found fit for human consumption, and has been both heated and acidified during processing.)

The slurry of washed bacteria is drained into sterilized trays, which are placed in a batch lyophilizer maintained at -50°C. Lyophilization is performed under negative pressure in an atmosphere filtered through high-efficiency particulate air filters and under sterile conditions. After lyophilization is complete, the identity of the bacteria is re-confirmed via either API 50CH or DNA fingerprinting. Finally, the bacteria are milled and packed in 5-kg polyethylene-lined containers and stored at temperatures not exceeding -18°C. The shelf life is 24 months from the date of production.

In-process samples are taken at each step and the final lyophilized bacteria are analyzed to confirm strain identity and purity from contamination by other microorganisms.

2.5. Specifications

All lots of *L. reuteri* 17938 from all suppliers meet the specifications set forth in Table 1 on the following page.

Table 1. Specifications for *L. reuteri* Strain 17938

Parameter	Unit	Specification	Method
Description		Freeze-dried powder	
Appearance		Light beige powder	BioGaia CM009
L. reuteri activity	cfu[1]/g	NLT[2] 2×10^{11}	BioGaia CM004
Identity		Confirmed	16Sr-RNA gene analysis
Water activity	%	NMT[3] 0.15	BioGaia CM007
Heavy metals			
Lead	mg/kg	NMT 0.05	NMKL[4] 161 mod; ICP-MS
Arsenic	mg/kg	NMT 0.1	NMKL 161 mod; ICP-MS
Cadmium	mg/kg	NMT 0.05	NMKL 161 mod; ICP-MS
Mercury	mg/kg	NMT0.05	NMKL 170 mod; AFS
Microbiological purity			
Total aerobic bacteria	cfu/g	NMT 10^3	NMKL 86:4 mod
Enterococci	cfu/g	NMT 10^2	Plating and incubation
Yeast and molds	cru/g	NMT 1	NMKL 98:4
Clostridium perfringens	cfu/g	NMT 1	NMKL 95:3
Bacillus cereus	cfu/g	NMT 1	NMKL 67:5
Coliforms (thermo tolerant)		Negative in 1 g	NMKL 125:3
Staphylococcus	cfu/g	NMT 1	NMKL 66:4
Escherichia coli		Negative in 1 g	NMKL 125:3
Listeria		Negative in 25 g	NMKL 16:2
Salmonellae		Negative in 40 g	NMKL 71:5

1. cfu = colony forming units
2. NLT = not less than
3. NMT = not more than
4. NMKL = Nordisk Metodik Komité for Levnedsmidler (Nordic Committee on Food Analysis)

3. Intended Use and Exposure

L. reuteri strain DMS 17938 is intended to be added to a variety of foods at concentrations needed to provide 10^8 cfu/serving throughout the shelf life of the product. The initial addition level may be as high as 10^9 cfu/serving in order to assure that at least 10^8 cfu/serving remain viable over the shelf life. The strain's function is to serve as a probiotic microorganism.

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

The foods to which *L. reuteri* is intended to be added are processed cheeses, yogurt, ice cream, fruit juices and drinks, processed vegetables and drinks, beverage bases, energy bars and drinks, and chewing gum. Some of these products are primarily intended for consumption by infants or children, and all of them are conventional foods that are available for consumption by infants and children as well as adults. It is also planned to incorporate *L. reuteri* in a drinking straw at the same level, 10^8 cfu, such that the probiotic bacteria can be ingested while consuming energy drinks or other cold or room-temperature beverages as an alternative to adding the probiotic directly to the beverage.

The drinking straw consists of a polypropene straw 154 mm in length and 5-6 mm in diameter containing freeze-dried *L. reuteri* suspended in a droplet of food-grade canola oil; each straw is packed in a welded aluminum foil laminate protective package to ensure stability over a shelf life of 15 months at $\leq$25°C. The straw initially contains 4 x 10^9 cfu L. reuteri in order to ensure that at least 10^8 cfu remain at the end of the shelf life.

3.2. Estimated Daily Intake of *L. reuteri*

The findings of U.S. Department of Agriculture (USDA) food-consumption surveys are reported in *Foods Commonly Eaten by Individuals* (Pao et al. 1982), which lists mean and 90th percentile daily consumption of each food category by survey respondents who reported consuming that food. The daily intakes of the foods intended for *L. reuteri* addition are:

- processed cheeses: 22 g and 46 g (mean and 90th percentile)
- yogurt: 99 g and 204 g
- ice cream: 57 g and 111 g
- fruit juice and drinks: 124 g and 234 g
- processed vegetables and drinks: 94 g and 182 g[3]

Pao et al. (1982) does not include chewing gum. Based on information from Wm. Wrigley Jr. Co., the mean daily consumption of chewing gum by those who chew gum is 1.5 g/day. From the USDA data in Pao et al. (1982), it is apparent that the 90th percentile daily intake of a food is most often about twice the mean daily intake, a fact recognized by FDA (2006). Using this multiplier for chewing gum gives an estimate of 3.0 g/day as the 90th percentile of intake.

3 Tomato juice

A "serving" of food is defined as the Reference Amount Customarily Consumed (RACC; 21 CFR §101.12). The RACC for processed cheese is 30 g, that for yogurt is 225 g, the RACC for ice cream is 66 g (½ cup); that for juices and drinks is 240 g (240 ml), and the RACC for chewing gum is 3 g. Mean daily intakes of the various foods thus range from 0.4 to 0.9 servings, and 90th percentile intakes from 0.9 to 1.7 servings.

One target product, an energy drink, is currently on the market without *L. reuteri*. Confidential market research by its manufacturer found that the product is consumed an average of 5.17 times per week, a mean of 0.7 servings per day. Applying the multiplier of 2 to the mean intake yields an estimated 90th percentile intake of 1.5 servings/day for the energy drink. It is anticipated that consumption of energy bars would be similar.

Adding up 90th percentiles of intake of the target foods for *L. reuteri* supplementation provides a greatly exaggerated estimate of the potential intake of *L. reuteri*. Nevertheless, the sum of the 90th percentile intakes is less than 10 servings. Since *L. reuteri* is expected to be present in these foods at between 10^8 and 10^9 cfu/serving, a conservative estimate of its likely maximum ingestion is 10^9 to 10^{10} cfu/day.

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). 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 biota 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. 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 2×10^9 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."

Connolly (2004) reported that, as of the time of writing, more than 200 million doses of 10^8 cfu *L. reuteri* ATCC 55730 had been sold with no reports of clinical infection or adverse side-effects. In addition to its use in dietary supplements in the U.S., this strain of *L. reuteri* has been added to numerous dairy products consumed by individuals of all ages and health status. *L. reuteri* is a heterofermentative species that normally resides in the gastrointestinal tract of humans and, indeed, is regarded as one of the few truly autochthonous *Lactobacillus* species in humans (Reuter 2001).

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, it does not call into question the safety of the intended use of *L. reuteri* strain 17938.

First, the subjects in this study were acutely ill with a condition the authors indicated has a 10-30% mortality rate and, second, it is far from clear that the probiotics administered to these acutely ill patients were responsible for any of the observed mortality. The group that received the probiotics had a significantly higher rate of multiorgan failure prior to enrollment than did the control group (27% v. 16%). Since most of the deaths were caused by multiorgan failure, this is potentially a 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.

4.2. History of Use of *L. reuteri* Strain DSM 17938 and Parent Strain

L. reuteri strain ATCC 55730, the parent strain of DSM 17938, has been sold both in the U.S. and abroad for human use both as a food ingredient and as a dietary supplement, as well as for addition to animal feed. Among other uses, in the U.S. and internationally it has been sold as an ingredient in oral and enteral formulas intended for the dietary management of post-surgical patients. Currently, the ATCC 55730 strain is being replaced with the plasmid-cured DSM 17938 strain.

L. reuteri is added to a variety of dairy products, including yogurts, in the U.S., Finland, Sweden, Japan, Taiwan, South Korea, and China, as well as to baby foods in Austria, Germany, Portugal, Sweden, Switzerland, Russia, Ukraine, Saudi Arabia, Taiwan, Vietnam, South Korea, Singapore, Malaysia, and Hong Kong. It has other food applications in the U.S., Venezuela, Spain, and China, as well as use in animal feeds in Japan and Thailand.

In a greater number of countries, *L. reuteri* strain ATCC 55730 has been used as a dietary supplement in the form of tablets or capsules, dispersed in oil drops, or coated on the inside of a straw. Countries in which such uses are current include the U.S., Canada, Austria, the Czech Republic, Slovakia, Finland, Sweden, Norway, Belgium, France, Germany, United Kingdom, Ireland, Spain, Portugal, Italy, Bulgaria, Rumania, Slovenia, Hungary, Poland, Russia, Ukraine, Lebanon, Jordan, Namibia, Swaziland, Botswana, South Africa, Australia, New Zealand, Hong Kong, Indonesia, Japan, Indonesia, Malaysia, Singapore, South Korea.

4.3. Safety Parameters

4.3.1. Ability to Adhere to Intestinal Cells

Some concern has been expressed that high adhesion capability (a characteristic of pathogens as well as probiotics) may predispose bacteria to platelet aggregation and 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 either infectivity or platelet aggregation and adherence to Caco-2 cells, ileostomy glycoproteins, or human intestinal mucosa

A surface protein of *L. reuteri* 104R, mucus adhesion promoting protein (MapA), is considered to be an adhesion factor of this strain. Miyoshi et al. (2006) investigated the relation between MapA and adhesion of *L. reuteri* to human intestinal (Caco-2) cells. Quantitative analysis of the adhesion of *L. reuteri* strains to Caco-2 cells showed that various *L. reuteri* strains bind not only to mucus but also to intestinal epithelial cells. In addition, purified MapA bound to Caco-2 cells, and this binding inhibited the adhesion of *L. reuteri* in a concentration-dependent manner. Based on these observations, the authors concluded that the adhesion of *L. reuteri* appears to be due to the binding of MapA to receptor-like molecules on Caco-2 cells. Further analysis indicated the existence of multiple receptor-like molecules in Caco-2 cells.

Ouwehand et al. (2001) compared the adhesion capabilities of *L. reuteri* ATCC 55730, *Lactobacillus brevis* PEL1, *L. rhamnosus* VTT E-800, and *L. rhamnosus* LC-705 with *L.*

rhamnosus GG using an *in vitro* human intestinal mucus model. *L. reuteri* ATCC 55730 showed significant adhesion properties in this model.

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. In this research, the strain of *L. reuteri* tested was the least capable of epithelial adhesion of the 3 lactobacilli (*L. reuteri, L. acidophilus*, and *L. rhamnosus*) and 1 *Bifidobacterium* (*B. animalis*) tested. It was also the least likely to translocate in this immunodeficient model, although even the strains that did translocate did not result in bacteremia. The likelihood that adherence to intestinal mucosa may also increase bacterial translocation was noted by Boyle et al. (2006).

Ouwehand et al. (2003) studied adhesion of 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 it to healthy control tissue. All strains were observed to adhere better to immobilized mucus than to whole intestinal tissue. Two strains (*L. rhamnosus* strain GG and *L. reuteri*) were found to exhibit disease-specific adhesion to intestinal tissue. All tested strains, with the exception of *L. rhamnosus* GG, displayed disease-specific adhesion to intestinal mucus. These results suggest that strains with optimal binding characteristics for a particular intestinal disease can be selected.

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. reuteri* ATCC 55730 and 2 other *L. reuteri* strains, DC 20 and DSM 12246. This last strain proved to have the highest adhesion of the 11 strains tested—38%--while DC 20 had only 4.2% and ATCC 55730 only 3.5%. 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. *L. reuteri* ATCC 55730 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.

4.3.2. Ability to Degrade Mucin

As noted earlier, it has not been established whether probiotic bacteria adhere to epithelial cell surfaces or to the mucus layer covering the intestinal mucosa. Mucins, released from intestinal goblet cells, are highly complex polysaccharides 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 microorganisms. 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.

Ouwehand et al. (2002) enrolled 28 elderly subjects in an open parallel study of the effects of probiotic bacteria on constipation, which included consideration of the effect on the mucosal barrier. The control group received fruit juice while the test group received fruit juice supplemented with 3.6×10^6 cfu/day *L. reuteri* ATCC 55730 for 4 weeks. No changes in fecal pH or mucin excretion were observed and *L. reuteri* did not affect the mucosal barrier.

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).

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. (1996) 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.

In one of the studies discussed in a later section (Betta et al. 2007), premature infants confined to a Neonatal Intensive Care Unit were treated with either *L. reuteri* ATCC 55730 or *L. rhamnosus* strain GG; 64 of these infants had central venous catheters and 11 of them underwent surgery, but no infections were observed in any of these high-risk infants.

No case studies have appeared in the published literature that implicate *L. reuteri* as a cause of infection. However, seven case reports have been published on clinical infections in which *L. rhamnosus* strains, most often the most widely used strain, GG (LGG), are potentially implicated. 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 2×10^9 *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 replacement, leading to complete recovery. The authors concluded that the source of the endocarditis was unknown.

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 the LGG of the supplement.

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. It is clear that

all reported clinical cases of clinical infections with suspected *Lactobacillus* involvement occurred in subjects with one or more severe underlying diseases or health conditions who were ingesting probiotics. 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. 2008) 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)."

4.3.4. Presence of Antibiotic Resistance Genes and Likelihood of Transference

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*. 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 6 strains of *L. reuteri* tested, along with all strains of *L. acidophilus*, *L. rhamnosus*, *L. casei*, *L. johnsonii*, *L. lactis*, *Bifidobacterium longum*, and *B. lactis*; this kanamycin resistance is thus presumptively intrinsic. All strains of *L. reuteri* tested were resistant to tetracycline and penicillinG (Temmerman et al. 2003); no genetic basis for these resistances was identifiable and thus these resistances do not appear to be acquired.

Vescovo et al. (1982) tested 16 strains of *L. reuteri* (none of them ATCC 55730 or DMS 17938) and 20 strains of *L. acidophilus* for resistance to 22 antibiotics. All of the strains examined were resistant to the antibiotics of the aminoglycoside group but all except one strain of *L. acidophilus* were sensitive to penicillin and most were sensitive to chloramphenicol. All *L. reuteri* strains were resistant to vancomycin and cloxacillin. Except for DSM 20016, all *L. reuteri* strains were resistant to tetracycline and *L. reuteri* strains exhibited a widely distributed resistance to antibiotics of the peptide group.

Evidence suggesting linkage of identified resistances to plasmids was obtained by curing experiments with acridine dyes and high growth temperatures; loss of resistance after curing indicated the likelihood that the resistance was plasmid-based (Vescovo et al. 1982). The four *L. reuteri* strains tested in this phase (D109, 47S, D111, and D287) showed different sensitivities to the curing agent. Strain D109 lost resistance to gentamicin, aureomycin, chloramphenicol, and bacitracin in at least one condition. Strain 47S lost bacitracin and novobiocin resistances. Strain D111 lost resistance to erythromycin, oleandomycin, and spiramycin. Finally, strain D287 lost only resistance to oleandomycin and bacitracin.

Tannock (1987), in an *in vitro* study of antibiotic resistance transfer, found that the broad-host-range plasmid pAMβ1 (erythromycin resistance) was transferred conjugally from *Lactococcus lactis* (formerly designated as *Streptococcus lactis*) to *L. reuteri* strains 100-23, 100-151, and D237, *L. murinus*, and *L. fermentum*. Transfer of pAMβ1 between two *L. reuteri*

strains also occurred, and *Lactobacillus* transconjugants could act as donors of pAMβ1 in crosses with *Enterococcus faecalis* JH2-2.

In another early study, Morelli et al. (1988) determined the *in vivo* transferability of plasmid-mediated antibiotic resistance between two strains of enteric Gram-positive bacteria. Germ-free mice were associated with the donor *L. reuteri* DSM 20016 strain, carrying the broad host range pAMβ1 plasmid, and with the *Enterococcus faecalis* JH2SS recipient strain. Analysis of the fecal content of associated mice demonstrated that the *in vivo* transfer of this plasmid did occur.

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.

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 resistance.

Klein et al. (2000) investigated whether glycopeptide resistance in lactobacilli has a similar genetic basis to that found in enterococci. Five *L. reuteri* strains (including ATCC 55730) and one *L. rhamnosus*, as well as four *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. reuteri* and *L. rhamnosus* 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.

Under contract with BioGaia, the Norwegian Food Research Institute undertook conjugation experiments with *L. reuteri* ATCC 55730 (Holck et al. 2001, unpublished report). These experiments followed the recommendations of the Scientific Committee on Animal Nutrition (EC Scientific Committee on Animal Nutrition 2003) calling for conjugation experiments first to be conducted with a recipient of the same species, using a positive control

donor to demonstrate the validity of the test system (and possibly a second positive control donor if conjugation does not occur with the first positive control donor), and then cross-checked with a promiscuous donor and well performing recipient to verify that the experimental setup is appropriate and the mating method has high sensitivity.

Three conjugation methods were tested: mixing the cells and co-culturing them on MRS petri dishes, co-culturing them in MRS broth, and filter-mating conjugation. Resistance patterns were determined by plating approximately 1000 bacteria of the strain to be tested on petri dishes containing decreasing amounts of the appropriate antibiotic. In the initial set of experiments, *L. reuteri* ATCC 55730 was the donor organism and *L. reuteri* 1063 rifR, str R 1.4 was the recipient strain. The first positive control donor was *L. reuteri* T1 pAMβ1, which harbors the promiscuous pAMβ1 plasmid, with *L. reuteri* 1063 rifR, str R 1.4 again serving as the recipient. No transconjugants were detected in *L. reuteri* 1063 rifR, str R 1.4 with either donor, *L. reuteri* ATCC 55730 or *L. reuteri* T1 pAMβ1. Therefore a second donor organism, *Enterobacter faecalis* JH2-2 (pAMβ1), was used as a positive control donor in order to show that *L. reuteri* 1063 rifR, str R 1.4 can act as a recipient. Use of this alternate positive control donor produced approximately 1 transconjugant per 8.7 x 10^7 donor cells.

Table 2. Conjugation Studies with Recipient *L. reuteri* 1063 rifR, str R 1.4

Donor	Test System	Transfer Frequency Per Donor	Transfer Frequency Per Recipient
L. reuteri ATCC 55730	Petri dish	<1/4.0 x 10^6	<1/1.2 x 10^8
L. reuteri ATCC 55730	MRS broth	<1/2.0 x 10^8	<1/3.6 x 10^8
L. reuteri ATCC 55730	Filter	<1/1.4 x 10^7	<1/7.0 x 10^6
L. reuteri ATCC 55730	Filter	<1/1.1 x 10^8	<1/1.5 x 10^8
L. reuteri ATCC 55730	Filter	<1/2.2 x 10^8	<1/2.0 x 10^7
L. reuteri T1 pAMβ1	Petri dish	<1/3.0 x 10^6	<1/1.5 x 10^7
L. reuteri T1 pAMβ1	MRS broth	<1/1.5 x 10^8	<1/3.0 x 10^9
L. reuteri T1 pAMβ1	Filter	<1/1.0 x 10^7	<1/2.0 x 10^7
L. reuteri T1 pAMβ1	Filter	<1/4.3 x 10^8	<1/2.0 x 10^8
L. reuteri T1 pAMβ1	Filter	<1/2.0 x 10^7	<1/4.0 x 10^7
E. faecalis JH2-2 (pAMβ1)	Filter	1/3.1 x 10^5	1/1.2 x 10^5
E. faecalis JH2-2 (pAMβ1)	Filter	1/8.7 x 10^7	1/4.9 x 10^6
Source: Holck et al. 2001.			

Finally, as the recommended cross-check to verify the sensitivity of the experimental protocol, Holck et al. (2001) used the same donor strain with *E. faecalis* OGRIF:Tn916 as the recipient. This experiment produced approximately 1 transconjugant per 10^5-10^8 cells,

confirming the validity and sensitivity of the methods and indicating that transfer of tetracycline or ampicillin resistance from *L. reuteri* ATCC 55730 occurs rarely if ever under these experimental conditions, although it must be recognized that *in vitro* conditions are never identical to *in vivo* conditions. The results of these conjugation experiments are shown in Table 2.

In an investigation of the safety of feeding probiotic bacteria to chicks to stabilize the indigenous microflora, Klose et al. (2006) isolated a number of bacterial strains from the gastrointestinal tracts of healthy chickens. Over 120 representatives were selected based on differences in whole-cell protein patterns and screened for antagonistic properties. Five effective strains (*Lactobacillus reuteri* CE4, *Pediococcus acidilactici*, *Enterococcus faecium*, *Bifidobacterium animalis* ssp. *animalis*, and *Lactobacillus salivarius* ssp. *salivarius*) exhibited *in vitro* the ability to inhibit a range of common pathogens and were evaluated with regard to the risks associated with genetic transfer of antibiotic resistances from animals to humans via the food chain. The probiotic strains were sensitive to several clinically effective antibiotics, though some of them showed single resistances. None of the vancomycin-resistant strains carried the enterococcal *van(A)* gene. Since the resistance was not associated with plasmids, the risk of transfer to other organisms was considered as minimal. Two tetracycline resistant strains (but not *L. reuteri*) were shown to harbor a *tet*(M)-associated resistance. The strains contained no extrachromosomal DNA and were not able to transfer the resistance by means of conjugation. On the basis of the collected data the presence of easily transferable resistances was excluded and the chicken strains were considered to be suitable for use as feed additives.

Analysis of the genome sequence of *L. reuteri* ATCC 55730 by Roos and Rosander (2005) identified five genes encoding penicillin-binding proteins. The genes from three penicillin resistant and three sensitive strains of lactobacilli were amplified and sequenced. Alignment of the deduced protein sequences showed differences at four positions that correlated with penicillin resistance. Two of these are located in PBP1a, where aspartic acid in the sensitive strains is replaced by valine at position 399 of the sequence of *L. reuteri* ATCC 55730 and glutamine is replaced by leucine at position 479. The aspartic acid at position 399 is highly conserved in other lactobacilli (i.e., it is present in all publicly available sequences) and its replacement by valine was regarded by the authors as a good candidate to explain altered penicillin resistance.

Of the other two polymorphisms identified by Roos and Rosander (2005), one is located in PBP2a, where phenylalanine appears in place of valine at position 147. This exchange is not located in a conserved region. The fourth alteration is located in PBP2x, where alanine replaces threonine at position 526. This alteration is in a highly conserved region in that the corresponding sequences of all sequenced lactobacilli have a glycine at this position. Alanine has properties similar to glycine, while threonine has substantially different properties, and thus Roos and Rosander (2006) regarded this polymorphism also as a good candidate to explain altered penicillin resistance. The differences seen in the protein sequences are the result of point mutations of the corresponding genes: E1 is caused by a mutation of a GAT codon to GTT; E2, CAA to CTA; E3, TTC to GTC; E4, GCA to ACA. The other two penicillin-binding proteins (PBP2a and PBP class C) did not show any differences that correlated with resistance to penicillin.

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L. reuteri ATCC 55730	Filter	<1/1.1 x 10^8	<1/1.5 x 10^8
L. reuteri ATCC 55730	Filter	<1/2.2 x 10^8	<1/2.0 x 10^7
L. reuteri T1 pAMβ1	Petri dish	<1/3.0 x 10^6	<1/1.5 x 10^7
L. reuteri T1 pAMβ1	MRS broth	<1/1.5 x 10^8	<1/3.0 x 10^9
L. reuteri T1 pAMβ1	Filter	<1/1.0 x 10^7	<1/2.0 x 10^7
L. reuteri T1 pAMβ1	Filter	<1/4.3 x 10^8	<1/2.0 x 10^8
L. reuteri T1 pAMβ1	Filter	<1/2.0 x 10^7	<1/4.0 x 10^7
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Roos and Rosander (2005) noted that alterations in both PBP1a and PBP2x are often reported to cause resistance to ß-lactams in streptococci. High-level resistance is often caused by alterations in more than one of the proteins (Coffey et al, 1995; Hiramatsu et al., 2004). The penicillin-binding proteins are "housekeeping" and found in all bacteria. In *L. reuteri* ATCC 55730 the corresponding genes are located on the chromosome and are not coupled to any mechanisms for transfer to other bacteria. In addition there is no other gene present in the genome sequence with similarities to known penicillin resistance genes. Roos and Rosander (2005) concluded that the ß-lactam resistance in *L. reuteri* ATCC 55730 most probably is caused by 1 to 4 point mutations in the genes encoding PBP1a, PBP2a and/or PBP2x, that this resistance is intrinsic, and that it is not transferable.

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. reuteri* ATCC 55730 exhibited resistance to 15 antibiotics (cefotaxime, clindamycin, nitrofurantoin, ofloxacin, oxacillin, penicillin G, streptomycin, tetracycline, tobrmycin, vancomycin, fusidic acid, kanamycin, lincomycin, methicillin, and nalidixic acid) but not to 5 others (erythromycin, chloramphenicol, gentamicin, novobiocin, and rifampicin).

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 reuteri* ATCC 55730 (residing on a plasmid), and the lincosamide resistance gene *lnu(A)* (formerly *lin(A)*) in *L. reuteri* ATCC 55730 (Kastner et al. 2006). Gel electrophoresis and hybridization experiments with DNA isolated from *L. reuteri* ATCC 55730 originating from 2 different

sources confirmed the presence of a plasmid more than 10 kb in size containing the *tet(W)* gene. Since no other tetracycline resistance genes were found by the microarray approach it was evident that the tetracycline resistance phenotypes were caused by the *tet(W)* determinant. Although the experiments were not described in detail, Kastner et al (2006) stated that conjugation experiments by filter mating using *L. reuteri* ATCC 55730 as the donor strain and *E. faecalis* JH2-2 and *L. lactis* Bu2-60 as recipients failed to show transferability of the *tet(W)* gene. The lincosamide resistance gene *lnu(A)* was also detected in *L. reuteri* ATCC 55730. The sequence of the 324 base pair *lnu(A)* fragment amplified from *L. reuteri* ATCC 55730 was found to be 96% identical to the published *lin(A)* sequence of *Staphylococcus haemolyticus*.

It was in response to these (and other similar) data that Roos and Rosander (2005, 2006) undertook the curing of the plasmids harboring these antibiotic resistance genes in *L. reuteri* ATCC 55730, even though efforts to demonstrate transference of the resistance to *E. faecalis* and *L. lactis* had failed.

Two reports appeared in 2007 regarding antibiotic susceptibility profiles of *Lactobacillus* species. Klare et al. (2007) studied a variety of *Lactobacillus*, as well as *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic use, while Egervarn et al. (2007) focused on *Lactobacillus reuteri* and *L. fermentum*. For *L. reuteri* ATCC 55730, Egervarn et al. (2007) reported minimum inhibitory concentrations (MIC) to tetracycline, erythromycin, and ampicillin of >256, 1, and 16 μg/ml, respectively; only tetracycline's MIC represented non-susceptibility. Klare et al. (2007) tested 416 isolates of *Lactobacillus* representing 21 species including *reuteri*, 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, erythromycin, clindamycin, and oxytetracycline—3 isolates each for the first 3 antimicrobials and 12 isolates for oxytetracycline. None of these was an *L. reuteri* isolate. While a great number of other resistances were identified, they were classified as intrinsic and therefore less capable of conjugation.

A recent investigation found potential antibiotic resistance tranferability in LAB and bifidobacteria of African and European origin (Ouoba et al. 2008). One African strain of *L. reuteri*,strain 12002, isolated from pig feces, was found to harbor the *erm*(B) gene encoding from erythromycin resistance, and to be capable of transferring this gene *in vitro* to enterococci. This was the only *L. reuteri* strain tested in this study. However, *L. reuteri* ATCC 55730 was previously found to be susceptible to erythromycin (Kastner et al. 2006, Egervarn et al. 2007) and, as described below, has been shown not to harbor the *erm*(B) gene.

At the request of BioGaia, a genomic analysis of *L. reuteri* strain DSM 17938 was undertaken at the Center for Microbial and Plant Genomics of the University of Minnesota (O'Sullivan 2008). The report of this analysis, *Safety Assessment of* Lactobacillus reuteri

DSM17938 Based on an Analysis of Its Gene Content, appears in the Appendix to this monograph. A 21x shotgun sequencing, estimated to represent over 98% of the gene content of the strain based on comparison with genomes of other *L. reuteri* strains, predicted a total of 2,299 potential genes. Predicted functions for the encoded genes were determined using the COG database at the National Center for Biotechnology Information. The number of genes with motifs associated with antibiotic resistance was similar to other sequenced lactobacilli and none of these genes was part of a predicted mobile element. A gene resembling a known hemolysin gene was found, but this gene is present in the majority of lactobacilli and other lactic acid bacteria that have been sequenced to date. No gene or gene cluster was identified that is known to be involved in virulence or antibiotic resistance. The author concluded, "There is nothing unusual from a safety perspective about the *L. reuteri* DSM 17938 genome when compared to other lactobacilli genomes" (O'Sullivan 2008, p2).

4.3.5. Impact on Pathogens

In a review of a number of strains of *L. reuteri* and their effects on the gastrointestinal tract, Dobrogosz (2005) elucidated the mechanisms by which *L. reuteri* affects the intestinal immune response to both beneficial and pathogenic bacteria. As is discussed in detail in the sections on animal and human studies of *L. reuteri*, a number of animal studies and human clinical trials have established its value in lowering overall pathogen loads and controlling or eradicating infections of *Salmonella typhimurium, Enterococcus faecalis, Cryptosporidium parvum, Helicobacter pylori*, and *Streptococcus mutans*, as well as other microorganisms such as *Candida albicans* and rotavirus (e.g., Kasravi et al. 1997, Edens et al. 1997, Alak et al. 1999, Balish and Warner 2002, Saggioroa et al. 2005).

In the presence of glycerol, most strains of *L. reuteri* produce reuterin (3-hydroxypropionaldehyde), a soluble broad-spectrum antimicrobial substance active in a wide range of pH values against Gram-positive and Gram-negative bacteria, especially *E. coli*, yeasts, fungi, protozoa, and viruses (Cleusix et al. 2007b). Reuterin production occurs during the anaerobic growth of *L. reuteri* in the presence of glycerol and low concentrations of glucose; it is not clear to what extent reuterin is produced in the human gut. Cleusix et al. (2007a; abstract only) tested *in vitro* the activity of reuterin produced by *L. reuteri* ATCC 55730 against a representative panel of intestinal bacteria. Reuterin was purified and used to test the minimal inhibitory and minimal bacteriocidal concentrations (MIC and MBC, respectively), finding broad range antibacterial efficacy with most intestinal bacteria showing MIC below that of *E. coli*, a sensitive indicator. On the other hand, *Clostridium clostridioforme* and other strains of lactobacilli were more resistant. This work corroborates earlier published research by Yunmbam and Roberts (1993) in which it was found that mice infected with *Trypanosoma brucei* ssp *brucei* treated with reuterin showed significant reduction in the levels of parasitemia and prolonged survival.

4.3.6. Production of D(-)-Lactic Acid

All lactic-acid-producing bacteria (LAB), by definition, produce lactate from carbohydrate fermentation. Lactate exists in two enantiomeric forms, a dextrorotary enantiomer (D-lactate) and a levorotary enantiomer (L-lactate). Many LAB produce only L-lactate, but a number of species of lactobacilli, including *L. reuteri*, produce both L- and D-lactate (Mack 2004, Connolly et al. 2005). Other indigenous intestinal bacteria also produce D-lactate,

including *E. coli*, *Klebsiella* spp., and *Bacteriodes* spp. (Duzgun et al. 2007). Only nanomolar concentrations of D-lactate are produced endogenously in mammals due to the absence of the isomer-specific enzyme D-lactate dehydrogenase (D-LDH) needed for its production (Ewaschuk et al. 2005; Petersen 2005), but D-lactate may be present in serum due to exogenous sources such as fermented foods and microbial fermentation in the colon. Many intestinal bacteria possess D-LDH and produce D-lactate directly and others possess the enzyme DL-lactate racemase and are able to convert L-lactate to D-lactate (Petersen 2005). Under normal circumstances, lactate generated by bacterial fermentation in the intestine does not result in clinically significant elevation of lactate in the blood or stool of humans. The normal serum concentration of lactate, nearly entirely L-lactate, is about 500-6000 µmol/L (Anderson et al. 1997; Ewaschuk et al. 2005). The normal serum concentration of D-lactate is variously estimated as 0-250 µmol/L (McLellan et al. 1992; De Vrese and Barth 1991; Hove and Mortensen 1995; Vella and Farrugia 1998; Connolly et al. 2005) or as about 1.5% of the L-lactate concentration (Anderson et al. 1997)

In the past, it was believed that D-lactate in humans is metabolized only slowly by the enzyme D-α-hydroxy-acid dehydrogenase and is mainly excreted in the urine, but newer studies have identified putative human mitochondrial D-lactate dehydrogenases, most importantly D-2-hydroxy acid dehydrogenase (D-2-HDH), which is found in high concentration in the kidney cortex and the liver and provides a large capacity to metabolize D-lactate to pyruvate (Petersen 2005, Ewaschuk et al. 2005). As a result, most of the studies of the factors resulting in D-lactic acidosis and other effects of severely elevated serum levels of D-lactate have concluded that D-lactate is metabolized and hence does not accumulate (Hove and Mortensen 1995; Uribarri et al. 1998) and is unlikely to occur absent impaired D-lactate metabolism (Uribarri et al. 1998). In humans who do not have impaired D-lactate metabolism, de Vrese et al. (1990) found that with bolus consumption of up to 12.8 mmol/kg bw of racemic DL-lactic acid, D-lactate reached a maximum plasma concentration of 0.45 mmol/l and was eliminated from plasma with an average half-life of 40.4 minutes. Daily consumption of 6.4 mmol/kg bw/day of racemic DL-lactic acid for 5 weeks did not result in the accumulation of plasma D-lactate (de Vrese et al. 1990). Uribarri et al. (1998), after reviewing the literature, concluded that "impaired metabolism of D-lactate is almost a prerequisite for the development of the syndrome." However, the activity of D-2-HDH is inhibited by oxalate and by low pH (Petersen 2005), and the presence of these conditions may lead to accumulation of D-lactate. Additionally, the 2 organs that provide the highest concentrations of D-2-HDH, the kidney and liver, are often compromised in short-bowel patients and it is not uncommon for both renal and hepatic function to fluctuate significantly in these patients (Petersen 2005).

Nevertheless, some concern was expressed by Mack (2004) regarding the use of probiotic bacteria that produce D-lactic acid. (It is to be noted, however, that no case of D-lactic acidosis due to an intake of food containing D-lactic acid producing bacteria has been reported in the literature [Haschke-Becher et al. 2000]. This statement remains true as of 2008, based on a Medline search.) The author noted that there are no reports of healthy infants or children developing D-lactic acidosis, but urged that controlled clinical studies involving primary analysis of this issue be undertaken to set aside this concern.

Such a study has been completed. Connolly et al. (2005), reported in more detail in Section 4.2.3.3.1, compared the blood D-lactic acid levels of 14 infants who had received 10^8

cfu/day *L. reuteri* ATCC 55730 from birth with those of 10 infants who had received placebo, at the age of 6 months and at 12 months. In both groups, blood D-lactate levels were within the normal range; they were insignificantly higher in the *L. reuteri* treated group at 6 months, but insignificantly lower at 12 months. The authors concluded that the findings provide evidence that there is no elevation of D-lactic acid in the blood of healthy infants given *L. reuteri* at a dose of 10^8 cfu/day from birth to 12 months. However, the safety of the use of a probiotic that produces D-lactate in formula consumed by infants with short-bowel syndrome leading to bacterial overgrowth has not been evaluated.

4.4. Studies of *L. reuteri*

4.4.1. Animal Studies

A number of animal studies of *L. reuteri* were conducted in order to investigate potential beneficial effects, including protection from *Candida albicans* and other infective microbes, prevention or amelioration of colitis, and anticholesterolemic effects, as well as to demonstrate safety. As discussed below, the evidence for such benefits is variable. Most important, though, is that these studies—in which *L. reuteri* was given orally to mice, rats, and pigs—have consistently found an absence of any observable adverse effects due to administration of *L. reuteri* at daily doses as high as 10^{12} cfu, 1,000 to 10,000 times the exposure likely to occur from its intended use in foods. The studies discussed below are summarized in Table 4 at the end of the section.

Molin et al. (1992) fed rats freeze-dried oatmeal soup fermented by six different *Lactobacillus* strains including *L. reuteri* strains R21c, Hj108, and Hj108^{ery}. After 10 days, serum cholesterol levels were lower for rats eating fermented oatmeal as compared to a commercial non-fermented product. The colonizing ability of the administered strains was evaluated *in vivo*. Only *L. reuteri* R21c was shown to colonize the mucosa; *L. reuteri* represented about 30% of the *Lactobacillus* population 24 days after termination of the administration. No adverse effects were reported.

The potential beneficial effect of exogenous administration of *L. reuteri* R2LC (rat-derived) or HLC (human-derived) on acetic acid-induced colitis was evaluated in the rat (Fabia et al. 1993). Colitis was induced by instillation of 4% acetic acid in an exteriorized colonic segment for 15 seconds. Intracolonic administration of 3.5×10^8 cfu *L. reuteri* R2LC or HLC immediately after acetic acid administration prevented the development of colitis. No adverse effects were observed from the administration of either strain of *L. reuteri*.

In a study intended to evaluate the effects of rat-derived *L. reuteri* R2LC on methotrexate-induced enterocolitis in rats, Mao et al. (1996) provided continuous intragastric infusion of control diet or control diet with supplementation of oatbase, *L. reuteri*, or *L. plantarum* DSM 9843 from the beginning of the study. Methotrexate (20 mg/kg) was injected intraperitoneally on day 3 and the sampling was performed on day 6. Both *L. reuteri* and *L. plantarum* and oatbase decreased body weight loss and intestinal permeability and increased bowel mucosal mass in enterocolitic rats. Administration of lactobacilli, but not oatbase, decreased the intestinal myeloperoxidase level, re-established intestinal microecology, and reduced bacterial translocation to extraintestinal sites. Both lactobacilli and oatbase reduced

plasma endotoxin levels. *L. plantarum* was more effective in reducing intestinal pathogens than *L. reuteri*, and neither bacterium caused any adverse effects.

Adawi et al. (1997) investigated the ability of a variety of *Lactobacillus* strains to reduce bacterial translocation after acute liver injury. Five *Lactobacillus* strains, including 2 strains of *L. reuteri* (R2LC and 108), were administered rectally for 8 days to 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. 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. reuteri* 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.

In a similar but more extensive study, Kasravi et al. (1997) gave oral supplements of 5 x 10^9 cfu/day *L. reuteri* R2LC, 5 x 10^9 cfu/day *L. plantarum* DSM 9843, 5 ml/day of 20% lactulose solution, 20 mg/day neomycin sulfate, or saline solution (control) to 40 Sprague-Dawley rats (6 animals/group) for 1 week before inducing liver injury by intraperitoneal administration of D-galactosamine; a 6th group of rats received only saline solution as a control. After 24 hours the rats were killed and subjected to analysis of bacterial translocation to blood, mesenteric lymph nodes, and liver; intestinal bacterial count and intestinal mucosal histology; liver histology; and serum endotoxin and the liver enzymes bilirubin, alkaline phosphatase (ALP), aspartate transaminase (AST), and alanine transaminase(ALT).

Neither pretreatment nor induced liver injury affected serum levels of endotoxin or ALP or intestinal histology. Injection of D-galactosamine profoundly increased levels of bilirubin, AST, and ALT, but all of the pretreatments except administration of *L. reuteri* reduced the levels of these enzymes toward normal levels; *L. reuteri* had no effect. Similarly, all treatments except *L. reuteri* lessened the massive hepatocellular necrosis induced by D-galactosamine. Pretreatment with neomycin or either strain of *Lactobacillus* significantly decreased the prevalence of *Enterobacteriaceae* in the small intestine and cecum. With regard to reducing translocation of bacteria to the liver, mesenteric lymph nodes, or portal and arterial blood, only pretreatment with neomycin or lactulose were effective. The authors concluded that the impact of probiotics on the effects of acute liver injury was small, but they observed no indication of any adverse effects.

In studies of competitive exclusion as an alternative to antibiotic use in animal husbandry, Edens et al. (1997) investigated the effects of administration of *L. reuteri* to chicken and turkey poults. The *L. reuteri* was added to the feed at a level of $5x10^5$ cfu/g feed. The results of the studies suggested that *L. reuteri* has the potential to control many enteric pathogens in poultry. Additionally, the authors concluded that *L. reuteri* has been shown to be safe in not affecting hatchability and in causing no adverse effects on the hatched chicks or poults.

Alak et al. (1997) studied the efficacy of *L. reuteri* as a probiotic for the control of *Cryptosporidium parvum* infection. Female C57BL/6 mice were immunosuppressed by intraperitoneal inoculation with the LP-BM5 leukemia virus. Four months after inoculation, the mice developed lymphadenopathy, splenomegaly, and susceptibility to *C. parvum* infection. After daily prefeeding with 10^8 cfu/day *L. reuteri* for 10 days, mice were challenged with 6.5×10^6 *C. parvum* oocysts. They continued to be fed *L. reuteri* during the entire study. Mice supplemented with *L. reuteri* and challenged with *C. parvum* cleared parasite loads from the gut epithelium. However, unsupplemented animals developed persistent cryptosporidiosis and shed high levels of oocysts in the feces. *L. reuteri* feeding increased its colonization of the intestinal tract, which was inversely related to the fecal shedding of oocysts. These findings suggest that *L. reuteri* may help prevent *C. parvum* infection in immunodeficient subjects. Further, the body weights did not differ between the *L. reuteri* and control groups nor was there any indication of infection or other adverse effects from administration of 10^8 cfu/day *L. reuteri* to immunodeficient animals.

Wagner et al. (1997a) assessed the capacity of *L. reuteri* (strain not specified), *L. acidophilus*, *L. casei*, 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.

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. casei*. 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. casei*, 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. reuteri* or *L. casei*. Six of 28 pups born to *L. reuteri* colonized dams and 19 of 53 pups born to *L. casei* 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. 2008) considered that use of the immunocompromised mouse model is too premature and recommended against it, and thus it might be appropriate to

regard the results of Wagner et al. (1997a) as suggestive rather than conclusive. 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.

L. reuteri (strain not specified), *L. acidophilus*, *L. casei*, 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 immunodeficient animal model.

The effect of feeding live *L. reuteri* cells containing active bile salt hydrolase (BSH) on plasma cholesterol levels was studied in pigs using an unregistered strain of *L. reuteri* (De Smet et al. 1998). During an experiment lasting 13 weeks, 20 pigs were fed a high-fat, high-cholesterol diet for the first 10 weeks, and a regular pig diet for the last 3 weeks. One group of animals received 1.18×10^{11} cfu/day *L. reuteri* for 4 weeks (from week 3 until week 7). From week 8 onwards, the treated group was again fed the same diet as the control group without additions. The total fecal *Lactobacillus* counts were significantly higher in the treated pigs during the first 2 weeks of *L. reuteri* feeding. Based on limited data, it was suggested that the administered *L. reuteri* had caused a temporary shift within the indigenous *Lactobacillus* population rather than permanently colonizing the intestinal tract. The probiotic feeding brought about significant lowering ($p < 0.05$) of total and LDL-cholesterol concentrations in the treated pigs compared with the control pigs, while no change in HDL cholesterol concentration was observed. Although the blood cholesterol levels went up in both groups during the 3 weeks following the *Lactobacillus* administration period, significantly lower serum total- and LDL-cholesterol levels were observed in the treated pigs. During the final 3 weeks of normalization to the regular diet, cholesterol concentrations significantly decreased in both animal groups and the differences in total and LDL-cholesterol concentrations between the groups largely disappeared. The administration of this high dose of *L. reuteri* caused no adverse effects, did not affect body weight, and did not result in increased growth of pathogenic bacteria in the treated pigs.

Taranto et al. (1998) fed Swiss Albino mice a diet enriched with fat to produce hypercholesterolemia. The administration of 10^4 cfu/day *L. reuteri* CRL 1098 to hypercholesterolemic mice for 7 days decreased total cholesterol by 38%, producing serum cholesterol concentrations similar to that of the control group. This low dose of *L. reuteri* caused a 40% reduction in triacylglycerols and a 20% increase in the ratio of high density lipoprotein to low density lipoprotein without bacterial translocation of the native microflora into the spleen

and liver. In a follow-up study, Taranto et al. (2000) administered 10^4 cfu/day *L. reuteri* CRL 1098 to mice for 7 days before inducing hypercholesterolemia by feeding a fat-enriched diet for the following 7 days. At this low dose, *L. reuteri* was effective in preventing hypercholesterolemia in mice, producing a 17% increase in the ratio of high-density lipoprotein to low-density lipoprotein. Total cholesterol and triacylglycerols increased by 22 and 33%, respectively, in the group that was not fed the lactobacilli. No adverse effects were reported due to the *L. reuteri* treatment.

Three *Lactobacillus* strains isolated from pig feces, identified by DNA-DNA hybridization as 2 strains of *L. johnsonii* and 1 strain of *L. reuteri* (designated BFE 1058), were administered to pigs at a daily dose of 2×10^{12} cfu for 5 weeks (du Toit et al. 1998). The experimenters isolated 297 *Lactobacillus* strains from pig feces and tested them for bile-salt hydrolase activity, bile-salt resistance, tolerance for low pH, and production of antimicrobial substances, selecting 3 strains for further study. Six male Göttingen minipigs age 3-6 years and weighing about 55 kg were put on a "Western-style" diet for 17 weeks in order to induce hypercholesterolemia. They were then maintained on this diet with the probiotic supplement mixed in for 5 weeks, followed by 3 weeks of the diet without the probiotic supplement. Bacterial enumerations were carried out on aliquots of the pigs' feces to assess total LAB and lactobacilli; pH and water content were also evaluated. Blood samples were taken before probiotic feeding, after 3 weeks of probiotic feeding, and 2 weeks after terminating probiotic feeding and analyzed for total cholesterol, lipoproteins, and triacylglycerol.

Although a slight rise was seen in fecal counts of *Lactobacillus* cells after 2 weeks of probiotic feeding, no change was observed in total LAB. The water content of the feces was slightly but temporarily increased by probiotic administration, but there was no effect on fecal pH. The probiotic feeding reduced total cholesterol levels, but cholesterol returned to the previous levels by 2 weeks after cessation of probiotic administration. There was no change in lipoproteins or triacylglycerols. No diarrhea or other adverse effects were observed in response to this extremely high dose (2×10^{12} cfu/day) of *Lactobacillus* spp.

In a follow-up to earlier research, Alak et al. (1999) compared the effect of supplementation with *L. reuteri* or *L. acidophilus* with regard to reduction of fecal shedding of *Cryptosporidium parvum* oocysts. Female C57BL/6 mice were immunosuppressed by murine leukemia virus (strain LP-BM5) inoculation. Twelve weeks after LP-BM5 inoculation, 15 immunosuppressed mice each were randomly assigned to one of the following treatment groups: historical control (group A), LP-BM5 control (group B), *C. parvum* (group C), *L. reuteri* plus *C. parvum* (group D) or *L. acidophilus* plus *C. parvum* (group E). Mice were pre-fed the *L. reuteri* or *L. acidophilus* bacteria strains daily for 13 days, challenged with *C. parvum* oocysts, and thereafter fed the specified *Lactobacillus* regimens daily during the experimental period. Animals supplemented with *L. reuteri* shed significantly fewer ($p < 0.05$) oocysts on day 7 after *C. parvum* challenge compared to controls. Mice supplemented with *L. acidophilus* also shed fewer ($p < 0.05$) oocysts on days 7 and 14 post-challenge compared to controls. Overall, *Lactobacillus* supplementation reduced *C. parvum* shedding in the feces but failed to suppress the production of T-helper type 2 cytokines [interleukin-4 (IL-4) and IL-8)], which are associated with immunosuppression. Additionally, *Lactobacillus* supplementation did not restore T-helper type 1 cytokines [IL-2 and gamma interferon (IFN-γ)], which are required for recovery from

parasitic infections. Altered T-helper types 1 and 2 cytokine production as a consequence of immunodysfunction permitted the development of persistent cryptosporidiosis while mice with intact immune system were refractory to infection with *C. parvum*. No adverse effects were evident as a result of administration of either *Lactobacillus* species.

In a study of the ability of precolonization with *L. reuteri* (strain not specified) to protect mice against *Salmonella typhimurium*, Waters et al. (1999) found that *L. reuteri* significantly reduced mortality, decreased translocation of *S. typhimurium*, and reduced gut epithelial damage. Waters et al. (1999) also used gnotobiotic TCR-α deficient mice to learn that precolonization with *L. reuteri* reduced colonization and adverse effects from later inoculation with *Cryptosporidium parvum*. There were no indications of adverse effects from the administration of *L. reuteri*.

Simpson et al. (2000) studied the diversity and stability of the fecal bacterial microbiota in weaning pigs after introduction of *L. reuteri* MM53, using a combination of cultivation and techniques based on genes encoding 16S rDNA. Nine piglets were assigned to 3 treatment groups (control, daily dosed, and 4th-day dosed), and fresh fecal samples were collected daily. Dosed animals received 2.5×10^{10} cfu *L. reuteri* daily or every 4th day. Mean *Lactobacillus* counts for the three groups ranged from 1×10^9 to 4×10^9 cfu/g feces. Enumeration of *L. reuteri* MM53 showed that the introduced strain fluctuated between 8×10^3 and 5×10^6 cfu/g feces in the two dosed groups. Analysis of denaturing gradient gel electrophoresis (DGGE) banding profiles indicated that each individual pig maintained a unique fecal bacterial population that was stable over time, suggesting a strong host influence. In addition, individual DGGE patterns could be separated into distinct time-dependent clusters. Primers designed specifically to restrict DGGE analysis to a select group of lactobacilli allowed examination of interspecies relationships and abundance. Based on relative band migration distance and sequence determination, *L. reuteri* was distinguishable within the V1 region 16S rDNA gene patterns. Daily fluctuations in specific bands within these profiles were observed, which revealed an antagonistic relationship between *L. reuteri* MM53 (band V1-3) and another indigenous *Lactobacillus* assemblage (band V1-6).

Germ-free beige-nude (bg/bg-nu/nu) and beige-heterozygous (bg/bg-nu/+) mice were colonized with *Candida albicans* and with *L. reuteri* (strain not specified), *L. acidophilus*, *L. casei*, or *Bifidobacterium infantis* (Wagner et al. 2000). Colonized mice were subsequently challenged orally with *C. albicans* and the effect of prior colonization with probiotic bacteria on the antibody responses of the immunodeficient mice to alimentary tract colonization with *C. albicans* was compared to the antibody responses of the gnotobiotic mice colonized only with *C. albicans*. Although the probiotic bacteria did not induce a vigorous antibody response to their own antigens, they altered the antibody responses of mice to *C. albicans*. In T-cell competent bg/bg-nu/+mice, *B. infantis* enhanced and focused IgG1, IgG2A, and IgA responses to *C. albicans* antigens. Some of the probiotic bacteria also enhanced the IgG1 and IgG2A antibody responses of bg/bg-nu/nu mice to *C. albicans* antigens. No infections or other adverse effects were seen as a result of the administration of any of the probiotic strains of bacteria.

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. LGG, *L. reuteri*, or sulphasalazine were given orally to the rats. 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.

Germ-free interleukin-10 knockout (IL-10 KO) mice developed inflammatory bowel disease (IBD) after they were colonized with a pure culture of *Enterococcus faecalis* (Balish and Warner 2002). *E. faecalis* induced not only IBD, but also but rectal dysplasia and adenocarcinoma in the IL-10 KO mice. Conventional (complex-intestinal flora) IL-10 KO mice developed IBD within 10 to 15 weeks of age and showed more pathology in the cecum than was observed with *E. faecalis*-induced IBD in gnotobiotic IL-10 KO mice. Conversely, neither germ-free IL-10 mice nor IL-10 KO mice colonized as adults with pure cultures of *Candida albicans*, *Escherichia coli*, *Lactobacillus casei*, *L. reuteri*, *L. acidophilus*, *Bifidobacterium*, *Lactococcus lactis*, or a *Bacillus* sp. developed IBD during the 25- to 30-week study. *E. faecalis* is a common intestinal microbe of man and animals that can trigger IBD, dysplasia, and carcinoma in a genetically susceptible murine host. No adverse effects resulted from treatment with *L. reuteri* or other probiotic bacteria.

Metaxas et al. (2002; article in Greek with English abstract) investigated whether *L. reuteri* would be effective in reducing bacterial translocation in Zymosan-induced non-septic peritonitis in the rat. Eighty male Wistar rats received either *L. reuteri* (10^7 cfu/day) or placebo for 5 days in drinking water. On day 5, half the rats of each group were subjected to intraperitoneal (IP) injection of Zymosan (500 mg/kg body weight) for induction of non-septic peritonitis, while the remaining rats received IP normal saline. Enteric mucosal microcirculation and adherent mucus gel thickness were assessed 18 hours later and the mesenteric lymph nodes were processed under aseptic conditions for evaluation of bacterial translocation. *L. reuteri* pretreatment enhanced enteric mucosal barrier strength by means of increasing enteric mucosal microcirculation and adherent mucus gel thickness and thus reduced bacterial translocation. There were no indications of infection or other adverse effects from the administration of 10^7 cfu/day *L. reuteri*.

Kelleher et al. (2002) used an infant rhesus monkey model to study the effects of *L. reuteri* M164 supplementation of infant formula, with or without supplemental zinc, on nutritional status, gut colonization, and the ability to resist gastrointestinal infection. Infant monkeys were fed control infant formula, control formula with *L. reuteri*, or control formula with *L. reuteri* and supplemental zinc from birth to 4 months. Growth, nutritional status, mineral absorption, intestinal colonization, and frequency and severity of enteropathogenic *Escherichia coli*-induced gastroenteritis were monitored. *L. reuteri* colonization was achieved, based on enumeration of fecal swabs, and was associated with increased ileal villous surface area and improved hematocrit, with no adverse effects on growth or nutritional indices. Infant monkeys fed *L. reuteri*-supplemented formula had reduced diarrhea severity throughout the study period and recovered more rapidly from acute diarrhea than the other groups. The authors concluded

that *L. reuteri* supplementation of infant formula is safe, improves iron status, and decreases diarrhea severity in infant rhesus monkeys.

Colitic severe combined immunodeficiency (SCID) mice were treated for 1 week with the antibiotics vancomycin and meropenem, followed by a 3-week administration of $2x10^{10}$ cfu/day *L. reuteri* DSM12246 and *L. rhamnosus* 19070-2 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. reuteri* and *L. rhamnosus* to these severely immunodeficient animals.

Kamiya et al. (2006) explored the effects of live, heat-killed, or gamma-irradiated *L. reuteri* ATCC 23272 on cardio-autonomic response and single fiber unit discharge in dorsal root ganglia to colorectal distension in healthy Sprague-Dawley rats. The effects of treatment on somatic pain were also examined. Doses of 10^9 bacteria were given by gavage for 9 days. Colorectal distension was performed under anesthesia and heart rate was measured through continuous electrocardiography. Single fiber unit discharge was recorded from the 6th left lumbar dorsal root ganglion. Somatic pain was evaluated by the tail flick and paw pressure tests. Colorectal distension caused a pressure dependent bradycardia in the control group. Treatment with live, heat-killed, or gamma-irradiated *L. reuteri* prevented the pain response even during the maximum distension pressure (80 mm Hg). Both viable and non-viable bacteria significantly decreased dorsal root ganglion single unit activity to distension. No effects on somatic pain were seen with any treatment, and no adverse effects were observed from any treatment.

Effects of *L. reuteri* administration on hematological parameters were studied in Sprague-Dawley rats by Anukam et al. (2004). The strain tested was *L. reuteri* RC-14 (classified at the time of the study as *L. fermentum*). Daily doses of 10^9 cfu of *L. reuteri* RC-14 and a strain of *L. rhamnosus* were fed via oro-gastric 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 3 on the next page, 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 3. Hematological Parameters Of Rats Given *L. reuteri* and *L. rhamnosus*.

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

*All values were within the normal range and no statistically significant differences were observed between probiotic-fed and control rats for any parameter.
Source: Anukam et al. 2004

In conclusion, *L. reuteri*, both BioGaia's strain ATCC 55730 and other strains, has been administered to a variety of animal species in numerous studies, with dose levels as high as 10^{12} cfu/day. *L. reuteri* appeared to produce adverse effects in only one study (Wagner 1997a). It is not clear why adverse effects were seen in this study, because numerous other experiments involving administration of high doses of *L. reuteri* to severely immunodeficient animals have found no indication of infection or other adverse effects. As was noted, the EU-PROSAFE committee (Vankerckhoven et al. 2008) regarded use of this model as premature, but it seems clear that the immunodeficient mouse model exhibited a number of features dissimilar to those seen in humans and other animal models, included long-duration high-concentration gastrointestinal colonization with all probiotic species tested and a high incidence (over 50% with 2 species, although not *L. reuteri*) of translocation of bacteria to internal organs.

Table 4. Studies of *L. reuteri* in Animals

Reference	Objective	Study Design	Animal Model	*L. reuteri* Dose and Source	Duration	Safety-Related Results
Adawi et al. 1997	Investigate the ability of a variety of lactobacillus strains 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.	Sprague-Dawley rats	Rectal dose not reported; *L. reuteri* strains R2LC and 108	8 days	The probiotic treatment reduced bacterial translocation without any reported adverse effects.
Alak et al. 1997	Assess ability to control *Cryptosporidium parvum* infection in immunodeficient mice	Mice were immunosuppressed with leukemia virus, given *L. reuteri* or control and challenged with *C. parvum*	Female C57BL/6 mice	10^8 cfu/day; strain not specified	10 days pre-*C. parvum* infection	Reduced cryptosporidiosis, no difference in body weight, no adverse effects noted.
Alak et al. 1999	Compare the effects of *L. reuteri* and *L. acidophilus* with regard to reduction of fecal shedding of *Cryptosporidium parvum* oocysts.	Immunosuppressed mice were pre-fed *L. reuteri* or *L. acidophilus* daily for 13 days, challenged with *C. parvum* oocysts, and fed the specified lactobacillus regimens daily during the experimental period.	75 C57BL/6 female mice immunosuppressed by murine leukemia virus inoculation	10^8 cfu/day; strain not specified	13 days	Lactobacillus supplementation reduced *C. parvum* shedding in the feces but did not suppress the production of T-helper type 2 cytokines [interleukin-4 (IL-4) and IL-8)]. No adverse effects were evident as a result of administration of either lactobacillus species.

Table 4. Studies of *L. reuteri* in Animals

Anukam et al. 2004	Determine the effect of *L. reuteri* 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 *L. reuteri* RC-14 and *L. rhamnosus* via oro-gastric 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.
Balish and Warner 2002	Investigate the effect of several bacterial strains on the development of inflammatory bowel disease (IBD)	Mice were colonized with *Enterococcus faecalis*, *Candida albicans*, *Escherichia coli*, *Lactobacillus casei*, *L. reuteri*, *L. acidophilus*, *Bifido-bacteria*, *Lactococcus lactis*, or a *Bacillus* spp., without effect.	Germ-free interleukin-10 knockout (IL-10 KO) mice	Dose and strain of *L. reuteri* were not specified. Fecal samples had 10^8 to 10^9 cuf/g.	25-30 weeks	Colonization with *Enterococcus faecalis*, induced IBD, rectal dysplasia, and adenocarcinoma. Colonization with *Candida albicans*, *Escherichia coli*, *Lactobacillus casei*, *L. reuteri*, *L. acidophilus*, *Bifidobacteria*, *Lactococcus lactis*, or a *Bacillus* spp. No adverse effects were seen from colonization of this immunodeficient host with *L. reuteri*.
De Smet et al. 1998	Investigate the effect of feeding live *L. reuteri* cells containing active bile salt hydrolase (BSH) on plasma cholesterol levels in pigs.	Pigs were fed a high-fat, high-cholesterol diet for the first 10 weeks, and a regular pig diet for the last 3 weeks; half received *L. reuteri* from week 3 to week 7 and half did not.	20 pigs. 10 in the test group and 10 controls	1.18×10^{11} cfu/day of an unregistered strain of *L. reuteri*	13 weeks	The administered *L. reuteri* caused a temporary shift within the indigenous *Lactobacillus* population rather than permanently colonizing the intestinal tract. The probiotic feeding brought about significant lowering ($p < 0.05$) of total and LDL-cholesterol concentrations while no change in HDL cholesterol concentration was observed. The administration of this high dose of *L. reuteri* caused no adverse effects, did not affect body weight, or result in increased growth of pathogenic bacteria in the treated pigs.

Table 4. Studies of *L. reuteri* in Animals

du Toit et al. 1998	Test the ability of *Lactobacillus* strains exhibiting high bile-salt hydrolase activity, bile-salt resistance, tolerance for low pH, and production of antimicrobial substances to reduce serum cholesterol in hypercholesterolemic minipigs.	Uncontrolled prospective study in which minipigs were put on a "Western-style" diet for 17 weeks in order to, then were given a mixture of 3 *Lactobacillus* strains	6 male Göttingen minipigs age 3-6 years and weighing about 55 kg with induced hypercholesterolemia	*L. reuteri* BFE 1058 isolate from pig feces along with 2 strains of *L. johnsonii*; total dose $2x10^{12}$ cfu/day	5 weeks	No change was observed in total fecal LAB. The probiotic feeding reduced total cholesterol levels, but cholesterol returned to the previous levels by 2 weeks after cessation of probiotic administration. No diarrhea or other adverse effects were observed in response to this extremely high dose ($2x10^{12}$ cfu/day) of *Lactobacillus* spp.
Edens et al. 1997	Study the use of L. reuteri administration to reduce pathogens in chicken and turkey poults via competitive exclusion.	Test pigs were given L. reuteri in their feed while control pigs were not. Pathogens were enumerated and safety-related endpoints were measured.	Chicken and turkey poults	$5x10^5$ cfu/g feed of unspecified *L. reuteri*	Not specified	The results suggested that *L. reuteri* has the potential to control many enteric pathogens in poultry. Additionally, the authors concluded that *L. reuteri* has been shown to be safe in not affecting hatchability and in causing no adverse effects on the hatched chicks or poults.
Fabia et al. 1993	Evaluate the potential benefit of administration of *L. reuteri* R2LC or HLC on acetic acid-induced colitis.	Colitis was induced by instillation of 4% acetic acid in an exteriorized colonic segment for 15 seconds; followed immediately by intracolonic administration of *L. reuteri* strains.	Rat (strain not specified)	$3.5x10^8$ cfu *L. reuteri* R2LC (rat-derived) or HLC (human-derived) immediately after administration of acetic acid	Single dose	Intracolonic administration of *L. reuteri* R2LC or HLC immediately after acetic acid administration prevented the development of colitis. No adverse effects were observed from the administration of either strain of *L. reuteri*.

Table 4. Studies of *L. reuteri* in Animals

Holma et al. 2001	Compare the effects of *L. rhamnosus* GG (LGG), rat-derived *L. reuteri* R2LC, and sulphasalazine on acetic acid-induced colitis in rats.	Colitis was induced by colonic instillation of acetic acid, followed immediately by oral administration of *L. reuteri*, LGG, or sulphasalazine; ulceration was measured.	Rat (strain not specified)	Unspecified dose of *L. reuteri* R2LC (rat derived)	Single dose	*L. reuteri* significantly antagonized body weight loss caused by inflammation, decreased edema formation in the colon,. reduced the median value of macroscopic ulceration, and reduced the protein content of inducible nitric oxide synthase and inducible cyclooxygenase. The authors concluded that *L. reuteri* R2LC reduces the severity of acetic acid-induced colitis in rats and noted that neither LGG nor *L. reuteri* produced any observable side effects.
Kamiya et al. 2006	Explore the effects of live, heat killed, or gamma irradiated *L. reuteri* ATCC 23272 on cardio-autonomic response and single fiber unit discharge in dorsal root ganglia to colorectal distension.	After dosing the rats with bacteria for 9 days, colorectal distension was performed and heart rate was measured through continuous electrocardiography. Single-fiber unit discharge was recorded and somatic pain was evaluated.	Sprague-Dawley rats	10^9 cfu/day of live, heat killed, or irradiated *L. reuteri* ATCC 23272	9 days	Colorectal distension caused a pressure dependent bradycardia in the control group. Treatment with live, heat killed, or γ-irradiated *L. reuteri* prevented the pain response even during the maximum distension pressure. Both viable and non-viable bacteria significantly decreased dorsal root ganglion single unit activity to distension. No effects on somatic pain were seen with any treatment, and no adverse effects were observed from any treatment.
Kasravi et al. 1997	Investigate the ability of *L. reuteri* R2LC or *L. plantarum* DSM 9843 to reduce bacterial translocation and other adverse effects of induced acute liver injury.	Pretreated rats with *L. reuteri* R2LC, *L. plantarum* DSM 9843, 5 ml/day of 20% lactulose solution, or 20 mg/day neomycin sulfate for 1 week; then induced acute liver injury via injection of D-galactosamine	48 Sprague-Dawley rats	5×10^9 cfu/day *L. reuteri* R2LC or 5×10^9 cfu/day *L. plantarum* DSM 9843 (or 5 ml/day of 20% lactulose solution or 20 mg/day neomycin sulfate)	8 days, 7 before inducing acute liver injury and 1 day after	The authors concluded that the impact of probiotics on the effects of acute liver injury was small, but they observed no indication of any adverse effects.

Table 4. Studies of *L. reuteri* in Animals

Kelleher et al. 2002	Investigate the effects of *L. reuteri* M164 supple-mentation, with or without supplemental zinc, on nutritional status, gut colonization, and the ability to resist gastrointestinal infection	Infant monkeys were fed control infant formula, formula with *L. reuteri*, or formula with *L. reuteri* and zinc from birth to 4 months. Growth, nutritional status, mineral absorption, intestinal colonization, and enteropathogenic *Escherichia coli*-induced gastroenteritis were monitored.	Infant rhesus monkey	9×10^6 cfu/g *L. reuteri* M164	4 months	*L. reuteri* colonization was achieved and was associated with increased ileal villous surface area and improved hematocrit, with no adverse effects on growth or nutritional indices. Infant monkeys fed *L. reuteri*-supplemented formula had reduced diarrhea severity throughout the study period and recovered more rapidly from acute diarrhea than the other groups. The authors concluded that *L. reuteri* supplementation of infant formula is safe, improves iron status, and decreases diarrhea severity in infant rhesus monkeys.
Mao et al. 1996	Evaluate the effects of rat-derived *L. reuteri* R2LC on methotrexate-induced enterocolitis	Provided continuous intragastric infusion of control diet or diet with oatbase, *L. reuteri*, or *L. plantarum* DSM 9843. Injected methotrexate intraperitoneally on day 3 and sampled on day 6.	Rat (strain not specified)	Rat-derived *L. reuteri* R2LC, unspecified dose	6 days	*L. reuteri* decreased loss of body weight and intestinal permeability, increased bowel mucosal mass, decreased intestinal myeloperoxidase level, reestablished intestinal microecology, reduced bacterial translocation to extraintestinal sites and plasma endotoxin levels, and reduced intestinal pathogens in enterocolitic rats. Neither bacterium caused any adverse effects.

Table 4. Studies of *L. reuteri* in Animals

Metaxas et al. 2002 (article in Greek with English abstract)	Investigate whether *L. reuteri* is effective in reducing bacterial translocation in Zymosan-induced non-septic peritonitis.	Rats received *L. reuteri* or placebo for 5 days; half the rats received IP Zymosan injection to induce peritonitis; enteric mucosal microcirculation and adherent mucus gel thickness were assessed and the mesenteric lymph nodes were examined for bacterial translocation	80 male Wistar rats	10^7 cfu/day of an unspecified strain of *L. reuteri*	5 days	*L. reuteri* pretreatment enhanced enteric mucosal barrier strength by increasing enteric mucosal microcirculation and adherent mucus gel thickness and thus reduced bacterial translocation. There were no indications of any adverse effects from the administration of 10^7 cfu/day *L. reuteri*.
Molin et al. 1992	Evaluate the effect of fermented oatmeal soup on the cholesterol level and the *Lactobacillus* colonization of rat intestinal mucosa	Rats were fed freeze-dried oatmeal soup fermented by six different *Lactobacillus* strains including *L. reuteri* R21c, Hj108, and Hj108^{nxy}for 10 days; cholesterol levels were evaluated.	Male Sprague-Dawley rats	10^7 cfu/g *L. reuteri* R21c, Hj108, and Hj108^{nxy}; estimated intake was 2.3×10^8 cfu/day	10 days	Serum cholesterol levels were lower for rats eating fermented oatmeal v. a non-fermented product. The colonizing ability of the administered strains was evaluated *in vivo*. Only *L. reuteri* R21c was shown to colonize the mucosa; *L. reuteri* represented about 30% of the *Lactobacillus* population 24 days after termination of the administration. No adverse effects were reported.

Table 4. Studies of *L. reuteri* in Animals

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, followed by a 3-week administration of *L. reuteri*, *L. rhamnosus*, or placebo. After 12 weeks, rectums were removed for histology, and CD4 T cells from the mesenteric lymph node were polyclonally activated for cytokine measurements.	Colitic SCID mice	$2x10^{10}$ cfu/day *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*.
Simpson et al. 2000	Study the diversity and stability of the fecal bacterial microbiota in weaning pigs after introduction of *L. reuteri* MM53	Piglets were assigned to 3 treatment groups (control, daily dosed, and 4th-day dosed), and fresh fecal samples were collected daily	9 weanling pigs	$2.5x10^{10}$ cfu/day *L. reuteri* MM53	21 days	Each pig maintained a unique fecal bacterial population that was stable over time, suggesting a strong host influence. The GI microbiota population was not easily modified by administration of exogenous bacteria.
Taranto et al. 1998	Study the effect of *L. reuteri* CRL 1098 on the development of hypercholesterol-emia in mice	Mice were fed a diet enriched with fat to produce hyper-cholesterolemia, then given L. reuteri or placebo; blood lipoproteins were evaluated.	Swiss albino mice	10^4 cfu/day *L. reuteri* CRL 1098	7 days	This low dose of *L. reuteri* caused a 40% reduction in triglycerides and a 20% increase in the ratio of high density lipoprotein to low density lipoprotein without bacterial translocation of the native microflora into the spleen and liver. No adverse effects were seen

Table 4. Studies of *L. reuteri* in Animals

Taranto et al. 2000	Study the effect of pre-administration of *L. reuteri* CRL 1098 on the development of hypercholesterolemia in mice	Administered 10^4 cfu/day *L. reuteri* CRL 1098 to mice for 7 days before inducing hypercholesterolemia by feeding a fat-enriched diet for the following 7 days.	Swiss albino mice	10^4 cfu/day *L. reuteri* CRL 1098	14 days	*L. reuteri* produced a 17% increase in the ratio of high-density lipoprotein to low-density lipoprotein. Total cholesterol and triglycerides increased by 22 and 33%, respectively, in the group that was not fed the lactobacilli. No adverse effects were reported due to the *L. reuteri* treatment.
Wagner et al. 1997a	Assess the capacity of *L. reuteri*, *L. acidophilus*, *L. casei*, and *Bifidobacterium animalis* to colonize, stimulate immune responses, and affect growth and survival.	Mice were inoculated by swabbing their oral cavity and anal area with 10^8 cfu/ml. Colonization of the mice was monitored by enumeration of viable bacteria in the feces	Congenitally immuno-deficient gnotobiotic beige-athymic (*bg/bg-nu/nu*) and beige-euthymic (*bg/bg-nu/+*) mice	Strain not identified; administered via oral and rectal swabs and so the effective dose is uncertain	12 weeks	Some infant mortality occurred in beige-athymic pups born to mothers colonized with *L. reuteri* or *L. casei*. The probiotic bacteria manifested different capacities to adhere to epithelial surfaces, disseminate to internal organs, affect the body weight of adult mice and the growth of neonatal mice, and stimulate immune responses. Although the probiotic species were innocuous for adults, the authors regarded the safety of probiotic bacteria for immunodeficient neonates is less certain. However, it is questionable whether valid inferences can be made to immunocompetent humans from findings in double immunodeficient animal models.
Wagner et al. 1997b	Assess ability to protect athymic *bg/bg-nu/nu* and euthymic *bg/bg-nu/+* mice from mucosal and systemic candidiasis	Mice were colonized by inoculation with both *Candida albicans* and one of the following probiotics: *L. reuteri*, *L. acidophilus*, *L. casei*, and *Bifidobacterium animalis*. Both infection and mortality were assessed.	Athymic *bg/bg-nu/nu* and euthymic *bg/bg-nu/+* mice	10^7 cfu; strain not identified	Single ad-ministration and 12 weeks observation	All probiotics reduced the incidence and severity of infection and prolonged life. No adverse events were reported as a result of the inoculation of any of the probiotics.

Table 4. Studies of *L. reuteri* in Animals

Wagner et al. 2000	Assess antibody response to *Candida albicans* by athymic *bg/bg-nu/nu* and euthymic *bg/bg-nu/+* mice after colonization with probiotic bacteria.	Mice were colonized by inoculation with *Candida albicans* after previous colonization with one of the following probiotics: *L. reuteri*, *L. acidophilus*, *L. casei*, and *Bifidobacterium animalis*. Antibody response to the *C. albicans* were evaluated.	Athymic *bg/bg-nu/nu* and euthymic *bg/bg-nu/+* mice	10^7 cfu; strain not specified	Single administration	The probiotic bacteria did not induce antibody response to their own antigens, they altered the antibody responses of mice to *C. albicans*. No adverse effects were seen as a result of the administration of any of the probiotic strains of bacteria.
Waters et al. 1999	Investigate the ability of pre-colonization with *L. reuteri* to give protection against later inoculation with pathogens.	1. Precolonized mice with *L. reuteri*, then inoculated with *Salmonella typhimurium*. 2. Precolonized mice with *L. reuteri*, then inoculated with *Cryptosproridium parvum*.	1. BALB/c mice 2. Gnotobiotic TCR-α deficient mice	Dose not specified; source not specified.	Not specified	Precolonization with *L. reuteri* was protective against both pathogens, limiting colonization, reducing epithelial damage, decreasing translocation, and reducing mortality, without causing adverse effects.

4.4.2. Human Studies

4.4.2.1. Studies in Adults

4.4.2.1.1. Study of BioGaia's *L. reuteri* DSM 17938 in Adults

As was discussed in Section 2.3.2.3, Roos and Rosander (2006), based on *in vitro* testing, established that *L. reuteri* daughter strain DSM 17938 is substantially equivalent to parent strain ATCC 55730 with respect to colony and cell morphology, fermentation pattern, production of reuterin, growth (generation time), mucus binding ability, acid tolerance, and bile tolerance. Melin et al. (2008), in a study that is currently unpublished, extended this work to demonstrate substantial equivalence through direct *in vivo* comparison of the two strains in a randomized, double-blind, placebo-controlled clinical trial. (This study is summarized in Table 6 at the end of the following section along with studies of *L. reuteri* ATTC 55730 in adults.)

Seventeen healthy adults age 27±7 years, 11 females and 6 males, were randomized into 4 groups: 10^9 cfu/day of *L. reuteri* ATCC 55730 (n=3), 10^9 cfu/day of *L. reuteri* DSM 17938 (n=5), 10^{11} cfu/day of *L. reuteri* DSM 17938 (n=5), or placebo (n=4) for 28 days. The doses of 10^9 cfu/day were chosen to represent the potential maximum exposure to *L. reuteri* in foods formulated to assure that 10^8 cfu remains viable at the end of their shelf life. The dose of 10^{11} cfu/day of *L. reuteri* DSM 17938 was intended to provide a 100-fold dose exaggeration. Participants were instructed to keep the sachets containing the freeze-dried powder (mixed with maltodextrin) refrigerated until the point of consumption in order to maintain bacterial viability.

All participants were given personal diaries and asked to complete them on days 0, 7, 14, 21, and 28, recording their experience during the previous 7 days. Fecal samples were taken on days 0, 7, 14, 28, 42, and 56 for detection and strain identification of *L. reuteri*; *L. reuteri* species were identified by production of reuterin and the specific strain was identified by analysis of specific plasmid content. Physical examinations were performed on days 0 and 28, including blood pressure, pulse rate, and body temperature, and blood was drawn on the same dates. Blood samples were analyzed for red blood cell (erythrocyte) counts, hemoglobin, erythrocyte particle concentration, mean corpuscular volume (MCV), total white blood cell (leukocytes) count, white blood differential cell counts (neutrophils, eosinophils, basophils, lymphocytes, and monocytes), total cholesterol, HDL-cholesterol, triacylglycerols (TG), albumin, glucose, iron, total iron-binding capacity (TIBC), calcium, sodium, potassium, phosphate, total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GT), alkaline phosphatase (ALP), creatinine, urea, urate, C-reactive protein (CRP), as well as for bacteremia using standard hospital blood-culture methods

The treatments were well tolerated and there were no reports of adverse events by the participants. Two individuals reported that they had common colds during the study. Three individuals had *L. reuteri* in their feces at baseline; these isolates were not identifiable as known strains based on plasmid analysis. Doses of 10^9 cfu/day of either ATCC 55730 or DSM 17938 produced similar levels of *L. reuteri*, 10^4 to 10^5 cfu/g feces. Increasing the dose 100-fold to 10^{11} cfu/day increased the level in the feces by about 100-fold. The detection levels reached maximum by day 7 and remained at that level through day 28, the end of administration. In nearly all cases, the detected *L. reuteri* were identifiable by specific plasmid content as the administered strain. In the placebo group, an unidentified strain of *L. reuteri* was detected in 1 individual. After a wash-out period of 14 days there was no evidence of either ATCC 55730 or DSM 17938 in the feces of any of the participants.

The health questionnaires in the personal diaries elicited few reports of problems. One member of the group that received ATCC 55730 reported a fever prior to the start of the study and continued to report poor health during the first 2 weeks of the study. One individual in the placebo group developed a transient fever that disappeared before the end of the study. Three participants, 1 in the ATCC 55730 group and 2 in the DSM 17938 low-dose group, reported diarrhea, but there were no reports of diarrhea in the DSM 17938 high-dose group or in the control group. There were rare sporadic reports of nausea in all groups except the DSM 17948 high-dose group. Stomach ache and gas were occasionally reported by members of all groups, but the incidence was just as high in the baseline diary (reporting health prior to the start of probiotic administration) as later. Vomiting was not observed in any study participant.

No bacteria were detected in any of the blood samples. The physical examination revealed no changes in weight, pulse rate, blood pressure, or body temperature in any individual in any group. Similarly, none of the numerous blood parameters measured showed any significant changes during the study nor were any significant differences observed among the groups. Table 5, on the next page, shows the blood measures for the DSM 17938 high-dose group before administration (day 0) and at the end of dosing (day 28), along with data for the placebo group on day 28.

Table 5. Blood Parameters After Placebo and DSM 17938 High-Dose Administration

Parameter	Unit	Placebo Day 28	DSM 17938 Day 0	DSM 17938 Day 28
ALP	µkat/L	0.93±0.15	1.07±0.28	1.05±0.28
GT	µkat/L	0.18±0.05	0.36±0.15	0.35±0.15
AST	µkat/L	0.50±0.11	0.36±0.04	0.36±0.06
ALT	µkat/L	0.30±0.02	0.29±0.07	0.29±0.06
Creatinine	µmol/L	68±11	70±13	72±12
Urea	mmol/L	5.7±2.3	5.9±0.5	6.5±0.9
Bilirubin	µmol/L	8±5	10±5	8±5
Hemoglobin	g/L	127±8	144±16	137±8
Erythrocytes	10^{12}/L	4.5±0.1	4.8±0.5	4.6±0.3
Leukocytes	10^{9}/L	7.0±2.1	6.0±0.6	6.0±1.0
Neutrophils	10^{9}/L	4.3±1.4	3.4±0.3	3.3±0.6
Eosinophils	10^{9}/L	0.1±0.1	0.1±0.1	0.1±0
Basophils	10^{9}/L	0.1±0.1	0.1±0	0.1±0
Lymphocytes	10^{9}/L	2.1±0.7	1.9±0.3	2.0±0.5
Monocytes	10^{9}/L	0.5±0.1	0.5±0.3	0.5±0.2
CRP	mg/L	1.0±0.4	0.8±0.1	0.8±0
MCV	fL	86±3	89±3	88±5
Na	mmol/L	141±2	141±3	141±3
K	mmol/L	4.0±0.2	3.9±0.3	3.9±0.1
Urate	µmol/L	257±102	294±65	281±23
Phosphate	mmol/L	1.2±0.1	1.1±0.04	1.1±0.6
Ca	mmol/L	2.30±0.05	2.37±0.07	2.33±0.11
Fe	µmol/L	13±6	17±8	14±5
TIBC	µmol/L	71±9	59±6	56±4
Glucose	mmol/L	4.6±0.3	4.9±0.3	4.8±0.2
TG	mmol/L	0.8±0.2	1.7±1.3	1.9±1.5
HDL-cholesterol	mmol/l	1.49±0.55	1.50±0.38	1.42±0.51
LDL-cholesterol	mmol/L	2.00±0.15	3.05±0.83	2.90±0.71
Albumin	g/L	42±1	42±1	40±3

Mean±SD
Source: Melin et al. 2008 (unpublished)

The findings of this study provide further evidence that daughter strain *L. reuteri* DSM 17938 is substantially equivalent to its parent ATCC 55730 with regard to human tolerance, gastro-intestinal residency, elimination from the gastrointestinal tract, freedom from adverse effects, and absence of deviations in blood hematological and biochemical parameters. Notably, even at a dose 100-fold higher than is intended for human use no test-article-related adverse effects were seen and there were no indications in the hematology data of transference and infectivity.

4.4.2.1.2. Studies of BioGaia's *L. reuteri* ATCC 55730 in Adults

In a randomized double-blind placebo-controlled trial, Wolf et al. (1995) evaluated the safety and tolerance of *L. reuteri* ingestion by healthy males. Thirty individuals received either 10^{11} cfu *L. reuteri* ATCC 55730 + a cryoprotectant (nonfat dry milk powder, maltodextrin, and sucrose) or placebo (the cryoprotectant alone) for 21 days followed by a 7-day washout. All participants kept a diary in which they recorded the occurrence and severity of any gastrointestinal symptoms: nausea, diarrhea, cramping, distention, flatulence, vomiting, constipation, burping, and reflux. Physical examinations were conducted on days 0, 21, and 28, including body weight, blood pressure (both systolic and diastolic), pulse rate, respiration rate, and temperature. Urinalysis, hematology, blood chemistries, fecal fat, and fecal microbial counts were assessed at baseline and on days 7, 14, 21, and 28; an additional fecal sample was collected on day 77.

Enumeration was performed for total *Lactobacillus* spp., *L. reuteri*, and *L. reuteri* ATCC 55730. Urinalysis parameters were clarity, color, specific gravity, pH, protein, glucose, occult blood, ketones, and leukocyte esterase. Hematological measures included red blood cell (RBC) count, white blood cell (WBC) count, differential leukocytes (neutrophils, lymphocytes, monocytes, eosinophils, and bands), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), and platelet count. The serum chemistry parameters were albumin, albumin/globulin ratio, alkaline phosphatase (ALP), alanine amino transaminase (ALT), aspartate amino transaminase (AST), total bilirubin, blood urea nitrogen (BUN), creatinine, BUN/creatinine ratio, calcium, iron, phosphorus, potassium, sodium, chloride, cholesterol, HDL cholesterol, LDL cholesterol VLDL cholesterol, total cholesterol/HDL ratio, gamma glutamyltranspeptidase (GGT), globulin, glucose, lactic acid dehydrogenase (LDH), total protein, triacylglycerol, and uric acid.

While there were many isolated differences in the measured or calculated parameters, they were not consistent. For example, iron was higher in the test group than the controls on day 28 and GGT on day 7; calcium was lower on day 14, creatinine on day 28, and potassium on day 7. Similar isolated differences were seen in several hematology and urinalysis parameters. No consistent clinically significant differences were seen between the test and control groups, and all values were within the normal ranges; the authors concluded that administration of *L. reuteri* did not compromise any serum chemistry, hematology, or urinalysis variables. There were no differences between the test and control groups in the reported frequency or severity of such manifestations of intolerance as cramping, flatulence, and diarrhea.

Several participants tested positive for *L. reuteri* at baseline, as did 3 control subjects at one or more sampling points. The subjects receiving *L. reuteri* had increased fecal level of *L.*

reuteri on days 7, 14, 21, and 28; 2 subjects still had detectable levels of *L. reuteri* on day 77. (It is not clear that either of these isolates was strain ATCC 55730; 1 of the subjects colonized on day 77 had also been colonized at baseline.) There was no effect, however, on total *Lactobacillus* spp. levels. The authors concluded that *L. reuteri* may be fed to healthy males at 10^{11} cfu/day without any clinically significant safety or tolerance problems. They pointed out that this is probably close to the maximum that would be fed, and expressed confidence in the tolerance and safety of *L. reuteri* ATCC 55730.

In a second study, Wolf et al. (1998) evaluated the safety and tolerance of *L. reuteri* ingestion by subjects infected with the human immunodeficiency virus (HIV). Thirty-nine subjects consumed a freeze-dried preparation of 10^{10} cfu/day *L. reuteri* ATCC 55730 or a placebo for 21 days in a double-blind parallel-design study. Physical examinations, conducted on days 1, 21, and 35, included body weight, oral body temperature, pulse rate, respiratory rate, systolic and diastolic blood pressure. Fecal samples taken during screening were evaluated for enteric pathogens including *Salmonella, Shigella, Yersinia, and Campylobacter; Clostridium difficile* toxin; and ova or parasites including *Cryptosporidium parvum*. Fecal samples were also taken for fecal fat analysis on days 1, 21, and 35 and for gut microbiota analysis on days 1, 7, 14, 21, 28, and 35. Fasting blood samples were drawn at screening and on days 21 and 35 and analyzed for clinical chemistries and hematology. Biochemistry parameters were glucose, phosphorus, calcium, sodium, potassium, chloride, magnesium, iron, triacylglycerols, total cholesterol, HDL cholesterol, LDL cholesterol, blood urea nitrogen (BUN), creatinine, uric acid, albumin, total protein, total bilirubin, direct bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyltranspepsidase (GGT), alkaline phosphatase (ALP), and lactate dehydrogen (LDH). Hematology parameters included red blood cell (RBC) count, total white blood cell (WBC) count, differential WBC count (neutrophils, lymphocytes, monocytes, eosinophils, basophils), platelets, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), red blood cell distribution width (RCDW), and mean platelet volume (MPV). Urine samples were taken at screening and on days 21 and 35 and analyzed for pH, specific gravity, color, bilirubin, ketones, glucose, nitrite, urobilinogen, leukocyte esterase, protein, blood, epithelial cells, bacteria, mucosal threads, WBC, and RBC. Participants also completed a daily questionnaire reporting occurrence and severity of nausea, diarrhea, cramping, distention, flatulence, vomiting, constipation, burping, and reflux as well as number and consistency of bowel movements.

No clinically significant changes were noted in any of the safety parameters measured. Overall, tolerance was good in both groups. Consumption of *L. reuteri* increased fecal levels of *L. reuteri*. However, fecal levels of *L. reuteri* and total *Lactobacillus* species were lower than levels previously observed in healthy male adults. The authors concluded that *L. reuteri* may be fed to HIV-positive individuals at 10^{10} cfu/day without any clinically significant safety or tolerance problems.

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 *reuteri* group received fruit juice supplemented with *L. reuteri* ATCC 55730, and the *rhamnosus* group received fruit juice supplemented with *L. rhamnosus* and *Propionibacterium freudenreichii*. 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.

A study designed to examine local colonization of the human gastrointestinal mucosa and subsequent immune response after dietary supplementation with *L. reuteri* ATCC 55730 was conducted by Valeur et al. (2004). In an open clinical investigation, 10 healthy volunteers and 9 volunteers with ileostomy underwent gastroscopy or ileoscopy and biopsy samples were taken from the stomach, duodenum, or ileum before and after supplementation with $4x10^8$ cfu/day *L. reuteri* ATCC 55730 for 28 days. Biopsy specimen colonization was analyzed using fluorescence *in situ* hybridization with a molecular beacon probe, and immune cell populations were determined by immunostaining. Blood samples taken at baseline and on day 28 were analyzed for hemoglobin, hematocrit, thrombocytes, leukocytes, C-reactive protein, potassium, sodium, creatinine, blood urea nitrogen, plasma glucose, total cholesterol, LDL cholesterol, VLDL cholesterols, triacylglycerols, total bilirubin, urate, alanine aminotransferase, alkaline phosphatase, and lactate. Endogenous *L. reuteri* was detected in the stomach of 1 subject and the duodenum of 3 subjects (out of 10 subjects). After *L. reuteri* ATCC 55730 supplementation, on day 28, the stomachs of 8 and the duodenums of all 10 subjects were colonized. Three ileostomy subjects (of 6 tested) had endogenous *L. reuteri* at baseline, while all 6 displayed colonization after *L. reuteri* supplementation. Gastric mucosal histiocyte numbers were reduced and duodenal B-lymphocyte numbers were increased by *L. reuteri* ATCC 55730 administration. *L. reuteri* administration also induced a significantly higher level of CD4-positive T-lymphocytes in the ileal epithelium. All blood parameters were normal and no systematic changes were observed following supplementation with *L. reuteri* ATCC 55730.

Nikawa et al. (2004) gave 40 healthy females age 20 or older 95 g yogurt that contained either *L. reuteri* ATCC 55730 or placebo for 2 weeks, then, in a crossover design, switched them for the next 2 weeks. There was apparently no washout period between the 2 treatments. The daily dose of *L. reuteri* was not specified. As compared to the group receiving the placebo yogurt, those receiving *L. reuteri* had significantly decreased salivary concentrations of *Streptococcus mutans*, a bacterium correlated with the risk of dental caries. No adverse effects were reported.

As part of a prospective study of decreasing the risk of allergy during the first years of life (available only in abstract form), Jakobsson et al. (2005) administered 10^8 cfu/day *L. reuteri* ATCC 55730 or placebo to 109 pregnant women during their last 4 weeks before delivery. In samples of colostrum taken within 3 days of delivery, the level of anti-inflammatory interleukin-10 (IL-10) was significantly increased and the level of transforming growth factor β2 (TGF-β2) was significantly decreased. There was no effect on TGF-β1, tumor necrosis factor-α, soluble cell surface-protein CD14, IgA, or secretory IgA. There was no report of any adverse effect on the mothers or their infants due to the administration of *L. reuteri*; specifically, the

sodium/potassium ratio, regarded as a marker of subclinical mastitis, was unchanged. By a month after termination of the treatment the composition of the breast milk was similar in the 2 groups of mothers.

Tubelius et al. (2005) conducted a randomized, double-blind, placebo-controlled study of 262 healthy employees at TetraPak in Sweden in order to investigate the effect of *L. reuteri* on workplace healthiness. A total of 181 individuals complied with the study protocol. Study participants received either 10^8 cfu/day *L. reuteri* ATCC 55730 or placebo for 80 days. In the placebo group, 26% reported sick-leave for the defined causes during the study as compared with 11% in the *L. reuteri* group ($p<0.01$). The frequency of sick-days was 0.9% in the placebo group and 0.4% in the *L. reuteri* group ($p<0.01$). Among the 53 shift-workers, 33% in the placebo group reported sick during the study period as compared with none in the *L. reuteri* group ($p<0.005$). The authors noted that no adverse effects were reported by study participants.

In a randomized, double-blind, placebo-controlled study investigating the effect of *L. reuteri* ATCC 55730 on treatment of *Helicobacter pylori* infection (published only in abstract form), Saggioroa et al. (2005) administered either 10^8 cfu *L. reuteri* 2x/day or placebo to 30 patients age 25-30 with confirmed cases of *H. pylori* infection for 30 days. All of the patients also received 20 mg/day omeprazole. In 60% of the patients receiving *L. reuteri* along with omeprazole the H. pylori infection was eliminated, while no eradication occurred in the group given omeprazole + placebo. The authors suggested that *L. reuteri* cell-surface protein may inhibit the binding of *H. pylori* to glycolipid receptors. No adverse effects were reported.

Krasse et al. (2005) studied the effect of 2 different *L reuteri* formulations on gum bleeding and gingivitis using a randomized double-blind placebo-controlled study. The *L. reuteri* strains were reported to be of human origin but were not further identified[4]. Twenty adults in good health but with moderate or severe gingivitis were randomly assigned to receive gum providing $2x10^8$ cfu/day of one *L. reuteri* strain, 21 patients were assigned to receive the same dose of the other *L. reuteri* strain, and 18 received placebo gum. After 14 days it was found that the probiotic treatment had reduced the gingival and plaque indices. No adverse effects were noted.

To assess the short- and long-term effects of *L. reuteri* administration on clinical symptoms of irritable bowel syndrome (IBS), Niv et al. (2005) conducted a double blind, placebo-controlled 6-month trial. Subjects consumed a tablet containing 10^8 cfu *L. reuteri* ATCC 55730 twice a day. The clinical severity of the IBS symptoms was evaluated by the Francis Severity score and the IBS quality-of-life score at study entry and then monthly. Fifty-four subjects age 19 to 74 years were randomized for treatment and 39 completed the study. Both treatment and placebo groups improved significantly in all studied parameters with no significant differences between groups. The treatment with *L. reuteri* produced no harmful effects; indeed, fewer adverse events were reported from the treatment group than from the control group.

A randomized, double-blind, placebo-controlled study of the effect of *L. reuteri* ATCC 55730 on salivary concentrations of *Streptococcus mutans* compared administration of 10^8 cfu/day either through a straw (along with 200 ml water) or in a daily tablet (Caglar et al. 2006).

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4 The two *L. reuteri* were actually BioGaia strains ATCC 55730 and ATCC PTA 5289.

A total of 120 young adults remained in one of these treatment groups, or in control groups receiving either a placebo straw or a placebo tablet, for 3 weeks. Both methods of administration of *L. reuteri* significantly reduced *S. mutans* concentrations, and no effects on saliva secretion or other side effects were observed.

Caglar et al. (2007) gave chewing gum containing 10^8 cfu of *L. reuteri* ATCC 55730 + 10^8 cfu of another *L. reuteri* strain, ATCC PTA 5289, 3 times a day to 40 healthy young adults age 20 years. Half of these individuals received gum that also contained 1.0 g xylitol. Another 40 young adults received gum that contained no probiotic, half with xylitol and half without. The primary endpoint was reduction of salivary *Streptococcus mutans* after 3 weeks. Salivary mutans streptococci were enumerated before and after treatment; a statistically significant reduction was seen due to both the probiotic and the xylitol as compared to controls, but there appeared to be no added advantage in combining xylitol and probiotic. The authors stated that no side effects were reported.

Using a randomized, double-blind, placebo-controlled, cross-over trial, Imase et al. (2007; article in Japanese with English abstract) administered an unreported[5] dosage of *L. reuteri* ATCC 55730 to 10 asymptomatic individuals suffering from *Helicobacter pylori* infection for 4 weeks while 10 others received a placebo; then the treatments were reversed, apparently with no washout period. The *L. reuteri* treatment significantly suppressed *H. pylori* urease activity and *H. pylori* density. No adverse events were reported.

Caglar et al. (2008) followed up their earlier studies (Caglar et al. 2006; Caglar et al. 2007) by testing a probiotic delivery device for the effect of a lozenge providing 10^8 cfu of *L. reuteri* ATCC 55730 + 10^7 cfu of strain ATCC PTA 5289 on salivary *Streptococcus mutans*. The delivery device was a plastic ring resembling a teething ring but including a perforated pouch into which a lozenge could be inserted. Twenty healthy young women with counts of salivary mutans streptococci of at least 10^5 cfu were assigned to suck the medical device containing either a probiotic or placebo lozenge once daily for 10 days. Enumerations of salivary mutans streptococci taken before treatment and one day after final ingestion showed a statistically significant reduction in the probiotic group but not among the placebo group; no adverse side effects on oral health were noted.

5 The article was in Japanese with only the abstract available in English.

Table 6. Studies of *L. reuteri* ATCC 55730 in Adults

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Caglar et al. 2006	Study the effect of 2 different methods (straw or tablet) of administering *L. reuteri* ATCC 55730 on the risk of dental caries	Randomized, double-blind, placebo-controlled trial with 2 test groups and 2 controls.	120 young adults	10^8 cfu/day *L. reuteri* ATCC 55730 either through a straw (along with 200 ml water) or in a daily tablet	3 weeks	Both methods of administration of L. reuteri significantly reduced *S. mutans* concentrations, and no effects on saliva secretion or other side effects were observed.
Caglar et al. 2007	Investigate the effect of gum containing either xylitol or *L. reuteri* on the reduction of salivary *Streptococcus mutans*	Randomized, double-blind, placebo-controlled trial with 3 test groups and 1 control	40 healthy young adults age 20 years	10^8 cfu of *L. reuteri* ATCC 55730 + 10^8 cfu of *L. reuteri*, ATCC PTA 5289 3x/day	3 weeks	A statistically significant reduction was seen due to both the probiotic and the xylitol as compared to controls, but there appeared to be no added advantage in combining xylitol and probiotic. The authors stated that no side effects were reported.
Caglar et al. 2008	Tested a device for the delivery of a lozenge providing *L. reuteri* on salivary *Streptococcus mutans*.	Randomized, double-blind, placebo-controlled trial	20 healthy young women with counts of salivary mutans streptococci of at least 10^5 cfu	10^8 cfu of *L. reuteri* ATCC 55730 + 10^7 cfu of ATCC PTA 5289	10 days	Found a significant reduction of salivary mutans streptococci in the probiotic group but not among the placebo group; no adverse side effects on oral health were noted.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Imase et al. 2007 (article in Japanese with English abstract)	Investigate the effect of probiotic treatment on *H. pylori* infection	Randomized, double-blind, placebo-controlled, cross-over trial.	20 asymptomatic individuals suffering from *Helicobacter pylori* infection	*L. reuteri* ATCC 55730; dose not reported	4 weeks	The *L. reuteri* treatment significantly suppressed *H. pylori* urease activity and *H. pylori* density. No adverse events were reported.
Jakobsson et al. 2005 (abstract)	Study the effect of *L. reuteri* on the immunologic composition of human breast milk. (Part of a larger study.)	Prospective, randomized, double-blind, placebo-controlled study; pregnant women received L. reuteri or placebo for the final 4 weeks of pregnancy.	109 pregnant women	10^8 cfu/day *L. reuteri* ATCC 55730	4 weeks	In samples of colostrum taken within 3 days of delivery, the level of the anti-inflammatory substance interleukin-10 (IL-10) was significantly increased and the level of transforming growth factor β2 (TGF-β2) was significantly decreased. There was no report of any adverse effects on the mothers or their infants due to the administration of *L. reuteri*.
Krasse et al. 2005	Study the effect of 2 different *L reuteri* formulations on gum bleeding and gingivitis	Randomized double-blind placebo-controlled study with 2 test groups and 1 control	59 gingivitis patients—20 received gum providing 21 patients were assigned to receive the same dose of the other *L. reuteri* strain, and 18 received placebo gum	$2x10^8$ cfu/day of 1 of 2 strains of *L. reuteri* (not identified in the article but actually strains ATCC 55730 and ATCC PTA 5289)	14 days	The probiotic treatment reduced the gingival and plaque indices. No adverse effects were noted.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Melin et al. 2008 (unpublished)	Compare colonization and effects of daughter strain *L. reuteri* DSM 17938 with parent strain *L. reuteri* ATCC 55730	Randomized, double-blind, placebo-controlled study; 1 dose of 55730, 2 doses of 17938, placebo	17 healthy adults age 27±7 years, 11 females and 6 males	10^9 cfu/day *L. reuteri* ATCC 55730 (n=3), 10^9 cfu/day *L. reuteri* DSM 17938 (n=5), 10^{11} cfu/day *L. reuteri* DSM 17938 (n=5)	28 days	All treatments were well tolerated and there were no reports of adverse events by the participants. After a wash-out period of 14 days there was no evidence of either ATCC 55730 or DSM 17938 in the feces of any of the participants. One subject in the ATCC 55730 group and 2 in the DSM 17938 low-dose group reported diarrhea; there were no reports of diarrhea in the DSM 17938 high-dose or control groups. There were rare sporadic reports of nausea in all groups except the DSM 17948 high-dose group. The physical examination revealed no changes in weight, pulse rate, blood pressure, or body temperature in any individual in any group. None of the blood parameters showed changes nor were any significant differences observed among the groups. It was concluded that ATCC 55730 and DSM 17938 were equally safe.
Nikawa et al. 2004	Study the effect of *L. reuteri* ATCC 55730 on the risk of dental caries	Randomized, double-blind, placebo-controlled, crossover trial with *L. reuteri* in yogurt consumed daily.	40 healthy females age 20 or older	Dose of *L. reuteri* ATCC 55730 not specified	2 weeks	Nikawa et al. (2004) gave 95 g yogurt that contained either *L. reuteri* ATCC 55730 or placebo for 2 weeks, then, in a crossover design, switched them for the next 2 weeks. The daily dose of *L. reuteri* was not specified. As compared to the group receiving the placebo yogurt, those receiving *L. reuteri* had significantly decreased mouth concentrations of *Streptococcus mutans*, a bacterium correlated with the risk of dental caries. No adverse effects were reported.
Niv et al. 2005	Assess the effect of *L. reuteri* on symptoms of irritable bowel syndrome (IBS).	Randomized, double-blind, placebo-controlled; IBS symptoms and quality-of-life scores compared.	Patients age 19-70 years with severe long-standing IBS; 54 screened, 39 completed study	10^8 cfu 2x/day *L. reuteri* ATCC 55730	6 months	Fewer adverse events were reported in the test group than among the controls and no adverse effects were observed.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Ouwehand et al. 2002	Study the effects of probiotic bacteria on constipation in the elderly.	Randomized open parallel study with *L. reuteri*, *L. rhamnosus*, or placebo; assessed defecation frequency, laxative use, fecal pH, mucin content and azoreductase activity.	28 elderly subjects with long-term constipation	3.6×10^6 cfu/day *L. reuteri* ATCC 55730	4 weeks with 3-week washout	The treatment with *L. reuteri* had no significant effect on constipation. No changes in fecal pH or mucin excretion were observed. *L. reuteri* ATCC 55730 did not affect the mucosal barrier, and no adverse effects were observed; the authors concluded that use of the tested bacterial strains is safe.
Saggioroa et al. 2005 (abstract)	Investigate the effect of *L. reuteri* ATCC 55730 on treatment of *Helicobacter pylori* infection	Randomized, double-blind, placebo-controlled study; patients got omeprazole with either *L. reuteri* or placebo.	30 patients age 25-30 with confirmed cases of *H. pylori* infection	10^8 cfu *L. reuteri* ATCC 55730 2x/day	30 days	In 60% of the patients receiving *L. reuteri* along with omeprazole the H. pylori infection was eliminated, while no eradication occurred in the group given omeprazole + placebo. No adverse effects were reported.
Tubelius et al. 2005	Investigate the effect of *L. reuteri* on workplace health.	Randomized, double-blind, placebo-controlled; measured sick leave.	Healthy adult employees of TetraPak in Sweden; 262 screened, 181 completed study	10^8 cfu/day *L. reuteri* ATCC 55730	80 days	Fewer sick days were taken in the treatment v. placebo group; no adverse events were reported by study participants.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Valeur et al. 2004	Assess colonization and effect on immune response.	Open clinical investigation; biopsy samples taken from stomach, duodenum, or ileum.	10 healthy volunteers and 9 volunteers with ileostomy	$4x10^8$ cfu/day *L. reuteri* ATCC 55730	28 days	Colonization was observed in both the stomach and duodenum. L. reuteri ingestion increased duodenal B-lymphocytes and CD4-positive T-lymphocytes and decreased gastric mucosal histiocytes. Blood parameters were normal and no adverse systematic changes were observed.
Wolf et al. 1995	Evaluate the safety and tolerance of ingestion of *L. reuteri* by healthy males.	Randomized, double-blind, placebo-controlled; urinalysis, hematology, and blood chemistry were assessed on days 7, 14, 21, and 28, as well as signs of intolerance.	30 healthy adult males	10^{11} cfu/day *L. reuteri* ATCC 55730	21 days	There were no differences between the test and control groups in the reported frequency or severity of such manifestations of intolerance as cramping, flatulence, and diarrhea. No consistent clinically significant differences were seen between the test and control groups, and all values were within the normal ranges. The authors concluded that *L. reuteri* may be fed to healthy males at 10^{11} cfu/day without any clinically significant safety or tolerance problems.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Wolf et al. 1998	Evaluate the safety and tolerance of ingestion of *L. reuteri* by individuals infected with human immunodeficiency virus (HIV).	Randomized, double-blind, placebo-controlled; urinalysis, hematology, immune profile, and blood chemistry were assessed as well as signs of intolerance and fecal bacteria.	39 individuals infected with HIV	10^{10} cfu/day *L. reuteri* ATCC 55730	21 days	There were no differences between the test and control groups in the reported frequency or severity of such manifestations of intolerance as cramping, flatulence, and diarrhea. No consistent clinically significant differences were seen between the test and control groups, and all values were within the normal ranges. Fecal levels of *Lactobacillus* spp. were lower than levels observed in healthy males. The authors concluded that *L. reuteri* may be fed to HIV-positive individuals at 10^{10} cfu/day without any clinically significant safety or tolerance problems.

4.4.2.1.3. Studies of Other *L. reuteri* Strains in Adults

The studies discussed below are summarized in Table 7 at the end of the section.

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. The tested strains included *L. reuteri* 108 (originally isolated from the human jejunum) and *L. reuteri* R2LC, originally derived from the rat colon. Both *L. reuteri* strains successfully colonized the mucosa, as did 4 other tested strains. No tolerance problems or adverse events were reported.

Jacobsen et al. (1999) investigated the probiotic potential of 47 selected strains of *Lactobacillus* spp., including *L. reuteri* DSM 12246. 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. reuteri* DSM 12246, *L. rhamnosus* 19070-2, *L. rhamnosus* GG, *L. delbrueckii* subsp. *lactis* CHCC 2329, and *L. casei* subsp. *alactus* CHCC 3137. The daily consumption by 12 healthy 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. The tested *L. reuteri* strain showed relatively high adhesion to Caco-2 cells as well as broad inhibition of the growth of pathogens. Among the tested strains, *L. reuteri* DSM 12246, *L. rhamnosus* 19070-2, and *L. rhamnosus* 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. reuteri* 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.

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. reuteri* RC-14 and *L. rhamnosus* GR-1 or 10^{10} cfu/day of *L. rhamnosus* GG 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.

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. reuteri* RC-14 and *L. rhamnosus* 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.

In a double-blind, placebo-controlled study, 10 women received either placebo or a combination of *L. reuteri* RC-14 and *L. rhamnosus* for 14 days at a dose of 5 x 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.

In a randomized, double-blind, placebo-controlled study, Anukam et al. (2006) studied the effect of *L. reuteri* RC-14 and *L. rhamnosus* on bacterial vaginitis. Premenopausal sub-Saharan women (n=125), age 18-44 years, suffering from bacterial vaginosis, were given orally 2 daily doses containing a total of 5 x 10^9 cfu each of *L. reuteri* and *L. rhamnosus* 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.

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 10^3 cfu/ml of *L. reuteri* RC-14 (and 2 x 10^7 cfu/ml of *L. rhamnosus* GR-1), providing a daily intake of 1.25 x 10^5 cfu of *L. reuteri* and 2.5 x 10^9 cfu of *L. rhamnosus*. 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.

The effect of *L. reuteri* ingested during late pregnancy on the immunological composition of breast milk was studied by Bottcher et al. (2008) in a prospective, double-blind, placebo-controlled trial. Women were treated with either *L. reuteri* (n=54) or placebo (n=55) from gestational week36 until delivery. Both colostrum and mature milk was analyzed for total IgA, secretory IgA, TGF-β1, TGF-β2, IL-10, TNF, soluble CD14, and Na/K ratio. Women's fecal samples were also tested for the presence of *L. reuteri*. The infants were followed prospectively

for 2 years to determine development of eczema and sensitization as defined by a positive skin-prick test or circulating allergen-specific IgE antibodies at 6, 12, and 24 months.

Mothers with positive tests for *L. reuteri* in their feces showed reduced levels of TGF-β2 and increased IL-10; none of the other tested parameters were affected. Infants receiving breast milk from *L. reuteri*-treated mothers showed decreased incidence of eczema and sensitization.

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. reuteri* RC-14 and 10^7 cfu/ml *L. rhamnosus* GR-1 on premenopausal women with HIV/AIDS. Twelve women consumed 100 ml/day of placebo yogurt (containing normal levels of *L. delbruekii* var *bulgaricus* and *Streptococcus thermophilus*), while 12 women consumed 100 ml/day of this same yogurt supplemented with *L. reuteri* and *L. rhamnosus*, a daily ingestion of 10^9 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.

Table 7. Studies of Other *L. reuteri* Strains in Adults

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose and Source	Duration	Safety-Related Results
Anukam et al. 2006a	Study the effect of *L. reuteri* RC-14 and *L. rhamnosus* on bacterial vaginitis.	Randomized, double-blind, placebo-controlled study	125 premenopausal sub-Saharan women age 18-44 years suffering from bacterial vaginitis	5 x 10^9 cfu/day each of *L. reuteri* RC-14 and *L. rhamnosus*	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. reuteri* and *L. rhamnosus* on women with HIV/AIDS	Prospective, double-blind, placebo-controlled trial of probiotic-supplemented yogurt v. placebo yogurt	24 women being treated for HIV/AIDS	10^9 cfu/day each of *L. reuteri* RC-14 and *L. rhamnosus* GR-1	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..
Baroja et al. 2007	Evaluate the anti-inflammatory effect of yogurt supplemented with *L. reuteri* RC-14 and *L. rhamnosus*	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	1.25 x 10^5 cfu of *L. reuteri* RC-14 and 2.5 x 10^9 cfu of *L. rhamnosus*		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.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose and Source	Duration	Safety-Related Results
Bottcher et al. 2008	Test the effect of *L. reuteri* ingested during late pregnancy on the immunological composition of breast milk and the incidence of eczema and sensitization in their infants	Prospective, double-blind, placebo-controlled trial	111 healthy women in late pregnancy	Not reported	From gestational week 36 to delivery	Mothers with positive tests for *L. reuteri* in their feces showed reduced levels of TGF-β2 and increased IL-10; none of the other tested parameters were affected. Infants receiving breast milk from *L. reuteri*-treated mothers showed decreased incidence of eczema and sensitization.
Jacobsen et al. 1999	Evaluate the probiotic potential of 47 strains of *Lactobacillus* spp. in resistance to acidity, adhesion to Caco-2 cells, and antimicrobial activity against enteric pathogenic bacteria.	Preliminary *in vitro* testing to identify 5 candidate strains. Open study. Fecal samples were taken at days 0 and 18 and twice during the washout period.	12 healthy adults	10^{10} cfu 2x/day *L. reuteri* DSM 12246	18 days with 17-day washout	Among the tested strains, *L. reuteri* DSM 12246 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. reuteri* 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.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose and Source	Duration	Safety-Related Results
Johansson et al. 1993	Study the ability of *Lactobacillus* strains to colonize human intestinal mucosa.	Administered 19 different strains of multiple species of *Lactobacilli, including L. reuteri* 108 and *L. reuteri* R2LC; took biopsies of the gut for enumeration.	13 healthy adult volunteers	$5x10^8$ cfu/day of each strain	10 days	Colonization was shown for both *L. reuteri* strains tested. No adverse effects or tolerance issues were reported.
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 urogenital infection	5×10^9 cfu/day each of *L. reuteri* RC-14 and *L. rhamnosus*	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.
Reid et al. 2001	Comparison of the effect on bacterial vaginitis of a combination of *L. reuteri* RC-14 and *L. rhamnosus* GR-1 v.*L. rhamnosus* GG	Randomized intervention trial	42 healthy women	6×10^9 cfu/day of a combination of *L. reuteri* RC-14 and *L. rhamnosus* GR-1	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.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose and Source	Duration	Safety-Related Results
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. reuteri* RC-14 and *L. rhamnosus*	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.
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. reuteri* RC-14 and *L. rhamnosus*	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.

4.4.2.2. Studies in Children

4.4.2.2.1. Studies of BioGaia's *L. reuteri* ATCC 55730 in Children

Studies of *L. reuteri* ATCC 55730 in children are summarized in Table 8 at the end of the section.

Ruiz-Palacios et al. conducted three studies (all available only in abstract form) among young children in Mexico City (Guerrero et al. 1996; Ruiz-Palacios et al. 1996a and 1996b). Guerrero et al. (1996) executed a prospective, randomized, double-blind, placebo-controlled study, enrolling 388 healthy children age 12-32 months to study the prevention of community-acquired diarrhea in Mexico City. The children received whole cow's milk (control), or whole cow's milk supplemented with *L. acidophilus* and *B. animalis*, or whole cow's milk supplemented with *L. reuteri* ATCC 55730, *L. acidophilus*, and *B. animalis* for up to 16 weeks (median = 111 days). The total daily intake of probiotics was 1.4×10^{10} or 8.9×10^{9} cfu, respectively, in the two formulas; the daily intake of *L. reuteri* was 1.5×10^{8} cfu. There were no tolerance issues and no effects on normal growth; no adverse effects were reported in the abstract by the study authors.

In a randomized blinded placebo-controlled community-based study (available only as an abstract), Ruiz-Palacios (1996a) administered cows-milk supplemented with *L. reuteri* ATCC 55730 (5×10^{7} cfu/day), *L. acidophilus*, and *B. animalis* for 14 weeks to 123 children age 12-36 months living in Mexico City while 120 children received unsupplemented milk. Tolerance, stool patterns, fecal counts of pathogens including rotavirus, and fecal counts of *Lactobacillus* spp. and *L. reuteri* were monitored. The probiotic treatment reduced the incidence of diarrhea, but not the severity; there was no report of any effect on duration. There were no issues with regard to tolerance and no reported adverse effects from the probiotics in the abstract.

In a second randomized blinded placebo-controlled community-based study (available only as an abstract), Ruiz-Palacios (1996b) administered cows-milk supplemented with *L. reuteri* ATCC 55730, *L. acidophilus*, and *B. animalis* for 21 days to 72 children age 12-36 months living in Mexico City. Three dose levels were tested: 10^{6}, 10^{8}, and 10^{10} cfu/day of the three bacteria. Tolerance, stool patterns, fecal counts of pathogens including rotavirus, and fecal counts of *Lactobacillus* spp. and *L. reuteri* were monitored. The probiotic treatments reduced the incidence of diarrhea in a dose-dependent fashion. There were no issues with regard to tolerance (vomiting, abdominal discomfort, gas, or stool characteristics) and no adverse effects from the probiotics at any tested dose were reported in the abstract.

Shornikova et al. (1997a and 1997b) conducted two randomized, double-blind, placebo-controlled studies to investigate the value of supplementation with *L. reuteri* ATCC 55730 in treating viral and bacterial diarrhea. In the first study (Shornikova et al. 1997a), children age 6-36 months with mild diarrhea received either a placebo or 10^{10} to 10^{11} cfu/day *L. reuteri* for 5 days or hospital release, whichever occurred sooner. The frequency and duration of diarrhea and the incidence of vomiting were decreased by the *L. reuteri* treatment. No adverse events were noted and there were no effects on weight gain, consumption of oral rehydration solution or

electrolyte, or on acid/base balance. The authors concluded that administration of 10^{11} cfu/day *L. reuteri* ATCC 55730 to children is safe.

In a second randomized, double-blind, placebo-controlled study (Shornikova et al. 1997b), children age 6-36 months with mild diarrhea received a placebo or either 10^7 or 10^{10} cfu/day *L. reuteri* for 5 days or hospital release, whichever occurred sooner. The frequency and duration of diarrhea and the incidence of vomiting were decreased by the *L. reuteri* treatment. No adverse events were noted at either dosage and there were no effects on weight gain, consumption of oral rehydration solution or electrolyte, acid/base balance, rotavirus IgA antibodies, or activity of β-glucuronidase or β-glucosidase. The authors concluded that administration of 10^{10} cfu/day *L. reuteri* ATCC 55730 to children is safe.

Kang et al. (2004), in an unpublished study, completed a prospective, randomized, placebo-controlled study of the value of *L. reuteri* as a therapeutic agent in acute diarrhea in young children. Fifty children age 6-36 months hospitalized in Seoul with acute diarrhea were enrolled; 40% of them tested positive for rotavirus. The test group received 10^8 cfu/day of *L. reuteri* ATCC 55730 for 5 days or until discharge from the hospital if sooner. The investigators did not report any adverse effects due to *L. reuteri*.

Cirillo et al. (2005) reported on a pilot study of the effect of treatment with *L. reuteri* ATCC 55730 on atopic dermatitis; this report is available only as an abstract. A total of 15 children age 3-5 years who had developed atopic dermatitis while ingesting cows' milk and who showed improvement when it was withdrawn were randomized to receive either placebo or 10^8 cfu 2x/day *L. reuteri* along with re-introduction of cows' milk. While all 7 children in the control group showed a relapse to eczema, none of the 8 children in the *L. reuteri* group did during the 3½ months of the study. No adverse effects were reported.

In a randomized placebo-controlled study of the therapeutic value of *L. reuteri* ATCC 55730 in the treatment of acute diarrhea in young children (Eom et al. 2005; article in Korean with English abstract), 50 patients age 6-36 months hospitalized with acute diarrhea received either 10^8 cfu *L. reuteri* twice a day or a matching placebo. The treatment continued for the duration of the hospital stay or up to 5 days. The *L. reuteri* treatment reduced both diarrhea and vomiting; no adverse events or tolerance issues were reported.

Lionetti et al. (2006) carried out a randomized, double-blind, placebo-controlled study to investigate the effect of *L. reuteri* ATCC 55730 on gastrointestinal side effects during and after treatment for *Helicobacter pylori* infection. A total of 40 dyspeptic children age 3-18 years with confirmed *H. pylori* infection were randomized to receive either 10^8 cfu/day *L. reuteri* or placebo for 20 days; they also received anti-*H. pylori* treatment for 10 days. The *L. reuteri*-supplemented children had significantly improved gastrointestinal health compared to the placebo group, with no interference with the *H. pylori* treatment or any adverse side effects.

Table 8. Studies of *L. reuteri* ATCC 55730 in Children

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Cirillo et al. 2005 (abstract)	Investigate the effect of *L. reuteri* on children with mild atopic dermatitis aggravated by the intake of cows' milk.	Pilot study of children without allergy to cows' milk who had a history of atopic dermatitis, received milk along with *L. reuteri* or placebo.	15 children age 3-5 years who had had cows' milk withdrawn after appearance of atopic dermatitis, but who tested negative for cows' milk allergy.	10^8 cfu 2x/day *L. reuteri*	3½ months	Administration of *L. reuteri* prevented relapse to eczema despite continued intake of milk. There were no adverse effects reported.
Eom et al. 2005 (article in Korean with English abstract)	Assess the value of *L. reuteri* in the treatment of acute pediatric diarrhea.	Randomized placebo-controlled trial; clinical diarrhea outcomes were evaluated.	50 children age 6-36 months hospitalized for acute diarrhea	10^8 cfu 2x/day *L. reuteri*	Duration of hospital-ization or up to 5 days	The treatment with *L. reuteri* was efficacious, and there were no reports of intolerance or adverse events.
Guerrero et al 1996 (abstract)	Prevention of community acquired diarrhea in healthy children in Mexico City	Prospective, randomized, double-blind, placebo-controlled; *L. reuteri* + *L. acidophilus* + *Bifidobacterium* spp, *L. acidophilus* + *Bifidobacterium* spp. without *L. reuteri*, + control	395 healthy free-living children age 12-32 months in Mexico City	1.5 x 10^8 cfu/day *L. reuteri* with unknown dose of other probiotics	16 weeks	Free of diarrhea: 62%, 68%, 71% of placebo, non-*L. reuteri* beverage, *L. reuteri* beverage Incidence of diarrhea per thousand days: 5.4, 4.1, 3.6 respectively [*L. reuteri* v other and placebo significant] Reported reduction for both probiotic groups in duration of diarrhea, days with watery stools, and number of watery stools during diarrhea.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Kang et al. 2004 (unpublished)	Investigate the value of L reuteri as a therapeutic agent in acute diarrhea in young children	Prospective, randomized, placebo controlled study	50 children age 6-36 months who were hospitalized with acute diarrhea; 40% tested + for rotavirus; n=25 each in test and control groups	10^8 cfu 2x/day *L reuteri*	Length of hospital-ization or up to 5 days	Duration of diarrhea not significantly different, but duration of hospital stay was lower in test group ($p = 0.04$). Significantly fewer cases of diarrhea on day 2 ($p = 0.006$) and day 3 ($p = 0.014$). Significantly less vomiting ($p < 0.01$)
Lionetti et al. 2006	Determine if *L. reuteri* can improve gastro-intestinal health during treatment for *H. pylori* infection in children.	Randomized, double-blind, placebo-controlled study. Children received either *L. reuteri* or placebo during treatment for *H. pylori* infection.	40 children age 3-18 years receiving treatment for confirmed infection with *Helicobacter pylori*	10^8 cfu/day *L. reuteri*	20 days	*L. reuteri* improved gastrointestinal health without adversely affecting treatment or producing adverse effects.
Ruiz-Palacios et al. 1996[a] (abstract)	Assess ability of milk with blends of probiotics to ameliorate community acquired diarrhea in healthy young children in Mexico City; study tolerance and dose-response to probiotic blend	Prospective, randomized, double-blind, placebo-controlled; blend of *L reuteri* + *L acidophilus* + *B lactis* at 3 dose levels; placebo	72 healthy children age 12-36 months: n=20 placebo n=18 low dose n=16 mid dose n=18 high dose	Daily intake of *L reuteri* in low dose = 2.2×10^7; mid = 9.3×10^8; high = 5.0×10^{11} Dose of other probiotics was not reported.	21 days	Probiotic blends were well tolerated. No differences were observed in intake, incidence of vomiting, abdominal discomfort, gas, or stool characteristics. No safety-related endpoints and no specific report regarding adverse effects.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Ruiz-Palacios et al. 1996b (abstract)	Prevention of community acquired diarrhea in healthy children in Mexico City	Prospective, randomized, double-blind, placebo-controlled; blend of *L reuteri* + *L acidophilus* + *B lactis*; placebo (flavored cows' milk)	239 healthy free-living children age 12-36 months in Mexico City: n=123 test group n=120 control	*L. reuteri* = 5.6 x 10^7; *L. acidophilus* = ~1.0 x 10^8; *B. lactis* = ~9.5 x 10^7.	14 weeks	Incidence of diarrhea: 42% v 27% in control v probiotic (significant at $p < 0.05$); no difference in severity of diarrhea No safety-related endpoints and no specific report regarding adverse effects.
Shornikova et al. 1997a	Investigate the value of L reuteri as a therapeutic agent in acute diarrhea in young children	Prospective, randomized, double-blind, placebo controlled study; Randomization was after rehydration treatment . Exclusion criteria: on immuno-suppressive therapy or with immune deficiency, allergy to cows' milk, or underlying disease	40 well-nourished children age 6-36 months who were hospitalized (in Tampere, Finland) for acute diarrhea of <7 days duration; n=19 in test group, n=21 in control; 75% of children had rotavirus antigen	10^{10-11} cfu/day *L. reuteri*	5 days or duration of stay if shorter	No difference in weight gain, correction of acidosis, or electrolyte levels. No other safety-related endpoints and no specific report regarding adverse effects.
Shornikova et al. 1997b	Investigate the value of high v. low doses of L reuteri as a therapeutic agent in rotavirus-associated acute diarrhea in young children	Prospective, randomized, double-blind, placebo controlled study; low and high dose groups + control Randomization was after rehydration treatment	66 well-nourished children age 6-36 months who were hospitalized for acute diarrhea of <7 days duration who tested + for rotavirus; n=21 in high-dose group, N=20 in low-dose group; n=25 in control	High dose = 10^{10-11} cfu/day; low dose = 10^7 cfu/day	5 days or duration of stay if shorter	No differences in weight gain, electrolyte or acid-base balance, or hospital stay. No differences in β-glucuronidase or β-glucosidase activity. No other safety-related endpoints and no specific report regarding adverse effects. Authors concluded that administration of *L reuteri* is a safe and effective therapy of acute rotavirus diarrhea in children.

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

Rosenfeldt et al. conducted a series of 4 prospective, randomized, double-blind, placebo-controlled trials (2002a, 2002b, 2003, 2004) using *L. reuteri* DSM 122460 provided by Chr Hansen. These 4 studies are summarized in Table 9 on the following page. In all 4 studies, doses of 10^{10} cfu of *L. reuteri*, along with similar doses of *L. rhamnosus* 19070-2, 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 age 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 age 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. reuteri* DSM 122460 and *L. rhamnosus* on children with atopic dermatitis. Forty-three children age 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 age 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 indicated by the reduced 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.

Table 9. Studies of Other *L. reuteri* Strains in Children

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose and Source	Duration	Safety-Related Results
Rosenfeldt et al. 2002a	Assess the value of *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. reuteri* DSM 122460 and *L. rhamnosus* 19070-2	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 *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. reuteri* DSM 122460 and *L. rhamnosus* 19070-2	5 days	The authors stated that "no serious adverse effects were registered."
Rosenfeldt et al. 2003	Assess the value of *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. reuteri* DSM 122460 and *L. rhamnosus* 19070-2	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 *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. reuteri* DSM 122460 and *L. rhamnosus* 19070-2	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.

4.4.2.3. Studies in Infants

4.4.2.3.1. Studies of BioGaia's *L. reuteri* ATCC 55730 in Infants

Table 10 at the end of the section summarizes the studies discussed in greater detail below.

Karvonen et al. (2001; available only as an abstract) investigated the safety of *L. reuteri* ATCC 55730 in neonates given 10^5, 10^7, or 10^9 cfu/day *L. reuteri* in 20 ml of breast milk or formula for 30 days. Ninety healthy term infants were randomized into a placebo group (which received breast milk or formula without added *L. reuteri*) or one of the three test groups; the study was double-blinded. Abdominal symptoms and stool consistency were recorded, and no adverse effects were noted. The authors concluded that it is safe to provide *L. reuteri* ATCC 55730 at doses up to 10^9 cfu/day to neonates.

A randomized, double-blind, placebo-controlled study was conducted by Alsheikh and Weizman (2003; available only in abstract form) to evaluate the safety of *L. reuteri* ATCC 55730 and *Bifidobacterium lactis* Bb12 in early infancy. A total of 60 healthy full-term infants age 3-65 days were assigned to receive infant formula supplemented with 10^8 cfu/day *L. reuteri* or *B. lactis* or neither for 60 days. Growth, feeding, stooling, and behavior characteristics were monitored with a weekly physical examination. There were no significant differences between groups regarding growth (weight, length, head circumference), feeding, stooling (effort and gas), or behavior (crying and restlessness). No adverse effects were noted, and the authors concluded that both probiotics are clinically safe and do not affect growth.

Asli et al. (2003) studied the effect of infant formulas supplemented with two strains of probiotics on the rate of acute infectious illnesses, growth, feeding, stooling, behavior, and adverse effects in daycare infants. A prospective, randomized, double-blind, placebo-controlled trial (available only as an abstract) was conducted with 194 healthy full-term infants age 4-10 months, who were assigned to receive 10^8 cfu/day *L. reuteri* ATCC 55730, *Bifidobacterium lactis* Bb12, or a placebo for 12 weeks. The infants were monitored at outset and at 4, 8, and 12 weeks by a physical examination that included growth measures; a weekly telephone interview was also used. Infants fed a probiotic supplemented formula exhibited fewer febrile episodes and fewer gastrointestinal illnesses. This effect was more pronounced with *L. reuteri*. There was no significant effect on respiratory illnesses. For the *L. reuteri* group there were significantly fewer visits to the doctor, fewer days with absence from day care center and less use of antibiotics compared to the other two groups. No significant differences were noted between groups regarding growth, or characteristics of feeding, stooling, and behavior (crying and restlessness). No adverse effects were noted in any subject.

Following up earlier work (Karvonen et al. 2001), Karvonen et al., in work as yet unpublished (Karvonen et al. 2005), further explored the safety of *L. reuteri* ATCC 55730 in neonates. They completed 3 consecutive prospective, randomized, double-blind, placebo-controlled trials, each for 28 days, with *L. reuteri* supplementation beginning within the first 3 days of life. In the first trial, 76 healthy full-term infants received either placebo (breast milk or formula), or 10^5, 10^7, or 10^9 cfu/day *L. reuteri* dissolved in breast milk or formula. In the second

trial, 35 healthy full-term infants received either a placebo powder suspended in 5 drops of oil or 10^8 cfu/day *L. reuteri* in 5 drops of oil. In the third trial, 43 premature (gestational age 31-36 weeks) but otherwise healthy infants weighing 1500-2500 g received the oil placebo or either 10^7 or 10^9 cfu/day *L. reuteri* in oil suspension. In all three trials, the primary endpoints were safety, specifically the absence of adverse reactions including abdominal symptoms such as stomach discomfort, pain, or cramps. Abdominal symptoms in placebo and *L. reuteri* supplemented full-term infants did not differ; in the premature infants more abdominal symptoms were reported in the low-dose *L. reuteri* group than in the controls, but this was not seen in the high-dose group and was not considered to be related to *L. reuteri* supplementation. Supplementation with *L. reuteri* did not result in significantly higher levels of total lactobacilli compared with placebo-treated infants. The levels of total lactobacilli after 28 days of *L. reuteri* supplementation were normal for infants with no signs of overgrowth. No adverse events related to *L. reuteri* were observed in any of the infants studied at any dose and with any delivery system.

Also in 2005, Weizman et al. performed a prospective, randomized, double-blind, placebo-controlled trial to determine the ability of *L. reuteri* ATCC 55730 or *B. lactis* Bb12 to reduce the incidence of infections in healthy infants in child-care centers. A total of 201 healthy full-term infants age 4-10 months (mean = 6.8 months) in 14 daycare centers were given Materna Premium formula alone (n=60) or with 10^7 cfu/ml of either *L. reuteri* (n=68) or *B. lactis* (n=73) for 12 weeks. There were no group differences in age, birth weight, gestational age, sex, prior breastfeeding, siblings, pets, parental smoking, or crowding. Each participant was given a physical examination at baseline and at 4, 8, and 12 weeks. Parents completed a daily questionnaire that included feeding (number of meals, response to food, daily formula volume, number of regurgitation and vomiting episodes), behavior (crying, night awakenings, restlessness), and stooling characteristics (number of bowel movements, stooling effort, stool consistency, presence of blood in stools, and gas), and were asked to report on any symptom, including respiratory (runny nose, cough, shortness of breath), gastrointestinal (watery stools), febrile episodes, absences, visits to the clinic, prescription of any medication, and adverse reactions. Rectal temperatures were also taken daily. Any reported illnesses resulted in a daily examination by a pediatrician on the research team. Formula consumption was recorded; the daily intake of *L. reuteri* was 1.2×10^9 cfu/day.

No differences were seen in any feeding characteristics or respiratory illnesses. The primary endpoints were febrile and diarrheal episodes and duration. Infants receiving *L. reuteri* were significantly healthier on all 4 endpoints than were the controls and had fewer days with fever, fewer visits to the clinic, and fewer prescriptions of antibiotics. "Side effects" were specifically included as outcome measures. The authors stated that "adverse effects were not noticed in any of the participants." There were no differences in the growth parameters of weight, length, or head circumference. The authors concluded that, based on the safety of *L. reuteri* as shown in other research, the natural occurrence of lactobacilli in the human intestine, and the absence in this study of any adverse effects or any effect on growth, 1.2×10^9 cfu/day *L. reuteri* is safe for administration to infants.

Connolly et al. (2005) reported on an ongoing double-blind, placebo-controlled, multi-center clinical trial in which either 10^8 CFU of *L. reuteri* ATCC 55730 or placebo was given every day from the first day of life to infants with a family history of allergy. The primary

endpoint of the study was the effect of *L. reuteri* on the development of allergic symptoms during the first year of life. (As of this date, the main study has not been published.) A sub-study was performed that included a randomly drawn sample of 24 6-month-old infants chosen for the analysis of D-lactic acid in the blood after 6 months of supplementation and again after 12 months. Since the main study was still locked, the group membership of the infants was not known at the time of drawing; an independent statistician found that 14 were from the *L. reuteri*-treated group and 10 were from the placebo-control group.

All 24 infants had low blood levels of D-lactic acid, ranging from 20-130 μM; well within the normal range in humans. At 6 months, the mean plasma D-lactic acid concentration was 49 μM/L in the *L. reuteri*-treated group and 42 μM/L in the placebo group; at 12 months, the group means were 34 μM/L and 40 μM/L, respectively. There were also no statistically significant differences in serum L-lactate concentrations between the *L. reuteri* and placebo groups at either 6 or 12 months. Additionally, the physicians monitoring the study reported no adverse effects in any of the 232 infants participating in the full study and, in particular, no symptoms that would be associated with acidosis. The authors concluded, "Our study provides evidence that there is no elevation of D(-)-lactic acid in the blood of infants given *L. reuteri* at a dose of 10^8 cfu/day from birth daily for 12 months. From this observation and after a careful review of the literature, we conclude that this D(-)-lactic acid producing probiotic can be safely given to infants."

Weizman and Alsheikh (2006) evaluated the safety and tolerance of infant formula containing either of two probiotic bacteria, *L. reuteri* ATCC 55730 or *B. lactis* Bb12, in a prospective, randomized, double-blind, placebo-controlled trial. A total of 59 full-term healthy infants age 3-65 days whose parents had elected not to breastfeed were randomized and 49 completed the study. By group, the number of infants beginning and completing the 4-week study were: *L. reuteri*—20 and 17; *B. lactis*—20 and 16; control—19 and 16. The exclusion criteria were prematurity, birth weight < 2500 g, congenital abnormality, chronic disease, failure to thrive, allergy or atopic disease, or exposure to pre-, pro-, or antibiotics within 4 weeks. The primary outcome measures were clinical adverse effects, including deviations of growth parameters. The experiment was powered to have 85% certainty of detecting at 95% confidence a 20% difference in the targeted growth parameters. Infants received physical examinations at baseline and completion of the trial. The parents completed a questionnaire daily during weeks 1 and 4, including questions on feeding, behavior, and stooling characteristics as described in Weizman et al. (2005). The probiotic bacteria were added at 10^7 cfu/g formula powder or 2.2×10^8 cfu/180 ml prepared formula; the mean daily formula consumption was about 660 ml, and thus the estimated mean daily ingested dose of probiotic microorganisms was approximately10^9 cfu/day.

There were no withdrawals based on formula-related complaints or adverse effects in the *L. reuteri*, the *B. lactis*, or the control group. The mean daily formula volume did not differ significantly between the groups, nor did food compliance, daily number of meals, or daily number of regurgitation and vomiting episodes. There were no significant differences in stooling scores (effort, fecal consistency, gas), number of bowel movements, crying or restlessness scores, number of severe crying episodes, number of night awakenings, or episodes of acute illness. No adverse effects were noticed throughout the study. There were no significant differences in

growth parameters—weight, length, or head circumference. In summary, the probiotic bacteria, at an estimated daily ingestion level of about 10^9 cfu/day *L. reuteri* ATCC 55730 or *B. lactis* Bb12, did not adversely affect either the health or the daily habits of the infants.

A randomized study of the value of *L. reuteri* ATCC 55730 in the treatment of infantile colic was conducted by Savino et al. (2007). Breastfed colicky infants aged 21-90 days, diagnosed according to Wessel's criteria, were randomly assigned to 2 treatments; 41 infants received 10^8 cfu/day *L. reuteri* ATCC 55730 and 42 received 6 mg/kg bw/day simethicone for 28 days, both in oil suspensions given 30 minutes after feeding. The parents completed daily questionnaires regarding crying and side effects such as constipation, vomiting, cutaneous reactions, etc., using a structured diary. Infants were examined by a pediatrician on days 1, 7, 14, 21, and 28. Both treatments reduced crying, with *L. reuteri* the more efficacious treatment, within 1 week of initiation of treatment with improvement continuing through day 28. No adverse side effects were observed in either group. There were no differences in parental reports of side effects except that inconsolable crying was significantly reduced in the probiotic group as compared with the simethicone group.

The ability of *L. reuteri* to reduce the incidence of IgE-associated eczema was investigated in a randomized, double-blind, placebo-controlled study (Abrahamsson et al. 2007). Pregnant women with a familial history of allergic disease (n = 232) were given 10^8 cfu/day *L. reuteri* ATCC 55730 or placebo beginning at gestational week 36 and continuing up to delivery. Their infants received the same supplement at the same dosage for 12 months. The primary outcome was allergic disease, with or without positive skin prick or circulating IgE to food allergens.

Many of the mothers receiving *L. reuteri* had detectible isolates in their colostrum; 80% of the infants receiving *L. reuteri* were colonized with this bacterium at 5-6 days v. only 19% in the placebo group. At 12 months, 63% of the test infants and 23% of the controls were colonized. No difference was seen in the incidence of eczema during the first year, but the *L. reuteri*-treated infants showed a significantly reduced incidence of IgE-associated eczema during the second year (8% v. 20%), and the incidence of positive reactions to skin pricks was also significantly lower, 14% v. 31%. There were no significant differences in adverse effects among the infants in the two groups except for a significantly higher incidence of acute otitis media in the *L. reuteri* group. Abrahamsson et al. (2007) regarded this as a chance difference not attributable to the *L. reuteri* treatment. Both groups maintained normal growth; the *L. reuteri*-treated infants were significantly heavier at 3 months of age but not at any other time point.

Betta et al. (2007; available only as an abstract) conducted a retrospective controlled study of high-risk premature infants confined to the Neonatal Intensive Care Unit (NICU)[6]. A total of 184 premature neonates (mean gestational age 34.8 weeks, mean birth weight 2188.8 g) were randomized to receive from their first day of life either 10^8 cfu/day of *L. reuteri* strain ATCC 55730 (n=67), $3x10^9$ cfu/day *L. rhamnosus* strain GG (LGG; n=55), or nothing (n=62). The infants remained on these treatments until they left the NICU. Most of the infants had been delivered by Caesarian section: 58/67 in the 55730 group, 44/55 in the LGG group, and 56/62 in

6 An earlier report of this study was presented at the 5th annual meeting of the Italian Society of Perinatal Medicine in June 2006 (Romeo 2006).

the control group. About half of the infants had central venous catheters: 37/67 in the 55730 group, 27/55 in the LGG group, and 28/62 in the control group. A small number of the infants received surgery (5/67 in the 55730 group, 6/55 in the LGG group, and 6/62 in the control group). The infants were swabbed and their feces examined for bacterial or *Candida* infection on days 0, 7, 14, 21, and 27 (or until they were discharged from the NICU).

Both probiotic interventions significantly reduced the incidence of both bacterial and *Candida* infections, particularly among the infants receiving surgery. Among the surgical patients, the *L. reuteri* group experienced 1 bacterial and 2 *Candida* infections, the LGG group had 2 cases of each, and the control group suffered 5 bacterial infections and 4 *Candida* infections. The probiotic treatments also greatly reduced the number of days infected infants remained on antimycotic therapy, from a mean of 36 days among the control to 17 days in the LGG group and only 10 days among those receiving *L. reuteri*. No adverse side effects were observed from the probiotic treatment; indeed, both probiotic groups experienced lower incidences than did the control group of reflux, vomiting, meteorism, diarrhea, and gastric distress. The premature infants receiving *L. reuteri* were released from the NICU in about half the time as the controls, a mean of 21.8 days v. 42.3 days. Finally, the *L. reuteri* and LGG groups had nonsignificantly faster growth than the controls due to lower post-delivery weight loss.

It is especially worthy of note in this study that 37 of the premature infants in the *L. reuteri* group and 27 in the LGG group had central venous catheters. The presence of such a central line is widely regarded as the "main risk factor" for invasive infections by probiotic bacteria (Vandenplas et al. 2007), yet no such infections were observed (Betta et al. 2007).

Indrio et al. (2008) investigated the effect of administration of *L. reuteri* strain ATCC 55730 on feeding tolerance and gastrointestinal motility in healthy formula-fed preterm infants (mean gestational age 34 weeks; mean birth weight 1890 g; mean Apgar score 8). In a randomized, double-blind, placebo-controlled study, 30 preterm neonates were enrolled on their 3rd to 5th day of life; 10 were breast-fed and the other 20 were randomly assigned to receive either an oil formulation providing 10^8 cfu/day of *L. reuteri* or a placebo for 30 days. During their hospital stay, the number of episodes of regurgitation, vomiting, inconsolable crying, and evacuations were recorded by the nurses; parents did the same recording after discharge. Gastric electrical activity (EGG) was recorded on day 4 and day 35 after birth for evaluation of mean frequency, instability coefficient, and percentages of dominant frequencies in the ranges defined as normal, bradygastric, and tachygastric. Gastric emptying was also recorded by ultrasound on the same 2 days.

The breast-fed infants and those receiving *L. reuteri* had significantly fewer episodes of regurgitation and inconsolable crying and an increased number of stools compared to the placebo group. No differences were noted among the groups in any of the EGG parameters. The breast-fed and *L. reuteri* groups had nonsignificantly more weight gain over the 30 days. The premature infants receiving *L. reuteri* more closely resembled the breast-fed infants in gastric emptying and motility. No adverse side effects were noted in any of the infants, including growth or behavioral effects.

Table 10. Studies of *L. reuteri* ATCC 55730 in Infants

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Abrahamsson et al. 2007	Determine the ability of *L. reuteri* to colonize the GI tract and to reduce the incidence of IgE-associated eczema in neonates.	Randomized, double-blind, placebo-controlled study. Pregnant women took *L. reuteri* or placebo beginning at gestational week 36 and the infants continued taking the same supplement to the age of 12 months	232 mothers and their infants with a familial history of allergies	10^8 cfu/day *L. reuteri* ATCC 55730	4 weeks for mothers, 12 months for infants	*L. reuteri* appeared in 12% of the colostrum of mothers taking it v. 2% of those receiving placebo. 80% of test infants (v. 19% of controls) were colonized by day 5-6, and 63% of test infants v. 23% of controls were colonized at 12 months. There were no significant differences in adverse effects among the infants in the two groups except for a significantly higher incidence of acute otitis media in the *L. reuteri* group,. regarded as a chance difference not attributable to the *L. reuteri* treatment. Both groups maintained normal growth; the *L. reuteri*-treated infants were significantly heavier at 3 months of age but not at any other time point.
Alsheikh and Weizman 2003 (abstract)	Evaluate the safety of the probiotic bacteria *L. reuteri* ATCC 55730 and *B. lactis* Bb12 for neonates.	Randomized, double-blind, placebo-controlled study; infants received either probiotic or placebo; safety and tolerance endpoints were evaluated.	60 healthy full-term infants age 3-65 days (20 per group)	10^8 cfu/day *L. reuteri*	60 days	There were no significant differences between groups regarding growth (weight, length, and head circumference), feeding, stooling (effort and gas), or behavior (crying and restlessness). No adverse effects were noted, and the authors concluded that both probiotics are clinically safe for neonates and do not affect growth.
Asli et al 2003 (abstract)	Prevention of acute infectious illness in healthy full-term formula-fed infants	Prospective, randomized, double-blind, placebo-controlled; *L. reuteri*, *Bifidobacterium*, + control	201 healthy full-term formula-fed infants age 4-10 months in a day-care center in Israel	10^8 cfu/day *L. reuteri*	12 weeks	Febrile events and GI illnesses were fewer with both probiotics, especially. *L. reuteri* Respiratory illnesses: no effect Doctor visits, days absence, and u se of antibiotics were all reduced with *L. reuteri* No adverse effects were observed.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Betta et al. 2007 (abstract)	Reduce the incidence and severity of bacterial and *Candida* infections among premature neonates in a Neonatal Intensive Care Unit.	Retrospective controlled study; *L. reuteri*, LGG, + control.	184 premature neonates (mean gestational age 34.8 weeks, mean birth weight 2188.8 g) confined to a NICU; most were Caesarian delivery, half had central venous lines, about 10% were surgical patients	10^8 cfu/day of *L. reuteri*, $3x10^9$ cfu/day LGG	Until discharge from the NICU, mean of 21.8 days for those receiving *L. reuteri* and 28.1 days for those receiving LGG	Both probiotic interventions significantly reduced the incidence of both bacterial and *Candida* infections, particularly among the infants receiving surgery. Among the surgical patients. The probiotic treatments also greatly reduced the number of days infected infants remained on antimycotic therapy. No adverse side effects were observed from the probiotic treatment; both probiotic groups experienced lower incidences than did the control group of reflux, vomiting, meteorism, diarrhea, and gastric distress. The premature infants receiving *L. reuteri* were released from the NICU in about half the time as the controls. The *L. reuteri* and LGG groups had nonsignificantly faster growth than the controls due to lower post-delivery weight loss. No infections were observed, even among the surgical and central-line infants.
Connolly et al. 2005	Determine the impact of feeding of *L. reuteri* ATCC 55730 on D-lactic acid in the blood of neonates.	Sub study from an ongoing double-blind, placebo-controlled, multi-center clinical trial (on the effects of *L. reuteri* on the development of allergic symptoms). Blood samples were drawn and analyzed at 6 and 12 months.	24 infants with a family history of allergy; 14 receiving *L. reuteri*, 10 receiving placebo	10^8 cfu/day *L. reuteri*	6 months and 12 months at the times of blood draws	At 6 months, the mean plasma D-lactic acid concentration was 49 μM/L in the *L. reuteri*-treated group and 42 μM/L in the placebo group; at 12 months, the group means were 34 μM/L and 40 μM/L, respectively. There were also no statistically significant differences in serum L-lactate concentrations between the *L. reuteri* and placebo groups at either 6 or 12 months. Additionally, the physicians monitoring the study reported no adverse effects in any of the 232 infants participating in the full study and, in particular, no symptoms that would be associated with acidosis.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Indrio et al. 2008	Investigate the effect of administration of *L. reuteri* strain ATCC 55730 on feeding tolerance and gastrointestinal motility in healthy formula-fed preterm infants	Randomized, double-blind, placebo-controlled study	30 healthy preterm neonates enrolled on their 3rd to 5th day of life; 10 were breast-fed, 10 received *L. reuteri*, and 10 received placebo	10^8 cfu/day	30 days	The breast-fed infants and those receiving *L. reuteri* had fewer episodes of regurgitation and inconsolable crying and an increased number of stools compared to the placebo group. No differences were noted among the groups in any of the EGG parameters. The breast-fed and *L. reuteri* groups had nonsignificantly more weight gain over the 30 days. The premature infants receiving *L. reuteri* more closely resembled the breast-fed infants in gastric emptying and motility. No adverse side effects were noted in any of the infants, including growth or behavioral effects.
Karvonen et al. 2001 (abstract)	Evaluate the safety of administration of *L. reuteri* ATCC 55730 to neonates	Randomized, double-blind, placebo-controlled study; 3 test doses of *L. reuteri* + placebo; measured safety-related outcome endpoints.	90 healthy term infants during the first 3 days of life	10^5, 10^7, or 10^9 cfu/day *L. reuteri*	30 days	Abdominal symptoms and stool consistency were recorded, and no adverse effects were noted. The authors concluded that it is safe to provide *L. reuteri* ATCC 55730 to neonates at doses up to 10^9 cfu/day.
Karvonen et al. 2005 (unpublished)	Evaluate the safety of administration of *L. reuteri* ATCC 55730 to neonates, including pre-term infants	3 randomized, double-blind, placebo-controlled studies with different doses of *L. reuteri* + placebo; measured safety-related endpoints.	1. 76 healthy full-term infants during the first 3 days of life 2. 35 healthy full-term infants during the first 3 days of life 3. 43 healthy pre-term infants during the first 3 days of life	1. 10^5, 10^7, or 10^9 cfu/day *L. reuteri* 2. 10^8 cfu/day *L. reuteri* 3. 10^7 or 10^9 cfu/day *L. reuteri*	28 days each	In all three trials, the primary endpoints were safety, specifically the absence of adverse reactions including abdominal symptoms such as stomach discomfort, pain, or cramps. No adverse events related to *L. reuteri* were observed in any of the infants studied at any dose and with any delivery system.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Savino et al. 2007	Investigate the value of *L. reuteri* ATCC 55730 in the treatment of infantile colic	Randomized open study with 2 treatment groups, *L. reuteri* and Simethicone, and no placebo group. Effect on colic was assessed by reports of crying.	46 breastfed colicky infants aged 21-90 days, diagnosed according to Wessel's criteria; 23 received *L. reuteri* and 23 received Simethicone	10^8 cfu/day *L. reuteri*	28 days	Both treatments reduced crying, with *L. reuteri* the more efficacious treatment, within 1 week of initiation of treatment with improvement continuing through day 28. No side-effects were reported on parental questionnaires and no adverse side effects were observed in either group by physicians' examinations.
Weizman et al. 2005	Determine the ability of probiotic bacteria (*L. reuteri* ATCC 55730 and *B. lactis* Bb12) to reduce infections in healthy day-care infants	Prospective, randomized, double-blind, placebo-controlled trial. Exclusion criteria were prematurity, BW < 2500 g, congenital abnormality, chronic disease, failure to thrive, allergy or atopic disease, or exposure to pre- pro- or antibiotics within 4 weeks. The experiment was powered to have 85% certainty of detecting at 95% confidence a 20% difference in the number of days with acute illness.	Healthy full-term formula-fed infants age 4-10 months (mean = 6.8 mo.) in daycare centers; Ns were: *L. reuteri*--68 *B. lactis*—73 Control—60	Probiotics added at 10^7 cfu/g formula powder Estimated mean daily ingested dose of probiotic microorganisms was 1.2×10^9 cfu/day	12 weeks	Febrile episodes: Control mean: 0.41 *L. reuteri* mean: 0.11 ($p < 0.001$) Fever duration: Control mean: 0.83 *L. reuteri* mean: 0.17 ($p < 0.001$) Diarrhea episodes: Control mean: 0.31 *L. reuteri* mean: 0.02 ($p < 0.001$) Diarrhea duration (days): Control mean: 0.59 *L. reuteri* mean: 0.15 ($p < 0.001$) Clinic visits: Control mean: 0.55 *L. reuteri* mean: 0.23 ($p < 0.002$) Absences: Control mean: 0.43 *L. reuteri* mean: 0.14 ($p < 0.015$ Antibiotic prescriptions: Control mean: 0.19 *L. reuteri* mean:0.06 ($p < 0.037$ Respiratory illnesses: No significant differences. There were no drop-outs due to adverse effects, and no differences in growth parameters (weight, length, head circumference). No adverse effects were reported.

Reference	Objective	Study Design	Subjects	*L. reuteri* Dose	Duration	Safety-Related Results
Weizman and Alsheikh 2006	Determine the safety and tolerance of infant formula containing probiotic bacteria (*L. reuteri* ATCC 55730 or *B. lactis* Bb12)	Prospective, randomized, double-blind, placebo-controlled trial. Exclusion criteria were prematurity, BW < 2500 g, congenital abnormality, chronic disease, failure to thrive, allergy or atopic disease, or exposure to pre- pro- or antibiotics within 4 weeks. The experiment was powered to have 85% certainty of detecting at 95% confidence a 20% difference in growth parameters.	59 full-term healthy infants age 3-65 days; 49 completed the study. Ns were: *L. reuteri*—20 (17) *B. lactis*—20 (16) Control—19 (16)	Probiotics added at 10^7 cfu/g formula powder or 2.2×10^8 cfu/180 ml prepared formula Estimated mean daily ingested dose of probiotic microorganisms was 10^9 cfu/day	4 weeks	There were no withdrawals based on formula-related complaints or adverse effects. The mean daily formula volume did not differ significantly between the groups, nor did food compliance, daily number of meals, or daily number of regurgitation and vomiting episodes. There were no significant differences in stooling scores (effort, fecal consistency, gas), number of bowel movements, crying or restlessness scores, number of severe crying episodes, number of night awakenings, or episodes of acute illness. No adverse effects were noticed throughout the study. There were no significant differences in growth parameters—weight, length, or head circumference. In summary, the probiotic bacteria did not affect either the health nor the daily habits of the infants.

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

No published studies were found in the literature involving administration to infants of strains of *L. reuteri* other than ATCC 55730.

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

Two Cochrane 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. Four of these studies included *L. reuteri*, 2 ATCC 55730 and 2 another strain, DSM 12246. 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 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 1-15 received probiotic treatment with *Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus* spp., or *Saccharomyces boulardii*. No trials involving *L. reuteri* were included. Five of the 10 trials (including 647 patients) monitored for adverse events; 3 reported that no adverse events had occurred and 2 reported such events, but equally prevalent among test and control groups and not attributable to the probiotic intervention.

4.6. Previous Review of *L. reuteri* ATCC 55730 by FDA

4.6.1. McNeil New Dietary Ingredient Notice and Response

On June 30, 2000, McNeil Consumer Healthcare notified the Food and Drug Administration (FDA) of its intention to market a dietary supplement containing the new dietary ingredient (NDI) *Lactobacillus reuteri* ATCC 55730 (McNeil Consumer Healthcare 2000). The dietary supplement would be in the form of a chewable tablet and would contain between 10^8 and 10^9 cfu *L. reuteri*. McNeil stated that the label directions would indicate a recommended daily intake of one tablet daily to support digestive function.

McNeil's notice included a dossier summarizing the basis on which McNeil had concluded that *L. reuteri* is safe for the intended use. This dossier provided an overview of lactic-acid bacteria and addressed the biological identification of the species and strain, results of animal and human studies of *L. reuteri* available at that time, and information regarding the history of food use of *L. reuteri* and other strains of *Lactobacillus*. McNeil concluded that "the safety of, and tolerance to, *L. reuteri* administration at levels up to 10^{11} cfu/day has been demonstrated in healthy adults, individuals with HIV infection, and children."

FDA responded to McNeil's notice on September 18, 2000 (FDA 2000). The agency did not question McNeil's conclusion that the intended use of *L. reuteri* is safe for populations other than infants and young children. However, with regard to infants and young children, FDA stated:

> "The agency has concerns about the adequacy of the evidence in your submission regarding whether a dietary supplement containing *Lactobacillus reuteri* will reasonably be expected to be safe for use by infants and young children. You provided two abstract reports of clinical studies on gastrointestinal effects of *Lactobacillus reuteri* in healthy children aged 12 to 36 months. However, one of these abstracts lacks information on the amount of *Lactobacillus reuteri* consumed by the children during the study. Both of the abstracts lack detailed information that is needed to fully evaluate the effect of *Lactobacillus reuteri* on clinical measurements (e.g., hematology, chemistry, immunology) that are generally needed to evaluate the chronic use of a substance. Your submission also includes two published studies and one unpublished study of *Lactobacillus reuteri* used in the treatment of infants and young children aged 6 to 36 months hospitalized with diarrhea. The subjects of these studies were sick infants and children. These studies are of limited relevance to evaluating the use of *Lactobacillus reuteri* in healthy infants and children. Additionally, these studies lack physiological and biochemical analyses. Furthermore, the duration of treatment with *Lactobacillus reuteri* is unclear in these studies. For these reasons, the information in your submission does not provide a sufficient basis to establish that a dietary supplement containing *Lactobacillus reuteri*, when used under the conditions recommended or suggested in the labeling of your product, will reasonably be expected to be safe for infants and young children" (FDA 2000).

It is important to recognize that FDA did not indicate suspicion that the intended use of *L. reuteri* would be unsafe for infants and young children; rather, the agency's expressed concern was simply the paucity of data demonstrating safety in these populations: the small number of studies of *L. reuteri* in healthy infants and young children and flaws in the design or reporting of the few existing studies that limited their value in assessing safety (FDA 2000).

The clinical trials of infants or young children submitted with the NDI notice were the following:

- Ruiz-Palacios et al. 1996a: Healthy children age 12-36 months. Duration: 3 weeks. Abstract only.
- Ruiz-Palacios et al. 1996b: Healthy children age 12-36 months. Duration: 14 weeks. Abstract only.
- Shornikova et al. 1997a: Children age 6-36 months with acute diarrhea.
- Shornikova et al. 1997b: Children age 6-36 months with acute diarrhea.

4.6.2. Additional Research Since the NDI Notice

Since the submission of the 2000 NDI notice, a considerable amount of new research in which *L. reuteri* was administered to infants and children has become available to address the concerns expressed by FDA. All of the available research was discussed above in Section 4.4. Section 4.4.2.2.1 includes reports of 9 clinical trials in which *L. reuteri* ATCC 55730 was given to children. These include the 4 trials submitted with the NDI notice, 1 additional trial from that time that was not included in the submission, and 4 clinical trials conducted since 2004. *L. reuteri* ATCC 55730 has been given to more than 1,000 children ranging in age from 6 months

(in 2 studies) to 18 years at doses as high as 10^{10} cfu/day and for periods as long as 16 weeks without adverse effects. An additional 4 studies with *L. reuteri* strains other than ATCC 55730 were also reported. No adverse effects were observed resulting from administration of *L. reuteri* in any of these studies.

The post-2000 research also includes 10 clinical trials involving over 1000 infants in which *L. reuteri* ATCC 55730 was administered for as long as 12 months in doses as high as 10^9 cfu *L. reuteri*/day (See Section 4.4.2.3.1.). Administration of the probiotic was begun as early as the first day or two after birth (and even to the mother before delivery and continuing with the infant after birth), and infant populations studied included both full-and pre-term infants. Six of the 10 trials were specifically designed to investigate the safety of the administration of *L. reuteri* to infants and included appropriate measures to allow observation of any adverse events or adverse effects on gastrointestinal function, behavior, or growth. One study was designed to determine the reality of the speculation that use of probiotic D-lactic-acid-producing bacteria might increase the risk of D-lactic acidosis in infants; this study showed that feeding infants 10^8 cfu/day *L reuteri* ATCC 55730 did not result in any indication of D-lactic acidosis; indeed, it did not even increase the infants' serum D-lactate concentration at all as compared with controls. No adverse effects resulting from *L. reuteri* have been identified in any of these trials. The study reported by Betta et al. (2007) is of particular significance since it involved administration of 10^8 cfu/day *L. reuteri* ATCC 55730 to 67 premature neonates confined to the Neonatal Intensive Care Unit, including 5 surgical patients and 37 infants with central venous catheters. The treatment provided beneficial effects and no adverse side effects were observed. The premature infants receiving *L. reuteri* were released from the NICU in about half the time as the controls, a mean of 21.8 days v. 42.3 days. Finally, the *L. reuteri* group had nonsignificantly faster growth than the controls due to lower post-delivery weight loss.

These studies cumulatively provide convincing evidence of the safety of administration of *L. reuteri* to infants and children, even to premature neonates at high risk.

4.7. Review of the Safety of *L. reuteri* by an Authoritative Body

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 envisioned 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. reuteri*. In listing *L. reuteri* 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 further 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).

5. Safety Assessment and GRAS Determination

5.1. Introduction

This section presents an assessment that demonstrates that *Lactobacillus reuteri* strain DSM 17938 (henceforth referred to simply as *L. reuteri*) is safe, and is GRAS, for addition to conventional foods—including foods intended for consumption by infants and children—as probiotic bacteria.

This safety assessment and GRAS determination entail two steps. In the first step, the safety of *L. reuteri* under its intended conditions of use is demonstrated. Safety is established by demonstrating a reasonable certainty that the exposure of adults, infants, and children to *L. reuteri* under its intended conditions of use is not harmful. In the second step, the intended use of *L. reuteri* 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 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. reuteri* to foods is safe and is GRAS. Once this use of *L. reuteri* is determined to be GRAS, it is permitted to be used for these purposes because it is by definition not a food additive and therefore does not require promulgation of a food additive regulation under 21 CFR prior to being marketed and sold in the United States.

5.2. Safety Evaluation

A large number of studies, in animals, human adults, human children, and human infants, have individually and collectively demonstrated the safety of *L. reuteri* ATCC 55730, the parent strain of *L. reuteri* DSM 17938. In the many experiments performed in a variety of animal species, *L. reuteri* was, with one exception, free of adverse effects. The single exception was the study by Wagner et al. (1997a) in congenitally immunodeficient gnotobiotic beige-athymic (*bg/bg-nu/nu*) and beige-euthymic (*bg/bg-nu/+*) mice. In this single study, exposure of dams to *L. reuteri* or *L. casei* had no adverse effects on the dams but resulted in some unexpected mortality in their pups. The authors had no explanation for this mortality as no effect could be discerned on the gastrointestinal systems of the pups. It must be recognized, however, that these athymic immunodeficient mice differ from normal mice in many ways, not all of them fully understood; it is partly for this reason that the EU-PROSAFE project has not recommended use of this model.

Fourteen studies have been published in which *L. reuteri* ATCC 55730 was administered to human adults at doses up to 10^{11} cfu/day for as long as 6 months, and no adverse effects have been observed. In addition to healthy adults, participants included pregnant women, elderly subjects with long-term constipation, those with irritable bowel syndrome, individuals with *Helicobacter pylori* infection, individuals affected with HIV, and ileostomized patients. The two randomized, double-blind, placebo-controlled studies by Wolf et al. (1995 and 1998) are particularly valuable in evaluating the safety of *L. reuteri* ATCC 55730 because these two studies, one in healthy adults and the other in HIV-infected persons, were specifically designed to assess safety (rather than efficacy, as are most human trials of probiotics). To this end, both studies included repeated measures of self-reported gastrointestinal problems or side-effects, vital signs, urinalysis parameters, blood chemistries, and hematological measures. No adverse effects were found on any of these parameters. In a similar vein, Anukam et al. (2008) studied the safety of the administration of a different strain of *L. reuteri* (along with *L. rhamnosus*) to women being treated for HIV/AIDS. This study included essentially the same safety parameters as did the studies by Wolf et al. (1995 and 1998) except for clinical chemistry; again, no adverse effects were observed.

L. reuteri ATCC 55730 has also been extensively studied (in 9 published papers) in children ranging in age from 6 months to 5 years; 1 study included participants up to 18 years of age. Children have received *L. reuteri* at doses up to 10^{11} cfu/day (in Shornikova et al. 1997a and 1997b). Durations have ranged from only a few days in hospitalized children for whom treatment ended upon discharge to 16 weeks among free-living children. Although the children chosen for some of these studies were healthy, in most cases they were afflicted with some disorder such as atopic dermatitis, *Helicobacter pylori* infection, or (most often) acute diarrhea. Few specific safety endpoints other than gastrointestinal health were evaluated in these studies, but none of them reported any adverse side-effects.

The use of *L. reuteri* ATCC 55730 has been well studied in infants, including neonates ingesting *L. reuteri* on their first day of life and also including both preterm and full-term infants, both healthy and suffering from severe disorders. Eleven studies including over 1200 infants have been published, with *L. reuteri* doses as high as 1.2×10^9 cfu/day (Weizman et al. 2005). In 2 of these studies (Abrahamsson et al. 2007 and Connolly et al. 2005), the duration of *L. reuteri* treatment was 12 months. In addition to healthy full-term infants, populations studied have

included infants with family histories of allergies, infants with colic, healthy preterm infants, and preterm infants confined to a Neonatal Intensive Care Unit (NICU) with severe health conditions. No behavioral or gastrointestinal side effects were noted in any of these studies and there were no adverse effects on growth or development. The study by Betta et al. (2007) is particularly significant because the population studied, premature neonates confined to an NICU, included about 10% surgical patients and about 50% with central venous catheters, both conditions widely recognized as posing the greatest risks of bacteremia. No adverse effects of any kind were observed even with these extremely sensitive neonates receiving 10^8 cfu/day *L. reuteri*..

With regard to the potential for ingestion of *L. reuteri* to cause D-lactic acidosis, it is widely believed that this condition results only in infants or children with severely impaired carbohydrate metabolism, nearly always due to short-bowel syndrome. Normal humans are known to be capable of metabolizing adequate quantities of D-lactate to prevent its accumulating in the blood serum. Nevertheless, Connolly et al. (2005) directly studied the effect of feeding 10^8 cfu/day *L. reuteri* ATCC 55730 to infants for 12 months. Not only were no symptoms of acidosis observed, but there was no rise in serum levels of D-lactic acid. (Indeed, the *L-reuteri*-treated group actually had nonsignificantly lower D-lactate levels after 12 months than did the placebo group.)

This extensive record of safety of *L. reuteri* ATCC 55730 in adults, children, and infants, demonstrates that there is no reason to suspect harm to individuals consuming conventional foods supplemented with this strain. The following information further demonstrates that ATCC 55730's daughter strain DSM 17938 is also without significant risk of harm.

It was shown (Roos and Rosander 2005, 2006; Melin et al. 2008) that the modified *L. reuteri* strains DSM 17686 and DSM 17938 are substantially equivalent to the *L. reuteri* ATCC 55730 parent strain. In its discussion of the proposed rule establishing a GRAS notification process, FDA (1997) quoted from a report of a joint Food and Agriculture Organization (FAO) and World Health Organization (WHO) consultation (FAO/WHO 1996):

> "... substantial equivalence embodies the concept that if a new food or food component is found to be substantially equivalent to an existing food or food component, it can be treated in the same manner with respect to safety (i.e. the food or food component can be concluded to be as safe as the conventional food or food component). Account should be taken of any processing that the food or food component may undergo as well as the intended use and the intake by the population."

FDA then noted that, "FDA believes that in certain instances the concept of substantial equivalence may have applicability to the technical element of a GRAS determination." Thus, the history of safe consumption of *L. reuteri* ATCC 55730, along with its demonstrated safety in both animal experiments and human clinical trials with adults, infants, and children, provides strong evidence of the safety of its daughter strain DSM 17938.

The safety of the daughter strain, based on its substantial equivalence to strain ATCC 55730, was corroborated by the colonization and safety study reported by Melin et al. (2008), in which healthy adults consumed daily either 10^9 or 10^{11} cfu *L. reuteri* DSM 17938 for 28 days with no adverse effects on gastrointestinal parameters, vital signs, blood chemistry, or hematology.

Finally, the genomic analysis by O'Sullivan (2008) confirmed that strain DSM 17938 does not harbor either virulence or antibiotic resistance genes.

5.3. General Recognition of the Safety of *L. reuteri*

The proposed use of *L. reuteri* strain DSM 17938, to be added as a probiotic to a variety of conventional foods, including foods intended for consumption by infants and children, 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. reuteri* DSM 17938 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. reuteri* to food has been made through the deliberations of an Expert Panel consisting of Berthold V. Koletzko, M.D., Mary Ellen Sanders, Ph.D., Daniel J. O'Sullivan, Ph.D., and John A. Thomas, 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. reuteri* DSM 17938 anticipated to result from its intended uses, and determined that no evidence exists in the available information on *L. reuteri* DSM 17928, its parent strain ATCC 55730, or other *L. reuteri* strains, that demonstrates or suggests reasonable grounds to suspect a hazard to adults or to infants or children under the intended conditions of use of *L. reuteri* DSM 17938.

5.4. Conclusion of the Expert Panel

We, the undersigned expert panel members, have individually and collectively critically evaluated the information summarized above, and unanimously conclude that the intended use of *L. reuteri* DSM 17938 in conventional foods, resulting in a maximum anticipated exposure of less than 10^{10} cfu *L. reuteri*/day, is safe.

We further conclude that the intended use of *L. reuteri* DSM 17938 in conventional foods, resulting in a maximum anticipated exposure of less than 10^{10} cfu *L. reuteri*/day, is generally recognized as safe (GRAS) based on scientific procedures

It is our opinion that other qualified experts would concur with these conclusions.

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Appendix

Safety Assessment of *Lactobacillus reuteri* DSM17938 Based on an Analysis of its Gene Content

Report prepared by:

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Executive Summary

The draft genome sequence of *Lactobacillus reuteri* DSM17938 was analyzed for any possible genes or structures that may be of safety interest. This draft genome sequence was contained in 289 contigs, which were derived from a total genome coverage of 21 fold. All contigs were annotated in each of their six possible open reading frames (ORFs) using standard computerized annotation programs and were manually checked, predicting a total of 2,299 potential genes. Comparison of this draft genome sequence, with that of a complete *L. reuteri* genome sequence available in GenBank, suggested that this draft genome sequence likely represents > 98% of the gene content of *L. reuteri* DSM17938. An analysis of the genome annotation did not reveal any gene or gene cluster known to be involved in virulence or antibiotic resistance. Predicted functions for the encoded genes were determined using the COG database at the National Center for Biotechnology Information (NCBI). The number of genes with motifs that are associated with antibiotic resistance was similar to other sequenced lactobacilli. None of these genes were part of a predicted mobile element. A gene similar to a hemolysin gene was found, but this gene is present in the majority of lactobacilli and other lactic acid bacteria that have been sequenced to date. It can be concluded from this genome safety analysis, that there is nothing unusual from a safety perspective about the *L. reuteri* DSM17938 genome, when compared to other lactobacilli genomes.

Objective:

To provide a safety assessment of *Lactobacillus reuteri* DSM17938 based on the predicted genes and structures contained in the 289 sequence contigs that represent the draft genome sequence of this bacterium.

L. reuteri DSM17938 Genome Sequence Annotation:

The 289 contig files containing the raw nucleotide sequence data from *L. reuteri* DSM17938 were obtained from Dr. Stephan Roos from the Swedish University of Agricultural Sciences. It should be noted that while the contigs are numbered up to 379, there are only 289 total contigs. In this report this numbering system as supplied by Dr. Roos will be utilized. These contigs were derived from a total of 21X genome coverage which included 4X coverage using standard dideoxy sequencing of shotgun clones and 17X coverage using the newer alternative 454 sequencing technology. This combined level of coverage is excellent and would be expected to reveal > 98% of the gene content. The DNA sequence of each of the 289 contig files was annotated using the gene prediction and annotation functions of the GAMOLA program (Altermann and Klaenhammer. 2003. GAMOLA: A New Local Solution for Sequence Annotation and Analyzing Draft and Finished Prokaryotic Genomes. OMICS A Journal of Integrative Biology 7:161-169).This program uses the Glimmer gene prediction program for its gene calls (http://cbcb.umd.edu/software/glimmer/). Following annotation of each of the 289 sequence files, potential genes were present in 268 contig files. The other 21 contigs were too small to encode genes as predicted by GAMOLA.

The annotation details from the 268 contig files that contained predicted genes were compiled in a single Table to provide the complete gene content of *L. reuteri* DSM17938. This Table contains the annotation data for 2,299 potential genes that were predicted. All genes were further analyzed using local BLAST engines and the BLAST database (http://www.ncbi.nlm.nih.gov/blast/). All analysis data were visualized and manually confirmed using the Artemis7 gene graphics software program (http://www.sanger.ac.uk/Software/Artemis/).

Comparison of the *L. reuteri* DSM17938 genome sequence with other *L. reuteri* genome sequences:

Recently, the complete genome sequence of *L. reuteri* F275 and a draft genome sequence of *L. reuteri* 100-23 were deposited in GenBank. A comparison of the draft genome sequence of *L. reuteri* DSM17938 with these two other genomes was undertaken to get a better evaluation of the extent of the gene coverage that the

draft sequencing of *L. reuteri* DSM17938 reveals. This comparison is depicted in Table 1. Based on the number of predicted genes and estimated genome size, it substantiates that the draft genome sequence of *L. reuteri* DSM17938 is indeed extensive and likely represents > 98% of its gene content. It should be noted that the number of estimated genes in a draft genome sequence is an over estimate as many genes at the ends of a contig are also on the end of another contig and thus counted twice. While this accounts for some of the genes predicted for this bacterium, it likely does not explain the 272 extra genes predicted compared to *L. reuteri* F275, whose genome is fully sequenced into a single contig. It is estimated that this genome is likely larger than that of strain F275. We therefore did a gene comparison of the genomes of DSM17938 with F275 and compiled the full complement of genes that are unique to strain DSM17938.

Table 1: Comparison of the *L. reuteri* DSM17938 draft genome sequence with two other *L. reuteri* strains whose genome sequence is available

	DSM17938	F275	100-23
Size (bp)	2278431	1999618	2174299
G+C content (%)	38.59	38.87	38.61
Contigs	289	1	103
Contigs containing genes	268	1	103
Encoded genes	2299	2027	2049
COG-assigned genes	1710	1658	1562
Status	Draft	Finished	Draft

Gene Function Predictions for *L. reuteri* DSM17938:

The COG database at the National Center for Biotechnology Information (NCBI) was developed to enable predictions to be made on potential functional characteristics of genes. This is widely used in genome projects to categorize genes based on predicted functions. Specifically it consists of orthologous gene clusters. This means genes are grouped in a COG cluster if they likely have a similar evolutionary origin. While this can also predict gene function in many cases, it does not always imply a function as different motifs can evolve into proteins with different functions.

The deduced amino acid sequences of all predicted genes in the 268 gene containing contig files of the *L. reuteri* DSM17938 genome sequence were formatted to FastA format using READSEQ and compared to the COG database using GAMOLA-COG. This analysis assigned 74% of genes to a predicted functional category based on COG. This rate of COG assignments is consistent with other lactobacilli draft genomes. The summary of functional predictions is depicted in Table 2 and

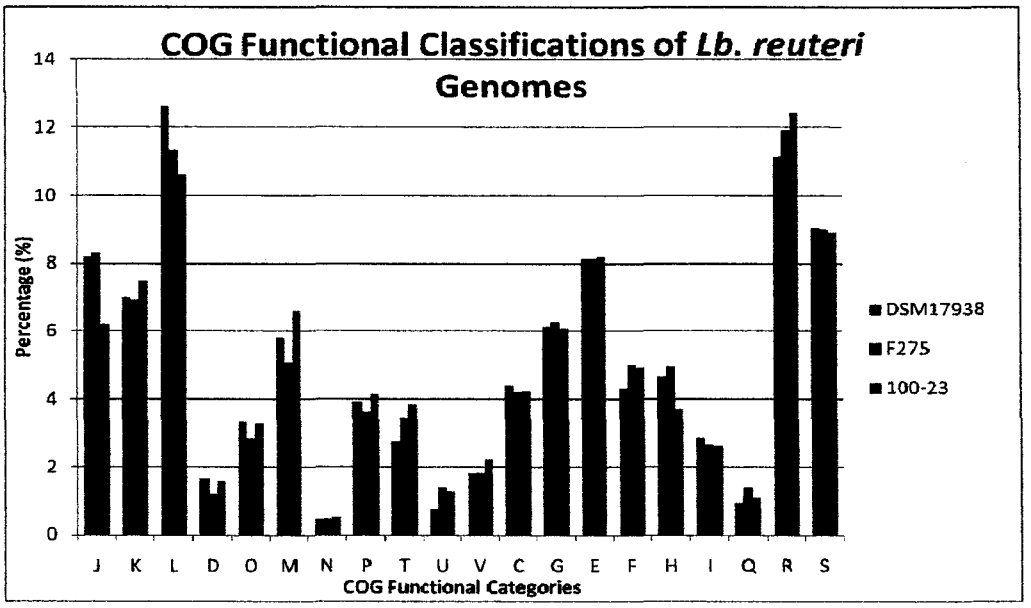
graphically represented in Fig. 1 to illustrate the relative concentration of each functional category in *L. reuteri*.

The overall COG analysis of the *L. reuteri* DSM17938 genome is comparable to other published lactobacilli genomes. Each of the COG categories listed in Table 2 consists of many individual COG specific functional groups. The V grouping (termed defense mechanisms) consists of many COGs that may have a potential safety interest, such as antibiotic resistance and will be analyzed in more detail below.

Table 2: COG functional categories of the *L. reuteri* DSM17938 genome and *L. reuteri* strains F275 and 100-23.

Class	Individual Function Categories	DSM17938		F275		100-23	
		Genes	%	Genes	%	Genes	%
	Information storage and processing						
[J]	Translation, ribosomal structure and biogenesis	140	8.19	138	8.32	97	6.21
[K]	Transcription	120	7.02	115	6.94	117	7.49
[L]	DNA replication, recombination and repair	216	12.63	188	11.34	166	10.63
	Cellular processes						
[D]	Cell division and chromosome partitioning	28	1.64	20	1.21	25	1.60
[O]	Posttranslational modification, protein turnover, chaperones	57	3.33	47	2.83	51	3.27
[M]	Cell envelope biogenesis, outer membrane	99	5.79	84	5.07	103	6.59
[N]	Cell motility and secretion	8	0.47	8	0.48	8	0.51
[P]	Inorganic ion transport and metabolism	67	3.92	60	3.62	65	4.16
[T]	Signal transduction mechanisms	47	2.75	57	3.44	60	3.84
[U]	Intracellular trafficking and secretion	13	0.76	23	1.39	20	1.28
[V]	Defense mechanism	31	1.81	30	1.81	35	2.24
	Metabolism						
[C]	Energy production and conversion	75	4.39	70	4.22	66	4.23
[G]	Carbohydrate transport and metabolism	105	6.14	104	6.27	95	6.08
[E]	Amino acid transport and metabolism	139	8.13	135	8.14	128	8.19
[F]	Nucleotide transport and metabolism	74	4.33	83	5.01	77	4.93
[H]	Coenzyme metabolism	80	4.68	82	4.95	58	3.71
[I]	Lipid metabolism	49	2.87	44	2.65	41	2.62
[Q]	Secondary metabolites biosynthesis, transport and catabolism	16	0.94	23	1.39	17	1.09
	Poorly characterized						
[R]	General function prediction only	191	11.17	198	11.94	194	12.42
[S]	Function unknown	155	9.06	149	8.99	139	8.90
	COG –assigned genes	**1710**	**74**	**1658**	**82**	**1562**	**76**

Figure 1:



Genome Based Safety Assessment of *L. reuteri* DSM17938

Each of the 2,299 predicted genes for *L. reuteri* and listed in Appendix 1 were manually assessed to see if any genes known to reflect virulence of an organism were present. No dedicated virulence genes or virulence gene clusters that may have been obtained from other organisms were detected. A few genes of potential safety interest were observed and will be discussed individually below. Genes of potential antibiotic resistance involvement predicted from COG are grouped in the COG V (defense) category. Those of potential safety interest were compiled in Table 3 below.

Table 3: Genes of potential safety interested in the COG defense category V of the *L. reuteri* DSM17938 genome

Contig	Gene	Start	End	Dir	COG	COG annotation
9	3	1105	2379		COG0534	Na+-driven multidrug efflux pump Length= 475 Score=248 Expect=2e-65
58	3	1760	3050	c	COG0534	Na+driven multidrug efflux pump Length= 443 Score=145 Expect=2e-34
44	20	17237	18011	c	COG0577	ABC-type antimicrobial peptide transport system, permease component Length=662 Score=122 Expect=1e -27
181	33	37534	38584	c	COG0577	ABC-type antimicrobial peptide transport system, permease component Length=357 Score=227 Expect=4e -59
163	13	16071	16719	c	COG0842	ABC-type multidrug transport system, per mease component Length=284 Score=112 Expect=1e-24
28	41	40581	41432		COG1131	ABC-type multidrug transport system, ATP ase component Length=295 Score=68.6 Expect=2e-11
119	3	2040	2838	c	COG1131	ABC-type multidrug transport system, ATP ase component Length=283 Score=160 Expect=4e-39
163	14	16879	17713	c	COG1131	ABC-type multidrug transport system, ATP ase component Length=292 Score=209 Expect=6e-54
196	1	241	370	c	COG1131	ABC-type multidrug transport system, ATP ase component Length=295 Score=38.5 Expect=0.004
284	2	1860	2571	c	COG1131	ABC-type multidrug transport system, ATP ase component Length=243 Score=301 Expect=7e-82
17	13	12897	14694	c	COG1132	ABC-type multidrug transport system, ATP ase and permease components Length=605 Score=575 Expect=e- 163
17	14	14686	16414	c	COG1132	ABC-type multidrug transport system, ATP ase and permease components Length=611 Score=529 Expect=e- 150
181	31	35017	35857	c	COG1136	ABC-type antimicrobial peptide transport system, ATPase component Length=290 Score=364 Expect=e-10 0
181	32	36835	37501	c	COG1136	ABC-type antimicrobial peptide transport system, ATPase component Length=225 Score=198 Expect=9e-5 1
259	3	1796	2539		COG1136	ABC-type antimicrobial peptide transport system, ATPase component Length=259 Score=273 Expect=2e-7 3
297	17	14117	14725		COG1136	ABC-type antimicrobial peptide transport system, ATPase component Length=312 Score=95.9 Expect=1e- 19
2	4	2402	3301		COG1680	Beta-lactamase class C and other penicil lin binding proteins Length=391 Score=89.7 Expect=9e-18
12	28	23619	24519	c	COG1680	Beta-lactamase class C and other penicil lin binding proteins Length=391 Score=89.7 Expect=9e-18
224	8	7112	8080		COG1680	Beta-lactamase class C and other penicil lin binding proteins Length=335 Score=206 Expect=6e-53
48	18	10885	11103		COG4767	Glycopeptide antibiotics resistance prot ein Length=196 Score=42.7 Expect=4e-04
266	1	136	289	c	COG4767	Glycopeptide antibiotics resistance prot ein Length=159 Score=47.0 Expect=1e-05

As can be seen from Table 3, the COG annotation of these genes refers to components of antibiotic resistance components. As COG groupings refer to protein evolutionary families, it doesn't imply that these genes are involved in antibiotic resistance. However, some may be and need to be further analyzed to evaluate the potential. As a reference, this can be compared to the other *L. reuteri* genomes as well as another intestinal *Lactobacillus* genome to see if there is anything unusual. Table 4 below compares the number of each of these COGs between the *L. reuteri* strains and *L. johnsonii.*

Table 4: Comparison of the number of selected category V COG's between *L. reuteri* and *L. johnsonii*

Function	COG	DSM17938	F275	100-23	*L. johnsonii*
ABC-type multidrug transport system ATPase component ATPase and permease component Permease component	COG1131 COG1132 COG0842	5 2 1	5 3 0	6 3 1	6 22 0
ABC-type antimicrobial peptide transport system ATPase component Permease component	COG1136 COG0577	4 2	4 1	4 1	3 0
ABC-type bacteriocin transporter	COG2274	0	1	3	3
Beta-lactamase	COG1680	3	2	2	3
Penicillin-binding protein, transpeptidase	COG2367	0	1	1	2
VanZ family protein	COG4767	2	1	1	1
Multi antimicrobial extrusion protein, MatE	COG0534	2	2	2	0
Total		20	20	24	40

As can be seen from this analysis, there is nothing unusual about the number of these COG's in *L. reuteri* DSM17938. A further analysis of each of the genes listed in Table 3 revealed that two of them are clustered with predicted transposase genes suggesting they may be associated with potential IS elements. These are contig 2 gene number 4 (2402 – 3301) and contig 9 gene number 3 (1105 – 2379).

An analysis of contig 2 gene number 4 reveals a predicted transposase gene directly downstream from it. An analysis of this entire contig sequence for inverted repeats (IR) using EINVERTED software did not reveal any IR associated with this transposase gene consistent with it being an insertion sequence (IS) remnant. The only potentially active IS element on this contig was located over 6 kb downstream and this appears to be a composite transposon (Fig. 2). However, there are no predicted safety issues with any of the genes associated with this putative mobile element. It is shown solely as an example of what a potential mobile element may

look like. It can therefore be concluded that gene number 4 on contig 2 is not associated with any mobile elements.

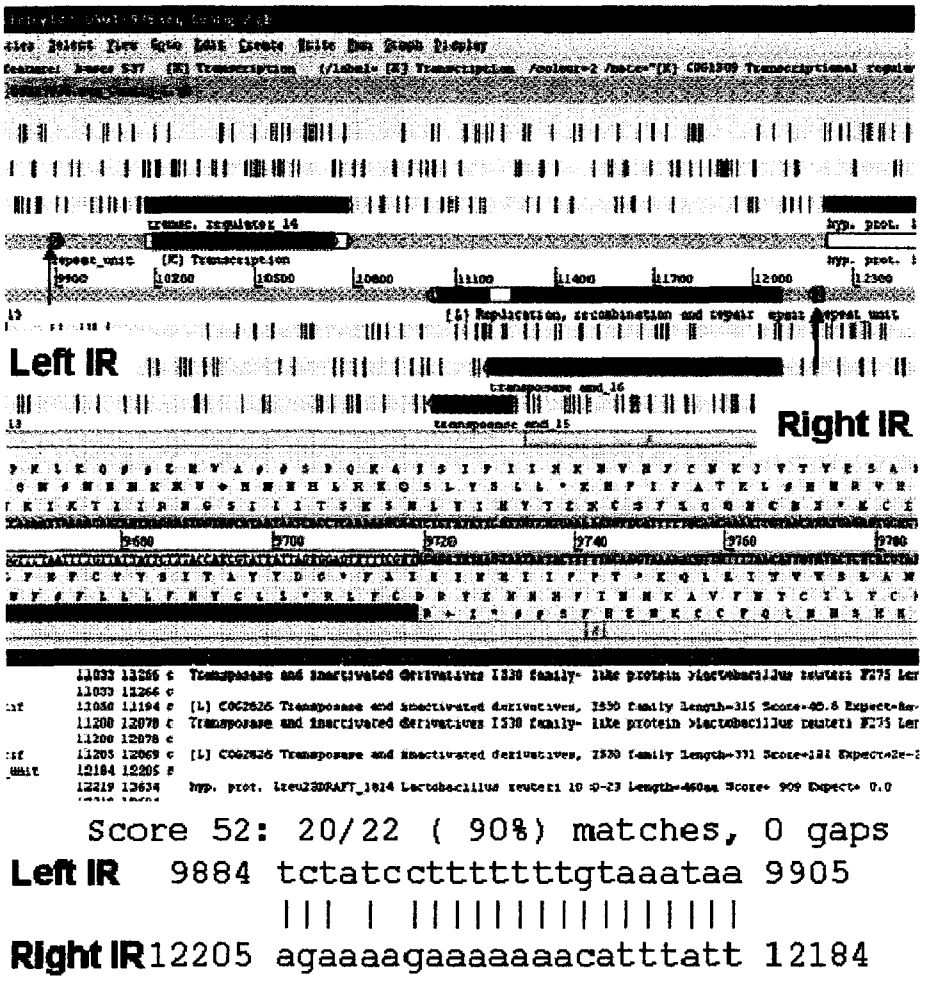
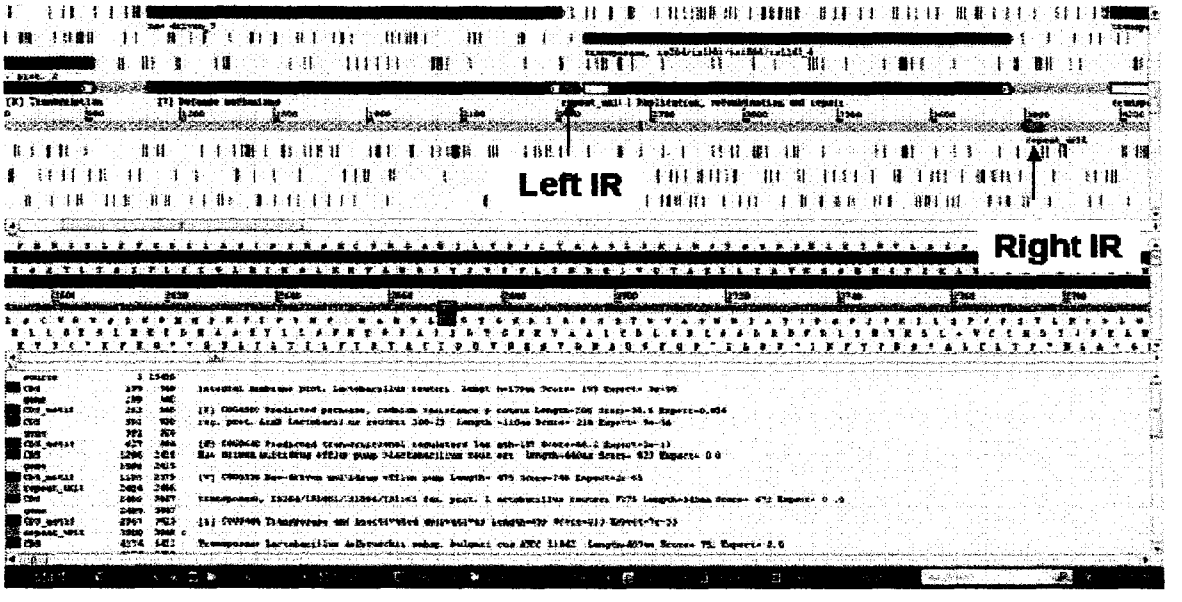


Figure 2:
Artemis graphical view of a potential mobile element on contig 2 of *L. reuteri* DSM17938. The left and right repeats sandwich putative transposase genes and another gene predicted to be a transcription regulator.

An analysis of contig 9 gene number 3 also revealed it to be linked to a putative transposase gene. A similar analysis of contig 9 with the EINVERTED software did show one set of inverted repeats on this contig and these sandwiched this transposase gene (Fig. 3). There were a number of mismatches in the IR associated with this element suggesting it may not be active. However, IS elements can be

active with mismatches in their IR's. Gene number 3 was clearly outside this potential mobile element. It can therefore be concluded that none of the genes listed in Table 3 above is a part of a detectable mobile element. It is noteworthy that three of those genes are clustered on contig 181 and as that gene cluster also contains a transcriptional regulator and a short hypothetical protein, it may be involved in bacteriocin production (see Fig. 4 below). This is common among lactobacilli and is considered a beneficial trait of these bacteria. However, further analysis is needed to link this region to bacteriocin production.




```
Left IR  2418 aaatagattggctctacaatatctggtcttgatatagaaatatcacttt 2466
              ||||  |||||||||||   |  | |||||||||   |||||| | |||
Right IR 3948 tttagataaccgagatgcagttcatcacaactatgcgtttatacttaaa 3900
```

Figure 3:

Artemis graphical view of the putative IS element on contig 9, showing that the COG V annotated gene possible involved in an efflux pump is outside this element.

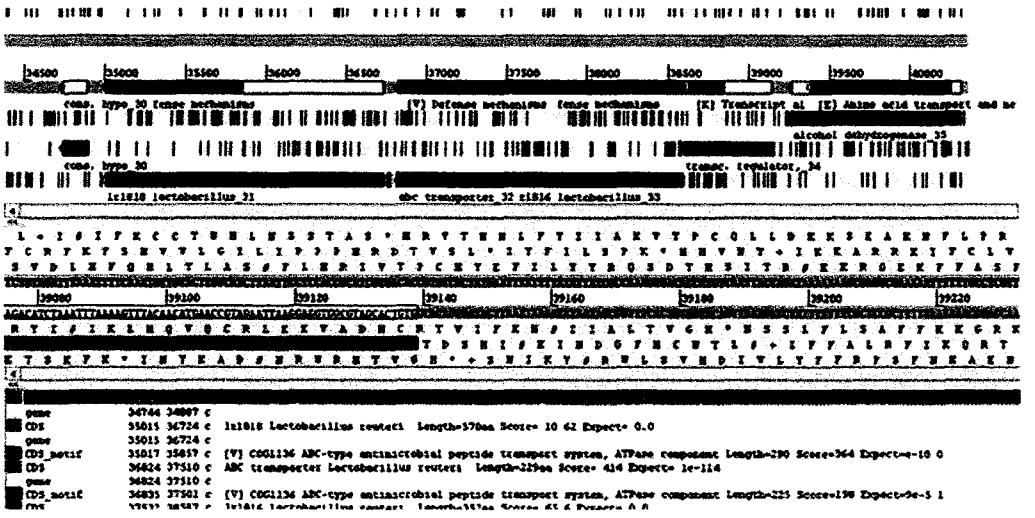


Figure 4:

Artemis graphical view of the region on contig 181 containing three genes in an apparent operon with motifs to ABC transporters linked with a short hypothetical encoding ORF upstream and a putative transcription regulator downstream.

Further analysis of the gene content of the *L. reuteri* DSM17938 genome, revealed a putative hemolysin gene on contig 257 gene number 15 (14959-15777). However, this gene homolog is also found in the majority of *Lactobacillus* genomes completed so far indicating it is not an unusual find. It is also present in several other lactic acid bacteria, including the well known cheese starter bacterium *Lactococcus lactis* subsp. *cremoris* SK11. While the actual function of this gene in lactic acid bacteria has not been investigated, it does not appear to be involved in virulence.

In summary, the gene content of *L. reuteri* DSM17938, based on a 21X total coverage likely revealing > 98% of its gene content, does not contain anything unusual from a safety perspective when compared with other *Lactobacillus* genomes.

SUBMISSION END

AM

Harry, Molly *

From: Jim Heimbach [jh@jheimbach.com]
Sent: Tuesday, October 28, 2008 1:29 PM
To: Harry, Molly *
Cc: Gaynor, Paulette M
Subject: Re: GRN 000254 (Lactobacillus reuteri)
Attachments: Waters et al 1999 [abstract].pdf

Dear Molly--

I appear to have omitted this citation in the reference list. Sorry. To the best of my knowledge, it is available only as an abstract on the USDA/ARS website. Here is a copy.

Regards,
Jim

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----- Original Message -----
From: Harry, Molly *
To: Jim Heimbach
Cc: Gaynor, Paulette M
Sent: Tuesday, October 28, 2008 12:30 PM
Subject: GRN 000254 (Lactobacillus reuteri)

Dear Dr. Heimbach,
We will like some clarification on the citation for the mice study by Waters et al. 1999 listed in Table 4, page 45 of GRN 000254. We are unable to locate this citation on the list of references in the notice. We will like you to provide this citation.
Thanks.
Molly Harry
Division of Biotechnology and
GRAS Notice Review

USDA ars Agricultural Research Service

NADC National Animal Disease Center

Title: Lactobacillus Reuteri and Intestinal Integrity

Authors

- Waters, Wade
- Harp, James - *jim*
- Wannemuehler, M - IOWA STATE UNIV., AMES
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Publication Date: N/A

Technical Abstract: Rodent models have helped to demonstrate that Lactobacillus reuteri plays a central role in maintenance of intestinal integrity. For example, rat models of colitis (induced by acetic acid or methotrexate) and acute liver failure have been used to show that L. reuteri improves intestinal epithelial permeability, decreases bacterial translocation, and decreases colonization by certain intestinal pathogens in the gastrointestinal tract. The information in this presentation deals with L. reuteri host protection from two pathogens: Salmonella typhimurium or Cryptosporidium parvum. BALB/c mice colonized with L. reuteri were challenged with S. typhimurium. L. reuteri significantly reduced mortality and decreased S. typhimurium translocation and gut epithelium damage. In an inflammatory bowel disease model usig gnotobiotic TCR-alpha deficient mice, fewer C. parvum were detected in ileal and cecal sections from L. reuteri-colonized mice than were detected in sections from mice not receiving L. reuteri (7 weeks post-crypto challenge). Inflammatory and hyperplastic cecal lesions due to C. parvum infection were also diminished by L. reuteri colonization. These results support the role of L. reuten in maintaining intestinal integrity in rodents, similar to effects previously observed in humans, avians, and non-rodent mammals.

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