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ORIGINAL SUBMISSION

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JHeimbach LLC

March 20, 2012

Paulette Gaynor, Ph.D.
Supervisory Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Paulette:

Pursuant to proposed 21 CFR 170.36 (62 FR 18960; April 17, 1997), Yakult Honsha Co., Ltd., through me as its agent, hereby provides notice of a claim that the use of *Lactobacillus casei* strain Shirota as described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because Yakult Honsha Co. has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

As required, three copies of the notification are provided along with three copies of the summary conclusion of the GRAS expert panel as well as the signatures of the three panel members.

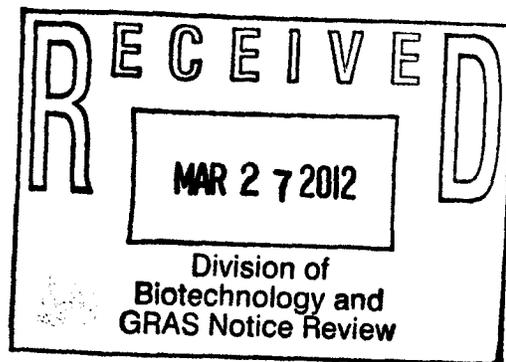
If you have any questions regarding this notification, please feel free to contact me at 804-742-5548 or jh@jheimbach.com.

Sincerely,

(b) (6)

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

Encl.



**Generally Recognized as Safe (GRAS)
Determination for the Use of
Lactobacillus casei Strain Shirota
As a Food Ingredient**

**Prepared for
Yakult Honsha Co., Ltd.
Tokyo, Japan**

**Prepared by
JHeimbach LLC
Port Royal VA**

**March
2012**

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1. GRAS Exemption Claim

Yakults Honsha Co., Ltd., through its agent JHEIMBACH LLC, hereby notifies the Food and Drug Administration that the use of *Lactobacillus casei* strain Shirota as an ingredient in fermented milk products as described below is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because Yakult Honsha Co., Ltd., has determined through scientific procedures that this use is generally recognized as safe (GRAS).

(b) (6)

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

March 20, 2012

Date

1.1. Name and Address of Notifier

Yakult Honsha Co., Ltd.
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1.2. Name of GRAS Organism

The subject of this Generally Recognized as Safe (GRAS) determination is the Shirota strain of the probiotic bacterium *Lactobacillus casei*.

1.3. Intended Use and Consumer Exposure

L. casei strain Shirota is intended for use as an ingredient in fermented milk products, including but not limited to the fermented milk drink sold under the trade name "Yakult." The strain's function is to serve as a probiotic microorganism.

1.4. Basis for GRAS Determination

Yakult's GRAS determination for the intended use of *L. casei* strain Shirota is based on scientific procedures as described under 21 CFR §170.30(b). Determination of the safety and GRAS status of the intended use of *L. casei* strain Shirota was made through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., and Daniel J. O'Sullivan, 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. casei* strain Shirota resulting from its intended use and individually and collectively concluded that no evidence exists in the available information on *L. casei* strain Shirota or other *L. casei* strains that demonstrates, or suggests reasonable grounds to suspect, a hazard to adults or children under the intended conditions of use of *L. casei* strain Shirota.

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

1.5. Availability of Information

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

2. Identity of the Organism

2.1. Name of the GRAS Organism

The subject of this Generally Recognized as Safe (GRAS) determination is the Shirota strain of the probiotic bacterium *Lactobacillus casei*. The strain is one of the approximately 300 strains of bacteria which Dr. Minoru Shirota, the founder of Yakult, collected from many foods. This isolation occurred in 1930, but details of the isolation have been lost during the three-quarters of a century that have elapsed since that time. The strain was given the designation Shirota in 1966, but has often been referenced in the scientific literature as *L. casei* YIT 9018

At some time, the bacterium isolated in 1930 became infected with a prophage insertion into its DNA; the phenotypic effect of this contamination was occasional under-production of lactic acid during fermentation. In order to stabilize the production process, a prophage-removed derivative of the original strain (initially designated as C239 in the relevant reference, Shimizu-Kadota and Sakurai 1982) was selected in 1980 (Shimizu-Kadota et al. 1983), designated YIT 9029, and deposited in the culture collection of the International Depository Authority, Agency of Industrial Science and Technology of Japan, with registration number FERM BP-1366. This strain, YIT 9029, is currently designated *L. casei* strain Shirota and used to manufacture fermented milk products, including the drink sold worldwide under the name Yakult.

The taxonomy of the genus *Lactobacillus* is currently in a state of flux, and nowhere is taxonomic placement less certain than in the complex of *L. casei* strains. According to the most recent opinion (as of this writing) of the Judicial Commission of the International Committee on Systematics of Bacteria in 2008 (JCICSB 2008), *L. casei* strain Shirota would now be regarded as a strain of *L. paracasei* rather than *L. casei*. However, the current state of the taxon is widely regarded as unsatisfactory, including the phenotypically distinct former *L. zae* and possessing a type strain, *L. casei* ATCC 393, which is dissimilar to most members of the species. As a result, the species names *L. casei* for novel *L. paracasei* strains and *L. zae* for novel *L. casei* strains are still often used in scientific publications due to the long history of use in scientific communities, and as the common and usual name for the strains in commercial use, advertising, and labeling, again due to a long history of such use and the likelihood that further evolution of the taxonomic classification and nomenclature lies in the near future.

2.2. Description and Species Identification of the GRAS Organism

Lactobacillus casei is a Gram-positive bacterium that is a member of the broad classification of lactic acid bacteria (LAB). LAB comprise a group of microbes related by common metabolic functionality—the production of lactic acid as the major metabolic end product of carbohydrate metabolism—and common physiological traits. LAB are Gram-positive, non-spore-forming, and catalase-negative and are devoid of cytochromes (Holzapfel et al. 2001). They are preferential nonaerobes but are aerotolerant, acid-tolerant, and strictly fermentative. Although they are not a tightly defined taxonomic grouping, LAB generally are considered to include the following phylogenetically related genera, which have several biochemical and ecological features in common (Axelsson 1998): *Aerococcus*, *Alloicoccus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenoccus*, *Vagococcus*, and *Weissella*. Due to similarities in its biochemistry, physiology, and ecology, the genus

Bifidobacterium is often considered to be a LAB as well, even though it is phylogenetically unrelated (Axelsson 1998). With the exception of some *Streptococcus* species and possibly some *Enterococcus* strains, most LAB strains are considered to have little or no pathogenic potential (Donohue and Salminen 1996; Adams 1999). LAB have a long history of use in fermented and non-fermented foods and have been noted for their ability to inhibit other microorganisms capable of causing foodborne illness or food spoilage (Donohue and Salminen 1996; Adams, 1999). Furthermore, some LAB are ubiquitous as minor components in the intestinal epithelium and the gastrointestinal tract of humans of all ages. All of these factors lead to the reasonable conclusion that most LAB strains are safe for use in conventional foods that may be consumed by all members of the general population.

Lactobacillus is a non-pathogenic genus that consists of over a hundred species. A report by the European Food Safety Authority in November 2007 (EFSA 2007b) identified 112 species, while Bernardeau et al. (2008), writing the same year, suggested that the genus contains some 135 species and 27 subspecies. *Lactobacillus* is a heterogeneous genus with a large variety of phenotypic, biochemical, and physiological properties; it has been suggested that the extreme diversity of the *Lactobacillus* genomes would justify recognition of new subgeneric divisions (Bernardeau et al. 2008). Lactobacilli are rod-shaped, non-motile, and non-sporulating. *L. casei* are typically straight, measuring about 1 x 3 µm, and arranged in chains. Lactobacilli grow under reduced oxygen conditions in habitats where ample nutrients exist. Members of the genus may be either homo- or heterofermentative. The former convert carbohydrates to lactic acid through the glycolytic pathway, while the latter convert carbohydrates using phosphoketolase to produce lactic acid, acetic acid, and carbon dioxide. *L. casei* is homofermentative (Cogan 1996). Lactobacilli are used in commercial applications for the fermentation of dairy products, fruits, vegetables, and meats (Aguirre and Collins 1993; Gasser 1994). Some *Lactobacillus* strains are found in the gastrointestinal tract of healthy humans of all ages, where they are among the “normal” bacteria (Saxelin et al. 1996b; Goldin et al. 1992).

The classification of the *Lactobacillus* genus has evolved over the past several decades as a number of advances in molecular biology, particularly widespread use of 16S rDNA gene sequence analysis, have allowed further differentiation within groupings previously regarded as single species. Thus, over 100 species are now recognized while only a decade ago the number was barely over 50 (Axelsson 1998). Bernardeau et al. (2006) observed that the process of nomenclature change is continuous and ongoing. The *L. casei* group, previously regarded as a single species, is a case in point.

2.2.1. Morphology and Growth

L. casei strain Shirota cells are rod-shaped, 0.4–0.6 × 2–3 µm, and occur singly, in pairs, or in short chains comprising three or four cells. The strain is capable of growing at temperatures between 27°C and 43°C with an optimum of 37°C. After anaerobic growth at 37 °C for 48 hours, colonies on Man Rogosa Sharpe (MRS) agar are 2–3 mm in diameter, beige, smooth, and circular with entire (smooth) edges. In MRS broth, growth occurs at 15 °C but not at 45 °C. While it can grow at a pH as low as 3.5, its optimal pH is 6.5.

The strain is a facultative homofermentative lactic acid bacterium, producing 2 lactic acid molecules from every glucose molecule (although it also produces small quantities of acetate, ethanol, and diacetyl). Only the L-enantiomer of lactic acid is formed. The strain is auxotrophic for 12 amino acids, requiring a rich medium for growth (Morishita et al 1981).

With regard to the fermentative ability of *L. casei* strain Shirota, lactic acid is produced from ribose, adonitol, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, mannitol, sorbitol, methyl- α -D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, trehalose, turanose, D-lyxose, and D-tagatose, and is weakly produced from inositol, sucrose, β -gentiobiose, gluconate and 5-keto-gluconate, but not from glycerol, erythritol, D- or L-arabinose, D- or L-xylose, methyl- β -xyloside, rhamnose, galactitol, methyl- α -D-mannoside, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, D- or L-fucose, D- or L-arabitol or 2-keto-gluconate. Aesculin is hydrolyzed, but dextran is not produced from sucrose, ammonia is not produced from arginine, and nitrate is not reduced.

2.2.2. 16S rRNA Sequence

The 16S rRNA gene sequence of *L. casei* strain Shirota is 100% identical to those of *L. casei* strains designated ATCC 334 and NCDO 151. In contrast, the degree of 16S rRNA gene sequence similarity between *L. casei* strain Shirota and *L. zae* strains designated ATCC 393 and ATCC 15820, and the *L. rhamnosus* strain ATCC 7469, are 99.23, 99.23 and 98.9%, respectively. The 16S rRNA gene sequence has been registered in public DNA databases with accession number AB531131.

2.2.3. DNA-DNA Hybridization

The levels of DNA–DNA relatedness between *L. casei* strain Shirota and *L. casei* ATCC 334 are 78–84%. Based on these data, *L. casei* strain Shirota was classified as belonging to the species *L. casei*. In contrast, the relatedness between *L. casei* strain Shirota and the type strains for the species *L. zae* (ATCC 393) and *L. rhamnosus* (ATCC 7469) is only 17% and 9%, respectively. The DNA G+C contents of *L. casei* strain Shirota and strains in the *L. casei* group (ATCC 334, NCDO 151, ATCC 393, ATCC 15820 and ATCC 7469) are 46.1, 44.2, 45.7, 47.8, 44.9 and 45.7 mol%, respectively.

2.2.4. Summary

In summary, the phenotypic and genotypic features of *L. casei* strain Shirota described above confirm that the strain belongs to the species *L. casei* (Dicks et al. 1996) or current species name *L. paracasei*. The methods of species identification are described in detail in Appendix 1.

2.3. Strain Identification

2.3.1. Randomly Amplified Polymorphic DNA

A special PCR-based method was developed for the identification and quantification of *L. casei* strain Shirota using a *L. casei* strain Shirota-specific primer set (pLcS) derived from randomly amplified polymorphic DNA (RAPD) analysis (Fujimoto et al. 2008). In a human feeding study, *L. casei* strain Shirota was quantified in the feces after ingestion of the strain by quantitative PCR (qPCR) using pLcS. The method's ability to identify the strain matched that of an ELISA using a monoclonal antibody and a RAPD analysis in a representative sample of colonies cultured from human faeces. After 14 healthy subjects ingested 10^{11} cfu of *L. casei* strain Shirota daily for 7 days, $10^{9.1\pm 0.5}$ cfu/g of *L. casei* strain Shirota were detected by qPCR in the fecal samples of all subjects, while $10^{8.0\pm 0.9}$ cfu/g were detected by selective culture

methods. The *L. casei* strain Shirota-specific DNA sequence obtained in this study was registered in public DNA databases with accession number AB246299.

2.3.2. Direct DNA Comparison

Identification of *L. casei* strain Shirota is possible directly via its DNA sequence, since the entire chromosome is known (Sato et al. 2004). Although its actual base sequence remains confidential, various parts of the *L. casei* strain Shirota genome sequence have been made public. These DNA sequences are registered in DNA databases under the accession numbers X02734 (Shimizu-Kadota et al. 1985), AB023773 (Shimizu-Kadota et al. 2000), and AB470649 (Yasuda et al. 2008).

2.3.3. Pulse-Field Gel Electrophoresis

In a study of the survival of *L. casei* strain Shirota through the gastrointestinal tract, discussed in detail in Section 4.4.2.1.1, Tuohy et al. (2007) showed the feasibility of utilizing pulse-field gel electrophoresis to identify *L. casei* strain Shirota among *L. casei*-like strains in feces.

2.3.4. Immunodetection

Yuki et al. (1999) established a detection and confirmation method for *L. casei* strain Shirota using a specific culture medium and specific monoclonal antibodies raised against this strain. This method is particularly useful in case *L. casei* strain Shirota must be distinguished from other bacteria and enumerated within a complex sample, such as a fecal specimen.

2.3.5. Summary

In summary, identification of the strain as *L. casei* strain Shirota is achieved by comparing the RAPD pattern and PFGE pattern as well as by direct DNA sequencing of the specific genome regions. In addition, the identification is also achieved by culturing the strain with a selective agar medium followed by confirmation with a specific monoclonal antibody. Additionally, Maze et al. (2010) sequenced the genome of *L. casei* strain BL23, determining a size of 3,079,196 base pairs with a G+C content of 46.34%. They compared their findings with available sequence information for the neotype strain, ATCC 334, and the Shirota strain, concluding that the 3 strains “seem to be very similar,” with most differences attributable to prophage insertions or to genes encoding for carbohydrate utilization. This provides additional confirmation of the speciation of strain Shirota.

2.4. Genomic Analysis

2.4.1. Shotgun Sequencing

Sato et al. (2004) determined the whole genome shotgun sequence of *L. casei* strain Shirota and the resulting sequence was annotated and analyzed for genes that could be possible safety concerns. *L. casei* strain Shirota genomic DNA sequencing resulted in approximately 60,000 reads giving 31 megabases (Mb). A previous estimate of the genome size based on PFGE data was about 3.1 Mb, giving 10-fold coverage of the genome. Assembly of these data produced 120 contigs of 3,104,260 base pairs (bp); the average size of the contigs was 25,868 bp. The final

estimated size of the genome is 3,035,755 bp plus a single plasmid (pLY101) containing 66,801 bp. This analysis was published in full in a Japanese scientific journal (Sato et al. 2004); an abbreviated translation of the article appears in Appendix 2.

2.4.2. Annotation of the Genome and Plasmid

Sato et al. (2004) compared the genome with the sequences of several strains of *Lactobacillus* and *Lactococcus*, including *L. casei* ATCC 334 and *L. casei* BL23, both closely related to *L. casei* strain Shirota. The result of this analysis was a list of pathways and biochemical processes that describe the basic metabolic capacity of *L. casei* strain Shirota. A total of 2,985 open reading frames (ORF) was identified, including 2,918 ORF in the genome and 67 ORF in the plasmid, with assigned functions for 2,301 (77%) of them—2,255 in the genome and 46 in the plasmid.

A second annotation of the genome sequence was conducted by O’Sullivan (2012) using the gene prediction and annotation functions of the GAMOLA program (Altermann and Klaenhammer 2003) and an updated BLAST database. This later annotation revealed 4,269 potential genes in the genome, which includes potential ORFs within other genes and allows analysis of all identified functional motifs, as well as 83 potential genes in the plasmid. The report by Dr. O’Sullivan appears in Appendix 3.

2.4.3. Results of the Genomic Analysis

2.4.3.1. Antibiotic Resistance

No genes encoding for antibiotic resistance, nor any sequences showing significant homology with known antibiotic resistance genes, were identified by Sato et al. (2004) in either the genome or the plasmid. Although *L. casei* strain Shirota, like most lactobacilli, is resistant to vancomycin, this resistance is not genetically based. The absence of identified antibiotic resistance genes is consistent with findings, discussed below, that the strain is susceptible to most antibiotics and shows no clear resistance except to vancomycin. The O’Sullivan (2012) annotation identified multidrug transporters that may encode resistance to Daunorubicin, a chemotherapeutic drug used in cancer treatment rather than infectious medicine, and to the neomycin/kanamycin family. These genes are common among Gram-positive bacteria, including lactobacilli, and are not associated with mobile elements. They consequently do not pose a safety risk.

2.4.3.2. Synthesis of Biogenic Amines

Microbial biogenic amine formation occurs via the decarboxylation of amino acids. While this is a common function of microorganisms, high concentrations of biogenic amines can cause undesirable physiological effects. The primary precursor amino acids and the amines produced by specific decarboxylases are histidine (histamine), tyrosine (tyramine), hydroxytryptophane (serotonin), tryptophane (tryptamine), lysine (cadaverine), ornithine (putrescine), and arginine (spermine/spermidine).

The genome of *L. casei* strain Shirota was searched for genes that might encode the production of any of these specific decarboxylases. The strain has the genome sequence of ornithine decarboxylase isozyme that catalyzes the conversion of ornithine into putrescine. However, the sequence of this enzyme exists in many strains of *Lactobacillus*, e.g., *L. casei*

BL23, *L. casei* ATCC 334, *L. rhamnosus* ATCC 53013, *L. rhamnosus* GG, *L. rhamnosus* Lc705, *L. salivarius* UCC118, *L. delbrueckii* ssp. *bulgaricus* BAA-365, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, *L. acidophilus* NCFM, *L. helveticus* CNRZ32, *L. helveticus* DPC4571, *L. gasseri* ATCC 33323, *L. johnsonii* FI9785, and *L. johnsonii* NCC533). It is clear that possession of this gene does not result in a safety concern.

2.4.3.3. Virulence/Infectivity

The genome of *L. casei* strain Shirota was examined for genes encoding factors putatively associated with virulence, including production of enterotoxins, outer-membrane or cytoskeleton-distending proteins, hemolysins, enterohemolysins, cell invasion and modulation proteins, endotoxins, cell-surface proteins, exotoxins, and inclusion proteins. While the genomic analysis identified several genes encoding for proteins related to known cell-invasion proteins, such as internalin, these same genes and their proteins are found in a range of other bacteria with no known pathogenic activity. Some of these are general housekeeping genes, encoding proteins such as collagen or fibronectin binding protein. One sequence shows homology to a putative hemolysin. With regard to this gene, O'Sullivan (2012) noted that, while hemolysins are considered to be virulence factors, homologs of them are common to many lactobacilli and their true function has yet to be investigated in the LAB.

As noted above, and as shown in Table 1, the virulence-associated genes found in *L. casei* strain Shirota are widely distributed among lactobacilli with no indication of pathogenic capabilities.

Table 1. Presence of Virulence-Associated Genes in Lactobacilli.

Strain	LCS0638	LCS1519	LCS2324	LCS1467	LCS1704	LCS1832	LCS2231
	Collagen Binding Protein	Fibronectin Binding Protein	Internalin	Hemolysin III Homolog	Hemolysin	Hemolysins and Related Proteins	Hemolysins and Related Proteins
<i>L. acidophilus</i> NCFM	o	o	o	o	o	o	o
<i>L. brevis</i> ATCC 367	o	o	o	o	o	o	o
<i>L. casei</i> ATCC 334	o	o	o	o	o	o	o
<i>L. casei</i> BL23	o	o	o	o	o	o	o
<i>L. delbrueckii</i> ssp. <i>bulgaricus</i> ATCC 11842	o	o		o	o	o	o
<i>L. delbrueckii</i> ssp. <i>bulgaricus</i> BAA 365	o	o		o	o	o	o
<i>L. fermentum</i> IFO 3956	o	o	o	o	o	o	o
<i>L. gasserii</i> ATCC 33323	o	o	o	o	o	o	o
<i>L. helveticus</i> DPC4571	o	o		o	o	o	o
<i>L. johnsonii</i> NCC 533	o	o	o	o	o	o	o
<i>L. plantarum</i> WCFS1	o	o	o	o	o	o	o
<i>L. reuteri</i> DSM 20016	o	o		o	o	o	o
<i>L. reuteri</i> JCM 1112	o	o		o	o	o	o
<i>L. rhamnosus</i> GG	o	o	o	o	o	o	o
<i>L. rhamnosus</i> Lc 705	o	o	o	o	o	o	o
<i>L. sakei</i> 23K		o		o	o	o	o
<i>L. salivarius</i> UCC118	o	o	o	o	o	o	o

2.5. Production Process of *L. casei* Strain Shirota

2.5.1. Maintenance of Seed Stock and Preparation of Master Stock

As shown in Figure 1, the production master stock is renewed approximately once every two years. When the master stock is renewed, it is inoculated in liquid culture medium which is incubated at 37°C until the culture reaches stationary phase. The broth culture is transferred to agar media and again incubated at 37°C until the culture reaches stationary phase, when it is chilled and stored at -20°C. This provides an approximately 2-year supply of master stock.

The liquid culture medium contains enough nitrogen, carbon, salts and other nutrients for the growth of *L. casei* strain Shirota.

Agar media also contains these same ingredients along with agar, and milk broth contains only food-grade skim milk powder and water.

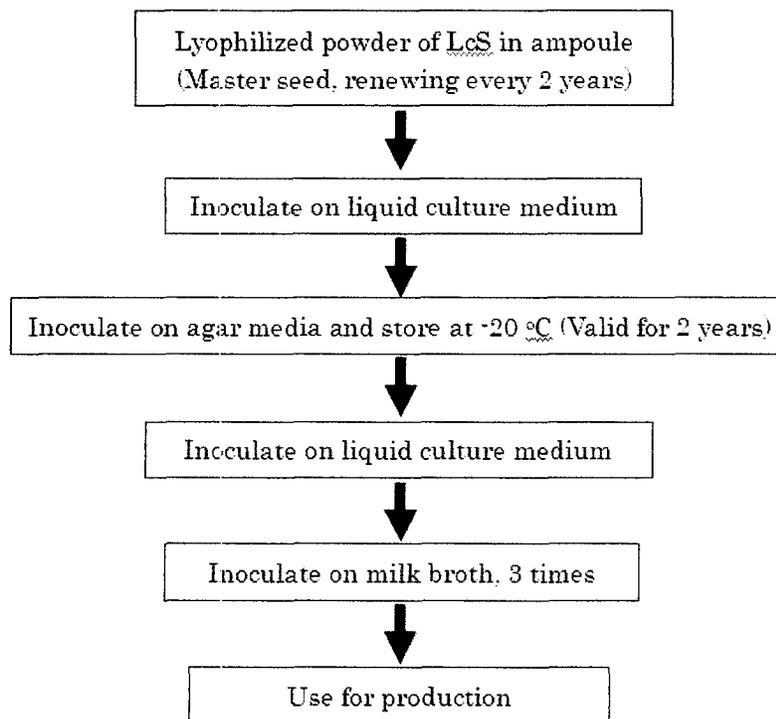


Figure 1. Maintenance of Seed Stock and Preparation of Master Stock.

2.5.2. Production Process

Production of probiotics as ingredients in fermented milk products is performed in a high-technology, highly sanitary environment in order to avoid contamination by any other microorganisms and to ensure that the product is safe for human consumption. It is also important to maintain a level of viable cultures in the product for which a health benefit has been demonstrated. For these reasons, all production is done under strict conditions. The production

process of *L. casei* strain Shirota as an ingredient in fermented milk products is an automated integrated production based on the use of Hazard Analysis and Critical Control Points (HACCP) to control the management systems. The culturing of *L. casei* strain Shirota is carried out through the following steps:

1. Food-grade skim milk powder and sugars are mixed with filtered distilled water in a mixing tank to make a milky solution.
2. The sweet milky solution is pumped to another tank where it is subjected to a high-temperature process similar to Ultra Heat Treatment Short Time (UHTST) and maintained at elevated temperature for an extended period of time before cooling.
3. All machines are computerized and the production flows are controlled by the control panel.
4. Live *L. casei* strain Shirota, cultured and tested under precise conditions in the laboratory located at the plant, is tested to ensure a high number of colony forming units (cfu).
5. The solution is cooled to about 37°C and introduced aseptically via closed pipes and valves into a fermentation tank. *L. casei* strain Shirota is added and incubated at 37°C for about a week. To break the milk curd and to make it smooth, the fermented milk is homogenized.
6. The cultured skim-milk solution is transferred to a storage tank to which sterile food-grade syrup and flavors permitted in the U.S. are added and the solution thoroughly mixed at a temperature of ~10°C. Prior to bottling, the component concentration is adjusted as needed to achieve the desired concentration of *L. casei* strain Shirota.
7. The finished products are stored in refrigerator under 10°C and checked for quality.
8. QC measures are in place to maintain standards for personnel and factory hygiene, equipment cleaning, processing methods and parameters, and product handling.

QC activities involve sampling, testing, and inspection of the product, bottles, and packaging. Individual bottles are randomly inspected to check for incorrect printing, undesirable markings, and proper lid sealing. Packages are checked to ensure that they are sealed correctly. Extensive product inspection, including microbiological quality, composition analysis, and sensory testing are done before product is released.

Chemical tests include specific gravity, Brix, and titratable acidity. Microbiological tests include enumeration of *L. casei* strain Shirota, standard plate count, yeasts and mold counts, coliform counts, *Escherichia coli* count, *Salmonella* spp. counts, and *Staphylococcus aureus* counts. Each batch of Yakult is also analyzed for its taste and aroma through sensory analyses.

These tests are designed to assure both quality and microbiological purity and are carried out on every lot produced. Results of analyses of microbiological purity as well as heavy metals in five samples of Yakult beverage are shown in Table 2. All samples were in compliance with all microbiological and heavy-metals specifications.

Table 2. Tests of Microbiological Purity and Heavy Metals In Five Samples of Yakult Beverage with *L. casei* Strain Shirota.

Parameter	Specification	Sample Identifier					Method
		RU0503101	RU0503102	RU0503103	RU0503104	RU0503105	
Microbiological Purity							
<i>Bacillus cereus</i>	<10 cfu/ml	Complies	Complies	Complies	Complies	Complies	AOAC 980.31
Coliforms	<10 cfu/ml	Complies	Complies	Complies	Complies	Complies	SMEDP 17 th ed.
<i>Escherichia coli</i>	<10 cfu/ml	Complies	Complies	Complies	Complies	Complies	SMEDP 17 th ed.
<i>Enterobacteriaceae</i>	<10 cfu/ml	Complies	Complies	Complies	Complies	Complies	AOAC 2003.01
<i>Listeria</i> spp.	Negative in 25 ml	Complies	Complies	Complies	Complies	Complies	AOAC 999.06
<i>Salmonella</i> spp.	Negative in 25 ml	Complies	Complies	Complies	Complies	Complies	AOAC 2004.03
Coagulation + Staphylococci	<10 cfu/ml	Complies	Complies	Complies	Complies	Complies	AOAC 975.55
Yeast	<10 cfu/ml	Complies	Complies	Complies	Complies	Complies	FDA-BAM 7 th ed.
Mold	<10 cfu/ml	Complies	Complies	Complies	Complies	Complies	FDA-BAM 7 th ed.
Heavy Metals							
Arsenic	<0.1 mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	EPA 3050/6020
Cadmium	<0.1 mg/kg	<0.001	<0.001	<0.001	<0.001	<0.001	EPA 3050/6020
Lead	<0.1 mg/kg	<0.01	<0.01	<0.01	<0.01	<0.01	EPA 3050/6020
Mercury	<0.1 mg/kg	<0.005	<0.005	<0.005	<0.005	<0.005	EPA 3050/6020

000020

2.6. Specifications

All lots of Yakult beverage containing *L. casei* strain Shirota meet the specifications set forth in Table 3.

Table 3. Specifications for Yakult Beverage with *L. casei* Strain Shirota.

Parameter	Unit	Specification	Method
<i>L. casei</i> strain Shirota activity	cfu ^a /ml	NLT ^b 10 ⁸	MRS media
Color		Beige	Inspection
Acidity	% lactic acid	0.49 – 0.67	
pH		3.5 – 3.7	
Heavy Metals			
Arsenic	mg/kg	NMT ^c 0.1	EPA 3040/6020 USP730
Cadmium	mg/kg	NMT 0.1	EPA 3040/6020 USP730
Lead	mg/kg	NMT 0.1	EPA 3040/6020 USP730
Mercury	mg/kg	NMT 0.1	EPA 3040/6020 USP730
Microbiological Purity			
<i>Bacillus cereus</i>	cfu/ml	NMT 10	AOAC 980.31
<i>Escherichia coli</i>	cfu/ml	NMT 10	SMEDP ^d 17 th ed.
Coliforms	cfu/ml	NMT 10	SMEDP ^d 17 th ed.
<i>Enterobacteriaceae</i>	cfu/ml	NMT 10	AOAC 2003.01
Coagulase+ <i>Staphylococci</i>	cfu/ml	NMT 10	AOAC 975.55
Yeasts	cfu/ml	NMT 10	FDA-BAM, 7 th ed.
Molds	cfu/ml	NMT 10	FDA-BAM, 7 th ed.
<i>Listeria</i> spp.	in 25 ml	Absent	AOAC 999.06
<i>Salmonella</i> spp.	in 25 ml	Absent	AOAC 2004.03
a. cfu = colony forming units b. NLT = not less than c. NMT = not more than d. SMEDP = Standard Methods for the Evaluation of Dairy Products			

2.7. Stability

The intended shelf life of Yakult beverage is 30 days. Five samples of product produced between February 22 and March 1, 2010, were stored under refrigerated conditions for this period of time with the acidity and probiotic concentration assayed weekly. The results of this study are shown in Table 4. As can be seen, all samples were within the specified ranges of acidity and *L. casei* strain Shirota concentration throughout their shelf life. Since the bacteria remained viable, although their numbers decreased slightly, acidity tended to increase with time.

Table 4. 30-Day Viability of *L. casei* Strain Shirota in Yakult Beverage.

Parameter	Sample				
	1	2	3	4	5
Date produced	2/22/10	2/22/10	2/26/10	2/27/10	3/1/10
Acidity (% lactic acid)					
Day 0	0.50	0.50	0.50	0.51	0.50
Day 7	0.51	0.52	0.52	0.53	0.52
Day 14	0.53	0.53	0.54	0.54	0.54
Day 21	0.54	0.55	0.56	0.56	0.55
Day 30	0.56	0.56	0.58	0.59	0.59
<i>L. casei</i> strain Shirota concentration (cfu/ml)					
Day 0	3.7x10 ⁸	4.0x10 ⁸	3.6x10 ⁸	3.3x10 ⁸	3.2x10 ⁸
Day 7	3.2x10 ⁸	3.5x10 ⁸	2.9x10 ⁸	2.9x10 ⁸	3.0x10 ⁸
Day 14	2.9x10 ⁸	3.2x10 ⁸	2.5x10 ⁸	2.5x10 ⁸	2.7x10 ⁸
Day 21	2.6x10 ⁸	2.8x10 ⁸	2.2x10 ⁸	2.2x10 ⁸	2.5x10 ⁸
Day 30	2.4x10 ⁸	2.2x10 ⁸	2.0x10 ⁸	2.0x10 ⁸	2.3x10 ⁸
Standards: % lactic acid = 0.49—0.67					
<i>L. casei</i> strain Shirota concentration ≥ 1.0x10 ⁸ cfu/ml					

3. Intended Use and Consumer Exposure

L. casei strain Shirota is intended for use as an ingredient in fermented dairy products manufactured by Yakult Honsha, Co., Ltd. and other overseas group companies. The most widely sold product is a fermented milk beverage sold under the trade name “Yakult”¹, where *L. casei* strain Shirota is present at a concentration of 10^8 cfu/ml. Yakult is sold in 80-ml bottles, each bottle providing 8×10^9 cfu/serving, with recommended consumption of one to two bottles per day. The strain’s function is to serve as a probiotic microorganism.

Yakult Honsha Co., Ltd. and other overseas group companies sell fermented milk products containing *L. casei* strain Shirota in over 30 countries and regions. The specific food-grade ingredients and serving sizes of bottles of fermented food products may vary slightly in different countries. The food grade ingredients that may be present in the subject fermented dairy products include water, skim milk powder, sugars (sucrose, glucose, glucose-fructose syrup), sweeteners (maltitol syrup, sucralose), dietary fiber (polydextrose), natural and artificial flavors, and *Lactobacillus casei* strain Shirota.

Yakult’s fermented milk products are served in 65- to 200-ml bottles depending on the country of manufacturing. The highest concentration of *L. casei* strain Shirota in Yakult’s fermented milk products is 4×10^{10} cfu/bottle. The recommended daily consumption is one to two bottles. If two bottles of fermented milk product are consumed, the expected intake of *L. casei* strain Shirota is 8×10^{10} cfu/day.

The recommended daily consumption for the fermented dairy drink, Yakult, sold in the U.S. is one to two 80-mL (2.7-oz) bottles, which provide 8×10^9 to 1.6×10^{10} cfu *L. casei* strain Shirota. The maximum initial concentration of *L. casei* strain Shirota in Yakult, intended to assure the presence of the labeled number of viable organisms through the 30-day shelf life of the product, is 4×10^8 cfu/ml, allowing for up to 75% loss of viability over the shelf life. An individual consuming two servings of Yakult containing this maximum potential concentration of *L. casei* strain Shirota would consume 6.4×10^{10} cfu per day.

The most widely consumed fermented dairy product in the U.S. is yogurt. According to data from the USDA Continuing Surveys of Food Intakes by Individuals, the mean per-capita consumption of yogurt by the U.S. population is 8 g/day, with 4.0% of the population reporting consumption (USDA/ARS 1997). From these numbers, it can be calculated that mean daily consumption of yogurt by users is 200 g/day (8g/0.04). The Reference Amount Customarily Consumed (RACC) for yogurt is 225 g, so 200 g represents 0.89 servings. It is widely accepted (see FDA 2006) that the 90th percentile daily intake of a substance can be estimated by doubling the mean, and thus the estimated 90th percentile of daily yogurt consumption is 1.78 servings. This is approximately the same as the maximum recommend consumption of Yakult fermented milk products, 2 servings, and a maximum intake of 8×10^{10} cfu *L. casei* strain Shirota/day may be accepted as a reasonable estimate of the 90th percentile daily intake of the strain.

¹ The current ingredient statement for Yakult Dairy Beverage lists water, sugar, skim milk powder, glucose, natural and artificial flavors, and *Lactobacillus casei* Shirota. All ingredients are food grade and the flavors are permitted for use in the U.S. The serving size listed in the Nutrition Facts panel is 1 bottle (80 ml), 2.7 oz., providing 50 calories with 0 g fat, 12 g carbohydrate (including 11 g sugars), and 1 g protein, along with 2% of the daily need for calcium.

4. Safety

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

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

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

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

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

Vandenplas et al. (2007) observed that lactobacilli and other probiotics “do not colonize the gastro-intestinal tract as they become undetectable a few days after stopping the administration. This results in the absence of any risk for long-term side effects” (p1212). As is discussed in more detail later, many studies have demonstrated that lactobacilli are not recovered from feces by 1-2 weeks after administration ceases.

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

“Lactobacilli and bifidobacteria are ubiquitous in the diet and in the healthy large intestine soon after birth. A classical risk assessment approach, similar to that used for pathogens, is not possible or warranted. Some studies of lactobacilli have attempted to define virulence factors. Such classical approaches, although useful for known pathogens, are inherently flawed when applied to normal commensals, lactobacilli, or bifidobacteria. In the case of

the risk assessment approach for pathogens, pathogenicity is demonstrated and is normally a consequence of several properties, including colonization factors and virulence factors, acting in concert. Frequently, such factors as adhesion are considered to be virulence factors when pathogens are studied. However, mucosal adhesion and other colonization factors are essential features of most commensals. For example, there is a distinct mucosal-associated flora in the gastrointestinal tract. There is little value in screening organisms of low clinical significance and with no proven virulence determinants for such characteristics as potential virulence factors, particularly in the absence of gastrointestinal commensals as comparative controls” (p777).

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

In a similar vein, Bernardeau et al. (2008) suggested that, “The bibliographical data support the hypothesis that the ingestion of *Lactobacillus* is not at all hazardous since lactobacillemia induced by food, particularly fermented dairy products, is extremely rare and only occurs in predisposed patients.”

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

First, the subjects in this study were acutely ill with a condition the authors indicated has a 10-30% mortality rate. Second, the route of administration of the probiotic strains in this study was nasojejunal rather than oral. Third, the group that received the probiotics had a significantly higher rate of multiorgan failure on the first day of enrollment than did the control group (27% v. 16%), suggesting a potentially serious confounder. Finally, there was no difference between the probiotic and control groups in the risk of developing infectious complications and no infectious complications in either group were caused by the lactobacilli or bifidobacteria used in the study.

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

4.2. History of Consumption of *L. casei* Strain Shirota

As previously noted, *L. casei* strain Shirota is one of the ~300 strains of bacteria collected from foods by Dr. Minoru Shirota in 1930. As the original list of this collection of isolates no longer exists, details of the origin of *L. casei* strain Shirota are unclear. Dr. Shirota introduced the dairy drink Yakult (from the Esperanto word for “yogurt”), containing the strain, to the Japanese market in 1935. The Yakult Honsha Company, Ltd., was founded in 1955, and Yakult was first sold outside of Japan in 1964.

At some time prior to 1980, the bacterium isolated in 1930 became infected with a prophage insertion into its DNA; the phenotypic effect of this contamination was occasional under-production of lactic acid during fermentation. Through selective replication, a strain without the integrated prophage was obtained and has been the source for all *L. casei* strain Shirota production since 1980.

Yakult entered the European market in 1994 and the U.S. in 1999, and is now sold in more than 30 countries. According to *Euromonitor International* (2006), Yakult is the world’s best-selling health and wellness food brand. Worldwide, according to company figures, sales of Yakult beverage are about 30 million bottles a day, including over 100,000 bottles a day in the U.S.

4.3. Safety Parameters

4.3.1. Ability to Adhere to Intestinal Cells

Although adherence of probiotic bacteria to intestinal surfaces is not confirmed to be required for health benefits, it has been hypothesized to be involved in establishing residence, for stimulation of the immune system, and for antagonistic activity against enteropathogens (Gopal et al. 2001). Nevertheless, some concern has been expressed that high adhesion capability—a characteristic of pathogens—may facilitate platelet aggregation and bacterial infectivity (Kirjavainen et al. 1999).

In an *in vitro* study, Lee et al. (2000) compared the adhesion to Caco-2 cells and intestinal mucus as well as the dissociation process of *L. casei* strain Shirota, *L. rhamnosus* GG, and *E. coli* TG1. The adhesion capacity of *L. rhamnosus* GG was about 10x that of *L. casei* strain Shirota, with *E. coli* TG1 intermediate. Nevertheless, both strains of *Lactobacillus* were successful in competitively excluding *E. coli* when they were exposed simultaneously to Caco-2 cells, with about 10 *Lactobacillus* cells adhering for each *E. coli* cell. When *E. coli* cells were permitted to adhere first, neither *L. casei* strain Shirota nor *L. rhamnosus* GG was successful at displacing them, while previously adhered *L. casei* strain Shirota or *L. rhamnosus* GG cells were quickly displaced by *E. coli* TG1 cells. The authors noted that, “The observation that adhered *Lactobacillus* cells in the GI tract were gradually replaced by enterobacteria suggests that the lactobacilli used were not able to grow sufficiently rapidly to establish permanent residence in the GI tract.”

Juntunen et al. (2001) also studied adhesion to human intestinal mucus *in vitro* using mucus derived from fecal samples from 20 infants during and after rotavirus diarrhea and 10 healthy aged-matched infants. Both monoculture inocula of *L. casei* strain Shirota, *L. paracasei* F19, *L. rhamnosus* GG, *L. acidophilus* LA5, and *B. animalis* ssp. *lactis* Bb12 and combinations of these bacteria were tested. Adherence was highest for *L. rhamnosus* GG (34%) and *B.*

animalis ssp. *lactis* Bb12 (31%) and lowest for *L. casei* strain Shirota (1%). Some probiotic combinations appeared to result in synergistically enhanced adherence. There were no significant differences in adhesion between the samples from infants with rotavirus diarrhea, infants after rotavirus diarrhea, or healthy infants.

In an expanded version of the research by Lee et al. (2000) discussed above, Lee et al. (2003) studied the adhesion to Caco-2 cells and intestinal mucus of *L. casei* strain Shirota and *L. rhamnosus* GG in competition with 8 strains of *E. coli* and *Salmonella* spp. As was previously found, when incubated simultaneously the lactobacilli were able to compete with the gastrointestinal bacteria on both human mucin glycoprotein and the surface of Caco-2 cells, although there were some GI strains with which the lactobacilli could not compete. On the other hand, if the *Lactobacillus* strains were incubated first, they could generally exclude GI bacteria to some degree. On the other hand, when the GI bacteria were incubated first, displacement by lactobacilli was an extremely slow process measured in multiple hours, far longer than food or beverage remains in the small intestine.

The next year, Lee et al. (2004) took a different approach to assessing adhesion of *L. casei* strain Shirota by estimating its colonization potential in mouse intestine based on using carboxy-fluorescein diacetate succinimidyl ester to assess the doubling time in different parts of the intestine. These doubling times were estimated as 4.10, 4.78, 4.56, and 5.59 days in the duodenum, jejunum, ileum, and colon, respectively, while the average half-lives before washout of the lactobacilli at these sites were 3.98, 1.55, 1.34, and 2.48 days, respectively. The authors concluded that reproduction rates of *L. casei* strain Shirota would have to be 2 to 3 times faster than they are in order to achieve long-term colonization.

More recently, Nissen et al. (2009) posited that *in vitro* studies using pig intestinal epithelial cells would be more valid indicators of *in vivo* adhesion than studies with Caco-2 cells because the latter are much more heavily glycosylated than normal epithelial cells. These authors studied adhesion of *L. casei* strain Shirota and 12 other strains—11 lactobacilli and one *Enterococcus faecium* strain. *L. casei* strain Shirota exhibited exactly average adhesion to pig intestinal epithelial cells, with 6 of the comparison strains showing stronger adhesion and 6 showing weaker adhesion.

4.3.2. Ability to Degrade Mucin

As noted earlier, it has not been established whether probiotic bacteria adhere to epithelial cell surfaces, to the mucus layer covering the intestinal mucosa, or to both. Mucins, released from intestinal goblet cells, are highly complex glycoproteins that provide structure and viscosity to the mucus layer that covers the intestinal epithelial surface. The primary function of this layer is to protect the underlying epithelial cells from corrosive gastric acids, shear forces generated by the digestive process, and invasion by pathogenic microflora. Thus, the potential for probiotic bacteria to degrade intestinal mucins is often evaluated as a potential virulence factor since damage or disturbance to the mucus layer could compromise the barrier function and lead to intestinal or other clinical infections.

Bernardeau et al. (2006) reported that one *L. paracasei* strain tested for mucin degradation, *L. paracasei* Immunitas (like *L. casei* strain Shirota regarded as an *L. casei* prior to the recent taxonomic reclassification) was unable to do so. Indeed, O'Brien et al. (1999) noted

that all of the “probiotic lactic acid bacteria that have been evaluated do not have the ability to degrade gastric mucin.”

4.3.3. Infectivity

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

Eleven case reports have been published on clinical infections in patients consuming probiotics, most commonly *L. rhamnosus* strains. However, in only some of these cases was the strain isolated from the infection confirmed to be identical to the strain that was consumed. It is clear that all reported cases of clinical infections with suspected *Lactobacillus* involvement occurred in subjects with one or more severe underlying diseases or health conditions. While these reports indicate that strains of *L. rhamnosus* have the potential to be opportunistic pathogens in severely compromised subjects, it is equally clear that all lactobacilli, including *L. rhamnosus*, are safe in healthy subjects and those with less severe medical conditions, where adverse events have never been reported.

Ze-Ze et al. (2004) reported on a case of aortic-valve endocarditis putatively caused by *L. casei*. The patient, a 53-year-old man with a history of rheumatic fever and regular consumption of yogurt, developed symptoms after a dental extraction. Blood, bone marrow, and the replaced aortic valve all tested positive for *Lactobacillus*, identified by 16S rRNA sequencing as *L. casei*. This microorganism was regarded as the cause of the bacteremia because it was the only strain consistently isolated from all three reservoirs, but no link was ever established between the isolated strain of *L. casei* and the yogurt and its source remains unknown.

A second case of *L. casei* bacteremia was reported by Tommasi et al. (2008). A 66-year-old male suffering from hypertension, diverticulosis, hemorrhoidal bleeding, chronic obstructive pulmonary disease, and a history of febrile episodes presented with a persistent fever. A blood culture isolate a specimen identified by 16S rRNA sequencing as *L. casei*. He was treated with antibiotics and later blood samples yielded no further *L. casei* isolates. The source of the bacteria was never determined, although the authors speculated that the intestinal diverticulosis with local mucositis may have provided an entry point.

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

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

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

Adams and Marteau (1995) observed that no case has been described of a *Lactobacillus* infection derived from food or feed fermented with *Lactobacillus* cultures; this statement remains true in 2012. The participants in the 2007 EU-PROSAFE project (Vankerckhoven et al. 2007) 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).”

In vitro research by Asahara et al. (2003) has shown that *L. casei* strain Shirota is even less virulent than other *Lactobacillus* strains. Seven *Lactobacillus* strains representing 4 species were evaluated for pathogenicity in a rabbit infective endocarditis model in which specific-pathogen-free male Japanese white rabbits weighing about 2.5 kg were catheterized and vegetations composed of platelets and fibrin were allowed to form on the tricuspid valve or endocardium at points of contact. After 24 hours, one of the *Lactobacillus* strains (or *Staphylococcus aureus* or *Streptococcus mitis* as positive controls) was inoculated into the marginal ear vein.

The positive controls induced lethal infections even at low doses. Additionally, all rabbits developed infective endocarditis when inoculated with 3.4×10^7 cfu *L. rhamnosus* ATCC 53103, 6.0×10^8 cfu *L. rhamnosus* PHLS A103/7, 3.0×10^9 cfu *L. rhamnosus* ATCC 7469 (the type strain), or 4.1×10^8 cfu *L. casei* PHLS A357/84, while no rabbits developed infection when inoculated with 1.3×10^9 cfu *L. casei* strain Shirota (or with 1.5×10^9 cfu *L. acidophilus* ATCC 4356 or 2.4×10^9 cfu *L. gasseri* DSM 20243). The authors regarded the virulence of these latter strains as “negligible” under the conditions tested. Additionally, the non-infective strains proved to be

more susceptible to intracellular killing activity by mouse macrophages *in vitro* and to bactericidal nitrogen intermediates such as nitric oxide.

4.3.4. Undesirable Metabolic Activity

Some metabolic products of lactobacilli may have adverse effects on human safety. For example, the production of D-lactate by some species of *Lactobacillus*, although not *L. casei*, has been identified as a possible safety issue (Mack 2004). Similarly, it has been questioned whether a safety issue may arise in fermented dairy products due to production by lactobacilli of biogenic amines, primarily histamine or tyramine, through amino acid decarboxylase activity. However, Bernardeau et al. (2006) observed that, although biogenic amines may be harmful to consumers, “no such potentially harmful compounds have been found in fermented milk prepared with probiotic lactobacilli.” In a later article, Bernardeau et al. (2008) noted that, if production of biogenic amines is indeed a safety issue, it is linked to spoilage or to long fermentation processes and pertains to the use of *Lactobacillus* species in the production of fermented dairy products and not to their use as probiotics. Indeed, Naidu et al. (1999) pointed out that, by producing lactic acid, probiotic lactobacilli reduce intestinal pH, which in turn limits the growth of many potential putrefactive bacteria that produce harmful biogenic amines.

As discussed in Section 2.4.3.2, the genome of *L. casei* strain Shirota was searched for genes that might encode the production of decarboxylases that might generate harmful biogenic amines. The only such gene found was the genome sequence of ornithine decarboxylase isozyme that may catalyze the conversion of ornithine into putrescine. However, the sequence of this enzyme exist in many strains of *Lactobacillus*, e.g., *L. casei* BL23, *L. casei* ATCC 334, *L. rhamnosus* ATCC 53013, *L. rhamnosus* GG, *L. rhamnosus* Lc705, *L. salivarius* UCC118, *L. delbrueckii* ssp. *bulgaricus* BAA-365, *L. delbrueckii* ssp. *bulgaricus* ATCC 11842, *L. acidophilus* NCFM, *L. helveticus* CNRZ32, *L. helveticus* DPC4571, *L. gasseri* ATCC 33323, *L. johnsonii* F19785, and *L. johnsonii* NCC533), and it is clear that possession of this gene does not result in a safety concern.

Like most other probiotic bacteria, *L. casei* strain Shirota produces bacteriocin (Avonts and De Vuyst 2001). The compound produced is of low molecular weight (about 6000 Da), is rapidly degraded by proteinases, and has a narrow inhibitory spectrum. There is no indication that the strain’s bacteriocin is harmful to the beneficial intestinal microbiota. The region of the genome encoding for production of this bacteriocin was identified by O’Sullivan (2012), who concluded that “This potential bacteriocin region is not a safety issue, but rather a positive attribute of a probiotic culture.”

In follow-up work, Avonts et al. (2004) compared bacteriocin production of *L. casei* strain Shirota, *L. casei* Immunitas, *L. acidophilus* ACC, *L. acidophilus* IBB801, *L. gasseri* K7, *L. johnsonii* La1, and *L. rhamnosus* GG in both MRS medium and milk medium. For all strains, bacteriocin production was higher in MRS medium than in milk. The highest production of bacteriocin was seen in *L. acidophilus* IBB801, *L. gasseri* K7, and *L. johnsonii* La1. Only low levels were observed in *L. casei* strain Shirota or *L. casei* Immunitas, and no bacteriocin activity at all was noted for *L. acidophilus* ACC or *L. rhamnosus* GG.

Jimenez-Serna and Hernandez-Sanchez (2011) investigated the effects of the non-steroidal anti-inflammatory drugs (NSAIDs) acetylsalicylic acid, sodium acetylsalicylate, acetaminophen, sodium naproxen, and sodium ibuprofen on the growth of *L. casei* strain Shirota,

as well as whether the microorganism can use NSAIDs as a carbon source. The bacteria were isolated from Yakult® beverage and cultured in MRS broth; species confirmation was achieved by its fermentation patterns with galactose, glucose, fructose, mannitol, cellobiose, maltose, lactose, trehalose, raffinose, arabinose, and xylose.

The strain could not grow when any of the NSAIDs replaced glucose in the growth medium, indicating an inability of the bacteria to utilize these drugs as carbon sources. While none of the NSAIDs at low doses had a significant inhibitory effect on bacterial growth, the presence of sodium naproxen and sodium ibuprofen at higher concentrations significantly inhibited the growth of the strain. The results of the testing indicate that “the consumption of NSAIDs simultaneous with a dairy fermented product with [*L. casei*] does not affect the bioavailability of the drugs.”

4.3.5. Antibiotic Resistance and Likelihood of Transference

Ammor et al. (2007) observed that “lactobacilli are usually sensitive to penicillins and β -lactamase inhibitors, but more resistant to oxacillin and cephalosporins; cell wall impermeability seems to be the main mechanism of resistance.” In a detailed evaluation of the safety of the *Lactobacillus* genus, Bernardeau et al. (2008) addressed all issues pertaining to safety and concluded that, “transferable antibiotic resistance is the only relevant cause for caution . . . Safety assessment requirements for *Lactobacillus* strains of technological interest should be limited to an antibiotic profile and a study to determine whether any antibiotic resistance(s) of medical interest detected is (or are) transferable.”

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. Most 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 safety concern is associated with intrinsic type of resistance.

Like all bacteria, lactobacilli 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*.

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

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

Many strains of lactobacilli—including *L. casei* strain Shirota—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).

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.

Klare et al. (2007) studied a variety of *Lactobacillus* as well as *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic use. A total of 416 isolates of *Lactobacillus* representing 21 species, including 3 isolates of *L. casei* strains and 90 of *L. paracasei*, were tested against 16 antimicrobial agents encompassing nearly all important classes to determine the distribution of minimum inhibitory concentrations (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.

ECOFFs were tentatively established for *L. paracasei* with regard to 12 antimicrobials (Klare et al. 2007): penicillin, ampicillin, ampicillin/sulbactin, gentamicin, streptomycin, quinupristin/dalfopristin, erythromycin, clindamycin, oxytetracycline, chloramphenicol, fusidic acid, linezolid, and trimethoprim.

In similar research, D’Aimmo et al. (2007) determined MIC for 55 bacterial strains regarded as either probiotics or dairy starter cultures representing 3 genera and 5 species—22 *Bifidobacterium* spp. (all but one *B. animalis* ssp. *lactis*), 6 *L. acidophilus*, 6 *L. casei*, 11 *L. bulgaricus*, and 10 *S. thermophilus*. For each species tested, the range of MIC was reported as well as the median, mode, and 90th percentile.

The susceptibility of *L. casei* strain Shirota to a variety of antibiotics was tested and published by Yuki et al. (1999). In Table 5, this susceptibility is compared with that of 42 other strains of *L. casei*. This comparison shows clearly that *L. casei* strain Shirota shows no total resistance to any antibiotic other than vancomycin, and no atypical increased resistance to any tested antibiotic. Further, *L. casei* strain Shirota exhibits no resistance higher than the tentative ECOFFs established for *L. paracasei* by Klare et al. (2007) or the median MIC of *L. casei* reported by D’Aimmo et al. (2007). This indicates an absence of acquired antibiotic resistance and is consistent with the finding, discussed in Section 2.4.3.1, that the genomic analysis of *L. casei* strain Shirota found no evidence of genes encoding for antibiotic resistance in either the genome or the plasmid.

Table 5. Antibiotic Susceptibility of *L. casei* Strains.

Antibiotics	Minimum Inhibitory Concentration (µg/ml)		
	Minimum ¹	Maximum ¹	<i>L. casei</i> strain Shirota
Gentamicin	2	32	8
Kanamycin	32	256	64
Streptomycin	16	128	32
Neomycin	4	128	16
Tetracycline	1	16	2
Erythromycin	0.25	1	0.25
Clindamycin	0.25	2	0.25
Chloramphenicol	4	32	8
Ampicillin	1	4	1
Penicillin	0.25	1	0.25
Vancomycin	> 128	> 128	> 128
Virginiamycin	0.25	4	0.5
Linezolid	2	16	2
Trimethoprim	1	> 64	64
Ciprofloxacin	4	16	4
Rifampicin	0.25	> 64	0.25
Quinurpistin/Darfopristin	0.25	2	0.5

1. Of 42 *L. casei* strains tested by microdilution in LSM for 48 hours at 37°C and CO₂ = 10 %.

As discussed in the previous section, Jimenez-Serna and Hernandez-Sanchez (2011) isolated bacteria from Yakult® beverage and cultured them in MRS broth in their investigation of the interaction of *L. casei* and NSAIDs. They also assessed the resistance of the bacteria to tetracycline, ampicillin, erythromycin, cephalothin, cefuroxime, penicillin, cefotaxime, dicloxacillin, sulfamethoxazole with trimethoprim, ceftazidime, pefloxacin, and gentamicin using Multidiscs® against Gram+ microorganisms on MRS agar. The strain proved to be somewhat resistant to sulfamethoxazole with trimethoprim, pefloxacin, and gentamicin and sensitive to the other 9 antibiotics tested, results generally in accord with the findings of Yuki et al. (1999).

4.4. *In Vivo* Studies of *L. casei* Strain Shirota

4.4.1. Animal Studies

A number of animal studies of *L. casei* strain Shirota were conducted in order to demonstrate safety and to investigate potential beneficial effects. These studies are summarized in Table 6 at the end of the section.

4.4.1.1. Published Studies

4.4.1.1.1. Mice

Matsuzaki et al. (1997) studied the effect of *L. casei* strain Shirota on the incidence of diabetes in an insulin-dependent diabetes mellitus (IDDM) model, nonobese diabetic mice. Four-week-old inbred specific pathogen-free female NOD mice (weight not reported) were fed mouse chow containing 0 or 500 ppm heat-killed *L. casei* strain Shirota cells. Feed and water were available *ad libitum* and treatment continued for about 36 weeks. Plasma glucose was measured periodically to diagnose diabetes. After sacrifice, the pancreas was excised from 3 mice/group for immuno-histochemical examination of islet cells. Spleens were removed from 3 mice/group/week for cytokine assessment.

Disappearance of insulin-secreting β cells in Langerhans islets and the incidence of diabetes in the treatment groups was significantly lower than in the control group. The treated group also showed an increased ratio of CD45R+ B-cells relative to CD8+ T-cells in the spleen as well as higher production of IL-2 and lower production of IFN- γ . The authors concluded that administration of heat-killed *L. casei* strain Shirota prevented the development of diabetes through regulation of immunoresponse. No adverse effects were reported.

In a series of experiments, Kato et al. (1998) investigated the effect of *L. casei* strain Shirota on the development of type II collagen-induced arthritis in DBA/1 mice. Arthritis was induced in the mice by intradermal injection of Bovine type II collagen into the base of the tail. All experiments used 8-week-old male inbred DBA/1 mice that were housed in plastic cages and given free access to feed and water. In the first experiment, mice in the test group (n=5) received by gavage 10^9 cfu *L. casei* suspended in distilled water 5 times per week while arthritis control mice (n=5) received equal volumes of distilled water.. A third group of n=5 mice were not subjected to induced arthritis or given *L. casei*. Clinical symptoms of arthritis were observed 1-3 times weekly for 10 weeks and blood was collected weekly for measurement of anti-CII antibodies titer. In the second experiment, 2 groups of n=5 mice received either the same dose of *L. casei* strain Shirota or distilled water for 22 days, after which their blood was tested for IFN- γ and IL-4 activity. In the third experiment, 5 groups of mice received different doses of *L. casei* by gavage 5 times per week for 10 weeks: 0 (n=13), 0.25×10^9 cfu (n=10), 0.5×10^9 cfu (n=12), 1×10^9 cfu (n=12), or 2×10^9 cfu (n=12).

In the first experiment, administration of *L. casei* strain Shirota significantly delayed or prevented the development of induced arthritis and significantly reduced anti-CII antibodies. The second experiment resulted in significantly reduced secretion of IFN- γ from splenocytes. In the third experiment, a significant delay in the development of induced arthritis and a reduction in the severity of the symptoms were seen in response to administration of *L. casei*, but no dose-response relationship was apparent. The authors concluded that “oral administration of [*L. casei* strain Shirota] was able to modify the humoral and cellular immune responses to [type II collagen].” No adverse effects were reported attributable to the administration of *L. casei* strain Shirota.

Matsuzaki et al. (1998) evaluated the effect of oral administration of heat-killed *L. casei* strain Shirota on immunoglobulin E (IgE) and cytokine production in BALB/c mice. Twenty 7-week-old inbred male BALB/c mice were placed in plastic cages with *ad libitum* access to feed and water and injected intraperitoneally with ovalbumin and aluminum hydroxide. Their diets

were supplemented with 0%, 0.05%, or 0.1% *L. casei* strain Shirota cell mass, resulting in *L. casei* strain Shirota mass intakes of 1-1.25 and 2-2.5 mg/day. The mice consumed their assigned diet for 21 days and were re-injected with ovalbumin and aluminum hydroxide on day 14. A reference group of 5 mice was left untreated. On day 21, blood was collected for analysis of IgE activity and spleen cells were excised for testing of cytokines.

Administration of heat-killed *L. casei* strain Shirota significantly inhibited production of both total and ovalbumin-specific IgE in a dose-dependent manner. *L. casei* strain Shirota also significantly stimulated splenic production of Th1 cytokines such as IFN γ and IL-2 (which augment cell-mediated immunity) as well as IL-12, while inhibiting production of Th2 cytokines such as IL-4, IL-5, IL-6, and IL-10 (which augment humoral immunity). The authors attributed the inhibition of IgE production to the regulation of Th1 and Th2 cells.

Using a murine model, Takagi et al. (2001) investigated the ability of *L. casei* strain Shirota to stimulate production of natural killer (NK) cells in response to 3-methylcholanthrene-induced carcinogenesis. Seven-week-old C3H/HeN male mice and C57BL/6J and C57BL/6 (beige) female mice, maintained 6 mice/cage with *ad libitum* feed and water, were injected with 3-methylcholanthrene to induce tumor formation. Mice consumed either a control diet (n=12) or a diet supplemented with *L. casei* (n=12) for 15 weeks. Feed intake, body weight, and tumor growth were measured weekly. After sacrifice, spleens were removed for analysis of NK cells.

There were no significant differences between the test and control groups in feed intake, body weight, or liver or spleen weights. In the control group, tumor incidence at 6 and 11 weeks post-induction was 33% and 83%, respectively, while in the *L. casei*-treated group it was 0% and 42% at the same time points, both statistically significant reductions. The number of NK cells and NK cell cytotoxicity were significantly enhanced in splenocytes from the *L. casei* group. However, no tumor-suppressive effect of *L. casei* was evident in the beige mouse model, indicating that the delayed tumor onset mediated by the probiotic depended on NK cells. The authors suggested that *L. casei* strain Shirota may be degraded in gut-associated lymphoid tissue, and that the signal from immunocompetent cells then leads to a systemic effect. No adverse effects were noted.

Hori et al. (2002) investigated the effect of oral administration of *L. casei* strain Shirota on the cellular immune system in aged mice exposed to influenza virus. Fifteen-month-old female BALB/c mice (number not reported) were fed MM-3 diet either with or without added lyophilized *L. casei* (dose not reported) for 4 months and then either sacrificed for analysis of NK cell activity and cytokine concentrations or intranasally exposed to influenza virus and sacrificed 3 days later for measurement of viral titer.

NK cell activity in the *L. casei* strain Shirota group was significantly higher than in the control group, as were the concentrations of IFN- γ and TNF- α . In the mice exposed to influenza virus, the viral titer in the *L. casei* group was significantly lower than in the control group. No adverse effects were reported, and the authors concluded that "oral administration of *L. casei* strain Shirota activated not only the systemic immune system but also the local immune system and that it ameliorated [influenza virus] infection in aged mice."

In the study discussed above (Hori et al. 2002), the NK cell activity of blood mononuclear cells was not assessed because of the small number of mononuclear cells per mouse. In order to address this issue, Hori et al. (2003) fed a diet containing 0.05% heat-killed *L. casei* strain Shirota cells or a control diet to 15-month-old female BALB/c mice (n=19 per treatment)

for 2 months. After sacrifice, the NK cell activity of blood mononuclear cells and splenocytes was measured. It was found that ingestion of *L. casei* strain Shirota significantly increased NK cell activity in both mononuclear cells and splenocytes. The authors interpreted this finding as indicating that *L. casei* strain Shirota may augment the NK activity of peripheral blood mononuclear cells in healthy low-NK individuals and the elderly. No adverse effects were reported due to the treatment with *L. casei* strain Shirota.

De Waard et al. (2003) studied the effects of orally administered *L. casei* strain Shirota on immunological memory to *Listeria monocytogenes* infection in BALB/c mice and in both Wistar and Brown Norway rats. The latter studies are discussed below in the section dedicated to research using the rat model; only the mouse study is described here. Male BALB/c mice, 6-7 weeks of age, were kept 2 mice/cage and given food pellets and water *ad libitum*. Six mice/group received a daily gavage of 0.5 ml saline or 10^9 cfu viable *L. casei* strain Shirota suspended in saline. After 10 days they were infected with *L. monocytogenes*, and 10 days after that the *L. monocytogenes*-specific delayed sensitivity was measured. After sacrifice, spleens were removed and *L. monocytogenes* numbers were counted.

The mice treated with *L. casei* strain Shirota, as compared to controls, showed significantly higher antigen-specific delayed sensitivity, but there was no difference in the *L. monocytogenes* loads in the spleen. The authors concluded that this finding, in combination with the results of the studies with Wistar and Brown Norway rats discussed below, demonstrates that the immunological effect of *L. casei* strain Shirota is not dependent on host genetics.

Yasui et al. (2004) orally administered *L. casei* strain Shirota to neonatal and infant mice to assess its ability to protect against influenza infection. The neonatal group included male and female BALB/c mice aged 2 days, while the infant groups included female BALB/c mice aged 2, 3, 5, 7, and 13 weeks old. Mice (number not reported) were given 4×10^8 cfu *L. casei* strain Shirota or saline by gavage 5 times a week for over 3 weeks to a total of 17 administrations; then they were infected by influenza virus administered intranasally. One day later, NK activity in the lung and IL-12 production in the mediastinal lymph nodes were measured. After 3 days the viral titer of nasal washings was measured, saline was administered intranasally to disseminate the virus from the nasal cavity to the lower respiratory tract, and the survival rate of the mice was determined.

Administration of *L. casei* strain Shirota significantly increased pulmonary NK cell activity and IL-12 production in the mediastinal lymph nodes, lowered the viral titers, decreased the severity of influenza symptoms, and lowered the mortality rate (14.3% of treated mice v. 40.0% of control mice) in all age groups. The authors did not report any adverse effects and concluded that *L. casei* strain Shirota activates innate local immune response and may protect against respiratory infection in infants and children.

In a study of the inhibitory effect of *L. casei* strain Shirota on *Helicobacter pylori*, Sgouras et al. (2004) gave 10^8 cfu of the probiotic/ml in the drinking water to 25 6-week-old female C57BL/6 mice, previously infected, with *H. pylori* for 9 months. The same dose of *L. casei* strain Shirota was given to 25 uninfected mice, a third group of 25 mice was infected with *H. pylori* and not treated, and a final group of 25 mice was not infected or treated. Caging arrangements were not reported, but all mice had feed and water freely available. Water consumption was monitored daily and averaged about 6.0 ml/day/mouse; thus, the daily intake of *L. casei* strain Shirota was 6×10^8 cfu. Blood samples were taken at 1, 2, 3, 6, and 9 months; at

each collection 5 animals/group were sacrificed and dissected for examination of the stomach for *H. pylori* colonization and the intestines for *L. casei* strain Shirota.

Tests for *L. casei* strain Shirota detected the strain in feces at populations of 10^7 - 10^8 cfu/g, but it was not detected in intestinal tissue samples. The authors concluded that the strain does not colonize the gut epithelium and therefore is present in the biome only as long as it is continuously administered. Treatment with *L. casei* strain Shirota significantly reduced *H. pylori* populations and gastric mucosal inflammation. No adverse reactions were reported due to the long-term administration of *L. casei* strain Shirota.

Herias et al. (2005) gave 8-week-old male BALB/cOlaHsd mice daily gavage administrations of 10^8 cfu *L. casei* strain Shirota suspended in 100 μ l phosphate-buffered saline or the saline alone. Ulcerative colitis was induced by adding 5% dextran sodium sulfate into the drinking water. In the prophylactic condition (8 test mice and 6 controls), colitis induction was started on day 11 after initiation of *L. casei* strain Shirota administration and continued for 7 days. In the simultaneous condition (8 test mice and 6 controls), colitis induction and *L. casei* administration began at the same time. In the short-term post-colitis condition (6 test and 6 control mice), *L. casei* strain Shirota administration was begun 7 days after colitis induction and continued for another 7 days, while in the long-term post-colitis condition (7 test and 6 control mice), *L. casei* strain Shirota administration continued for 17 days. The animals were clinically evaluated and weighed daily. After sacrifice, blood was collected and analyzed for red and white blood cell counts, hemoglobin, hematocrit, and differential leukocyte counts. Spleens, kidneys, mesenteric lymph nodes, and ceca were removed and weighed, and the colon was measured and examined histologically.

L. casei strain Shirota was isolated from the feces of all mice receiving it for 17 days, but only in some of those who received it for shorter periods. Induction of colitis resulted in a significant increase in the population of *Enterobacteriaceae*, a rise that was not different in the *L. casei* strain Shirota and control groups. However, treatment with *L. casei* strain Shirota significantly reduced the weight loss and the gain in spleen weight that resulted from colitis, as well as the loss of red blood cells and decreases in hemoglobin and hematocrit. There was no difference in histopathology between the test and control groups with induced colitis. There was no indication of adverse effects from administration of *L. casei* strain Shirota, and the authors concluded that the strain has the potential for reversing some of the deleterious effects of colitis.

Matsumoto et al. (2005) used a murine model to study the effect of *L. casei* strain Shirota on chronic inflammatory bowel disease (IBD). Twenty 10-week-old female BALB/c mice and 20 15-week-old female SAMP1/Yit mice were administered dextran sodium sulfate in their drinking water in 4 cycles of 7 days with treated water and 10 days with untreated water to induce chronic colitis in the BALB/c mice and chronic ileitis in the SAMP1/Yit mice. Simultaneously, half of the mice (10 of each strain) received standard rodent chow while the remaining mice (10 of each strain) received chow containing 0.05% heat-killed *L. casei* strain Shirota. Feeding continued until the mice were 25 weeks old. Mice were assessed for disease activity (change in body weight, stool consistency, and intestinal bleeding) Blood samples were taken for analysis of cytokines.

As compared to controls, BALB/c mice treated with *L. casei* strain Shirota showed significantly less weight loss, diarrhea, and occult blood, as well as significantly reduced mortality. The serum analyses showed that production of IL-6 and IFN- γ was significantly down-

regulated while IL-4 was up-regulated in BALB/c mice treated with *L. casei* strain Shirota. In the SAMP1/Yit induced-ileitis mice, ingestion of *L. casei* strain Shirota significantly improved histological scores compared to controls. The authors concluded that *L. casei* strain Shirota may be a safe and useful probiotic for the treatment of IBD.

To further elucidate the role of *L. casei* strain Shirota in modulation of T helper 1 (Th1) mediated immune response, Baken et al. (2006) gavaged 6-8-week-old male BALB/c mice once a day with either 2×10^8 cfu *L. casei* strain Shirota suspended in 0.1 ml saline/peptone (n=16) or saline/peptone alone (n=16) for 8 days prior to sensitization with 4 different doses (4 mice each) of 2,4-dinitrochlorobenzene (DNCB). DNCB application was done daily for 3 days, and 3 days after the end of application the mice were euthanized and auricular lymph nodes were excised.

At the highest concentration of DNCB, the treatment with *L. casei* strain Shirota significantly reduced the cell proliferation in the lymph nodes; differences at lower levels of DNCB were not significant. This apparent inhibition of Th1 activity is inconsistent with previous findings of a stimulatory effect. The authors noted that the development of T cells is determined by the extent of maturation of dendritic cells and suggested that probiotics, including *L. casei* strain Shirota, may affect maturation and function of dendritic cells, thus resulting in either inhibitory or stimulatory effect.

Ezendam and van Loveren (2008) tested the effect of administration of *L. casei* strain Shirota during lactation on later respiratory allergy. Both 6-8-week-old and 2-week old male and female BALB/c mice were studied to compare effects of early administration of *L. casei* strain Shirota. The adult mice (12 mice/sex/group) were given chow and water *ad libitum* while the pups (12 mice/sex/group) were cross-suckled among the dams; both adult mice and pups were gavaged with $2-4 \times 10^8$ cfu *L. casei* strain Shirota/day or saline alone for 21 days. Eight adult mice/sex/group and 8 pups/sex/group were sensitized by intraperitoneal injection of ovalbumin while 4 adult mice and 4 pups/sex/group received only saline, and all mice were challenged by inhalation of ovalbumin. Two days later the mice were killed, blood was collected for measurement of ovalbumin-specific IgE and IgG1 titers, and spleens were collected for ovalbumin stimulation of cell suspensions and measurement of Th1 and Th2 cytokines.

Mice that were sensitized with ovalbumin showed significantly increased numbers of eosinophils and lymphocytes on challenge than did controls, but ingestion of *L. casei* strain Shirota did not have a consistent significant effect. Nor did administration of *L. casei* strain Shirota have a significant effect on titers of ovalbumin-specific IgE or IgG1. Administration of *L. casei* strain Shirota during lactation had no effect on cytokine production, but *L. casei* strain Shirota ingested by adult mice resulted in significant increases in Th2 cytokines (IL-4, IL-5, IL-10, and IL-13) but not in IFN- γ . On the other hand, administration of *L. casei* strain Shirota during lactation, but not during adulthood, significantly enhanced the inflammatory lung response. The authors interpreted the data as confirming that *L. casei* strain Shirota has modest immunostimulatory properties and suggested that these effects are complex and not well understood.

Kobayashi et al. (2011) studied the safety of *L. casei* strain Shirota in dysregulated immune conditions using 2 chronic murine models of experimental autoimmune encephalomyelitis (EAE), a relapse/remission model in 7-week-old female SJL/J mice and a durable model in 7-week-old female C57BL/6 mice. Mice were individually caged with free access to feed and water. The SJL/J mice were administered proteolipid protein peptide and *M.*

tuberculosis subcutaneously as well as pertussis toxin intraperitoneally. The probiotic was administered by gavage at a daily dose of 0 (control; n = 15) or $0.6-1.2 \times 10^9$ cfu (n = 15) beginning 1 week before immunization. The C57BL/6 mice received subcutaneous injections of myelin oligodendroglial glycoprotein peptide and *M. tuberculosis* and intraperitoneal injections of pertussis toxin. Again, *L. casei* strain Shirota was administered by gavage at a daily dose of 0 (control) or $0.6-1.2 \times 10^9$ cfu beginning 1 week before immunization.

Neurological symptoms of the mice were scored daily and fourth lumbar segments of the spinal cord were collected periodically for histopathological analysis—on days 7, 12, 27, and 50 post-immunization from the SJL/J mice and on days 7, 18, and 29 from the C57BL/6 mice. On day 3 after immunization, inguinal lymph nodes and spleens were taken for analysis of cell-surface antigens and determination of cytokine concentrations and levels of IL-17A, IFN- γ , and IL-10. On the same day, mRNA was extracted from the third lumbar segment of the spinal cord for evaluation of cytokine genes.

Among the SJL/J mice, 2 control and 1 test-group mice died of acute EAE. Administration of *L. casei* strain Shirota resulted overall in nonsignificantly improved neurological symptoms with no exacerbation of any neurological symptom or histopathological changes. No deaths were reported among the C57BL/6 mice and there were no significant differences in any endpoints between the test and control groups. Although ingestion of *L. casei* strain Shirota transiently increased IL-17 production by inguinal lymph nodes (but not the spleen), this had no effect on histopathology or neurological symptoms. The authors concluded that “oral administration of [*L. casei* strain Shirota] does not exacerbate but instead may improve EAE.”

4.4.1.1.2. Hamsters

Nishino et al. (2000) hypothesized that suppression of oxidative stress in the colon would be beneficial and studied the ability of both heat treatment of milk products and fermentation by *L. casei* strain Shirota to increase radical scavenging activity. Eighteen 6-week-old male golden Syrian hamsters were randomly assigned to receive one of 3 diets (n=6 individually caged hamsters per treatment) for 14 days. Control hamsters received skim milk with added glucose and fructose, the Maillard reaction group received the same skim milk after it had been heated at 100°C for 90 minutes, and the probiotic group received the heat-treated milk product fermented with 10^9 cfu *L. casei* strain Shirota at 37°C until it reached a pH of 3.7. The hamsters had free access to feed and water; feed consumption was measured every 2-3 days and bodyweight every week. After sacrifice, the hamsters' ceca were excised and the cecal content collected for measurement of radical scavenging activity.

The heat treatment significantly increased the radical scavenging activity of the skim-milk product, and fermentation with *L. casei* strain Shirota further significantly increased it. There were no differences in feed consumption or feed-efficiency ratio, and no adverse effects were apparent.

4.4.1.1.3. Rats

De Waard et al. (2001) tested the effect of orally administered *L. casei* strain Shirota on immune parameters in the Wistar and Brown Norway rat models. Thirty-two 4-week-old male outbred Wistar (U:Wu) rats and 16 inbred Brown Norway (Rij:Hsd) rats were double-caged in Macrolon cages with free access to standard rat chow and water. In all experiments, 8 test rats

received 10^9 cfu *L. casei* strain Shirota suspended in 0.5 ml distilled water via gavage while control rats received an equal volume of cornstarch in distilled water 5 days per week for 6 weeks. On day 14, rats were infected by oral administration of 10^3 viable *Trichinella spiralis* muscle larvae, and a *T. spiralis* antigen DTH assay was done 3 weeks later. One week after that, the rats were sacrificed and analyzed for serum *T. spiralis*-specific antibodies, the number of larvae in tongue muscle tissue, severity of inflammation, and body, thymus, and spleen weights and histologies.

Administration of *L. casei* strain Shirota had no effect on terminal body weight, nor on absolute or relative thymus or spleen weights, in either Wistar or Brown Norway rats. Nor was any difference between test and control animals noted during histological examination of thymus and spleen. Both Wistar and Brown Norway rats that received *L. casei* strain Shirota showed significantly augmented *T. spiralis* antigen-specific DTH response at both 24 and 48 hours as compared to controls. Both strains of rat also showed significantly enhanced serum IgG2b anti-*T. spiralis* titers associated with ingestion of *L. casei* strain Shirota, although other isotypes (IgM, IgG1, IgG2a, IgA, and IgE) did not differ between test and control animals. Nevertheless, no effect of the probiotic treatment was noted on parasitic load or on inflammatory response around the larvae. The authors concluded that *L. casei* strain Shirota has a significant effect on Th1 cell activity, and that this effect appears to be independent of the host's genetic background.

De Waard et al. (2003) studied the effects of orally administered *L. casei* strain Shirota on immunological memory to *Listeria monocytogenes* infection in both Wistar and Brown Norway (BN) rats and in BALB/c mice. The mouse studies were discussed above; only the rat studies are described here. Male Wistar and BN rats, 6-7 weeks of age, were double-caged and given food pellets and water *ad libitum*. In the first experiment, 6 Wistar and BN rats/group received daily gavage of 0.5 ml saline or 10^9 cfu viable *L. casei* strain Shirota suspended in saline. After 10 days they were infected with *L. monocytogenes*, and 10 days after that the *L. monocytogenes*-specific delayed sensitivity was measured. In the second experiment, control and test rats (n=6/group) received saline or 10^9 cfu *L. casei* strain Shirota beginning either 10 days before or 7 days after *L. monocytogenes* infection; again, *L. monocytogenes*-specific delayed sensitivity was measured 10 days after infection. In the third experiment, groups of 4 rats were gavaged daily with 0.5 ml saline, heat-killed *L. casei* strain Shirota obtained by heating a suspension of 2×10^9 cfu/ml suspended in 0.5 ml saline, or a dose of 10^7 , 10^8 , or 10^9 cfu of viable *L. casei* suspended in saline, beginning 7, 8, or 9 days after the *L. monocytogenes* infection; *L. monocytogenes*-specific delayed sensitivity was again measured 10 days after infection. In this experiment, the rats continued to receive saline or *L. casei* for an additional 8 weeks, after which they were re-infected with *L. monocytogenes* and killed 36 hours later. After sacrifice, spleens and livers were removed and analyzed for bacterial burden, and serum alanine aminotransferase levels were measured.

Ingestion of 10^9 cfu *L. casei* strain Shirota/day significantly enhanced the *L. monocytogenes*-specific delayed sensitivity response at 24 and 48 hours in both Wistar and BN rats. Further, this significant enhancement was similar regardless of whether administration of *L. casei* strain Shirota began 10 days prior or 7 days post-infection. This effect was not seen with administration of heat-killed *L. casei* strain Shirota, nor with lower doses of 10^7 - 10^8 cfu viable *L. casei* strain Shirota/day. Long-term (8-week) administration of 10^9 cfu viable *L. casei* strain Shirota/day, but not 10^7 - 10^8 cfu viable *L. casei* strain Shirota or heat-killed *L. casei* strain Shirota, reduced alanine transaminase activity and enhanced the immune response to *L. monocytogenes*

re-infection as measured by reduced counts of viable *L. monocytogenes* in the spleen and liver. No adverse effects of ingestion of *L. casei* strain Shirota were reported for any of the rats of either strain. The authors concluded that orally administered *L. casei* strain Shirota enhances cell-mediated immunological memory responses, that this effect is seen across species and genetic backgrounds, that it is dependent on the timing of administration, is dose-dependent, and possibly is imparted through a heat-sensitive constituent of *L. casei* strain Shirota.

Baken et al. (2006) studied immune modulatory effects of *L. casei* strain Shirota. Sixteen 6-8-week-old SPF male Lewis rats (LEW/HanHsd), housed individually with free access to feed and water, were divided into 2 groups (n=8/group) to receive daily gavage of 10^9 cfu *L. casei* strain Shirota suspended in 1 ml saline solution or saline alone. After 8 days, experimental autoimmune encephalomyelitis was induced by subcutaneous injection of a combination of guinea pig spinal cord homogenate and *Mycobacterium tuberculosis* type H37RA. Feeding continued for 28 days, and body weight and neurological signs were recorded daily.

The animals receiving *L. casei* strain Shirota showed signs of experimental autoimmune encephalomyelitis sooner than did the controls and continued to exhibit symptoms longer; the symptoms were also significantly more severe. The authors suggested that this was a result of alteration of the Th1/Th2 balance, and noted that, "Although Th1-inhibiting properties of probiotics could be beneficial in some immune related diseases, Th1 stimulation may have harmful consequences for the development of certain autoimmune disorders."

Because *L. casei* strain Shirota may modulate the production of cytokines, which may affect drug metabolism, Kato et al. (2007) investigated the effect of orally administered *L. casei* strain Shirota on the absorption and pharmacokinetics of nifedipine. Seven-week-old male Wistar ST rats (number not reported) were gavaged daily for 4 weeks with 10^9 cfu *L. casei* strain Shirota in 0.5 ml water or were not treated. A 10-cm segment of jejunal intestine was ligated to make a closed intestinal loop and nifedipine was administered into the loop. Blood samples were taken at time 0 and at 5, 10, 15, 20, 30, 45, 60, 90, 120, and 180 minutes after administration. For comparison, two other groups of rats were treated similarly but were administered nifedipine intravenously.

Ingestion of *L. casei* strain Shirota significantly increased the absorption of nifedipine administered into the intestinal loop, but the disposition of nifedipine after intravenous administration was not altered. The authors suggested that the increased absorption might reflect a reduction in the metabolic extraction by CYP3A in the intestinal mucosa, but conceded that this is not yet clear. No adverse effects were reported as a result of the 4-week exposure of the rats to *L. casei* strain Shirota.

Ezendam and van Loveren (2008) tested the effect of administration of *L. casei* strain Shirota during lactation on later induced autoimmune encephalomyelitis. Two-week-old male and female Lewis rats (LEW/HanHsd) were cross-suckled among the dams and received a daily gavage of $2-4 \times 10^8$ cfu *L. casei* strain Shirota or saline/peptone alone until weaning at 21 days. Eight pups/sex/group were injected subcutaneously with guinea pig myelin basic protein and mycobacterium tuberculosis to induce acute autoimmune encephalomyelitis while 4 pups/sex/group received only saline/peptone. After induction, the pups remained on their assigned *L. casei* strain Shirota treatment and body weight and neurological signs were assessed daily.

There was no difference between rats treated with *L. casei* strain Shirota and vehicle-control rats in the day of onset of experimental autoimmune encephalomyelitis; several measures

of severity showed increases due to ingestion of *L. casei* strain Shirota, but differences were most often not significant. However, the duration of symptoms was significantly extended in the *L. casei* strain Shirota group. The authors interpreted the data as confirming that *L. casei* strain Shirota has moderate immunostimulatory properties and suggested that immune effects are not necessarily beneficial and are poorly understood, especially in infants.

de Jonge et al. (2008) used the Brown Norway rat model to investigate the ability of *L. casei* strain Shirota to modulate the allergic response to peanut extract. Brown Norway rats were bred for 3 generations on a peanut and soy protein-free diet; 3-4 week-old female rats, housed two-to-a-cage with free access to feed and water, were used for the study. The rats were gavaged with $1-2 \times 10^9$ cfu *L. casei* strain Shirota suspended in saline or saline solution alone; after 7 days they began sensitization by daily gavage with 0, 1, or 10 mg peanut extract for 42 days (8 rats/treatment/ sensitization level). Blood samples were taken biweekly for analysis of allergen-specific IgE and IgG titers and rat mast cell protease II. On day 49, rats were orally challenged by gavage with 100 mg peanut extract. Blood samples were taken for hematology, and 30 minutes later the animals were sacrificed by exsanguination and spleens and mesenteric lymph nodes were removed.

Rats receiving *L. casei* strain Shirota showed modest but significantly higher titers of antigen-specific IgE, IgG1, and IgG2a. There was also a significant shift of the Th1/Th2 ratio toward Th2. No difference was seen in lymphocytes, neutrophils, monocytes, or eosinophilic granulocytes, nor in release of rat mast cell protease II. With regard to cytokines, treatment with *L. casei* strain Shirota significantly increased IL-4 levels but had no effect on IFN- γ . The authors reported no adverse effects on the rats from administration of *L. casei* strain Shirota, but concluded that the data did not support a hypothesis of a down-regulation of food allergic responses.

Oishi et al. (2008) investigated whether the probiotic bacteria *Bifidobacterium breve* or *L. casei* strain Shirota could have a protective effect against dietary exposure to bisphenol A. In the first of 2 experiments, 10-week-old female Fischer 344 rats, individually housed in metal cages with free access to feed and water, were divided into 5 groups (8 rats/group) and fed for 4 days on AIN-76 diet alone (control), AIN-76 diet containing either 2.5% or 5% lyophilized heat-killed *B. breve*, or AIN-76 diet containing either 2.5% or 5% lyophilized heat-killed *L. casei* strain Shirota. The rats then were fed 10 g feed containing 0.1 mg bisphenol A and resumed their assigned diets for 6 additional days. Feces were collected each day to measure free bisphenol A, and feed consumption and body weight were recorded every 2 days. In the second experiment, the rats were divided into 3 groups of n=6 to receive AIN-76 alone for 5 days or AIN-76 with 5% lyophilized heat-killed *B. breve* or 5% lyophilized heat-killed *L. casei* strain Shirota. They then received 10 mg bisphenol A/kg bw by gavage and blood was collected from the tail vein after 0.5, 2, 4, and 6 hours for measurement of bisphenol A.

In the first experiment, no difference was seen in feed consumption or body weight gain. Ingestion of either *B. breve* or *L. casei* strain Shirota reduced absorption of bisphenol A in a dose-dependent manner. As compared to the control group, the amount of bisphenol A excreted in the feces of rats receiving diets containing 2.5% probiotic was 1.5-1.8 times higher, while excretion in the rats receiving 5% probiotic was 2.4-2.5 times higher; only the latter difference was statistically significant. The total weight of feces as well as the concentration of bisphenol A in the feces also increased dose-dependently, again reaching statistical significance only for the 5% probiotic group v. the controls. As would be expected from the increase in bisphenol A excretion

resulting from probiotic ingestion, direct measurement of blood levels of bisphenol A confirmed that feeding diets with 5% either *B. breve* or *L. casei* strain Shirota significantly reduced its concentration. The authors reported no adverse effects from the administration of probiotics and suggested that the reduction of bisphenol A entry into the circulating blood was due to promotion of excretion into the feces.

To evaluate the safety of the probiotic bacterial strains *L. casei* strain Shirota and *B. breve* strain Yakult, Kobayashi et al. (2010) administered them orally to male and female Lewis (LEW/CrI) rats with experimental autoimmune encephalomyelitis, the experimental model of human multiple sclerosis, in 3 experiments. In the first experiment, using homogenate of guinea pig spinal cord as the sensitizing antigen, $1-2 \times 10^9$ cfu *L. casei* strain Shirota was administered daily to eight 6-week-old male rats by gavage. After 7 days, the antigen was injected into their hind footpads along with *Mycobacterium tuberculosis* type H37RA, after which probiotic treatment continued for 28 more days. Control rats (n = 8) received an equal volume of saline/peptone. In the second experiment, guinea pig myelin basic protein was used as the sensitizing antigen; the experimental procedure and administration of *L. casei* strain Shirota was the same as that of the first experiment. The third experiment also used guinea pig myelin basic protein as the sensitizing antigen, but the experimental animals were newborn male and female Lewis rats (8 male and female rats/group) that received daily gavage containing $9.2-10.1 \times 10^9$ cfu *L. casei* strain Shirota, $5.0-6.9 \times 10^9$ cfu *B. breve* strain Yakult, or saline/peptone alone beginning at 2 weeks of age. The rats were inoculated with myelin basic protein and *M. tuberculosis* type H37RA at 7 weeks of age, and probiotic treatment continued until the rats reached the age of 11 weeks. In all 3 experiments, the rats were weighed and neurological symptoms scored during the experimental period. In experiments 1 and 2, myelin basic protein specific IgG antibody levels in sera were measured on day 28. After sacrifice, spinal cord tissue was collected for examination of histopathology.

In none of the three experiments did the body weights of the rats differ between experimental groups and controls. While there was a tendency for probiotic treatment to delay the appearance of neurological symptoms, this never reached statistical significance. Probiotic treatment had no effect on the levels of IgG antibodies nor on the severity of spinal cord histopathology. The authors concluded that these results “support the notion that neither [*L. casei* strain Shirota] nor [*B. breve* strain Yakult] exacerbates autoimmune disease.”

4.4.1.1.4. Rabbits

Ogawa et al. (2001) used the infant rabbit infection model with Shiga toxin-producing *Escherichia coli* to study the effects of oral administration of *L. casei* strain Shirota. One-day-old neonatal Japanese White rabbits were isolated from their dams, housed individually in polypropylene cages, and fed milk by gavage twice a day in the following amounts: days 1 and 2, 3 ml; days 3 and 4, 5 ml; days 5 and 6 and the morning of day 7, 7 ml. Fourteen neonatal rabbits received only milk, while 18 received milk with 10^8 cfu *L. casei* strain Shirota/ml. On day 3, all animals were orally inoculated with *E. coli* and thereafter were weighed daily and checked for diarrhea until day 10 (7 days after inoculation), when they were sacrificed. The GI tract was dissected for enumeration of *E. coli* and *L. casei* strain Shirota; determination of pH and concentrations of short-chain fatty acids; assay for Shiga toxin concentration; titration of specific IgA against Shiga toxin and intact *E. coli* cells; and histological examination.

Most of the rabbits began to show diarrhea within 3 days of *E. coli* inoculation; by day 7 after inoculation 77.3% of the control rabbits but only 16.0% of the rabbits treated with *L. casei* strain Shirota suffered from severe diarrhea. Although *L. casei* strain Shirota did not delay the onset of diarrhea, it significantly reduced its severity. GI tract counts of viable *E. coli* were significantly (about 100-fold) lower in the *L. casei* strain Shirota group than among the controls. No difference was seen in the pH of the GI contents between test and control animals, although lactic acid levels were higher in the rabbits that had ingested *L. casei* strain Shirota. Concentrations of Shiga toxin were significantly lower in treated rabbits than in controls; specific anti-Shiga IgAs were not different in the small intestine, but were significantly higher in the colon among the rabbits treated with *L. casei* strain Shirota than among the controls. Finally, there was little histopathological change in the treated animals but extensive vacuolation of epithelial cells and necrosis in the controls.

No adverse effects due to ingestion of *L. casei* strain Shirota were reported in this study, and the authors concluded that preventive administration of this strain of bacteria to infants may lead to enhanced resistance to acute *E. coli* infection due to acceleration of specific humoral immune response to the organism and its toxin.

4.4.1.2. Unpublished Studies

A series of toxicity studies was conducted in rats to evaluate the oral toxicity of *L. casei* strain Shirota. Since these studies have not been published they are regarded only as corroborative of the safety of the strain. In the first unpublished study, 5 male and 5 female 6-week-old Crj:CD(SD) rats (males weighing 168.8—177.8 g and females weighing 130.9—143.1 g) received by gavage a single dose of 20 ml skim milk containing 0 or 10^{12} cfu *L. casei* strain Shirota/kg bw (Takahashi et al. 1993a). No deaths or changes in general condition or body weights were noted throughout the 14-day observation period, and the necropsy revealed no abnormalities attributable to administration of the test substance.

In an unpublished study, groups of 10 male and 10 female 6-week-old Crj:CD(SD) rats were assigned to five test conditions: an untreated control group and groups that had free access to 10 ml/kg bw/day of water with dissolved skim-milk powder and sucrose and providing 0, 10^9 , 10^{10} , or 10^{11} cfu/day of *L. casei* strain Shirota for 30 days (Takahashi et al. 1993b). Male rats weighed 155.0—182.7 g and females weighed 122.2—153.7 g at the start of the experiment. The rats were individually caged in wire-mesh cages and given free access to rat chow and water. The stability of the test article was tested throughout the study; no deviations were found.

The animals were observed twice daily and weighed weekly; water and feed consumption were measured weekly and feed efficiency was calculated. Urine was collected in the final week and tested for volume, pH, specific gravity, osmotic pressure, sodium, potassium, creatinine, protein, sugars, ketone bodies, bilirubin, urobilinogen, and occult blood. Ophthalmologic examinations were conducted before treatment and one week before necropsy; the anterior segment of the eye was grossly observed and fundus photography was performed. After the final dosing, blood was collected by abdominal aortal cannulation and the following hematological tests conducted: prothrombin time, activated partial thromboplastin time, red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, differential white blood cell count, and reticulocyte count. Clinical chemistry measures included transaminase, alkaline phosphatase, creatinine, urea nitrogen, total bilirubin, total cholesterol,

triacylglycerol, glucose, calcium, inorganic phosphorus, sodium, potassium chloride, total protein, albumin, and globulin. After sacrifice, all organs and tissues were grossly observed and the following organs were excised and weighed: brain, pituitary, thyroid and parathyroid, submandibular gland, thymus, lung and bronchus, heart, liver, spleen, kidney, adrenal gland, testis, seminal vesicle, ventral prostate, ovary, and uterus. The following were subjected to histopathological examination: skin and mammary gland, submandibular gland, mesenteric lymph node, bone and bone marrow (sternum and right femur), thymus, trachea, lung and bronchus, heart, thyroid and parathyroid, tongue, esophagus, stomach, duodenum, small intestine, large intestine, liver, pancreas, spleen, kidney, adrenal gland, urinary bladder, seminal vesicle, prostate, testis, ovary, uterus, vagina, brain, pituitary, thoracic marrow, eyeball, and Harderian gland.

There were no deaths, no differences in general conditions, and no differences in feed or water intake, weight gain, or feed efficiency. Ophthalmoscopic examinations found no differences between groups. Some incidental differences were seen in the urinalysis, hematology, and clinical chemistries, but none exhibited dose-response relationships and were not judged toxicologically significant. No differences in absolute or relative organ weights were judged to be test-article related or toxicologically significant. The NOAEL in this study was the highest dose tested, 10^{11} cfu *L. casei* strain Shirota/day, equivalent to approximately 5×10^{11} cfu/kg bw/day.

In a second repeated-dose study, groups of 10 male and 10 female 6-week-old Crj:CD(SD) rats were randomly assigned to five test conditions: an untreated control group and groups that had free access to 10 ml/kg bw/day of water with dissolved skim-milk powder and sucrose and providing 0, 10^9 , 10^{10} , or 10^{11} cfu/day of *L. casei* strain Shirota for 6 months (Takahashi et al. 1997). Male rats weighed 195.5—238.3 g and females weighed 135.1—164.5 g at the start of the experiment. The rats were individually caged in wire-mesh cages and given free access to rat chow and water. The stability of the test article was tested throughout the study; no deviations were found. Dosing and measurements were as described for the 1-month study above (Takahashi et al. 1993b), except that measurement of body weight, feed consumption, and water intake was performed weekly only through treatment week 13, and then every 4 weeks thereafter.

One male rat in the lowest dose group was found dead on day 115 of treatment and one high-dose female was moribund and sacrificed on day 164; neither case was regarded as test-article related. No significant differences were evident between groups in general conditions on observation; the few clinical observations recorded were not dose dependent and were regarded as unlikely to have been influenced by the administration of the test article. There were no significant differences between groups in water or feed intake, in feed efficiency, or in body weight gain. Neither urinalysis nor ophthalmology revealed any significant differences between groups, nor did the hematology or clinical chemistry measures. There were no dose-related differences in absolute or relative organ weights. Although a number of isolated lesions were found during histopathological examination of rats in the groups receiving *L. casei* strain Shirota (including 2 cases of mammary gland carcinoma and 1 case each of buccal ameloblastoma, trigeminal nerve neurinoma, and pituitary adenoma), none were judged to be test-article related. The NOAEL in this study was the highest dose tested, 10^{11} cfu *L. casei* strain Shirota/day, equivalent to approximately 5×10^{11} cfu/kg bw/day.

To further investigate the possible effect of oral administration of *L. casei* strain Shirota on the development or progression of tumors, the 6-month oral toxicity study was repeated (Takahashi et al. 1998). Four groups of 10 male and 10 female 6-week-old Crj:CD(SD) rats were randomly assigned to have free access to 10 ml/kg bw/day of water with dissolved skim-milk powder and sucrose and providing 0, 10^9 , 10^{10} , or 10^{11} cfu/day of *L. casei* strain Shirota for 6 months. Male rats weighed 206.5—231.2 g and females weighed 144.0—175.0 g at the start of the experiment. The rats were individually caged in wire-mesh cages and given free access to rat chow and water. The stability of the test article was tested throughout the study; no deviations were found. Dosing and measurements were as described for the 6-month study discussed above (Takahashi et al. 1997),

There was no mortality, although one low-dose male was judged to be moribund and sacrificed on day 108; post-mortem examination revealed malignant reticulosis of the cerebrum. The clinical observations did not differ between groups, and there were no differences in weight gain. Feed and water intake results were not reported. There were no dose-related differences in absolute or relative organ weights. All organs were examined macroscopically with no significant findings; those organs with suspected neoplastic lesions (based on organ weight or gross examination) were examined microscopically. Including the moribund male, the following four lesions were identified:

- 1 control-group female: hyperplasia of the adrenal glands
- 1 low-dose male: malignant reticulosis of the brain
- 1 low-dose female: hyperplasia of mammary and pituitary glands
- 1 high-dose female: hyperplasia of the mammary gland

These findings were regarded as spontaneous events not attributable to administration of the test article because they occurred with low incidence and were not dose-dependent. The NOAEL in this study was again the highest dose tested, 10^{11} cfu *L. casei* strain Shirota/day, equivalent to approximately 5×10^{11} cfu/kg bw/day.

Table 6. Studies of *L. casei* Strain Shirota in Animals.

Reference	Objective	Study Design	Animal Model	<i>L. casei</i> Strain Shirota Dose and Administration	Duration	Safety-Related Results
Mouse Studies						
Baken et al. (2006)	Study the role of <i>L. casei</i> strain Shirota in modulation of Th1-mediated immune response	<i>L. casei</i> strain Shirota suspended in saline or saline alone prior to sensitization with different doses of 2,4-dinitrochlorobenzene	6-8-week-old male BALB/c mice	0 or 2×10^8 cfu via gavage	8 days	<i>L. casei</i> strain Shirota treatment significantly reduced the cell proliferation in the lymph nodes, inconsistent with previous findings of a stimulatory effect. The authors suggested that probiotics, including <i>L. casei</i> strain Shirota, may affect maturation and function of dendritic cells, resulting in either inhibitory or stimulatory effect.
De Waard et al. (2003)	Studied the effects of <i>L. casei</i> strain Shirota on immunological memory to <i>L. monocytogenes</i> infection	<i>L. casei</i> strain Shirota suspended in saline or saline alone prior to infection with <i>L. monocytogenes</i>	6-7-week-old male BALB/c mice	0 or 10^9 cfu via gavage	10 days	<i>L. casei</i> strain Shirota-treated mice showed significantly higher antigen-specific delayed sensitivity, but there was no difference in the <i>L. monocytogenes</i> loads in the spleen.
Ezendam and van Loveren (2008)	Test the effect of administration of <i>L. casei</i> strain Shirota during lactation on later respiratory allergy	<i>L. casei</i> strain Shirota suspended in saline or saline alone prior to sensitization with ovalbumin	Both 6-8-week-old and 2-week old male and female BALB/c mice	0 or $2-4 \times 10^8$ cfu via gavage	21 days	Mice sensitized with ovalbumin showed significantly increased numbers of eosinophils and lymphocytes on challenge; ingestion of <i>L. casei</i> strain Shirota did not have a consistent significant effect. Nor did <i>L. casei</i> administration have a significant effect on titers of ovalbumin-specific IgE or IgG1. Administration of <i>L. casei</i> strain Shirota during lactation had no effect on cytokine production, but <i>L. casei</i> ingested by adult mice resulted in significant increases in Th2 cytokines but not in IFN- γ . <i>L. casei strain Shirota</i> administration during lactation, but not during adulthood, significantly enhanced the inflammatory lung response.

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Table 6. Studies of *L. casei* Strain Shirota in Animals.

Reference	Objective	Study Design	Animal Model	<i>L. casei</i> Strain Shirota Dose and Administration	Duration	Safety-Related Results
Herias et al. (2005)	Study the effect of <i>L. casei</i> strain Shirota on induction of ulcerative colitis by dextran Na sulfate	<i>L. casei</i> strain Shirota suspended in saline or saline alone prior to induction of ulcerative colitis by dextran Na sulfate	8-week-old male BALB/c-OlaHsd mice	0 or 10 ⁸ cfu via gavage	17 days	<i>L. casei</i> strain Shirota was isolated from the feces of all mice receiving it for 17 days, but only in some of those who received it for shorter periods. Induction of colitis resulted in a significant increase in the population of <i>Enterobacteriaceae</i> , a rise that was not different in the <i>L. casei</i> and control groups. <i>L. casei</i> strain Shirota treatment significantly reduced the weight loss and the gain in spleen weight that resulted from colitis, as well as the loss of red blood cells and decreases in hemoglobin and hematocrit. There was no difference in histopathology between the test and control groups with induced colitis. There was no indication of adverse effects from <i>L. casei</i> strain Shirota administration.
Hori et al. (2002)	Assess the effect of administration of <i>L. casei</i> strain Shirota on the cellular immune system in aged mice exposed to influenza virus	Fed either <i>L. casei</i> strain Shirota or control and intranasally exposed to influenza virus	15-month-old female BALB/c mice	Dose not reported; given in the diet	4 months	NK cell activity in the <i>L. casei</i> strain Shirota group was significantly higher than in the control group, as were the concentrations of IFN- γ and TNF- α . In the mice exposed to influenza virus, the viral titer in the <i>L. casei</i> group was significantly lower than in the control group. No adverse effects were reported.
Hori et al. (2003)	Assess the effect of administration of <i>L. casei</i> strain Shirota on NK cell activity of blood mononuclear cells	Fed either <i>L. casei</i> strain Shirota or control and intranasally exposed to influenza virus	15-month-old female BALB/c mice	0 or 0.05% dietary concentration of heat-killed <i>L. casei</i> cells	2 months	<i>L. casei</i> ingestion significantly increased NK cell activity in both mononuclear cells and splenocytes. The authors interpreted this finding as indicating that <i>L. casei</i> strain Shirota may augment the NK activity of peripheral blood mononuclear cells in healthy low-NK individuals and the elderly. No adverse effects were reported due to the <i>L. casei</i> treatment.

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Table 6. Studies of *L. casei* Strain Shirota in Animals.

Reference	Objective	Study Design	Animal Model	<i>L. casei</i> Strain Shirota Dose and Administration	Duration	Safety-Related Results
Kato et al. (1998)	Assess the effect of <i>L. casei</i> strain Shirota on development of type II collagen-induced arthritis	Administered <i>L. casei</i> strain Shirota suspended in saline or saline alone prior to injection of Bovine type II collagen	8-week-old male inbred DBA/1 mice	0, 0.25x10 ⁹ , 0.5x10 ⁹ , 10 ⁹ , or 2x10 ⁹ cfu via gavage	10 weeks	<i>L. casei</i> strain Shirota significantly delayed or prevented the development of induced arthritis, significantly reduced anti-CII antibodies, significantly reduced secretion of IFN-γ from splenocytes, significantly reduced the severity of the symptoms. The authors concluded that "oral administration of [<i>L. casei</i> strain Shirota] was able to modify the humoral and cellular immune responses to [type II collagen]." No adverse effects were reported attributable to the administration of <i>L. casei</i> strain Shirota.
Kobayashi et al. (2010)	Evaluate the safety of <i>L. casei</i> strain Shirota and <i>B. breve</i> strain Yakult in auto-immune disease	Administered <i>L. casei</i> strain Shirota or <i>B. breve</i> strain Yakult or saline/peptone prior to and following injection of sensitizing allergen & <i>M. tuberculosis</i> type H37RA	2- or 6-week-old male & female Lewis (LEW/Crl Crlj) rats	1-2x10 ⁹ or 9.2-10.1x10 ⁹ cfu <i>L. casei</i> strain Shirota/day	35 days or 63 days	The body weights of the rats did not differ between experimental groups and controls. While there was a tendency for probiotic treatment to delay the appearance of neurological symptoms, this never reach statistical significance. Probiotic treatment had no effect on the levels of IgG antibodies nor on the severity of spinal cord histopathology. The authors concluded that these results "support the notion that neither [<i>L. casei</i> strain Shirota] nor [<i>B. breve</i> strain Yakult] exacerbates autoimmune disease."
Kobayashi et al. (2011)	Assess the safety of <i>L. casei</i> strain Shirota in dysregulated immune conditions	2 models of experimental autoimmune encephalomyelitis (EAE), <i>L. casei</i> strain Shirota given beginning a week before immunization	7-week-old female SJL/J and C57BL/6 mice	0.6-1.2x10 ⁹ cfu <i>L. casei</i> strain Shirota/day	Up to 8 weeks	2 control and 1 test-group SJL/J mice died of acute EAE. <i>L. casei</i> strain Shirota resulted in nonsignificantly improved neurological symptoms with no exacerbation of any symptom or histopathological changes. No deaths were reported among the C57BL/6 mice and there were no significant differences in any endpoints between the test and control groups. Although <i>L. casei</i> strain Shirota transiently increased IL-17 production by inguinal lymph nodes (but not the spleen), this had no effect on histopathology or neurological symptoms. The authors concluded that "oral administration of [<i>L. casei</i> strain Shirota] does not exacerbate but instead may improve EAE."

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Table 6. Studies of *L. casei* Strain Shirota in Animals.

Reference	Objective	Study Design	Animal Model	<i>L. casei</i> Strain Shirota Dose and Administration	Duration	Safety-Related Results
Matsumoto et al. (2005)	Study the effect of <i>L. casei</i> strain Shirota on chronic inflammatory bowel disease	Fed either <i>L. casei</i> strain Shirota or control prior to induction of ulcerative colitis by dextran Na sulfate	10-week-old female BALB/c mice and 20 15-week-old female SAMP1/Yit mice	0 or 0.05% dietary concentration of heat-killed <i>L. casei</i> cells	25 weeks	<i>L. casei</i> -treated mice showed significantly less weight loss, diarrhea, and occult blood, as well as significantly reduced mortality. Production of IL-6 and IFN- γ was significantly down-regulated while IL-4 was up-regulated. In the SAMP1/Yit induced-ileitis mice, ingestion of <i>L. casei</i> strain Shirota significantly improved histological scores compared to controls. The authors concluded that <i>L. casei</i> strain Shirota may be a safe and useful probiotic for the treatment of IBD.
Matsuzaki et al. (1997)	Study the effect of <i>L. casei</i> strain Shirota on the incidence of diabetes in an IDDM model	Fed either <i>L. casei</i> strain Shirota or control	4-week-old inbred specific pathogen-free female nonobese diabetic mice	0 or 0.05% dietary concentration of heat-killed <i>L. casei</i> strain Shirota cells	36 weeks	Disappearance of insulin-secreting β cells in Langerhans islets and the incidence of diabetes in the treatment groups was significantly lower than in the control group. The treated group also showed an increased ratio of CD45R+ B-cells relative to CD8+ T-cells in the spleen as well as higher production of IL-2 and lower production of IF- γ . No adverse effects were reported.
Matsuzaki et al. (1998)	Evaluate the effect of <i>L. casei</i> strain Shirota on immunoglobulin E (IgE) and cytokine production	Fed either <i>L. casei</i> strain Shirota or control prior to injection with ovalbumin and aluminum hydroxide	7-week-old inbred male BALB/c mice	0, 0.05, or 0.1% dietary concentration of <i>L. casei</i> strain Shirota cell mass	21 days	Heat-killed <i>L. casei</i> strain Shirota significantly inhibited production of both total and ovalbumin-specific IgE in a dose-dependent manner. <i>L. casei</i> also significantly stimulated splenic production of Th1 cytokines such as IFN γ and IL-2 (which augment cell-mediated immunity) as well as IL-12, while inhibiting production of Th2 cytokines such as IL-4, IL-5, IL-6, and IL-10 (which augment humoral immunity).
Sgouras et al. (2004)	Study the inhibitory effect of <i>L. casei</i> strain Shirota on <i>Helicobacter pylori</i>	<i>L. casei</i> strain Shirota suspended in drinking water or water alone after infection with <i>Helicobacter pylori</i>	6-week-old female C57BL/6 mice	0 or 10 ⁸ cfu in the drinking water	9 months	<i>L. casei</i> strain Shirota was detected in feces but not in intestinal tissue samples. The authors concluded that the strain does not colonize the gut epithelium and therefore is present in the biome only as long as it is continuously administered. Treatment with <i>L. casei</i> strain Shirota significantly reduced <i>H. pylori</i> populations and gastric mucosal inflammation. No adverse reactions were reported due to the long-term administration of <i>L. casei</i> strain Shirota.

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Table 6. Studies of *L. casei* Strain Shirota in Animals.

Reference	Objective	Study Design	Animal Model	<i>L. casei</i> Strain Shirota Dose and Administration	Duration	Safety-Related Results
Takagi et al. (2001)	Investigate the ability of <i>L. casei</i> strain Shirota to stimulate production of NK cells in response to induced carcinogenesis	Fed either <i>L. casei</i> strain Shirota or control prior to injection with 3-methyl-cholanthrene	7-week-old C3H/HcN male mice and C57BL/6J and C57BL/6 (beige) female mice	Dose not reported; given in the diet	15 weeks	There were no significant differences between the test and control groups in feed intake, body weight, or liver or spleen weights. Tumor incidence at 6 and 11 weeks post-induction was 33% and 83% in controls vs. 0% and 42% in the <i>L. casei</i> strain Shirota group. The number of NK cells and NK cell cytotoxicity were significantly enhanced in splenocytes from the <i>L. casei</i> strain Shirota group. However, no tumor-suppressive effect of <i>L. casei</i> strain Shirota was evident. No adverse effects were noted.
Yasui et al. (2004)	Assess the ability of <i>L. casei</i> strain Shirota to protect against influenza infection	<i>L. casei</i> strain Shirota suspended in saline or saline alone prior to influenza infection	2-day-old male and female BALB/c mice and 2-, 3-, 5-, 7-, and 13-week-old female BALB/c mice	0 or 4×10^8 cfu via gavage	3 weeks	<i>L. casei</i> strain Shirota significantly increased pulmonary NK cell activity and IL-12 production in the mediastinal lymph nodes, lowered the viral titers, decreased the severity of influenza symptoms, and lowered the mortality rate (14.3% of treated mice v. 40.0% of control mice) in all age groups. The authors did not report any adverse effects.
Hamster Studies						
Nishino et al. (2000)	Study the ability of <i>L. casei</i> strain Shirota to increase oxygen scavenging activity	Received skim milk, heat-treated skim milk, or heat-treated skim milk with <i>L. casei</i> strain Shirota	6-week-old male golden Syrian hamsters	0 or 10^9 cfu in heat-treated skim milk	14 days	The heat treatment significantly increased the radical scavenging activity of the skim-milk product, and fermentation with <i>L. casei</i> strain Shirota further significantly increased it. There were no differences in feed consumption or feed-efficiency ratio, and no adverse effects were apparent.

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Table 6. Studies of *L. casei* Strain Shirota in Animals.

Reference	Objective	Study Design	Animal Model	<i>L. casei</i> Strain Shirota Dose and Administration	Duration	Safety-Related Results
Rat Studies						
Baken et al. (2006)	Study immune modulatory effects of <i>L. casei</i> strain Shirota	<i>L. casei</i> strain Shirota suspended in saline or saline alone prior to induction of experimental autoimmune encephalomyelitis	6-8-week-old SPF male Lewis rats (LEW/Han Hsd)	0 or 10 ⁹ cfu via gavage	36 days	The animals receiving <i>L. casei</i> strain Shirota showed symptoms of experimental autoimmune encephalomyelitis sooner than did the controls and continued to exhibit symptoms longer; the symptoms were also significantly more severe. The authors suggested that this was a result of alteration of the Th1/Th2 balance, and that changes which may be beneficial in some immune related diseases may be detrimental in the development of certain autoimmune disorders.
de Jonge et al. (2008)	Investigate the ability of <i>L. casei</i> strain Shirota to modulate the allergic response to peanut extract	<i>L. casei</i> strain Shirota suspended in saline or saline alone prior to sensitization with 0, 1, or 10 mg peanut extract	3-4 week-old female Brown Norway rats	0 or 1-2x10 ⁹ cfu via gavage	49 days	Rats receiving <i>L. casei</i> strain Shirota showed significantly higher titers of antigen-specific IgE, IgG1, and IgG2a and a significant shift of the Th1/Th2 ratio toward Th2. No difference was seen in lymphocytes, neutrophils, monocytes, or eosinophilic granulocytes, nor in release of rat mast cell protease II. <i>L. casei</i> strain Shirota significantly increased IL-4 levels but had no effect on IFN-γ. The authors reported no adverse effects on the rats from administration of <i>L. casei</i> strain Shirota.
De Waard et al. (2001)	Test the effect of <i>L. casei</i> strain Shirota on immune parameters	<i>L. casei</i> strain Shirota suspended in saline or saline alone prior to administration of 10 ³ viable <i>Trichinella spiralis</i> muscle larvae	4-week-old male outbred Wistar (U:Wu) rats and inbred Brown Norway (Rij:Hsd) rats	0 or 10 ⁹ cfu via gavage	7 weeks	<i>L. casei</i> strain Shirota had no effect on terminal body weight, nor on absolute or relative thymus or spleen weights, in either Wistar or Brown Norway rats. Nor was any difference between test and control animals noted during histological examination of thymus and spleen. Both Wistar and Brown Norway rats that received <i>L. casei</i> strain Shirota showed significantly augmented <i>T. spiralis</i> antigen-specific DTH response at both 24 and 48 hours. Both strains of rat showed significantly enhanced serum IgG2b anti- <i>T. spiralis</i> titers with ingestion of <i>L. casei</i> , although other isotypes (IgM, IgG1, IgG2a, IgA, and IgE) did not differ between test and control animals. No effect of the probiotic treatment was noted on parasitic load or on inflammatory response around the larvae.

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Table 6. Studies of *L. casei* Strain Shirota in Animals.

Reference	Objective	Study Design	Animal Model	<i>L. casei</i> Strain Shirota Dose and Administration	Duration	Safety-Related Results
De Waard et al. (2003)	Study the effects of <i>L. casei</i> strain Shirota on immunological memory to <i>L. monocytogenes</i> infection	<i>L. casei</i> strain Shirota suspended in saline or saline alone prior to infection with <i>L. monocytogenes</i>	6-7-week-old male Wistar and Brown Norway rats	0, 10 ⁷ , 10 ⁸ , or 10 ⁹ cfu via gavage	8 weeks	10 ⁹ cfu <i>L. casei</i> strain Shirota/day significantly enhanced the <i>L. monocytogenes</i> -specific delayed sensitivity response at 24 and 48 hours in both Wistar and BN rats. This enhancement was similar whether <i>L. casei</i> administration began 10 days prior or 7 days post-infection. This effect was not seen with administration of heat-killed <i>L. casei</i> , nor with lower doses of 10 ⁷ -10 ⁸ cfu viable <i>L. casei</i> /day. Long-term (8-week) administration of 10 ⁹ cfu viable <i>L. casei</i> /day, but not 10 ⁷ -10 ⁸ cfu viable <i>L. casei</i> or heat-killed <i>L. casei</i> , reduced alanine transaminase activity and enhanced the immune response to <i>L. monocytogenes</i> re-infection as measured by reduced counts of viable <i>L. monocytogenes</i> in the spleen and liver. No adverse effects of <i>L. casei</i> strain Shirota ingestion were reported for any of the rats of either strain.
Ezendam and van Loveren (2008)	Test the effect of <i>L. casei</i> strain Shirota given during lactation on later induced autoimmune encephalomyelitis	<i>L. casei</i> strain Shirota suspended in saline or saline alone prior to induction of acute autoimmune encephalomyelitis	2-week-old male and female Lewis rats (LEW/Han HsD)	0 or 2-4x10 ⁸ cfu via gavage	21 days	There was no difference between <i>L. casei</i> strain Shirota-treated and untreated rats in the day of onset of experimental autoimmune encephalomyelitis; several measures of severity showed increases due to <i>L. casei</i> ingestion, but differences were most often not significant. However, the duration of symptoms was significantly extended in the <i>L. casei</i> group. The authors interpreted the data as confirming that <i>L. casei</i> strain Shirota has moderate immunostimulatory properties and suggested that immune effects are not necessarily beneficial and are poorly understood, especially in infants.
Kato et al. (2007)	Investigate the effect of <i>L. casei</i> strain Shirota on absorption and pharmacokinetics of nifedipine	<i>L. casei</i> strain Shirota suspended in saline or saline alone prior to injection of nifedipine	7-week-old male Wistar ST rats	0 or 10 ⁹ cfu via gavage	4 weeks	<i>L. casei</i> strain Shirota ingestion significantly increased the absorption of nifedipine administered into an intestinal loop, but the disposition of nifedipine after intravenous administration was not altered. No adverse effects were reported as a result of the 4-week exposure of the rats to <i>L. casei</i> strain Shirota.

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Table 6. Studies of *L. casei* Strain Shirota in Animals.

Reference	Objective	Study Design	Animal Model	<i>L. casei</i> Strain Shirota Dose and Administration	Duration	Safety-Related Results
Oishi et al. (2008)	Investigate if <i>B. breve</i> or <i>L. casei</i> strain Shirota has a protective effect against dietary exposure to bisphenol A	Fed AIN-76 diet alone, with <i>B. breve</i> , or with <i>L. casei</i> strain Shirota	10-week-old female Fischer 344 rats	0, 2.5 or 5% dietary concentration of lyophilized heat-killed <i>L. casei</i>	10 days	No difference was seen in feed consumption or body weight gain. Ingestion of either <i>B. breve</i> or <i>L. casei</i> strain Shirota reduced absorption of bisphenol A in a dose-dependent manner. Bisphenol A excreted in the feces of rats receiving 2.5% probiotic was 1.5-1.8 times higher while excretion in the rats receiving 5% probiotic was 2.4-2.5 times higher. The total weight of feces as well as the concentration of bisphenol A in the feces also increased dose-dependently. Blood levels of bisphenol A confirmed that feeding diets with 5% either <i>B. breve</i> or <i>L. casei</i> strain Shirota significantly reduced its concentration. The authors reported no adverse effects from the administration of probiotics.
Takahashi et al. 1993a [Unpublished]	Assess the safety of <i>L. casei</i> strain Shirota	Acute oral toxicity study	6-week-old male and female Crj:CD (SD) rats	0 or 10^{12} cfu/kg bw via gavage	Single dose	No deaths or changes in general condition or body weights were noted over 14 days; necropsy (macroscopic examination) revealed no abnormalities.
Takahashi et al. 1993b [Unpublished]	Assess the safety of <i>L. casei</i> strain Shirota	30-day oral toxicity study	6-week-old male and female Crj:CD (SD) rats	0, 10^9 , 10^{10} , or 10^{11} cfu in water with dissolved skim-milk powder and sucrose	30 days	Ophthalmoscopic examination, urinalysis, clinical observations, hematological analysis, clinical chemistries, necropsy, and histological examination revealed no abnormalities. The NOAEL in this study was the highest dose tested, 10^{11} cfu <i>L. casei</i> strain Shirota/day, equivalent to approximately 5×10^{11} cfu/kg bw/day.
Takahashi et al. 1997 [Unpublished]	Assess the safety of <i>L. casei</i> strain Shirota	6-month oral toxicity study	6-week-old male and female Crj:CD (SD) rats	0, 10^9 , 10^{10} , or 10^{11} cfu in water with dissolved skim-milk powder and sucrose	6 months	Ophthalmoscopic examination, urinalysis, clinical observations, hematological analysis, clinical chemistries, necropsy, and histological examination revealed no abnormalities. Although a number of isolated lesions were found among rats receiving <i>L. casei</i> strain Shirota (including 2 cases of mammary gland carcinoma and 1 case each of buccal ameloblastoma, trigeminal nerve neurinoma, and pituitary adenoma), none were judged to be test-article related. The NOAEL in this study was the highest dose tested, 10^{11} cfu <i>L. casei</i> strain Shirota/day, equivalent to approximately 5×10^{11} cfu/kg bw/day.

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Table 6. Studies of *L. casei* Strain Shirota in Animals.

Reference	Objective	Study Design	Animal Model	<i>L. casei</i> Strain Shirota Dose and Administration	Duration	Safety-Related Results
Takahashi et al. 1998 [Unpublished]	Assess the safety of <i>L. casei</i> strain Shirota; replication to more fully investigate development or progression of tumors	6-month oral toxicity study	6-week-old male and female Crj:CD (SD) rats	0, 10 ⁹ , 10 ¹⁰ , or 10 ¹¹ cfu in water with dissolved skim-milk powder and sucrose	6 months	Ophthalmoscopic examination, urinalysis, clinical observations, hematological analysis, clinical chemistries, and necropsy revealed no abnormalities. All organs were examined macroscopically with no significant findings; organs with suspected neoplastic lesions were examined microscopically. Four lesions were identified: control-group female with hyperplasia of the adrenal glands; low-dose male with malignant reticulosis of the brain; low-dose female with hyperplasia of mammary and pituitary glands; high-dose female with hyperplasia of the mammary gland. These findings were regarded as spontaneous events not attributable to the test article because they occurred with low incidence and were not dose-dependent. The NOAEL in this study was the highest dose tested, 10 ¹¹ cfu <i>L. casei</i> strain Shirota/day, equivalent to approximately 5x10 ¹¹ cfu/kg bw/day.
Rabbit Studies						
Ogawa et al. (2001)	Study the effect of <i>L. casei</i> strain Shirota on the severity of <i>E. coli</i> infection	Given milk with <i>L. casei</i> strain Shirota or milk alone prior to injection with <i>E. coli</i>	1-day-old neonatal Japanese White rabbits	10 ⁸ cfu/ml in milk given as follows: days 1 and 2, 3 ml; days 3 and 4, 5 ml; days 5 and 6 and the morning of day 7, 7 ml	7 days	By day 7 after inoculation 77.3% of the controls and 16.0% of the <i>L. casei</i> -treated rabbits suffered from severe diarrhea. <i>L. casei</i> strain Shirota significantly reduced the severity of diarrhea. GI tract counts of viable <i>E. coli</i> were significantly lower in the <i>L. casei</i> group than among the controls. Lactic acid levels were higher in the rabbits that had ingested <i>L. casei</i> . Concentrations of Shiga toxin were significantly lower in treated rabbits; specific anti-Shiga IgAs were not different in the small intestine, but were significantly higher in the colon. There was little histopathological change in the treated animals but extensive vacuolation of epithelial cells and necrosis in the untreated controls. No adverse effects due to ingestion of <i>L. casei</i> strain Shirota were reported in this study.

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4.4.2. Human Studies

4.4.2.1. Studies in Adults

4.4.2.1.1. Healthy Adults

These studies are summarized in Table 7 at the end of this section.

In an article discussing the effects of probiotics on the intestinal microbiome and urinary metabolites, Tanaka et al. (1981) included a brief report of a study in which 10^{10} cfu/day of *L. casei* strain Shirota was orally administered to 5 healthy 25-32-year-old adults for 5 weeks. The administered strain was recovered at levels of 10^7 to 10^8 cfu/g wet feces, but disappeared within a week after administration ceased. An increase was also seen in counts of bifidobacteria. Urinalysis found significant reductions in indican and phenol concentrations, indicating inhibition of putrefactive fermentation. The authors did not report any adverse findings.

In an investigation of the effect of *L. casei* strain Shirota on the intestinal microbiota and immune parameters, Spanhaak et al. (1998) assigned 20 healthy males aged 40-65 years (mean age = 55.8 years) to receive 100 ml fermented milk 3 times a day, providing either 0 or 3×10^9 cfu *L. casei* (n=10 per treatment) for 4 weeks, with a 2-week follow-up period. Participants' body weight, temperature, blood pressure, and heart rate were measured along with hematological parameters (white and red blood cell counts, platelet counts, hemoglobin concentration, hematocrit, sedimentation rate, and differential leukocyte counts), clinical chemistries (cholesterol, aspartate aminotransferase, alanine aminotransferase, γ -glutamylase, total protein, albumin, protein electrophoresis [albumin, α 1-, α 2-, β -, and γ -globulins], C-reactive protein, and α 1-antitrypsin), and immune indices (natural killer cell activity, cytokine assays including IL-1 β , IL-2, IFN- γ , specific antigen response, and humoral parameters IgM, IgG, IgA, IgC, IgE, and complement factors C3, C4, and B). Fecal samples were collected and analyzed for total anaerobic bacteria, bacteroidaceae, bifidobacteria, lactobacilli, *L. casei* strain Shirota, clostridia, staphylococci, enterococci, enterobacteriaceae, bacilli, and yeasts as well as β -glucosidase, β -glucuronidase, urease, and tryptophanase. The fecal moisture content, pH, and short-chain fatty acids were measured, and intestinal transit time was estimated by feeding carmine red and observing the appearance of red color in feces. Urine samples were collected for analyses of phenol and *p*-cresol concentrations.

No significant changes were observed in body weight, blood pressure, heart rate, temperature, hematological parameters, or clinical chemistries. Fecal bacterial enumerations showed significantly increased *L. casei* strain Shirota and bifidobacteria in those receiving the probiotic; numbers of other microorganisms were not significantly different. Ingestion of *L. casei* also significantly increased the activities of β -glucuronidase and β -glucosidase as well as the moisture content of feces. There was a slight but statistically significant reduction in intestinal transit time through the follow-up period. Levels of acetic, propionic, and butyric acids were significantly reduced through the treatment period, but the difference disappeared during follow-up. No significant effects were noted in any of the immune parameters measured.

The authors concluded that there were no potentially adverse effects on the intestinal microbiota or in immune functioning, and, indeed, that "there were no adverse effects in either the treatment or the control group throughout the study." The study also demonstrated the survival of the ingested *L. casei* strain Shirota in the GI tract.

In a study of the effects of Yakult fermented dairy drink containing *L. casei* strain Shirota on immune function of healthy adults, Nagao et al. (2000) gave 1 bottle per day of Yakult providing 4×10^{10} cfu *L. casei* to 4 healthy males and 5 healthy females aged 20-40 years (mean = 32.7 years) for 3 weeks while a control group of 3 males and 5 females drank a similar beverage not containing probiotic bacteria. Blood was drawn before the study began, 1 and 3 weeks after the initiation of probiotic intake, and 3 and 8 weeks after cessation of intake for analysis of NK cell activity. The group receiving the probiotic showed increased NK cell activity beginning 1 week after the start of intake, remaining elevated until 3 weeks after the end of intake, and returning to earlier levels by 8 weeks after intake ceased. No adverse effects were reported.

In an unpublished prospective, randomized, double blind, placebo-controlled study, Matsumoto et al. (2003) assigned 31 healthy adults (17 males and 14 females with mean age = 34.3 years) to receive 300 ml/day for 14 days of either an experimental version of Yakult beverage providing 3.6×10^{11} cfu *L. casei* strain Shirota (n = 16) or a milk-beverage placebo (n = 15). Participants completed questionnaires addressing stool frequency and characteristics and any side effects such as gastrointestinal effects. There were no differences between the test and placebo groups in stool frequency or the frequency of watery stool; reports of side effects were rare and no differences were seen in reports of abdominal pain or distress but the *L. casei* strain Shirota recipients were significantly more likely to report bloating or flatulence than were the controls.

A second unpublished prospective, randomized, double blind, placebo-controlled study was conducted by Matsumoto et al. (2004) to verify the safety of Yakult 400LT—a milk-based beverage differing from the standard Yakult beverage primarily in the sweetener content. Thirty healthy volunteers (15 of each sex) with mean age = 29.2 years received 240 ml/day of either Yakult 400LT providing 1.2×10^{11} cfu *L. casei* strain Shirota (n = 15) or a matched milk-beverage placebo (n = 15) for 14 days. Participants completed questionnaires addressing stool frequency and characteristics and any side effects such as gastrointestinal effects. There were no differences between the test and placebo groups in stool frequency or the frequency of watery stool; reports of side effects were rare and no differences were seen between the *L. casei* strain Shirota recipients and the controls.

Finally, a third unpublished prospective, randomized, double blind, placebo-controlled study was conducted by Asahara et al. (2004) to verify the safety of Yakult 300V—a milk-based beverage differing from the standard Yakult beverage in containing galactooligosaccharides in addition to 3×10^{10} cfu *L. casei* strain Shirota per 80-ml bottle. Healthy volunteers (n = 28, 15 males and 13 females) with mean age = 29.5 years received 240 ml/day of either Yakult 300V providing 9×10^{10} cfu *L. casei* strain Shirota + GOS (n = 15) or a milk-beverage placebo (n = 13) for 14 days. Participants completed questionnaires addressing stool frequency and characteristics and any side effects such as gastrointestinal effects. The test group had a significantly higher stool frequency than the controls at baseline and continued to have significantly higher frequency throughout the study, but there were no observations of watery stool. Reports of side effects were rare and no significant differences were seen between the *L. casei* strain Shirota + GOS recipients and the controls.

Morimoto et al. (2005) enrolled 99 habitual cigarette smokers in a randomized, double-blind, placebo-controlled trial of the effect of Yakult dairy drink with *L. casei* strain Shirota on natural killer (NK) cell activity. The smokers, all males, had a mean age of 45.5 years. Fifty men drank 80 ml fermented milk/day, providing 4×10^{10} cfu *L. casei* strain Shirota, while the

remaining 49 men drank a matched beverage without the probiotic for 3 weeks. Peripheral blood was drawn before and after the treatment and analyzed for NK activity. Consumption of *L. casei* significantly increased NK activity as compared to drinking the control dairy beverage with no apparent adverse effects.

In a placebo-controlled crossover trial, Takeda et al. (2006) investigated the effect of *L. casei* strain Shirota on NK cell activity in 10 healthy individuals (3 male, 7 female) aged 69-97 years. The participants consumed 80 ml of fermented milk containing 4×10^{10} cfu *L. casei* or placebo for 3 weeks. After a 7-week washout, they consumed the other preparation for 3 weeks. Peripheral blood was drawn before and after each intake period and NK cell activity was measured as well as biochemical parameters (total protein, albumin, creatinine, cholesterol, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, alkaline phosphatase, and γ -glutamyl transpeptidase).

NK cell activity was significantly higher following the probiotic period than the placebo period, but there was no effect on the measured biochemical parameters, indicating an absence of adverse system effects.

Matsumoto et al. (2006) administered 1 80-ml bottle of Yakult fermented dairy drink providing 4×10^9 cfu *L. casei* strain Shirota or a matched control beverage per day to 40 healthy individuals in a randomized, double-blind, placebo-controlled crossover trial. The participants included 7 males and 33 females with an average age of 39.2 years; although they were regarded as "healthy," they all exhibited a "tendency to constipation" as reflected in a less-than-average reported frequency of 3.6 bowel movements per week. Participants consumed either the test or control drinks for 2 weeks, followed by a 3-week washout period, and 2 weeks consumption of the other drink. The number of bowel movements, stool quantity, shape, color, and smell were recorded in journals. Stools were sampled before intake period 1, after each week of the intake period, at the end of the washout, and after each week of intake period 2. Enumerations were completed of total bacteria, *L. casei* strain Shirota, *Bifidobacterium*, *Lactobacillus*, *Bacteroides fragilis*, *Clostridium coccooides*, *Clostridium leptum*, *Atopobium*, *Veillonella*, *Enterobacteriaceae*, *Enterococcus*, *Staphylococcus*, *Candida*, and *Prevotella*. Additionally, the pH and organic-acid content (salts of acetic, butyric, isobutyric, formic, propionic, succinic, valeric, isovaleric, and lactic acids) of the stool were assessed, along with ammonia, indole, phenol, and *p*-cresol.

The probiotic-containing drink, but not the placebo, significantly increased the frequency of bowel movements, with a larger effect in those participants who showed more pre-study tendency toward constipation, but had no effect on stool quality or quantity, pH, or moisture content. Consumption of *L. casei* had no effect on fecal counts of total bacteria, but significantly increased enumerations of *L. casei* strain Shirota, total lactobacilli, bifidobacteria, and *Atopobium*, while significantly reducing counts of *Candida* and *C. leptum*. As compared with placebo, intake of the fermented drink significantly reduced total organic acids, acetic acid, and propionic acid, but had no effect on other organic-acids or on putrefactive metabolites. No adverse effects were reported due to consumption of either the test or placebo dairy drink.

Tuohy et al. (2007) studied the survivability and the impact on intestinal microbiota of *L. casei* strain Shirota in the gastrointestinal tract of healthy adults using a prospective, randomized, double-blind, placebo-controlled design. Twenty healthy adults aged 23 to 70 years were randomized to receive two 65-ml aliquots of either placebo acidified-milk drink (n = 10) or

fermented milk drink providing a daily dose of 6×10^8 cfu *L. casei* strain Shirota (n = 10) for 21 days. Fecal samples were provided on days 0, 7, 14, 21, and 28 and analyzed for counts of *L. casei* strain Shirota by DNA digestion and pulsed field gel electrophoresis (PFGE) and enumeration of selected groups of bacteria using 16S rRNA-targeted oligonucleotide probes and fluorescent *in situ* hybridization (FISH) for total bacteria, bifidobacteria, bacteroides, clostridia, eubacteria, lactobacilli, enterococci, and *E. coli*.

Survivability of *L. casei* strain Shirota was demonstrated: all participants receiving the probiotic strain showed stable and relatively high fecal populations of *L. casei* strain Shirota. Several volunteers still maintained detectable levels of the strain a week after feeding cessation. This of course resulted in a significant increase in the fecal population of lactobacilli, but no significant changes were noted in populations of other bacteria.

In a crossover design, Takeda and Okumura (2007) administered either placebo or 1 bottle of Yakult 400 containing 4×10^{10} cfu *L. casei* strain Shirota to 19 healthy middle-aged (30-45 years) and older (55-75 years) volunteers for 3 weeks, assessing their NK cell activity and other immune function parameters. Blood was taken for analysis at enrollment, 1 and 3 weeks after the start of intake, and 3 and 8 weeks after the completion of intake. In both middle-aged and elderly participants, NK activity was significantly higher in the treatment group than among the controls. Since the numbers of NK cells were not affected, the increased activity appears to be mediated by an increase in cytotoxicity. The authors noted that the health condition of all volunteers remained well during all experimental periods.

De Preter et al. (2007) studied both immediate and long-term effects of probiotics (*L. casei* strain Shirota and *B. breve* Yakult) and prebiotics (oligofructose-enriched inulin) on colonic nitrogen-protein metabolism in healthy humans. Twenty healthy young adults (10 of each sex, mean age = 21 years) were randomly assigned to 2 treatment groups in a placebo-controlled crossover trial. The trial included 3 ingestion periods of 4 weeks each, with 2-week washout periods in between. During the first ingestion period, half of the participants received 10^9 cfu *B. breve* twice a day along with a maltodextrin placebo resembling inulin while the others received 6.5×10^9 cfu *L. casei* twice a day along with the inulin-like placebo. During the second ingestion period, all participants received placebo milk product resembling their probiotics as well as 10 g inulin twice a day. During the third ingestion period, all participants received both probiotic and prebiotic placebos twice a day. Half of the participants also had a fourth ingestion period during which they received both 10 g inulin and 6.5×10^9 cfu *L. casei* twice a day. Urine and fecal samples were collected for analysis at the beginning and end of each ingestion period.

The oligofructose-enriched inulin significantly reduced proteolytic activity in the colon, while both probiotics had smaller but still statistically significant effects. Both of these effects were temporary and disappeared over the 2-week washout periods. No adverse effects were reported due to the interventions.

In a prospective, randomized, placebo-controlled crossover study of 53 healthy adults, De Preter et al. (2008) investigated the effect of several pre- and probiotics on fecal β -glucuronidase and β -glucosidase activity. Twenty-eight men and 25 women aged 19-26 years were randomly assigned to one of 5 treatment groups of n = 10 or 11 and underwent 3 (or 4, for group 5) 4-week treatment periods separated by washout periods. During the treatment periods they received various combinations daily of lactulose, Raftilose® Synergy1 oligofructose-enriched inulin, or

placebo along with *Saccharomyces boulardii*, 2×10^9 cfu *Bifidobacterium breve*, 1.3×10^{10} cfu *L. casei* strain Shirota, or placebo probiotic. Stools were collected during the last 72 hours before the study began and at the end of each treatment period for determination of fecal output, fecal dry weight, and measurement of β -glucuronidase and β -glucosidase activities.

Three participants withdrew because they developed illnesses requiring antibiotic treatment and one for “personal” reasons; no withdrawal was regarded as treatment related. β -glucuronidase activity was significantly reduced by lactulose, oligofructose-enriched inulin, *B. breve*, and *L. casei* strain Shirota; combinations of pre- and probiotics provided no more benefit than either alone. Since β -glucuronidase is believed to promote conversion of procarcinogens into potentially carcinogenic compounds, reduction in its activity is regarded as beneficial.

Yamada et al. (2009; report in Japanese with English abstract) studied risk management in a long-term elderly care facility by giving 39 residents a 65-ml bottle of Yakult a day during December, while 38 controls did not receive the intervention. The fermented dairy drink provided 6.5×10^9 cfu *L. casei* strain Shirota. The impact of the fermented dairy drink on winter-time norovirus-induced gastroenteritis was evaluated, showing no significant difference on incidence but a reduction in the duration of febrile episodes. No adverse effects were reported.

Matsumoto et al. (2010) studied the effect of drinking 1 bottle of Yakult (80 ml providing 4×10^{10} cfu *L. casei* strain Shirota) each day for 4 weeks on the intestinal health of healthy individuals with soft stools. In a prospective, randomized, double-blind, placebo-controlled study, 63 healthy adults who had reported by questionnaire that they had soft stools were screened to select 30 individuals (mean age = 42.6 years) with a high frequency of defecation, high-water-content stools, and high fecal number of enterococci. These participants were randomly assigned to receive either Yakult ($n = 14$, 10 males and 4 females) or a sham Yakult identical with the real product but without *L. casei* strain Shirota ($n = 16$, 10 males and 6 females). After a 2-week run-up, the study participants consumed 1 80-ml bottle of their assigned beverage each day for 4 weeks, and were observed for an additional 2-week follow-up. The volunteers maintained diaries recording defecation frequency, stool quantity and characteristics, and any abdominal pain. Stool samples were taken at the end of the run-up, in the middle and at the end of the 4-week feeding period, and at the end of the follow-up for analysis of fecal microbiota, organic acids, putrefactive metabolites, pH, and water content.

All participants completed the study. Daily ingestion of Yakult significantly reduced defecation frequency but had no effect on quantity. Water content was non-significantly reduced and the pH decreased significantly. Significant increases were seen in total fecal bacteria, bifidobacteria, *Staphylococcus*, and *Candida*, and decreases in enterobacteriaceae; no change was noted in clostridia or enterococci. Organic acids, especially acetate and butyrate, increased significantly in the Yakult group as compared to baseline and to the placebo group, but no difference was seen in the putrefactive metabolites indole, ammonia, phenol, or *p*-cresol. The authors regarded the effects of *L. casei* strain Shirota as a beneficial normalization of the stooling pattern in individuals with soft stools.

The impact of a synbiotic combination of 20 g oligofructose-enriched inulin and 1.3×10^{10} cfu *L. casei* strain Shirota/day on fecal metabolite profiles was evaluated over 4 weeks in 9 apparently healthy young adults, 4 males and 5 females aged 19-23 years (De Preter et al. 2011). Fecal samples were obtained from all participants at baseline, on the first day of the intervention,

and at the end of the intervention period and analyzed for volatile organic compounds as well as bacterial DNA for identification of predominant microbiota.

A total of 139 different volatile organic compounds was identified with a mean of 58 compounds per sample; the number of volatile compounds identified did not change over the intervention period. Ingestion of the synbiotic significantly reduced the levels of sulfur-containing compounds and increased concentrations of acetate and butyrate, particularly the former. There were no significant changes in the predominant microbiota. No adverse effects were reported.

In a randomized, double-blind, placebo-controlled trial (Reale et al. 2011), 72 apparently healthy male cigarette smokers aged 40-60 years (mean age = 50.3 years) were randomized to consume sachets providing 4×10^{10} cfu lyophilized *L. casei* strain Shirota or a placebo powder without the probiotic for 3 weeks; peripheral blood mononuclear cells were isolated and the activity of natural killer cells and the number of CD16⁺ cells were assessed at enrollment and at the end of the intervention period. Participants also completed questionnaires regarding anxiety, occupational stress, and such physical signs as headache, nausea, and stomach ache.

The probiotic group exhibited significantly increased levels of natural killer cell activity and numbers of CD16⁺ cells, but no differences were seen in questionnaire responses. No adverse effects were reported.

Sakai et al. (2011) enrolled 40 apparently healthy adults of both sexes aged 18-65 years (mean age = 33.7 years) who had a tendency to produce “hard or lumpy stools” as defined by the Bristol Stool Form Scale in a randomized, open-label, controlled study in which participants (20/group) received a daily treatment of 65 ml fermented milk drink providing 6.5×10^9 cfu *L. casei* strain Shirota or no intervention for 3 weeks. All participants maintained a diary and collected stool samples 3 times a week.

One individual in the probiotic group was removed from the study due to the use of a restricted medication; the other 39 participants completed the study. Those receiving the probiotic exhibited a significant reduction in the frequency of hard or lumpy stools, while those in the control group had a significant increase. There were no significant differences in stool water content, concentrations of short-chain fatty acids, or discomfort during bowel movements. No adverse effects were reported.

Table 7. Studies of *L. casei* Strain Shirota in Healthy Adults

Reference	Objective	Study Design	Subjects	<i>L. casei</i> Dose and Source	Duration	Safety-Related Results
Asahara et al. (2004) [unpublished]	Study the safety of excessive intake of Yakult containing <i>L. casei</i> strain Shirota + GOS	Prospective, randomized, double blind, placebo-controlled study	28 healthy adults (15 males and 13 females) with mean age = 29.5 years	240 ml/day of Yakult 300V providing 9×10^{10} cfu <i>L. casei</i> strain Shirota + GOS	14 days	The test group had a significantly higher stool frequency than the controls at baseline and continued to have significantly higher frequency throughout the study, but there were no observations of watery stool. Reports of side effects were rare and no differences were seen between the <i>L. casei</i> strain Shirota + GOS recipients and the controls.
De Preter et al. (2007)	Study the immediate and long-term effects of probiotics and prebiotics on colonic nitrogen-protein metabolism in healthy humans.	Randomized placebo-controlled crossover trial.	20 healthy young adults (10 of each sex) with mean age = 21 years	1 bottle of Yakult with 6.5×10^9 cfu <i>L. casei</i> strain Shirota 2x/day with or without oligofructose	4 weeks	<i>L. casei</i> strain Shirota caused a small but statistically significant reduction in proteolytic activity in the colon. The effect was temporary, disappearing within 2 weeks. No adverse effects were reported due to the intervention.
De Preter et al. (2008)	Investigate the effect of pre- and probiotics on fecal β -glucuronidase and β -glucosidase activity.	Prospective, randomized, placebo-controlled crossover study	53 healthy adults (28 males and 25 females) aged 19-26 years	2 x 65 ml Yakult providing 1.3×10^{10} cfu <i>L. casei</i> strain Shirota/day	4 weeks	β -glucuronidase activity was significantly reduced by lactulose, oligofructose-enriched inulin, <i>B. breve</i> , and <i>L. casei</i> strain Shirota; combinations of pre- and probiotics provided no more benefit than either alone.

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Table 7. Studies of *L. casei* Strain Shirota in Healthy Adults

Reference	Objective	Study Design	Subjects	<i>L. casei</i> Dose and Source	Duration	Safety-Related Results
De Preter et al. (2011)	Assess the impact of a synbiotic combination of oligofructose-enriched inulin and <i>L. casei</i> strain Shirota on fecal metabolite profiles	Open-label study	9 apparently healthy young adults, 4 males and 5 females aged 19-23 years	1.3x10 ¹⁰ cfu powdered <i>L. casei</i> strain Shirota + 20 g oligofructose-enriched inulin/ day	4 weeks	139 volatile organic compounds were identified (mean of 58 compounds per sample); the number of volatile compounds did not change over the intervention period. The synbiotic significantly reduced levels of sulfur-containing compounds and increased concentrations of acetate and butyrate, particularly the former. There were no significant changes in the predominant microbiota. No adverse effects were reported.
Matsumoto et al. (2003) [unpublished]	Study the safety of excessive intake of Yakult containing <i>L. casei</i> strain Shirota	Prospective, randomized, double blind, placebo-controlled study	31 healthy adults (17 males, 14 females) with mean age = 34.3 years	300 ml/day of an experimental version of Yakult beverage providing 3.6x10 ¹¹ cfu <i>L. casei</i> strain Shirota/ day	14 days	There were no differences between the test and placebo groups in stool frequency or the frequency of watery stool. Reports of side effects were infrequent and no differences were seen in reports of abdominal pain or distress but the <i>L. casei</i> strain Shirota recipients were significantly more likely to report bloating or flatulence than were the controls.
Matsumoto et al. (2004) [unpublished]	Study the safety of excessive intake of Yakult containing <i>L. casei</i> strain Shirota	Prospective, randomized, double blind, placebo-controlled study	30 healthy adults (15 males, 15 females) with mean age = 29.2 years	240 ml/day of a "light" version of Yakult beverage providing 1.2x10 ¹¹ cfu <i>L. casei</i> strain Shirota/ day	Not reported	There were no differences between the test and placebo groups in stool frequency or the frequency of watery stool; reports of side effects were rare and no differences were seen between the <i>L. casei</i> strain Shirota recipients and the controls.

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Table 7. Studies of *L. casei* Strain Shirota in Healthy Adults

Reference	Objective	Study Design	Subjects	<i>L. casei</i> Dose and Source	Duration	Safety-Related Results
Matsumoto et al. (2006)	Study the effect of <i>L. casei</i> strain Shirota on intestinal microbiome and on constipation.	Randomized, double-blind, placebo-controlled crossover trial	7 males and 33 females with an average age of 39.2 years regarded as "healthy" but with less-than-average frequency of bowel movements	1 80-ml bottle of Yakult fermented dairy drink per day, providing 4×10^9 cfu <i>L. casei</i> strain Shirota	2 weeks	Ingestion of <i>L. casei</i> strain Shirota significantly increased enumerations of <i>L. casei</i> strain Shirota, total lactobacilli, bifidobacteria, and <i>Atopbium</i> , and the frequency of bowel movements, while significantly reducing counts of <i>Candida</i> and <i>C. leptum</i> , and total organic acids, acetic acid, and propionic acid. It had no effect on stool quality or quantity, pH, moisture content, or fecal counts of total bacteria. No adverse effects were reported due to consumption of either the test or placebo dairy drink.
Morimoto et al. (2005)	Assess the effect of <i>L. casei</i> strain Shirota on NK cell activity in smokers.	Randomized, double-blind, placebo-controlled trial	99 male habitual cigarette smokers with a mean age of 45.5 years	80 ml Yakult per day, providing 4×10^{10} cfu <i>L. casei</i> strain Shirota	3 weeks	Consumption of <i>L. casei</i> strain Shirota significantly increased NK activity as compared to drinking the control dairy beverage with no apparent adverse effects.
Matsumoto et al. (2010)	Study the effect of <i>L. casei</i> strain Shirota on intestinal health of healthy individuals with soft stools	Prospective, randomized, double-blind, placebo-controlled study	30 individuals (mean age = 42.6 years) with a high frequency of defecation, high-water-content stools, and high fecal number of enterococci	80 ml Yakult per day, providing 4×10^{10} cfu <i>L. casei</i> strain Shirota	4 weeks	Ingestion of Yakult reduced defecation frequency but had no effect on quantity. Water content was non-significantly reduced and the pH decreased significantly. Significant increases were seen in total fecal bacteria, bifidobacteria, <i>Staphylococcus</i> , and <i>Candida</i> , and decreases in enterobacteriaceae; no change was noted in clostridia or enterococci. Organic acids, especially acetate and butyrate, increased in the Yakult group as compared to baseline and to the placebo group, but no difference was seen in the putrefactive metabolites indole, ammonia, phenol, or <i>p</i> -cresol. The authors regarded the effects of <i>L. casei</i> strain Shirota as beneficial normalization of the stooling pattern in individuals with soft stools.

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Table 7. Studies of *L. casei* Strain Shirota in Healthy Adults

Reference	Objective	Study Design	Subjects	<i>L. casei</i> Dose and Source	Duration	Safety-Related Results
Nagao et al. (2000)	Study the effects of Yakult fermented dairy drink containing <i>L. casei</i> strain Shirota on immune function of healthy adults	Randomized, double-blind, placebo-controlled trial.	7 healthy males and 10 healthy females aged 20-40 years (mean = 32.7 years)	1 bottle per day of Yakult providing 4×10^{10} cfu <i>L. casei</i> strain Shirota	3 weeks	The group receiving the probiotic showed increased NK cell activity beginning 1 week after the start of intake, remaining elevated until 3 weeks after the end of intake, and returning to earlier levels by 8 weeks after intake ceased. No adverse effects were reported.
Reale et al. (2011)	Assess the effect of lyophilized <i>L. casei</i> strain Shirota on NK cell activity and CD16 ⁺ cells in smokers	Randomized, double-blind, placebo-controlled trial	72 apparently healthy male cigarette smokers aged 40-60 years (mean age = 50.3 years)	Sachets providing 4×10^{10} cfu lyophilized <i>L. casei</i> strain Shirota/day	3 weeks	The probiotic group exhibited significantly increased levels of natural killer cell activity and numbers of CD16 ⁺ cells. No adverse effects were reported.
Sakai et al. (2011)	Study the ability of <i>L. casei</i> strain Shirota to normalize stool characteristics among healthy individuals with a tendency to produce hard or lumpy stools	Randomized, open-label, controlled study	40 apparently healthy adults of both sexes aged 18-65 years (mean age = 33.7 years)	65 ml fermented milk drink/day providing 6.5×10^9 cfu <i>L. casei</i> strain Shirota	3 weeks	Those receiving the probiotic exhibited a significant reduction in the frequency of hard or lumpy stools, while those in the control group had a significant increase. There were no significant differences in stool water content, concentrations of short-chain fatty acids, or discomfort during bowel movements. No adverse effects were reported.

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Table 7. Studies of *L. casei* Strain Shirota in Healthy Adults

Reference	Objective	Study Design	Subjects	<i>L. casei</i> Dose and Source	Duration	Safety-Related Results
Spanhaak et al. (1998)	Study the effect of <i>L. casei</i> strain Shirota on the intestinal microbiota, hematology and clinical chemistry, and immune parameters	Randomized, double-blind, placebo-controlled trial.	20 healthy males aged 40-65 years (mean age = 55.8 years)	100 ml fermented milk 3 times a day, providing 3×10^9 cfu <i>L. casei</i> strain Shirota	4 weeks	<p><i>L. casei</i> strain Shirota and bifidobacteria were significantly increased; other microorganisms were not significantly different. There was a statistically significant reduction in intestinal transit time and reduced levels of acetic, propionic, and butyric acids. Ingestion of <i>L. casei</i> also significantly increased the activities of β-glucuronidase and β-glucosidase as well as the moisture content of feces. No significant changes were observed in body weight, blood pressure, heart rate, temperature, hematological parameters (white and red blood cell counts, platelet counts, hemoglobin concentration, hematocrit, sedimentation rate, and differential leukocyte counts), or clinical chemistries (cholesterol, aspartate aminotransferase, alanine aminotransferase, γ-glutamylase, total protein, albumin, protein electrophoresis [albumin, α1-, α2-, β-, and γ-globulins], C-reactive protein, and α1-antitrypsin). No significant effects were noted in any of the immune parameters measured (natural killer cell activity, cytokine assays including IL-1β, IL-2, IFN-γ, specific antigen response, and humoral parameters IgM, IgG, IgA, IgC, IgE, and complement factors C3, C4, and B).</p> <p>The authors concluded that there were no potentially adverse effects on the intestinal microbiota or in immune functioning, and, indeed, that "there were no adverse effects in either the treatment or the control group throughout the study."</p>

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Table 7. Studies of *L. casei* Strain Shirota in Healthy Adults

Reference	Objective	Study Design	Subjects	<i>L. casei</i> Dose and Source	Duration	Safety-Related Results
Takeda et al. (2006)	Study the effect of <i>L. casei</i> strain Shirota on NK cell activity and clinical chemistries of healthy older individuals.	Randomized placebo-controlled crossover trial	3 males and 7 females aged 69-97 years	80 ml of fermented milk containing 4×10^{10} cfu <i>L. casei</i> strain Shirota	3 weeks	NK cell activity was significantly higher following the probiotic period than the placebo period, but there was no effect on the measured biochemical parameters (total protein, albumin, creatinine, cholesterol, glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, alkaline phosphatase, and γ -glutamyl transpeptidase), indicating an absence of adverse system effects.
Takeda and Okumura (2007)	Investigate the effect of <i>L. casei</i> strain Shirota on immune function.	Randomized, placebo-controlled crossover design	19 healthy middle-aged (30-45 years) and older (55-75 years) volunteers	1 bottle of Yakult 400 containing 4×10^{10} cfu <i>L. casei</i> strain Shirota	3 weeks	In both middle-aged and elderly participants, NK cell activity was significantly higher in the treatment group than among the controls, although numbers of NK cells were not affected. The health condition of all volunteers remained well during all experimental periods.
Tanaka et al. (1981)	Study the effect of <i>L. casei</i> strain Shirota on gut microbiome and putrefactive fermentation	Open-label study	5 healthy 25-32-year-old adults	10^{10} cfu <i>L. casei</i> strain Shirota/day	5 weeks	<i>L. casei</i> strain Shirota was recovered at levels of 10^7 to 10^8 cfu/g wet feces, but disappeared within a week after administration ceased. An increase was also seen in counts of bifidobacteria. Urinalysis found significant reductions in indican and phenol concentrations, indicating inhibition of putrefactive fermentation. The authors did not report any adverse findings.

290000

Table 7. Studies of *L. casei* Strain Shirota in Healthy Adults

Reference	Objective	Study Design	Subjects	<i>L. casei</i> Dose and Source	Duration	Safety-Related Results
Tuohy et al. (2007)	Study the survivability and impact on intestinal microbiota of <i>L. casei</i> strain Shirota	Prospective, randomized, double-blind, placebo-controlled design	20 healthy adults aged 23 to 70 years	130 ml dairy beverage providing 6×10^8 cfu <i>L. casei</i> strain Shirota/day	21 days	All participants receiving <i>L. casei</i> strain Shirota showed stable high fecal populations; several maintained detectable levels a week later. A significant increase was noted in the fecal population of lactobacilli, but no significant changes were noted in populations of total bacteria or in bifidobacteria, bacteroides, clostridia, eubacteria, enterococci, or <i>E. coli</i> .
Yamada et al. (2009; report in Japanese with English abstract)	Study risk management in a long-term elderly care facility	Randomized unblinded non-controlled trial.	77 residents of a long-term elderly care facility	1 65-ml bottle of Yakult a day providing 6.5×10^9 cfu <i>L. casei</i> strain Shirota	1 month	The treatment reduced the severity of winter-time norovirus-induced gastroenteritis but did not affect incidence. No adverse effects were reported.

890000

4.4.2.1.2. Compromised Adults

These studies are summarized in Table 8 at the end of this section.

Aso et al. (1995) randomly allocated 133 post-surgical patients in 29 medical centers, aged 15 to 80 years (but nearly all patients were aged 50+), with superficial transitional cell carcinomas of the bladder to receive BLP, a fermented dairy preparation providing 10^{10} cfu *L. casei* strain Shirota/g, or a placebo 3 times a day for 1 year or until tumor recurrence. The test group included 65 patients while the placebo group had 68 patients. Cytologic examinations of urine samples and endoscopies were performed every 3 months, and blood samples were taken for hematological and biochemical analysis at enrollment, after 1 month and 3 months, and every 3 months thereafter.

The average time to tumor recurrence was significantly extended in the probiotic group. There were 3 adverse events recorded among the 65 patients receiving *L. casei*—one case each of diarrhea, constipation, and elevation of hepatic transaminases. The placebo group had an equal number of adverse events—2 cases of diarrhea and 1 case of elevated alanine aminotransferase and creatinine. All of these events in both groups were transient and mild. The authors concluded that the similarity of the adverse-event profiles in the test and placebo groups indicate that “BLP treatment was very safe.”

Cats et al. (2003) enrolled 20 32-73-year-old *Helicobacter pylori* positive patients (8 males, 12 females; mean age = 48 years) for an intervention study in which 14 received 65 ml Yakult providing 10^8 cfu/ml of *L. casei* strain Shirota 3 times daily (daily dose of *L. casei* = 2×10^{10} cfu) for 3 weeks while 6 others served as controls. Reported symptoms decreased significantly in the *L. casei* group, while urease activity decreased non-significantly. One probiotic participant reported transient diarrhea.

Koebnick et al. (2003) enrolled 70 patients with symptoms of chronic constipation to participate in a randomized, double-blind, placebo-controlled study of the effect of Yakult with *L. casei* strain Shirota. The patients (32 men and 38 women aged 18-70 years; average age = 44 years) were randomly assigned to receive 65 ml/day of a dairy beverage containing either 0 or 6.5×10^9 cfu *L. casei* for 4 weeks.

Beginning in the second week of treatment, the treatment group showed a significant increase in defecation frequency and concomitant reduction in the occurrence of severe or moderately severe constipation symptoms. The authors noted that no side effects were reported.

In a study of the possible benefit of *L. casei* strain Shirota and fiber in reduction of the risk of recurrence of colorectal tumors, Ishikawa et al. (2005) enrolled 398 men and women aged 40-65 years who had had at least 2 tumors endoscopically removed within the past 3 months. The participants included 325 males and 73 females with a mean age of 55 years. Patients were randomized into 4 groups to follow different interventions for 4 years: 25 g wheat bran biscuits containing 7.5 g bran (n=99), a preparation providing 10^{10} cfu *L. casei* strain Shirota 3 times a day (n=99), both wheat bran and *L. casei* at the same dose levels (n=103), and control (n=97). Patients were examined every 3 months and colonoscopies were performed after 2 and 4 years.

Only 18 patients failed to complete the trial (4 from the bran group, 3 from the *L. casei* group, 7 from the combined-treatment group, and 4 from the control group). No serious adverse events occurred related to the interventions: 1 patient died of lung cancer and 1 of cerebral

hemorrhage, and 2 patients developed acute appendicitis. The authors suggested that *L. casei* intake suppressed the development of colorectal tumors, but the reduction was not statistically significant.

An uncontrolled trial of the effect of *L. casei* strain Shirota in the treatment of 10 patients suffering from HTLV-1 associated myelopathy/tropical spastic paraparesis was conducted by Matsuzaki et al. (2005). The patients, including 3 males and 7 females, ranged in age from 34 to 62 years (mean age = 49.7 years) and had suffered from the disease for a mean of 15.4 years. All participants received an oral preparation providing 8×10^{10} cfu *L. casei*/day for 4 weeks. Significant clinical improvement was seen in all 10 patients, particularly with regard to urinary symptoms, motor dysfunction and spasticity of lower extremities. NK cell activity was significantly increased, but the HTLV-1 virus load was not significantly affected. No adverse effects were reported, and the authors concluded that administration of *L. casei* strain Shirota is effective, easy, and safe.

Kanazawa et al. (2005) enrolled 44 biliary cancer patients (29 males and 15 females, mean age = 63.6 years) scheduled for hepatectomy in a randomized placebo-controlled trial of the ability of synbiotic therapy to reduce postoperative infectious complications. The synbiotic treatment (n = 21) included 10^8 cfu/day of each of 2 probiotic bacteria, *B. breve* Yakult and *L. casei* strain Shirota, and 12 g galactooligosaccharides/day for 14 days; the control (n = 23) was standard enteral feeding. All patients were fed enterally for 6-7 days postoperatively, after which oral feeding was introduced and gradually supplanted enteral feeding. Lactulose-mannitol tests of intestinal permeability were performed and feces were collected for bacteriological examination before surgery and on postoperative days 2, 7, and 28. Blood was drawn and analyzed for hematological parameters and diamine oxidase activity and records of infectious complications were kept for 30 days after surgery.

No differences were seen in serum diamine oxidase activity, hemoglobin, total protein, or bilirubin, but white blood cell counts and C-reactive protein concentration were significantly lower in the synbiotics group. Intestinal permeability did not differ between the treatment and control groups. The intestinal microbiota was significantly improved in the synbiotics group as compared with controls in increased numbers of bifidobacteria and lactobacilli and reduced numbers of enterobacteriaceae, *Pseudomonas*, and *Candida*. The synbiotics group had significantly higher fecal concentrations of the short-chain fatty acids acetic acid, propionic acid, and butyric acid. The synbiotics group also suffered significantly fewer postoperative infectious complications, resulting in less antibiotic therapy and shorter hospital stays. No adverse effects from the synbiotic therapy were reported.

Shioiri et al. (2006) investigated the effects of ingestion of a fermented milk beverage containing 3×10^{10} cfu *L. casei* strain Shirota and 2.5 g GOS per 80-ml bottle on defecation frequency of 35 female college students suffering from constipation and elderly individuals with abnormal levels of putrefactive metabolites. Thirty-five female students (mean age = 19.4 years) with defecation frequencies of <10 times in 2 weeks and 20 generally healthy elderly individuals (mean age = 74.4 years) were recruited into two prospective, randomized, double-blind, placebo-controlled, crossover trials. After a 2-week run-up, participants ingested 1 bottle/day of either the test article or a matched placebo for 2 weeks, followed by a 3-week washout and 2 week ingesting the other treatment. All participants maintained diaries recording defecation events, stool quantity and characteristics, health status, and food intake; in the study of elderly individuals, stools were collected on the final day of the run-up and the wash-out period and

weekly during the feeding periods. Stools were weighed, homogenized, and analyzed for total obligate anaerobes, bacteroidaceae, bifidobacteria, lecithinase+ clostridia, lactobacilli, enterobacteriaceae, enterococci, staphylococci, candida, and *L. casei* strain Shirota (using ELISA with a strain-specific monoclonal antibody). Additionally, stool pH, water content, and the concentrations of the following organic acids were measured: succinic, lactic, formic, acetic, propionic, isobutyric, butyric, isovaleric, and valeric. The concentrations of the fecal putrefactive metabolites indole, ammonia, phenol, and *p*-Cresol were assessed.

Among the female college students, ingestion of the synbiotic beverage significantly increased defecation frequency after 1 week, but the effect was no longer significant after 2 weeks; there was no effect on stool quantity or character (Shioiri et al. 2006). Among the elderly, synbiotic ingestion had no effect on defecation frequency or stool quantity, but significantly increased counts of fecal bifidobacteria and lactobacilli at both 1 and 2 weeks. Counts of both lecithinase+ clostridia and enterobacteriaceae were significantly lower after 1 week of synbiotic ingestion, but only the enterobacteriaceae count remained significantly lower after 2 weeks. Other microorganisms were not affected. With regard to organic acid, ingestion of *L. casei* strain Shirota and GOS significantly reduced fecal levels of succinic acid and increased those of acetic, butyric, and total organic acids. As a result, the pH level of the feces was significantly lower, but there was no effect on water content. After 2 weeks of ingestion of the synbiotic beverage, the fecal levels of ammonia and phenol were significantly reduced. The authors did not report any adverse effects in either the students or elderly study subjects.

In a randomized placebo-controlled trial of the effects of perioperative oral administration of a synbiotic on intestinal barrier function, microbiota, and immune response, Sugawara et al. (2006) enrolled 81 patients with biliary cancer scheduled for hepatectomy to receive synbiotic therapy either postoperatively (n = 40) or both pre- and postoperatively (n = 41). The enrolled patients included 46 males and 35 females with an average age of 63.2 years. The synbiotic treatment given for 2 weeks prior to surgery consisted of 1 daily 80-ml bottle of Yakult 400 containing 4×10^{10} cfu *L. casei* strain Shirota, 1 100-ml bottle of Bifiel with 10^{10} cfu *B. breve* Yakult, and 15 g galactooligosaccharides. The postoperative therapy included 10^8 cfu each of *L. casei* and *B. breve* and 15 g GOS daily for 2 weeks. Intestinal barrier function was measured before the trial, immediately before surgery, and 2, 7, and 21 days after the hepatectomy. Blood was taken before and after surgery for standard tests and measurement of diamine oxidase activity, NK cell activity, and IL-6 concentrations. Feces were sample before the trial, immediately before surgery, and 7 and 21 days after surgery for bacteriological examination and measurement of short-chain fatty acids. Daily records were maintained of patients' postoperative progress and any infectious complications.

Preoperative administration of the synbiotic significantly enhanced NK cell activity during treatment, and reduced IL-6 and C-reactive protein concentrations both pre- and post-operatively. Further, patients who had received preoperative as well as postoperative synbiotic therapy had a significantly reduced likelihood of postoperative infectious complications. The authors noted that, "No patient had problems related to synbiotic treatment," and concluded that "administration of synbiotics as a food supplement is safe."

The effects of *L. casei* strain Shirota on allergic rhinitis were investigated in a randomized, double-blind, placebo-controlled study (Tamura et al. 2007). A total of 109 patients (43 males and 66 females, mean age = 39.4 years) having specific IgE for Japanese cedar pollen were randomly assigned to receive either 80 ml fermented milk containing 4×10^{10} cfu *L. casei* or GRAS Determination for
Lactobacillus casei strain Shirota

80 ml nonfermented milk placebo for 8 weeks. Symptoms such as sneezing, runny nose, stuffy nose, itchy eyes, and watery eyes as well as the findings of otolaryngologic examinations were scored weekly; and blood samples were collected 4 times and analyzed for anti-Japanese cedar pollen IgE, eosinophil count and cationic protein, and the ratio of Th1 to Th2 cells.

No significant differences were seen between the test and control groups on any of the scored parameters or on the immunological measures from the blood samples. Ten participants developed colds, 3 developed diarrhea, and 1 vomited once during the study, but none of these events appeared to be related to the ingestion of fermented milk.

Barrett et al. (2008) investigated the effects of *L. casei* strain Shirota on intestinal fermentation patterns on irritable bowel syndrome (IBS) patients. Eighteen patients aged 20-70 years (mean age = 44 years), 5 males and 13 females, undertook a 1-2-week run-in period during which their symptoms were assessed, after which they received 1 65-ml bottle of Yakult per day, providing 6.5×10^9 cfu *L. casei*/day for 6 weeks. All patients received a lactulose breath-hydrogen test at study initiation and completion.

The patients showed an overall improvement in their symptoms, indicating a reduction in small intestinal bacterial overgrowth. Absent a control group, however, it is difficult to assess the significance of this finding. No adverse effects were observed due to probiotic therapy.

Naito et al. (2008) enrolled 202 bladder-cancer patients who had undergone transurethral resection and intravesical instillation of epirubicin in a multi-center, prospective, randomized, nonblinded, parallel-group trial. One hundred patients (78 males and 22 females; median age about 72) received 6 additional intravesical instillations of epirubicin during the first 3 months and 3×10^{10} cfu *L. casei* strain Shirota daily for 1 year, while 102 control patients (86 males and 16 females; median age about 74) received only the epirubicin instillations. Urinalysis including cytological assessment was performed monthly for 3 months, quarterly for 2 years, and semiannually thereafter; cystoscopy was performed quarterly for 2 years and semiannually thereafter; routine hematology and biochemistry analyses were performed at baseline and at 1, 3, 6, 9, and 12 months. All patients were followed for at least 3 years and the severity of adverse reactions was objectively assessed.

A total of 31 patients failed to complete the study, 24 from the probiotic group and 7 from the control group; the difference in dropout rates was not due to adverse reactions to the probiotic preparation. Compliance was fairly good with 80% of the patients ingesting 80% or more of the prescribed doses of *L. casei* over the first year. Bladder cancer recurred in 26% of the patients receiving probiotic adjunct therapy and in 41% of the patients receiving epirubicin alone; the 3-year recurrence-free survival rate was significantly higher in the probiotic group (75%) than in the control group (60%).

There were no significant differences in the incidence of adverse reactions between the groups, and no serious adverse reactions or abnormal laboratory findings were observed in either group. The most common adverse reactions involved local toxicity due to intravesical chemotherapy; 6% of the probiotic patients had constipation and 2% had diarrhea, both possibly related to ingestion of *L. casei* strain Shirota. The authors concluded:

“The safety of LC preparation is established, although it may cause gastrointestinal symptoms in rare cases. While constipation and diarrhea developed in patients receiving LC preparation in the current study, the incidence was low, severity was mild and compliance with LC preparation treatment was favorable. There were no

differences between the groups in the incidence or severity of adverse reactions, including those associated with micturition” (Naito et al. 2008).

Using a randomized, double-blind, placebo-controlled design, Rao et al. (2009) studied the effect of *L. casei* strain Shirota on the emotional symptoms of chronic fatigue syndrome. A total of 39 chronic fatigue syndrome patients aged 18-65 years were randomized to receive sachets containing either 8×10^9 cfu *L. casei* strain Shirota (n = 21) or placebo (n = 18) 3 times a day for 8 weeks. Patients completed depression and anxiety measures and provided stool samples both prior to and immediately after the intervention.

Four patients, 2 each from the test and control groups, withdrew for non-intervention-related reasons, leaving 35 patients (8 males and 27 females) in the two groups for analysis. The probiotic groups showed significantly greater increases in fecal bifidobacteria and lactobacilli. Probiotic ingestion had no effect on depression scores, but anxiety scores were significantly reduced. (One suggestion offered is, that since most of the patients suffered from some degree of irritable bowel syndrome, improved bowel function may have resulted in lowered anxiety.) The authors stated that the “probiotic powder was well tolerated and there were no significant adverse events reported in the probiotic or placebo groups.”

In an open-label study, Cassani et al. (2011) studied the effect of daily consumption of 65 ml fermented milk drink providing 6.5×10^9 cfu *L. casei* strain Shirota on the incidence and severity of constipation among 40 outpatients with Parkinson’s disease, 36 males and 4 females with a mean age of 71.9 years. The patients kept a daily record of their bowel movements, stool consistency, abdominal pain, and sensations of incomplete emptying or abdominal bloating for 6 weeks—the first week without the probiotic and the remaining 5 weeks with it.

No patients withdrew and all diaries were complete with entries for 42 days for each patient. While there were no differences in the number of bowel movements, significant improvements were seen in achieving normal stool consistency, reducing abdominal pain, and lessening sensations of incomplete emptying or abdominal bloating. No adverse effects were reported.

In an open label study of 9 patients (3 males and 8 females aged 38-82 years; median age = 63 years) undergoing hemodialysis for end-stage renal disease, Nakabayashi et al. (2011) provided no synbiotic treatment for 2 weeks of observation and then administered 1.67 g galactooligosaccharides, 10^8 cfu *L. casei* strain Shirota, and 10^8 cfu *B. breve* strain Yakult 3 times daily for 2 weeks. Patients completed daily questionnaires regarding defecation patterns and abdominal symptoms, while blood concentrations of *p*-cresol, phenol, and indoxyl sulfate were determined at the end of the 2-week observation period and the 2-week intervention period.

All patients completed the study. Those with high serum *p*-cresol levels tended to have hard stools and difficulty in defecation, all of which were significantly reduced during the synbiotic administration., although concentrations of phenol and indoxyl sulfate were not affected. The authors reported that “the [synbiotic] treatment did not adversely affect abdominal symptoms, including abdominal pain.”

Table 8. Studies of *L. casei* Strain Shirota in Compromised Adults

Reference	Objective	Study Design	Subjects	<i>L. casei</i> Dose and Source	Duration	Safety-Related Results
Aso et al. (1995)	Effect of <i>L. casei</i> strain Shirota on tumor recurrence in post-surgical patients with superficial transitional cell carcinomas of the bladder	Randomized, double-blind, placebo-controlled trial.	133 post-surgical patients aged 15 to 80 years (nearly all aged 50+) with superficial transitional cell carcinomas of the bladder	BLP, a fermented dairy preparation providing 10^{10} cfu <i>L. casei</i> strain Shirota 3x/day	1 year or until tumor recurrence	Time to tumor recurrence was significantly extended. 3 adverse events were recorded among the 65 patients receiving <i>L. casei</i> —one case each of diarrhea, constipation, and elevation of hepatic transaminase. The placebo group had an equal number of adverse events—2 cases of diarrhea and 1 case of elevated alanine aminotransferase and creatinine. All of these events in both groups were transient and mild. The authors concluded that the similarity of the adverse-event profiles in the test and placebo groups indicated that "BLP treatment was very safe."
Barrett et al. (2008)	Investigate the effects of <i>L. casei</i> strain Shirota on intestinal fermentation patterns of IBS patients.	Open label study	5 male and 13 female IBS patients aged 20-70 years (mean age = 44 years)	1 65-ml bottle of Yakult per day, providing 6.5×10^9 cfu <i>L. casei</i> strain Shirota	6 weeks	The patients showed an overall improvement in their symptoms, indicating a reduction in small intestinal bacterial overgrowth. No adverse effects were observed due to probiotic therapy.
Cassani et al. (2011)	Assess the effect of <i>L. casei</i> strain Shirota on the incidence and severity of constipation among patients with Parkinson's disease	Open label study	40 Parkinson's disease outpatients, 36 males and 4 females with a mean age of 71.9 years	65 ml fermented milk drink/day providing 6.5×10^9 cfu <i>L. casei</i> strain Shirota	6 weeks	There were no differences in the number of bowel movements, but significant improvements were seen in achieving normal stool consistency, reducing abdominal pain, and lessening sensations of incomplete emptying or abdominal bloating. No adverse effects were reported.

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Table 8. Studies of *L. casei* Strain Shirota in Compromised Adults

Reference	Objective	Study Design	Subjects	<i>L. casei</i> Dose and Source	Duration	Safety-Related Results
Cats et al. (2003)	Evaluate the effect <i>L. casei</i> strain Shirota on patients positive for <i>Helicobacter pylori</i>	Randomized unblinded trial.	8 male and 12 female <i>Helicobacter pylori</i> positive patients aged 32-73 years old (mean age = 48 years)	65 ml Yakult providing 10 ⁸ cfu/ml of <i>L. casei</i> strain Shirota 3 times daily (daily dose of <i>L. casei</i> = 2x10 ¹⁰ cfu)	3 weeks	Reported symptoms decreased significantly. One probiotic participant reported transient diarrhea.
Ishikawa et al. (2005)	Study the benefit of <i>L. casei</i> strain Shirota and fiber in reduction of the risk of recurrence of colorectal tumors.	Randomized, unblinded, placebo-controlled trial.	325 males and 73 females aged 40-65 years (mean = 55 years) who had at least 2 tumors removed within the past 3 months	10 ¹⁰ cfu <i>L. casei</i> strain Shirota 3x/day, with or without wheat bran	4 years	The authors suggested that <i>L. casei</i> strain Shirota intake suppressed the development of colorectal tumors, but the reduction was not statistically significant. No serious adverse events occurred related to the interventions: 1 patient died of lung cancer and 1 of cerebral hemorrhage, and 2 patients developed acute appendicitis.
Kanazawa et al. (2005)	Study the ability of synbiotic therapy including <i>L. casei</i> strain Shirota to reduce postoperative infectious complications	Randomized unblinded placebo-controlled trial	29 male and 15 female biliary cancer patients (mean age = 63.6 years) scheduled for hepatectomy	10 ⁸ cfu/day of each of 2 probiotic bacteria, <i>B. breve</i> Yakult and <i>L. casei</i> strain Shirota, and 12 g GOS/day	14 days	No differences were seen in serum diamine oxidase activity, hemoglobin, total protein, or bilirubin, but white blood cell counts and C-reactive protein concentration were significantly lower in the synbiotics group. Intestinal permeability did not differ between the treatment and control groups. The intestinal microbiota in the synbiotics group had increased bifidobacteria and lactobacilli and reduced enterobacteriaceae, <i>Pseudomonas</i> , and <i>Candida</i> . The synbiotics group had significantly higher fecal concentrations of the short-chain fatty acids acetic acid, propionic acid, and butyric acid and suffered significantly fewer postoperative infectious complications, resulting in less antibiotic therapy and shorter hospital stays. No adverse effects from the synbiotic therapy were reported.

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Table 8. Studies of *L. casei* Strain Shirota in Compromised Adults

Reference	Objective	Study Design	Subjects	<i>L. casei</i> Dose and Source	Duration	Safety-Related Results
Koebnick et al. (2003)	Study the effect of Yakult with <i>L. casei</i> strain Shirota on chronic constipation	Randomized, double-blind, placebo-controlled trial	32 men and 38 women aged 18-70 years (average age = 44 years) with symptoms of chronic constipation	65 ml/day of Yakult containing 6.5×10^9 cfu <i>L. casei</i> strain Shirota	4 weeks	Beginning in the second week of treatment, the treatment group showed a significant increase in defecation frequency and concomitant reduction in the occurrence of severe or moderately severe constipation symptoms. The authors noted that no side effects were reported.
Matsuzaki et al. (2005)	Study the effect of <i>L. casei</i> strain Shirota in the treatment of HTLV-1 associated myelopathy/tropical spastic paraparesis	Open label study.	3 males and 7 females aged 34-62 years (mean = 49.7 years) suffering from HTLV-1 associated myelopathy/tropical spastic paraparesis	oral preparation providing 8×10^{10} cfu <i>L. casei</i> strain Shirota/day	4 weeks	Significant clinical improvement was seen in all 10 patients, particularly with regard to urinary symptoms, motor dysfunction and spasticity of lower extremities. NK cell activity was significantly increased, but the HTLV-1 virus load was not significantly affected. No adverse effects were reported, and the authors concluded that administration of <i>L. casei</i> strain Shirota is effective, easy, and safe.
Naito et al. (2008)	Assess the value of <i>L. casei</i> strain Shirota adjunct therapy in reducing the incidence of recurrence of bladder cancer	Multi-center, prospective, randomized, nonblinded, parallel-group trial	202 bladder-cancer patients who had undergone transurethral resection and intravesical instillation of epirubicin	3×10^{10} cfu <i>L. casei</i> strain Shirota	1 year	Bladder cancer recurred in 26% of the patients receiving probiotic adjunct therapy and in 41% of the patients receiving epirubicin alone; the 3-year recurrence-free survival rate was significantly higher in the probiotic group (75%) than in the control group (60%). There were no significant differences in the incidence of adverse reactions between the groups, and no serious adverse reactions or abnormal laboratory findings were observed in either group. The most common adverse reactions involved local toxicity due to intravesical chemotherapy; 6% of the probiotic patients had constipation and 2% had diarrhea, both possibly related to ingestion of <i>L. casei</i> strain Shirota.

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Table 8. Studies of *L. casei* Strain Shirota in Compromised Adults

Reference	Objective	Study Design	Subjects	<i>L. casei</i> Dose and Source	Duration	Safety-Related Results
Nakabayashi et al. (2011)	Examine the effects of <i>L. casei</i> strain Shirota, <i>B. breve</i> strain Yakult, and GOS on serum <i>p</i> -cresol levels of hemodialysis patients.	Open-label study	9 patients (3 males and 8 females aged 38-82 years; median age = 63 years) undergoing hemodialysis for end-stage renal disease	Synbiotic with 1.67 g GOS, 10 ⁸ cfu <i>L. casei</i> strain Shirota, and 10 ⁸ cfu <i>B. breve</i> strain Yakult 3 times daily	2 weeks	All patients completed the study. Those with high serum <i>p</i> -cresol levels tended to have hard stools and difficulty in defecation, all of which were significantly reduced during the synbiotic administration, although concentrations of phenol and indoxyl sulfate were not affected. The authors reported that "the [synbiotic] treatment did not adversely affect abdominal symptoms, including abdominal pain."
Rao et al. (2009)	Study the effect of <i>L. casei</i> strain Shirota on the emotional symptoms of chronic fatigue syndrome	Randomized, double-blind, placebo-controlled trial	39 chronic fatigue syndrome patients aged 18-65 years	sachets containing 8x10 ⁹ cfu 3x <i>L. casei</i> strain Shirota/day	8 weeks	The probiotic group showed greater increases in fecal bifidobacteria and lactobacilli. Probiotic ingestion had no effect on depression scores, but anxiety scores were reduced. The authors stated that the "probiotic powder was well tolerated and there were no significant adverse events reported in the probiotic or placebo groups."
Shiomi et al. (2006)	Study the effects of <i>L. casei</i> strain Shirota and GOS on defecation frequency, fecal bacteria, organic acids, and putrefactive metabolites	2 prospective, randomized, double-blind, placebo-controlled, crossover trials	35 female college students (mean age = 19.4 years) suffering from constipation; 20 generally healthy elderly individuals (mean age = 74.4 years) with abnormal levels of putrefactive metabolites	3x10 ¹⁰ cfu <i>L. casei</i> strain Shirota and 2.5 g GOS per 80-ml bottle; 1 bottle/day	2 weeks	Among the college students, the synbiotic beverage increased defecation frequency after 1 week, but the effect disappeared by 2 weeks. Among the elderly, synbiotic ingestion had no effect on defecation frequency or stool quantity, but increased counts of fecal bifidobacteria and lactobacilli at both 1 and 2 weeks. Counts of both lecithinase+ clostridia and enterobacteriaceae were lower after 1 week of synbiotic ingestion, but only the enterobacteriaceae count remained lower after 2 weeks. Fecal levels of succinic acid were reduced and acetic, butyric, and total organic acids were increased, reducing pH. Fecal levels of ammonia and phenol were significantly reduced. The authors did not report any adverse effects in either the students or elderly study subjects.

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Table 8. Studies of *L. casei* Strain Shirota in Compromised Adults

Reference	Objective	Study Design	Subjects	<i>L. casei</i> Dose and Source	Duration	Safety-Related Results
Sugawara et al. (2006)	Study the effects of perioperative administration of a synbiotic on intestinal barrier function and microbiota and immune response in biliary cancer patients.	Randomized unblinded placebo-controlled trial comparing pre- + postoperative therapy with postoperative therapy alone.	46 male and 35 female biliary cancer patients (average age = 63.2 years) scheduled for hepatectomy	Preoperative: 80 ml Yakult 400 containing 4×10^{10} cfu <i>L. casei</i> strain Shirota, 100 ml Bifiel with 10^{10} cfu <i>B. breve</i> and 15 g GOS Postoperative: 10^8 cfu each of <i>L. casei</i> and <i>B. breve</i> and 15 g GOS	2 weeks before and 2 weeks after surgery	Preoperative administration of the synbiotic significantly enhanced NK cell activity and reduced IL-6 and C-reactive protein concentrations both pre- and post-operatively. Patients receiving pre- and postoperative synbiotic therapy had a significantly reduced likelihood of postoperative infectious complications. The authors noted that, "No patient had problems related to synbiotic treatment," and concluded that "administration of synbiotics as a food supplement is safe."
Tamura et al. (2007)	Study the effects of <i>L. casei</i> strain Shirota on allergic rhinitis	Randomized, double-blind, placebo-controlled trial	43 males and 66 females (mean age = 39.4 years) having specific IgE for Japanese cedar pollen	80 ml fermented milk containing 4×10^{10} cfu <i>L. casei</i> strain Shirota	8 weeks	No significant differences were seen between the test and control groups on any of the immunological measures (anti-Japanese cedar pollen IgE, eosinophil count and cationic protein, and the ratio of Th1 to Th2 cells). Ten participants developed colds, 3 developed diarrhea, and 1 vomited once during the study, but none of these events appeared to be related to the ingestion of fermented milk.

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4.4.2.2. Studies in Children

4.4.2.2.1. Healthy Children

In a community-based, randomized, double-blind, placebo-controlled field trial, Sur et al. (2010) tested the ability of *L. casei* strain Shirota to reduce the incidence of acute diarrhea. A total of 3758 healthy children aged 1-5 years old living in what the authors described as an “urban slum” community in Kolkata, India consumed 65 ml/day of a beverage providing either 6.5×10^9 cfu *L. casei* strain Shirota ($n = 1894$) or the same nutrients (sugars and skim milk) without the bacteria ($n = 1864$) for 12 weeks and were observed for an additional 12 weeks after cessation of treatment. Participants’ height, weight, and upper-arm circumference were taken at enrollment, at the end of the probiotic intake, and at the end of the follow-up observation period. The coded beverages were consumed under direct observation, and trained observers visited the children’s homes daily to identify any episodes of diarrhea, defined as 3 or more abnormally loose or liquid stools within a 24-hour period. Stool samples were taken of children with diarrhea and evaluated for viral, bacterial, and parasitic enteric pathogens.

Over 95% of the children consumed their assigned beverage for at least 80% of the planned period (67 days)—95.2% in the probiotic group and 95.9% in the placebo group. There were no differences in growth as measured by height, weight, and arm circumference between the two groups. Over the full 24-month study period, 608 children in the probiotic group and 694 in the placebo group suffered one or more incidents of diarrhea, a statistically significant difference. The enteric pathogens most often causing diarrhea—*E. coli*, *Vibrio* spp., *Campylobacter* spp, and *Shigella* spp.—did not differ between groups, but diarrhea due to *Aeromonas* spp. and *Cryptosporidium* spp. was relatively more common in the placebo group. The authors concluded that *L. casei* strain Shirota is efficacious in preventing acute childhood diarrhea.

4.4.2.2.2. Compromised Children

In a small open-label study, Kanamori et al. (2004) used synbiotic therapy to treat 7 patients (5 boys and 2 girls, mean age = 8.7 years) with short-bowel syndrome resulting from surgical resection. The treatment included 3×10^9 cfu/day of 2 probiotic bacteria, *B. breve* Yakult and *L. casei* strain Shirota, and 3 g galactooligosaccharides. After receiving treatment for 15 to 55 months, the patients showed significant improvement in their intestinal microbial milieu, evidenced by increased numbers of anaerobic bacteria and reduced numbers of pathogens. The changes in the microbiota resulted in an increase in fecal short-chain fatty acids from 27.8 to 65.1 $\mu\text{mol/g}$. The patients showed accelerated gain in body weight.

Srinivasan et al. (2006) studied the safety of administering *L. casei* strain Shirota to critically ill children confined to a pediatric intensive care unit. Safety was assessed by bacteriologic surveillance for the administered strain in surface swabs, endotracheal aspirates, and in blood, urine, and sterile body fluid cultures. The 56 children enrolled ranged in age from 6 months to 16 years with a mean of 2.7 years. Those randomized to the study group ($n = 28$) received 10^7 cfu/day in 3 doses via nasogastric tube for 5 days.

While *L. casei* strain Shirota was found in the feces of 5 of the 6 children for whom analyses were undertaken, there was no indication of colonization or bacteremia among any of the children in the ICU. The authors observed that, “The preparation was well tolerated with no

apparent side effects,” and concluded that “the use of [*L. casei* strain Shirota] as a probiotic in enterally fed critically ill children is safe.”

4.4.2.3. Studies in Infants

4.4.2.3.1. Healthy Infants

Shirota et al. (1966) fed 30 2-6-year-old healthy infants 50 ml fermented milk once a day, providing $1-2 \times 10^8$ cfu *L. casei*¹ strain Shirota for 35 days (n=30). For half of the infants the bacteria were viable while for the other half the drink was heated to 80°C for 30 minutes to kill the bacteria. Fecal samples were obtained at the end of the feeding period and weekly for a month afterwards and evaluated for lactobacilli, the Shirota strain, bifidobacteria, enterobacteriaceae, enterococci, yeast, and *Candida*.

Treatment had no effect on the prevalence of bifidobacteria, yeast, *Candida*, or total lactobacilli, but counts of the Shirota strain increased significantly. Numbers began to decline immediately after cessation of administration; it was detected in the feces of only 4 infants after 2 weeks and in none after 3 weeks. Administration of *L. casei* strain Shirota significantly reduced the prevalence of enterococci and enterobacteriaceae, but numbers recovered quickly following cessation of treatment. The authors reported that “no infants showed abnormality in their healthy conditions.”

4.4.2.3.2. Compromised Infants

In a small open-label study, Kanamori et al. (2010) treated 4 neonates with synbiotic therapy, using a combination of GOS, *L. casei* strain Shirota, and *B. breve* Yakult. The neonates had been diagnosed antenatally with meconium peritonitis with ileal atresia, complete urorectal septum malformation, or a complex of omphalocele, bladder exstrophy, and myelomeningocele, and were begun on therapy immediately after birth. Their initial daily dose was 10^8 — 10^9 cfu of each probiotic, administered in 4 daily doses; as the infants’ milk intake increased the dose was elevated to 3 daily doses of 10^9 — 10^{10} cfu of each probiotic/dose and 1.0 g GOS was added to each dose. The authors reported that this therapy produced excellent results in “encouraging and maintaining a normal intestinal microbiota,” in producing normal weight gain, and in preventing enterocolitis.

4.4.2.4. Case Studies

Candy et al. (2001) reported on the effect of treatment with *L. casei* strain Shirota on an infant with short bowel syndrome. The case involved a male infant who developed necrotizing enterocolitis necessitating resection of the ileum and colon, leaving him with 60 cm of jejunum. He developed sepsis which led to further surgical and pharmacological care. Among other issues, the infant’s sodium balance declined significantly. At 12 months of age he was started on 15 ml Yakult providing 1.5×10^9 cfu *L. casei* strain Shirota 3x/day. Within 3 days his stool showed abundant lactobacilli, which had previously been absent. Urine sodium increased significantly, and it became possible to remove the central venous feeding catheter. At the time of the published report, the patient had remained on probiotic therapy for 2 years and was showing normal development.

¹ At the time this study was performed and reported, *L. casei* was included within the species *L. acidophilus*.
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In the same year, Kanamori et al. (2001) reported on the successful treatment of an infant girl with short bowel syndrome with synbiotic therapy including GOS, *L. casei* strain Shirota, and *B. breve* Yakult. The girl had been diagnosed with gastroschisis *in utero* and underwent surgery to repair the abdominal wall defect and to perform a massive intestinal resection, leaving 25 cm of small intestine. She survived and progressed for 2 years, but with subnormal growth and with a high incidence of metabolic acidosis and febrile episodes, the latter caused by enterocolitis resulting from sepsis of a central venous catheter. The catheter was removed and synbiotic therapy was begun at a daily dose (administered in 3 daily increments) of 3 g GOS and 3×10^9 cfu each of the two probiotic strains. After a month her fecal microflora had normalized, with significantly reduced levels of *E. coli* and *Candida* and increased levels of total anaerobic bacteria, including both the administered and additional strains of bifidobacteria and lactobacilli. Fecal lactate levels remained high (although the authors speculated that the ratio of D- to L-lactate may have decreased), but the treatment increased levels of acetate, propionate, and butyrate and episodes of acidosis ceased. At the time of the report, the patient had remained on the synbiotic therapy for a year with continued improvement and no adverse effects of the treatment.

Kanamori et al. (2002) reported on use of the same therapy with another critically ill infant in the following year. The girl was born preterm with laryngotracho-esophageal cleft, resulting in repeated pneumonia and severe gastrointestinal abnormalities with non-normal bowel function and unsatisfactory growth. At 9 months of age her feces showed no lactobacilli or bifidobacteria and she was placed on synbiotic therapy as described in Kanamori et al. (2001). Normal bowel function was restored immediately, and within a month fecal short-chain fatty acids and microbiota appeared normal. She had only a single episode of pneumonia after the start of the synbiotic therapy and her weight doubled over 11 months from 3 to 6 kg.

The same authors reported a third pediatric case in which a similar synbiotic therapy was used successfully (Kanamori et al. 2003). This case involved a 3-month-old boy with Down syndrome diagnosed with methicillin-resistant *S. aureus*-induced enteritis. The infant became septic and was placed in intensive care. The only bacteria that could be isolated in his feces were methicillin-resistant *S. aureus* and vancomycin was administered for 16 days. In order to re-establish normal intestinal microbiota the patient was started on 3 g GOS and 3×10^9 cfu *L. casei* strain Shirota/day while vancomycin treatment continued. Six weeks later 3×10^9 cfu *B. breve* Yakult/ day was added. The patient's condition improved significantly--methicillin-resistant *S. aureus* disappeared from the feces, being replaced by a more normal microbiota including lactobacilli and bifidobacteria, concentrations of short-chain fatty acids improved, and the patient was able to be removed from therapy.

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

A number of articles has appeared in the literature reviewing the history of use of *Lactobacillus* species and citing research data, and in most cases concluding that, except in exceptional circumstances, the entire genus poses little risk of harm to humans at any reasonable level of oral exposure.

In 2006, after reviewing the safety of exposure to lactobacilli by animals, workers, consumers, and in the environment, Bernardeau et al. (2006) urged EFSA to accord *Lactobacillus* "Long-standing Presumption of Safety" status, a recommendation which was followed by EFSA. Bernardeau et al. (2006) suggested that new strains of *Lactobacillus* should GRAS Determination for

be subjected to a “single-criterion safety assessment” with the single criterion being transferable antibiotic resistance.

Chmielewska and Szajewska (2010) reviewed 5 randomized clinical trials of the effect of probiotics on constipation. Three of the trials, enrolling 266 patients, were of adults, one trial each of *L. casei* strain Shirota, *B. lactis* DN-173010, and *E. coli* Nissle 1917, while 2 trials with 111 children enrolled studied *L. rhamnosus* GG and *L. rhamnosus* Lcr35. The durations of the studies ranged from 2 to 12 weeks with probiotic doses of 10^8 to 2.5×10^{10} cfu/day. All tested probiotics except *L. rhamnosus* GG showed beneficial effects and, according to the authors, “The probiotics were well tolerated, and no adverse events associated with supplementation were reported in any of the trials.” The authors noted that this record of safety is important since the populations tested in these studies may be regarded as at-risk with greater liability for harmful events.

4.6. Previous Safety Evaluations of *L. casei* by Authoritative Bodies

4.6.1. U.S. Food and Drug Administration

On May 26, 1998, FDA filed without comment a New Dietary Ingredient notification from LaVida Corporation for its dietary supplement ingredient *L. casei* (FDA 1998). This filing constituted a finding by FDA that the notification provided reasonable assurance that the intended use of *L. casei* is safe under the terms of the Dietary Supplement Health and Education Act (DSHEA). The intended use of *L. casei* was described in the notification as the inclusion of 1.5×10^9 cfu *L. casei* in a tablet, gelatin capsule, or freeze-dried powder. Further, the recommended dosage was stated to be one or two units per day. Thus, FDA accepted as safe (as defined by DSHEA) an exposure to *L. casei* of 3×10^9 cfu/day.

4.6.2. European Food Safety Authority

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

EFSA’s Scientific Committee was asked to recommend organisms regarded as suitable for QPS status. The list of such organisms proposed by the Committee included both *L. casei* and *L. paracasei*. In listing *L. casei*, *L. paracasei*, and other species of *Lactobacillus* as suitable for QPS status, the Committee stated, “Where QPS status is proposed, the Scientific Committee is satisfied that the body of knowledge available is sufficient to provide adequate assurance that any potential to produce adverse effects in humans, livestock or the wider environment is understood and capable of exclusion” (EFSA 2007a, p8) and that the recommendations are “based on a thorough review of the available scientific literature and the knowledge and GRAS Determination for *Lactobacillus casei* strain Shirota

experience of the scientists involved” (EFSA 2007a, p8). With regard to *L. casei* (along with *L. rhamnosus*, *L. fermentum*, *L. plantarum*, *L. jensenii*, *L. salivarius*, *L. gasseri*, and *L. acidophilus*), the Committee noted that rare cases have occurred in which these species have been recovered from human clinical specimens, but noted that “many of the patients with apparent *Lactobacillus* infection were immunocompromised or had other severe underlying illnesses” (EFSA 2007b, p7). The Committee concluded that “most of the *Lactobacillus* species described to date can rightly be considered to be non-pathogenic to humans . . . Only certain strains of *L. rhamnosus* may be considered to be potential human opportunistic pathogens” (EFSA 2007b, p7)

In December 2008, EFSA’s Panel on Biological Hazards released an opinion reassessing the QPS status of *L. casei*, *L. paracasei*, and other *Lactobacillus* strains (EFSA 2008). The Panel determined that no changes were needed; no new evidence calling into question the QPS status of *L. casei* or *L. paracasei* was introduced. EFSA has repeated these same opinions in more recent updates through 2011 (EFSA 2009, 2010, 2011).

5. Safety Assessment and GRAS Determination

5.1. Introduction

This section presents an assessment that demonstrates that the ingestion of the probiotic bacteria *Lactobacillus casei* strain Shirota in fermented milk products, under the conditions of use described, is safe and is GRAS.

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

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

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

1. data and information relied upon to establish the scientific element of safety must be generally available; and
2. there must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a scientific-procedures GRAS determination are applied below in an analysis of whether the addition of *L. casei* strain Shirota to fermented milk products is safe and is GRAS.

5.2. Safety Evaluation

The body of evidence supporting the safety of oral administration of *Lactobacillus casei* and *Lactobacillus paracasei* strains in general, and *L. casei* strain Shirota in particular, is large and convincing. Numerous commentators—in addition to authoritative bodies such as EFSA—have noted the safe history of human ingestion of *L. casei* and other lactobacilli over many years. *L. casei* strain Shirota produces no deleterious metabolites and is not destructive of mucin. Any effects that this probiotic microorganism has on intestinal permeability appear to be beneficial in strengthening barrier function. While it is theoretically possible for biogenic amines to be

produced as a result of fermentation of dairy products by lactobacilli, this phenomenon has not been observed and, when *Lactobacillus* strains are ingested as probiotics, they produce lactic acid, lowering the intestinal pH and reducing the opportunity for production of harmful biogenic amines by putrefactive bacteria.

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

The extensive record of human exposure to *L. casei* strain Shirota (with over 30 million bottles of Yakult consumed daily worldwide) is strongly supported by an extremely large body of published research. In addition to *in vitro* work, the published literature includes 21 experimental studies in a variety of animal species (mice, hamsters, rats, and rabbits) as well as 32 studies in humans: 15 in healthy adults, 13 in compromised adults, 2 in compromised children, 1 in healthy infants, and 1 in compromised infants. Additionally, 4 case studies have been published in which high doses of *L. casei* strain Shirota were administered to severely compromised infants with no indication of adverse effects. These published studies are supported by 3 unpublished studies in healthy adults and 4 unpublished oral toxicity studies in rats (acute oral toxicity, 30-day repeated dose oral toxicity, and two 6-month repeated dose oral toxicity studies). The animal studies have included doses as high as 2×10^9 cfu/day in mice and 10^{11} cfu/day in rats, for periods up to 6 months. Doses in healthy adults reached 3.6×10^{11} cfu/day and those in compromised adults 8×10^{10} cfu/day for up to 4 years. No adverse effects were reported in any of this research. Thus, the estimated daily intake of *L. casei* strain Shirota from its intended use in fermented milk products, 8×10^{10} cfu/day, is within levels that have been shown to be without harm.

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

First, the research by Yuki et al. (1999) found that *L. casei* strain Shirota exhibits little phenotypic resistance other than to vancomycin, and no phenotypic resistance not widely found in other *Lactobacillus* strains. No MIC for *L. casei* strain Shirota exhibits resistance higher than the tentative epidemiological cut-off values (ECOFF) established for *L. paracasei* by Klare et al. (2007) or the median MIC of *L. casei* reported by D’Aimmo et al. (2007). This is evidence that the Shirota strain is of the “wild type” with regard to commonly used antibiotics. The principle behind the phenotypic test is that the wild-type strains of a species have a normal distribution of minimum inhibitory concentrations (MIC) when the number of strains is plotted against the MIC for a given antibiotic. If all strains fall within this distribution, then it is likely that the susceptibility or resistance of the species is intrinsic since it is found in all strains. On the other hand, if a separate cluster of strains is found with higher resistance, it is likely that the resistance of these strains is acquired. This is important, because acquired resistance is far more likely to be

transferable than intrinsic resistance (Vankerckhoven et al. 2007). The usefulness of susceptibility testing for indication of acquired resistance is increased as the numbers of both susceptible strains and resistant strains tested are increased. The number of *L. casei* and *paracasei* strains tested by Klare et al. (2007) or D’Aimmo et al. (2007) is insufficient to make this study determinative when taken alone, but it does provide strong evidence of the absence of transferable antibiotic resistance.

The second line of evidence is more direct, and that is that the genomic analysis of *L. casei* strain Shirota found no genetic basis for antibiotic resistance that is even potentially transferable in either the genome or in the single plasmid.

All of the available evidence demonstrates clearly that there is no reason to suspect harm to individuals consuming fermented milk products supplemented with *Lactobacillus casei* strain Shirota.

5.3. General Recognition of the Safety of *L. casei* Strain Shirota

The use of *L. casei* strain Shirota in fermented milk products 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. casei* strain Shirota 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 intended use of *L. casei* strain Shirota has been made through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., and Daniel J. O’Sullivan, 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. casei* strain Shirota anticipated to result from its intended use, and individually and collectively concluded that no evidence exists in the available information on *L. casei* strain Shirota or other *L. casei* strains that demonstrates, or suggests reasonable grounds to suspect, a hazard to consumers under the intended conditions of use of *L. casei* strain Shirota.

It is the Expert Panel’s opinion that other qualified scientists reviewing the same publicly available data would reach the same conclusion. Therefore, the intended use of *Lactobacillus casei* strain Shirota is GRAS based on scientific procedures.

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Appendix 1
Species Identification of *Lactobacillus casei* Strain
Shirota

MATERIALS and METHODS

Bacterial Strains and Growth Conditions

Bacterial strains were obtained from the Culture Collection of the Yakult Central Institute (YIT; Tokyo, Japan). All bacterial strains were grown in MRS broth (BD Difco, MD, USA) at 37 °C for 24 hours.

Phenotypic Characteristics

Morphological, cultural and biochemical tests were performed according to standard techniques at 37 °C unless otherwise stated. Cell shape, cell size and Gram staining were determined by using cultures grown in MRS broth at 37 °C for 24 hours. Motility was tested in MRS soft agar (0.15%). Catalase activity was determined by using cells grown on MRS agar. Gas production from glucose was measured with a Durham tube in MRS broth. Production of dextran was assessed on MRS agar in which glucose was replaced with 2% (w/v) sucrose. The methods of Barrow and Feltham (1993) were used to determine growth at various temperatures and the reduction of nitrate and production of ammonia from arginine. Carbohydrate fermentation tests were conducted by using the API 50 CHL system (bioMérieux) according to the manufacturer's instructions.

Cells are rod-shaped, 0.4–0.6 × 2–3 µm, and occur singly, in pairs or in short chains comprising three or four cells. They are Gram-positive, catalase-negative, non-motile, non-spore-forming and facultatively anaerobic. After anaerobic growth at 37 °C for 48 hours, colonies on MRS agar are 2–3 mm in diameter; they are beige, smooth and circular with entire edges. In MRS broth, growth occurs at 15 °C but not at 45 °C. Ammonia is not produced from arginine. Nitrate is not reduced. Acid is produced from ribose, adonitol, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, mannitol, sorbitol, methyl- α -D-glucoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, trehalose, turanose, D-lyxose, and D-tagatose. Acid is weakly produced from inositol, sucrose, β -gentiobiose, gluconate and 5-keto-gluconate. Aesculin is hydrolyzed. Dextran is not produced from sucrose. Acid is not produced from glycerol, erythritol, D- or L-arabinose, D- or L-xylose, methyl- β -xyloside, rhamnose, dulcitol, methyl- α -D-mannoside, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, D- or L-fucose, D- or L-arabitol or 2-keto-gluconate.

DNA Extraction and Sequencing of 16S rRNA Gene.

Genomic DNA was extracted using benzyl chloride as previously described (Watanabe et al. 2008) with some modifications. One ml stationary phase bacterial culture was harvested by centrifugation at 20,000 × g for 3 minutes. The cell pellet was suspended in 250 µl extraction buffer (100 mM Tris-HCl, 40 mM EDTA, pH 9.0). Subsequently, 50 µl 10% sodium dodecyl sulfate, 150 µl benzyl chloride, and 0.7 g glass beads (0.1 mm diameter) were added to the suspension, which was then vortexed vigorously for 30 seconds with a FastPrep FP120 (Qbiogene, Carlsbad, CA, USA) on a power setting of 6.5 m/s to physically break the cells. The mixture was cooled on ice for 15 minutes after the addition of 150 µl 3 M sodium acetate. After centrifugation of the mixture at 20,000 × g for 15 minutes, the supernatant was collected and DNA was obtained by isopropanol precipitation. Finally, the DNA was diluted to 10 µg/ml in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

For 16S rRNA gene sequencing, primers 8F (5'-AGAGTTTGATCATGGTCAG-3'; positions 8 to 27) and 15R (5'-AAGGAGGTGATCCAACCGCA-3'; positions 1541 to 1522) were used to amplify the full length of bacterial 16S rRNA genes (positions 8 to 1541 in the *Escherichia coli* numbering system) (Brosius et al. 1978). The PCR mixture (25 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 µM dNTP, 1 mM MgCl₂, 1 µg BSA, 0.5 U *Taq* DNA polymerase (Takara Bio Inc., Japan), 0.1 µM each primer, and 10 ng template DNA. The PCR amplification program consisted of an initial denaturation step at 94 °C for 2 minutes; 30 cycles of 94 °C for 20 seconds, 55 °C for 20 seconds, and 72 °C for 20 seconds; and a final extension step at 72 °C for 3 minutes. The PCR-amplified 16S rRNA genes were purified with a Montage PCR Filter unit (Millipore Corporation, Billerica, MA, USA) and then sequenced by using primers 8F, 520F (5'-CAGGAGTGCCAGCAGCCGCGG-3'; positions 515 to 530), 930F (5'-GCACAAGCGGTGGAGCATGTGG-3'; positions 933 to 954), 800R (5'-CAGGACTACCAGGGTATCTAAT-3'; positions 804 to 787), 1100R (5'-AGGGTTGCGCTCGTTG-3'; positions 1115 to 1110), and 15R with an ABI PRISM BigDye Terminator v. 3.1 cycle sequence kit (Applied Biosystems) on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Sequence assembly was performed using AutoAssembler software version 2.1 (Applied Biosystems) and GENETYX-Mac version 13.0.1 (Software Development Co., Japan). The alignments were analyzed to compare similarities among the sequences by the neighbor-joining method (Saitou and Nei 1987) in the Clustal_X software version 1.82 (Thompson et al. 1997).

As shown in Table 1, the 16S rRNA gene sequence of *L. casei* strain Shirota was 100% identical to those of strains of *L. casei*—YIT 0180^T and *L. casei* YIT 0209, whereas levels of 16S rRNA gene sequence similarity between *L. casei* strain Shirota and strains of *L. zaeae*—YIT 0078^T and YIT 0278, and *L. rhamnosus* YIT 0105^T, were 99.23, 99.23 and 98.9%, respectively.

Table 1. 16S rRNA gene sequence similarities among the strains in the *Lactobacillus casei* group

	Species	Strain	Other designation	16S rRNA gene sequence		% Sequence similarity				
				Accession No.	Length (bp)	1	2	3	4	5
1	<i>Lactobacillus casei</i>	Shirota	BP-01366		1559					
2	<i>Lactobacillus casei</i>	YIT 0180 ^T	ATCC 334 ^T	AB008204	1563	100				
3	<i>Lactobacillus casei</i>	YIT 0209	NCDO 151	AB008205	1561	100	100			
4	<i>Lactobacillus zaeae</i>	YIT 0078 ^T	ATCC 393 ^T	AB008212	1563	99.23	99.23	99.23		
5	<i>Lactobacillus zaeae</i>	YIT 0278	ATCC 15820	AB008213	1559	99.23	99.23	99.23	99.74	
6	<i>Lactobacillus rhamnosus</i>	YIT 0105 ^T	ATCC 7469 ^T	AB008211	1540	98.90	98.90	98.90	98.83	99.09

DNA–DNA Relatedness and DNA G+C Content

For the determination of DNA–DNA relatedness and the DNA G+C content, chromosomal DNA was extracted according to the method of Marmur (1961). DNA–DNA hybridization analyses were performed between *L. casei* strain Shirota and other strains in the *L. casei* group—*L. casei* YIT 0180^T, *L. casei* YIT 0209, *L. zaeae* YIT 0078^T, *L. zaeae* YIT 0278 and *L. rhamnosus* YIT 0105^T. The microdilution-well technique was used as described by Ezaki et al. (1989), using a Fluoskan II microplate reader (Labsystems) for fluorescence measurement. Reciprocal hybridization experiments were performed for every pair of strains at 45 °C for 15 minutes in the presence of 50% formamide, using biotinylated DNA and unlabelled ssDNA,

which was bonded non-covalently to microplate wells. The data were calculated by the mean values done in four wells per each experiment.

To assess DNA base composition (G+C content), DNA was enzymically degraded into nucleoside as previously described (Mebash et al. 1989) and then separated by HPLC.

As shown in Table 2, the levels of DNA-DNA relatedness between *L. casei* strain Shirota and *L. casei* YIT 0180^T were 78–84%, which showed *L. casei* strain Shirota classified into *L. casei*, whereas the relatedness between *L. casei* strain Shirota and the type strains—*L. zeae* YIT 0078^T and *L. rhamnosus* YIT 0105^T was 9–17%. The values among the reference strains of *L. zeae* and *L. rhamnosus* were well below the 70% cut-off value that indicates separate species (Wayne et al. 1987). The DNA G+C contents of *L. casei* strain Shirota and strains in the *L. casei* group—YIT 0180^T, YIT 0209, YIT 0078^T, YIT 0278 and YIT 0105^T were 46.1, 44.2, 45.7, 47.8, 44.9 and 45.7 mol%, respectively.

Table 2. DNA base composition and levels of DNA-DNA relatedness between the strains in the *Lactobacillus casei* group.

Species	Strain	Other designation	DNA G+C (mol%)	% DNA relatedness with labelled DNA from					
				1	2	3	4	5	6
1 <i>Lactobacillus casei</i>	Shirota	BP-01366	46.1	100	78	nt	nt	nt	nt
2 <i>Lactobacillus casei</i>	YIT 0180 ^T	ATCC 334 ^T	44.2	84	100	103	23	28	15
3 <i>Lactobacillus casei</i>	YIT 0209	NCDO 151	45.7	91	69	100	18	20	15
4 <i>Lactobacillus zeae</i>	YIT 0078 ^T	ATCC 393 ^T	47.8	17	18	20	100	64	10
5 <i>Lactobacillus zeae</i>	YIT 0278	ATCC 15820	44.9	15	15	17	65	100	13
6 <i>Lactobacillus rhamnosus</i>	YIT 0105 ^T	ATCC 7469 ^T	45.7	9	11	15	13	16	100

Reciprocal DNA-DNA hybridization experiments were carried out at 45 °C for 15 min in the presence of 50% formamide. Values are the means of four replicate experiments.

Appendix 2
Genomic Analysis of *Lactobacillus casei* Strain Shirota
by Sato et al. (2004)

Genome Analysis of Lactic Acid Bacteria and Bifidobacteria: Genome Analysis of *Lactobacillus casei* and *Bifidobacterium breve*

Introduction

In 1995, the complete genomic sequence of *Haemophilus influenzae* was determined. It was the first bacterium to have its entire chromosome sequenced. Since then a rapid advancement has occurred in the methods of determining the genomic sequences of bacteria. Genome analysis began with model organisms such as *Escherichia coli* and *Bacillus subtilis* and progressed to analysis of pathogenic bacteria. In recent years, genome analysis has been performed on useful bacteria such as lactic acid bacteria. The published results of these genome analyses are available online, including at the National Center for Biotechnology Information and the Institute for Genomic Research. Many useful bacteria, including lactic acid bacteria, have been applied to commercial use, but their genome analysis results are mostly unpublished.

We performed genome analysis of two types of lactic acid bacteria: *L. casei* strain Shirota and *Bifidobacterium breve* (*B. breve*) Yakult. If the nucleotide sequences of the entire genome can be determined and the entire gene can be identified, then the genetic characteristics of the organism can be elucidated. If the organism is a lactic acid bacterium, it might be possible to improve or modify its lactic acid fermentation and utility as a probiotic. In addition, the results should be useful in determining the differences among closely related bacteria and strains. In this paper, we will present our results of genome analysis of *L. casei* strain Shirota and *B. breve* Yakult.

Methods

We used the whole genome shotgun sequencing in our genome analysis. It is the most frequently used method for bacterial genome analysis. First, the entire DNA is cut into small fragments and their nucleotide sequences are determined. Homologous sequences are assembled into longer nucleotide sequences on the computer. The procedure is divided into 5 steps. 1) DNA is isolated from the bacteria and cleaved by restriction enzyme or sonication. The cleaved DNA fragments are cloned, and a cloning library is constructed. 2) A sequencer is used to read the DNA nucleotide sequences in the library. 3) Nucleotide sequence data are collected and an assembly is performed using a computer. The sequence data are used to create linked contigs. 4) Adjacent contigs are identified, and the sequences are determined in the intercontig gaps. The chromosome sequence is completed by determining the sequences of the unreliable regions. 5) The resulting chromosome sequences are checked for errors.

1. Library construction and determination of nucleotide sequence

We isolated genomic DNA from *L. casei* strain Shirota and *B. breve* Yakult. Three libraries were constructed with DNA insert sizes of 0.9-2 kb, 6-10 kb, and 10-23 kb. The library with 0.9-2 kb DNA inserts was used to read nucleotide sequences and the other two libraries were used to read nucleotide sequences in the gaps and contig links. The estimated chromosome sizes of *L. casei* strain Shirota and *B. breve* Yakult are 3.2 Mb and 2.4 Mb, respectively. The sequence data were read for 60,000 clones of *L. casei* strain Shirota (redundancy: 9.6-fold) and for 35,000 clones of *B. breve* Yakult (redundancy: 7-fold). A portion of the sequence reading was subcontracted to Shimadzu Corporation. We used the ABI PRISM 377 DNA Sequencer (PE Biosystems) for the portion that we performed.

2. Assembly of sequence data

We used Phred/Phrap and Genome Gambler (Mitsui Knowledge Industry) for the assembly of the obtained sequence data. A computer searches homologous sequences. Overlapping sequences are identified and the sequence data are linked. Sets of long sequences called “contigs” are constructed on the computer. When contig assembly was monitored over time as the sequence data were accumulated, the number of assembled contigs initially increased as the sequence data increased. When the number of contigs reached its peak at a certain point, it decreased thereafter. In principle, the contigs should eventually merge to 1 or 2 larger contigs. In reality, they often merge to 100 to 200 contigs. In our case, the final number of contigs remained mostly unchanged even when the redundancy in *L. casei* strain Shirota was increased from 8- to 9-fold. This finding indicated that further sequencing was unnecessary. The reason is the existence of repetitive sequences and sequences which are difficult to decode among genome sequences, hindering the extension of contigs. The number of contigs for *L. casei* strain Shirota and *B. breve* Yakult were 120 and 160, respectively. The total sequence lengths in the contigs were consistent with the expected genome size. When formation of contigs was examined for *B. breve* Yakult, it appeared as if the number of contigs had not reduced sufficiently. Even with an increase in the amount of sequence data, we determined that the number of contigs would not decrease accordingly. Thus, the sequencing was stopped.

3. Contig links and construction of circular sequence

For *L. casei* strain Shirota 120 contigs and 160 contigs for *B. breve* Yakult were assembled on the computer. It was necessary to determine the arrangements of these contigs and the sequences in the intercontig gaps. When the sequences at the ends of the contigs are examined, there are often ribosomal RNA genes (rRNA), repetitive sequences such as insertion sequences (IS), and sequences which are difficult to decode. Common sequences are interspersed in a genome. When contigs are assembled on the computer in the presence of repetitive sequences, sequences which are not normally adjacent might be linked, producing chimera (or artificial) contigs or the contig extension is hindered. In a sequence which is difficult to decode, sequencing reaction is difficult to extend because of the secondary structure of DNA. Such a sequence cannot be read under normal conditions. It is seen often in DNA with a high GC content.

A bridging clone is necessary to resolve these problems. A bridging clone includes the applicable region and regions on either side which overlap both neighbors. The sequences on both sides of the applicable region can be determined using a bridging clone. As a result, the correct positional relationship of contigs can be determined. The intercontig gaps and undefined sequences can also be determined. Thus, we selected necessary DNA clones from a library with relatively long inserts. Since sequences which are difficult to decode cannot be read under normal conditions, it is necessary to change the sequencing reaction itself. In this manner, the positional relationships of the contigs were determined, and the sequences in the intercontig gaps were determined. As a result, we were able to concatenate the sequences of *L. casei* strain Shirota and *B. breve* Yakult into a single circular structure each.

4. Confirmation of circular sequence

Although the resulting circular structures were consistent with their chromosomes, the majority of structures were sequences created by the computer. Thus, it was necessary to confirm that

there were no large inconsistencies. The overlapping of sequence data was examined, and the sequences were rechecked in insufficiently overlapped areas. We confirmed that in the overall structure, restriction fragment lengths expected from the sequence were consistent with those from the cleaved genomic DNA fragments. A library with long inserts was used, and the sequences at both ends of the insert were confirmed to be located at a distance consistent with the insert length in the chromosome sequence.

Results

There is a plasmid pLY101 of 66.8 kb in *L. casei* strain Shirota. The sequence of this plasmid was determined by the Mizobuchi laboratory of the University of Electro-Communications. Besides *L. casei* strain Shirota and *B. breve* Yakult, we determined the DNA sequence of temperate phage FSW which infects *L. casei* strain Shirota. There were 3,035,753 bp nucleotide sequences for the chromosome of *L. casei* strain Shirota and 2,354,268 bp nucleotide sequences for the chromosome of *B. breve* Yakult. They were estimated to have 2,760 and 1,964 genes, respectively. There were 5 and 2 copies of rRNA operons and 70 and 29 ISs, respectively. The lengths of many ISs were approximately 1 kb for *L. casei* strain Shirota. In contrast, they were mostly short for *B. breve* Yakult, and they were thought to be inactivated. There were 58 and 54 copies of tRNA for *L. casei* strain Shirota and *B. breve* Yakult, respectively. Some tRNAs were missing in both bacteria. The majority of the missing ones had U as the third nucleotide of the codon. In *B. breve* Yakult, only one had U as the third nucleotide of the codon. *L. casei* strain Shirota had only one type of valine tRNA. When gene sequences were examined, all codons were used in both bacteria. The codons without their corresponding tRNAs were also used. Such codons were very likely recognized by other existing substitute tRNAs. This finding suggested the use of wobbling in codon recognition.

Genes were identified using GeneHacker Plus. A homology search was performed for each gene, and the gene names with hits were used. There were no major differences in the composition of genes between *L. casei* strain Shirota and *B. breve* Yakult.

There were some regions with low GC content in the chromosome of *B. breve* Yakult. These regions were often rRNA and phage-related sequences. There was one region of low GC content which was 32 kb. This region had IS-related sequences at both ends and was suspected to be of foreign origin. When the whole chromosome was plotted by GCskew, chromosome replication origins and termini could be estimated in the sequences with low GC content. The chromosome of *L. casei* strain Shirota is divided into two large regions when it is plotted by GCskew. There are replication origins and termini within the borders of the two regions. We found a region where the following were located: DNA replication origin gene *dnaA* and *dnaN* gene, and *dnaA* box which is a binding site of the DnaA protein. Thus, the replication origin was established as the border where these elements existed. If *B. breve* Yakult is plotted by GCskew, its chromosome will not divide into two regions. However, there are regions with the *dnaA*, *dnaN* gene, and *dnaA* box. Therefore, we estimated its replication origin taking into account positional relationships of replication origins and these genes in other bacteria.

The origin was established as the restriction site near the replication origin, and the chromosomal map was constructed. Distributions were compared between the plus (+) genes on the DNA plus (+) strands and the minus (-) genes on the DNA minus (-) strands. The chromosomal replication is thought to progress from both directions from the origin. When chromosomal replication occurs, the leading strand is + strand for the right half and - strand for the left half. The direction

of DNA synthesis in the leading strand is the same as that of the progressing replication. Thus, the direction is the same as the transcription direction of + gene in the right half and - gene in the left half. It also applies to rRNA operons. The following are seen in many bacteria: +gene and - gene ubiquity and correlation between directions of transcription and DNA synthesis. In addition, the chromosome divides into roughly two halves: one with many regions of +genes and the other with many regions of - genes. The border roughly coincides with the chromosome replication origin and terminus. This convention fits *L. casei* strain Shirota. In *B. breve* Yakult, the + gene regions are larger than the - gene regions, and the border does not coincide with chromosome replication origin and terminus. The reason is unknown. Other bacterial genomes have been reported to have dissimilar sizes of the + gene region and the - gene region.

The entire nucleotide sequences of *L. plantarum* WCFS1 and *B. longum* NCC 2705 have been published. Both are closely related strains of *L. casei* strain Shirota and *B. breve* Yakult. We compared these sequences. At the nucleotide sequence level, there were no major homologous regions between *L. casei* strain Shirota and *L. plantarum* WCFS1. At the operon level, there were homologous regions but they were not major. In contrast, there were major homologous regions in the sequences between *B. breve* Yakult and *B. longum* NCC 2705. There were four major homologous regions in both strains. If two of these regions were transposed, the sequences become very similar. There were also minor homologous regions. The arrangements of these minor homologous regions need to be considered. Otherwise, the sequences of these bacterial strains would not completely match each other. It is very important that if two of the major four homologous regions were transposed, these sequences become very similar. In *B. longum*, the + gene region and the - gene region are in a mosaic pattern and they are not clearly divided as in other bacteria. If homologous region I and III were transposed, the mosaic distribution of +gene regions and -gene regions disappears. As a result, these regions become clearly divided. This finding indicated that *B. breve* and *B. longum* are very closely related. If they evolved from the same prototype, *B. breve* is thought to be closer to the prototype. *B. longum* is believed to have had large-scale chromosome reorganization in the process of evolution.

We would like to thank our laboratory's Ms. Ayako Yamamoto and Ms. Mika Miura whose cooperation was invaluable in the DNA isolation for the libraries. We would also like to express our appreciation to Prof. Kiyoshi Mizobuchi of the University of Electro-Communications and Prof. Hirotada Mori of the Nara Institute of Science and Technology for their advice.

Appendix 3
Genomic Analysis of *Lactobacillus casei* Strain Shirota
by O’Sullivan (2012)

Safety Assessment of *Lactobacillus casei* YIT 9029 Based on an Analysis of its Complete Genome Sequence

Compiled by:

**Dan O'Sullivan
Professor
Department of Food Science and Nutrition
Microbial and Plant Genome Institute
University of Minnesota**

**Signature: _____
Dan O'Sullivan**

Date: January 25, 2012

Executive Summary

The complete genome sequence of *Lactobacillus casei* YIT 9029, consisting of a chromosome with 3,035,755 bp and a plasmid of 66,801 bp, was analyzed for any functional predictions that might be related to virulence or antibiotic resistance. This analysis did not reveal anything associated with virulence. It did contain one gene predicted to be a hemolysin, but all of the sequenced lactobacilli genomes to date also contain homologs of this, indicating it does not present a new safety issue here. Similarly, there were a few genes that may encode antibiotic resistance (eg kanamycin resistance), but these are also present in other lactobacilli and other gram positive bacteria used in foods. In addition, none was associated with mobile elements, indicating their presence doesn't present a new safety issue for this strain. In conclusion, this genome safety analysis did not reveal anything unusual in the genome of *L. casei* YIT 9029 from a safety viewpoint.

Objective:

To provide a safety assessment of *Lactobacillus casei* YIT 9029 based on the predicted potential genes encoded in its complete genome sequence

L. casei YIT 9029 Genome Sequence Annotation:

The complete genome sequence of *L. casei* YIT 9029 was previously determined and summarized by Sato et al. (2004). This study reported one chromosome of 3,035,753 bp and one plasmid of 66,801 bp. This sequence was forwarded to me on a CD by Dr. Hideyuki Shibata and the file contained a chromosome of 3,035,755 bp (the extra two bases may reflect an error in the earlier report or a sequence update) and a plasmid of 66,801 bp. The sequences were annotated using the gene prediction and annotation functions of the GAMOLA program (Altermann and Klaenhammer, 2003). This program uses the Glimmer gene prediction program for its gene calls (<http://cbcb.umd.edu/software/glimmer/>). An updated BLAST database (<http://www.ncbi.nlm.nih.gov/blast/>) was downloaded to enable this annotation to be done in house. The annotation was conducted using the default parameters, which minimize restrictions on gene calling, thus resulting in as many potential genes as possible.

This annotation revealed 4,269 potential genes encoded on the chromosome ranging in size from 29 to 2,725 amino acids. This is much larger than the 2,760 genes reported by Sato et al. (2004). The reason for this discrepancy is that the Sato et al. study removed those ORFs that are mostly not genes, such as potential ORFs within other genes. This is standard practice as it reflects a number closer to the actual gene number in a genome. However for a safety analysis it is more thorough to keep all the potential predicted genes, such that any functional motifs identified can be further analyzed. This automated annotation also revealed 83 potential genes for the plasmid, giving a total genome of 4,352 possible genes.

Gene Function Predictions for L. casei YIT 9029:

While functional predictions from genome sequences are still in its infancy, given the paucity of experimental validations for these predictions, it is currently the best tool available for assessing the functional potential of genomes. The Clusters of Orthologous Groups (COG) database at the National Center for Biotechnology Information (NCBI) was developed to enable predictions to be made on potential functional characteristics and to categorize genes based on predicted functions. It should be noted that the presence of a particular COG in a predicted protein does not always imply a similar function as different motifs can evolve into proteins with different functions.

The deduced amino acid sequences of all predicted genes in the *L. casei* YIT 9029 chromosome were compared to the COG database using GAMOLA-COG. This analysis assigned COGs to 1,719 of the possible predicted genes, which is a comparable number to the other sequenced lactobacilli genomes. The plasmid revealed COGs for 32 potential genes, giving a total of 1,751 COGs for the *L. casei* YIT 9029 genome. There are currently 25 functional categories in the COG database and 20 of them are represented in the *L. casei* YIT 9029 genome. This is comparable to the other lactobacilli genomes that have been sequenced. The functional

categories that are not represented in the *L. casei* YIT 9029 genome are A (RNA processing and modification); B (Chromatin structure and dynamics); W (Extracellular structures); Y (Nuclear structure); and Z (Cytoskeleton). A summary of the functional COG predictions is depicted in Table 1. A Table compiling the complete COG annotations for the 1,719 potential predicted genes of the *L. casei* YIT 9029 chromosome, grouped by functional category, was compiled and is attached as Appendix 1. An analogous file was compiled for the 32 potential predicted genes of the *L. casei* YIT 9029 plasmid and attached as Appendix 2.

Table 1: COG Functional Categories of the *L. casei* YIT 9029 Genome

Class	Individual Function Categories	Genes		
		Chr	Plasmid	Total
	Information storage and processing			
[J]	Translation, ribosomal structure and biogenesis	68		68
[K]	Transcription	129	2	131
[L]	DNA replication, recombination and repair	145	10	155
	Cellular processes			
[D]	Cell division and chromosome partitioning	20	1	21
[O]	Post-translational modification, protein turnover, chaperones	39		39
[M]	Cell envelope biogenesis, outer membrane	104	2	106
[N]	Cell motility and secretion	3		3
[T]	Signal transduction mechanisms	49		49
[U]	Intracellular trafficking and secretion	9	3	12
[V]	Defense mechanism	77	2	79
	Metabolism			
[C]	Energy production and conversion	89		89
[G]	Carbohydrate transport and metabolism	246	4	250
[E]	Amino acid transport and metabolism	177	4	181
[F]	Nucleotide transport and metabolism	56		56
[H]	Coenzyme metabolism	37		37
[P]	Inorganic ion transport and metabolism	77		77
[I]	Lipid metabolism	48		48
[Q]	Secondary metabolites biosynthesis, transport and catabolism	12		12
	Poorly characterized			
[R]	General function prediction only	203	3	206
[S]	Function unknown	131	1	132
	COG -assigned genes	1719	32	1751

The overall COG analysis of the *L. casei* YIT 9029 genome is comparable to other published lactobacilli genomes. The COG G category (Carbohydrate transport and metabolism) is the largest group reflecting the ability of the organism to adapt to different nutritional sources. This is an important feature for a successful intestinal organism as the nutritional sources in the gut are not constant. The COG V grouping (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. It should be noted that all lactobacilli genomes contain many of these COGs and while the COG annotation may refer to antibiotic resistance, it doesn't necessarily imply the same function here, but rather that the evolutionary history of the predicted gene reflects this.

Genome Based Safety Assessment of *L. casei* YIT 9029

Prior to focusing on the 1,751 genes that were found to have functional predictions by the COG analysis, each of the automated annotations of the 4,352 potential genes were manually assessed to see if any of the annotations reflected virulence or were otherwise of a possible safety issue. This did not reveal any potential genes with obvious virulence or of antibiotic resistance, other than those regions identified by the COG analysis. One interesting finding from this scan was the location of a region on the chromosome encoding a possible bacteriocin. This was recognized as it was a conserved hypothetical protein sandwiched by an active transporter encoding gene on one side and a two component signal transduction regulatory system on the other. Furthermore, analysis of the predicted gene also revealed all the obvious features of a real gene and also features associated with bacteriocins. Figure 1 depicts this potential bacteriocin gene region.

```

RBS
AAAAGAARTGAACAGT TTGATGAGCAGAAATGOTCAATATGAGTGCACGAAGAAC TTTTGGAA
M K Q F D E Q K M V N M E D E E L L E

TTCAATTGGAGGOTGACAGCATCCCTGATGTTTCCCCAAACATTC AACAAATAGAGAGATGCTTTGATGCTCTG
F I G G D S I R D V S P T F N K I R R W P D G L

TTTAAA
F K
  
```

Figure 1: Nucleotide sequence region of the *L. casei* YIT 9029 chromosome encoding a possible bacteriocin. The ATG proposed start codon is positioned at base number 2,480,107 on the *L. casei* YIT 9029 chromosome sequence. A conserved ribosome binding site (RBS) is indicated in blue and a potential double glycine GG processing site is indicated in red.

The automated sequence analysis of this region predicted a larger potential gene initiating at an upstream 'TTG' start site. However, there is no RBS associated with this possible start site, or the potential for translational coupling, indicating that the proposed 'ATG' start codon is the most probable start site. As bacteriocins also have a prepeptide, which is cleaved during secretion often at a double glycine 'GG' motif, the likely cleavage site for this bacteriocin is at the 'GG' motif indicated in Figure 1. In addition, a BLAST Protein analysis of this potential bacteriocin revealed an identical one in *Lactobacillus paracasei* and one annotation suggests it is an antimicrobial peptide. While there was no COG associated with this potential bacteriocin gene, there was a COG prediction for the downstream active transporter, which was "ABC-type bacteriocin lantibiotic exporters, contain an N-terminal double-glycine peptidase domain". This further supports the association of this gene region with the production of a possible small bacteriocin with a double glycine processing site on the prepeptide. This potential bacteriocin region is not a safety issue, but rather a positive attribute of a probiotic culture.

Based on the manual analysis of the automated annotations of the 4,552 potential genes and the COG V grouping, a subset of potential genes were compiled for individual assessment to evaluate their probable function and their potential for mobility. This assessment consists of individual BLAST searches as well as viewing the gene arrangement, as genes involved in a similar function tend to cluster together. This subset of 53 predicted genes of potential safety interest, all from the chromosome, is listed in Table 2 below. The plasmid encoded genes primarily involved in metabolic, phage resistance, replication and conjugative functions and nothing of a predicted safety concern.

Table 2: Genes of potential safety interest in the *L. casei* YIT 9029 chromosome

No.	Start	End	COG Number	COG Annotation
1	54267	55769	COG1132	ABC-type multidrug transport system, ATPase and permease components Length=536 Score=222 Expect=2e-57
2	65677	69295	c COG1131	ABC-type multidrug transport system, ATPase component Length=231 Score=122 Expect=7e-26
3	123495	124130	COG1131	ABC-type multidrug transport system, ATPase component Length=295 Score=176 Expect=4e-44
4	137610	138669	c COG1619	Uncharacterized proteins, homologs of microcin C7 resistance protein Mccf Length=377 Score=336 Expect=1e-92
5	367805	368638	COG2367	Beta-lactamase class A Length=504 Score= 61.6 Expect=2e-09
6	475690	476356	c COG1132	ABC-type multidrug transport system, ATPase and permease components Length=394 Score=221 Expect=1e-57
7	476750	477734	c COG1132	ABC-type multidrug transport system, ATPase and permease components Length=611 Score=188 Expect=2e-47
8	477609	479244	c COG1132	ABC-type multidrug transport system, ATPase and permease components Length=577 Score=268 Expect=1e-77
9	500160	500991	c COG1680	Beta-lactamase class C and other penicillin binding proteins Length=414 Score=110 Expect=3e-24
10	523567	524241	COG1131	ABC-type multidrug transport system, ATPase component Length=235 Score=174 Expect=2e-43
11	525163	526537	COG1131	ABC-type multidrug transport system, ATPase component Length=294 Score=180 Expect=2e-45
12	663643	664369	c COG1131	ABC-type multidrug transport system, ATPase component Length=258 Score=192 Expect=1e-48
13	670485	672188	COG1132	ABC-type multidrug transport system, ATPase and permease components Length=573 Score=579 Expect=1e-165
14	672208	673983	COG1132	ABC-type multidrug transport system, ATPase and permease components Length=590 Score=633 Expect=0.0
15	699464	700295	c COG0842	ABC-type multidrug transport system, permease component Length=284 Score=124 Expect=3e-26
16	700360	701242	c COG1131	ABC-type multidrug transport system, ATPase component Length=292 Score=223 Expect=6e-56
17	803710	805104	COG1132	ABC-type multidrug transport system, ATPase and permease components Length=535 Score=140 Expect=6e-33
18	979476	980271	c COG1968	Uncharacterized bacitracin resistance protein Length=284 Score=263 Expect=2e-76
19	1134566	1135702	c COG0842	ABC-type multidrug transport system, permease component Length=371 Score=236 Expect=6e-62
20	1135712	1136408	c COG1131	ABC-type multidrug transport system, ATPase component Length=240 Score=238 Expect=7e-63
21	1244762	1245178	COG4767	Glycopeptide antibiotics resistance protein Length=306 Score=77.4 Expect=1e-14

22	1264323	1266002		COG1132	ABC-type multidrug transport system ATPase and permease components Length=570 Score=561 Expect=1e-159
23	1266059	1267900		COG1132	ABC-type multidrug transport system ATPase and permease components Length=605 Score=720 Expect=0.0
24	1270264	1271214		COG1650	Beta-lactamase class C and other penicillin binding proteins Length=328 Score=176 Expect=9e-44
25	1322660	1323539		COG1131	ABC-type multidrug transport system ATPase component Length=307 Score=219 Expect=6e-57
26	1333512	1334502	c	COG0842	ABC-type multidrug transport system permease component Length=245 Score=104 Expect=2e-22
27	1334624	1335467	c	COG1131	ABC-type multidrug transport system ATPase component Length=301 Score=137 Expect=4e-32
28	1447371	1449110		COG1132	ABC-type multidrug transport system ATPase and permease components Length=604 Score=336 Expect=1e-92
29	1653246	1653923	c	COG1131	ABC-type multidrug transport system ATPase component Length=293 Score=171 Expect=2e-42
30	1710231	1711956	c	COG1132	ABC-type multidrug transport system ATPase and permease components Length=608 Score=559 Expect=1e-159
31	1711906	1713652	c	COG1132	ABC-type multidrug transport system ATPase and permease components Length=605 Score=638 Expect=0.0
32	1834561	1835278	c	COG1131	ABC-type multidrug transport system ATPase component Length=243 Score=285 Expect=6e-77
33	1915776	1916718	c	COG1650	Beta-lactamase class C and other penicillin binding proteins Length=391 Score=128 Expect=2e-29
34	1918017	1918899	c	COG1650	Beta-lactamase class C and other penicillin binding proteins Length=396 Score=136 Expect=1e-31
35	1984003	1984872		COG1131	ABC-type multidrug transport system ATPase component Length=292 Score=347 Expect=2e-95
36	1992191	1992874		COG1131	ABC-type multidrug transport system ATPase component Length=232 Score=152 Expect=6e-37
37	2002623	2004251	c	COG1132	ABC-type multidrug transport system ATPase and permease components Length=562 Score=144 Expect=4e-34
38	2215693	2217394	c	COG1132	ABC-type multidrug transport system ATPase and permease components Length=564 Score=586 Expect=1e-167
39	2220922	2221657	c	COG1650	Beta-lactamase class C and other penicillin binding proteins Length=397 Score=110 Expect=6e-24
40	2260695	2261537		COG1131	ABC-type multidrug transport system ATPase component Length=262 Score=179 Expect=6e-45
41	2276791	2260372	c	COG1132	ABC-type multidrug transport system ATPase and permease components Length=556 Score=258 Expect=3e-68
42	2337587	2338352	c	COG1131	ABC-type multidrug transport system ATPase component Length=295 Score=157 Expect=2e-47
43	2416672	2417355		COG1131	ABC-type multidrug transport system ATPase component Length=318 Score=209 Expect=5e-54
44	2417612	2418049		COG0842	ABC-type multidrug transport system permease component Length=255 Score=50.4 Expect=4e-06
45	2418403	2419005		COG0842	ABC-type multidrug transport system permease component Length=275 Score=79.3 Expect=6e-15
46	2421599	2422235	c	COG1131	ABC-type multidrug transport system ATPase component Length=231 Score=203 Expect=1e-53
47	2433425	2434009		COG1131	ABC-type multidrug transport system ATPase component Length=312 Score=121 Expect=2e-27
48	2663175	2663767	c	COG4767	Glycopeptide antibiotics resistance protein Length=354 Score=194 Expect=4e-49
49	2676542	2678020		COG1132	ABC-type multidrug transport system ATPase and permease components Length=606 Score=179 Expect=1e-44
50	2678787	2679399		COG1132	ABC-type multidrug transport system ATPase and permease components Length=606

				Score=214 Expect=7e-55
51	2699071	2700400	COG0534	Na ⁺ -driven multidrug efflux pump Length=446 Score=390 Expect=e-108
52	2687210	2688162	COG1680	Beta-lactamase class C and other penicillin binding proteins Length=335 Score=173 Expect=2e-43
53	2320370	2321767	COG1253	Hemolysins and related proteins containing CBS domains Length=430 Score=261 Expect=2e-69

As can be seen from Table 2, many of the COG annotations of these genes refer to components of multidrug or antibiotic resistance components. As COG groupings refer to protein evolutionary families, it doesn't imply that these genes are involved in antibiotic resistance. There were other defense related genes identified in the COG V grouping that were omitted from this analysis as they were not of a safety interest. These included several potential antimicrobial peptide transporters, restriction modification systems and abortive infection systems for phage resistance. Each of these 53 genes in Table 2 was manually evaluated as described above and the pertinent details regarding location and proximity to mobile elements are compiled in Table 3 below.

Table 3: Analysis of the genes with potential safety interest (from Table 2) in the *L. casei* YIT 9029 chromosome

Gene No.	Neighboring genes predicted functions [†]	Clustered with mobile element
1	Carbohydrate and amino acid transport	no
2	Carbohydrate and amino acid transport	no
3	Inorganic ion transport; Ribonucleotide reductase	no
4	Lactate dehydrogenase	no
5	Galactose metabolism and transport	no
6	Oligosaccharide transport	no
7	Oligosaccharide transport	no
8	Oligosaccharide transport	no
9	Pyruvate oxidase	no
10	Two component signal transduction system; Sortase cell surface proteins	no
11	Two component signal transduction system; Sortase cell surface proteins	no
12	Coenzyme transport	no
13	Coenzyme transport	no
14	Coenzyme transport	no
15	Daunorubicin resistance	no
16	Daunorubicin resistance	no
17	Peptidase; malate permease	no
18	Thiamine biosynthesis; hypothetical proteins	no
19	Inorganic ion transport	no
20	Inorganic ion transport	no

21	Carbohydrate transport, phosphoglycerol transferase	no
22	Bleomycin resistance, Beta-lactomase family	no
23	Bleomycin resistance, Beta-lactomase family	no
24	Bleomycin resistance, Multidrug transporter	no
25	Amino acid transport, Signal transduction	no
26	Amino acid transport, Signal transduction	no
27	Amino acid transport, Signal transduction	no
28	Elongation factor Tu, ATP dependant protease	no
29	Quinone reductase	no
30	Amino acid metabolism and transport	no
31	Amino acid metabolism and transport	no
32	Aminoglycoside phosphotransferase	no
33	Coenzyme transport and metabolism	no
34	Coenzyme transport and metabolism	no
35	Flavin reductase, Amino acid metabolism and transport	no
36	Flavin reductase, Amino acid metabolism and transport	no
37	Flavin reductase, Amino acid metabolism and transport	no
38	Esterase, Phosphoglyceromutase	no
39	Phosphoglyceromutase, Pyruvate oxidase	no
40	Glucose-1-dehydrogenase, ABC transporters	no
41	Transcription antiterminator, Cytochrome syn transporter	no ¹
42	Hypothetical protein, proteases	no
43	Hypothetical proteins, Transporters	no
44	Hypothetical proteins, Transporters	no
45	Hypothetical proteins, Transporters	no
46	Hypothetical proteins, DNA repair	no
47	Hypothetical protein, Potassium uptake	no
48	Nucleotide metabolism [Purine biosynthesis and UDP-N-acetylglucosamine pyrophosphorylase N-acetylglucosamine-1-phosphate undyltransferase	no
49	Tagatose 1, 6 diphosphate aldolase, tRNA synthase, Transporters	no
50	Tagatose 1, 6 diphosphate aldolase, tRNA synthase, Transporters	no
51	Hypothetic protein, Methyltransferase	no
52	N-acetylmuramic acid 6-phosphate etherase, muconate cycloisomerase	no
53	Hypothetical, Transporter, DNA mismatch repair	no

1. Transcription regulators are not included as they are associated with all of the genes of interest.
2. possible integrase or transposase located ~ 3 kb downstream.

As can be seen from Table 3, the majority of the 53 predicted proteins with possible safety interest are clustered with metabolic genes, suggesting non antibiotic resistance functions in *L.*

casei YIT 9029. Genes no. 15 and 16 encode active transporters of the multidrug variety and are linked with a gene projected to encode resistance to Daunorubicin. This is a chemotherapeutic drug used to treat some cancers and not of significance to infectious medicine. This therefore would not represent a safety concern. Gene no. 18 is predicted to encode a bacitracin resistance protein that likely functions by phosphorylation of undecaprenol, but is not linked to any other genes with safety issues or mobility. It is also conserved among the lactobacilli indicating it does not present a new safety issue for *L. casei* YIT 9029. Genes no. 22, 23 and 24 encode a cluster involved in active transport, bleomycin resistance and a beta-lactamase predicted to be analogous to penicillin binding proteins. These genes are also common among other lactobacilli and as they are not associated with mobile elements in *L. casei* YIT 9029, they would not be considered to present a new safety risk. Gene no. 32 is an active transporter clustered with a gene encoding aminoglycoside phosphotransferase. This feature confers resistance to the neomycin kanamycin family of antibiotics (Badarau et al., 2008). It is common among Gram positive bacteria, including lactobacilli, and as it is not associated with mobile elements in *L. casei* YIT 9029 it therefore would not be considered to present a new safety risk. There was one hemolysin homolog (gene no. 53) detected in the *L. casei* YIT 9029 chromosome. While hemolysins are considered virulence factors, homologs of them are common to many of the lactobacilli genomes (as well as the *Lactococcus lactis* subsp. *cremoris* SK11 cheese starter culture) and their true function is yet to be investigated in the lactic acid bacteria.

Genomic islands:

Genomic islands are generally defined by genome regions with a significantly different G/C content. They represent recent horizontal gene transfer (HGT) events and are sometimes associated with the acquisition of virulence genes. However this has not yet been observed for the lactobacilli genomes. There is one example of a large genomic island in some strains of *L. reuteri* that encodes reuterin (broad spectrum antimicrobial) and cobalamin (vitamin B12) production, which are desirable probiotic attributes. A scan of the G/C content of the *L. casei* YIT 9029 chromosome revealed several small genomic islands with a G/C content ~ 10% less than the overall G/C content of 46.35%. This is to be expected as it shows close evolutionary contact with other bacteria of the Firmicute phylum. None of these small genomic islands is associated with genes of a safety concern. There is just one genomic island with a significantly higher G/C content. This genomic island of ~ 2.5 kb has a G/C content of ~57% and is positioned between nucleotides 610813 and 613647 (Figure 2). It encompasses just one gene predicted to be an inorganic ion transporter. There were no significant genomic islands associated with the plasmid, other than a few short regions with a ~ 9% lower G/C content

**CONCLUSION OF THE EXPERT PANEL:
GRAS DETERMINATION FOR THE USE OF
LACTOBACILLUS CASEI STRAIN SHIROTA
AS A FOOD INGREDIENT**

**Prepared for:
Yakult Honsha Co., Ltd.
Tokyo, Japan**

March 2012

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**CONCLUSION OF THE EXPERT PANEL:
GRAS DETERMINATION FOR THE USE OF
LACTOBACILLUS CASEI STRAIN SHIROTA
AS A FOOD INGREDIENT**

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

Summary

- The bacterium that is the subject of this GRAS determination is the Shirota strain of the probiotic bacterium *Lactobacillus casei*. Although under current but rapidly evolving taxonomic assignments the strain would be regarded as a strain of *L. paracasei* rather than *L. casei*, its common and usual name remains *L. casei*.
- The strain was isolated in 1930, but later became infected with a prophage insertion and was replaced in 1980 with a prophage-removed derivative, which was designated YIT 9029 and deposited in the culture collection of the International Depository Authority, Agency of Industrial Science and Technology of Japan. This derivative has borne the name *L. casei* strain Shirota since that time.
- *L. casei* strain Shirota is intended for use as an ingredient in fermented milk products, including but not limited to the fermented milk drink sold under the trade name "Yakult." The strain's function is to serve as a probiotic microorganism.
- Yakult's fermented milk products contain up to 4×10^{10} cfu *L. casei* strain Shirota/serving. The recommended daily consumption is one to two servings. If two servings of fermented milk product are consumed, the expected maximum intake of *L. casei* strain Shirota is 8×10^{10} cfu/day.
- The whole genome shotgun sequence of *L. casei* strain Shirota was determined and the resulting sequence was annotated and analyzed for genes that could be possible safety concerns; the resulting analysis was published. No genes encoding for antibiotic resistance, or any sequences showing significant homology with known antibiotic resistance genes, were identified in either the genome or its single plasmid. The strain has the genome sequence of ornithine decarboxylase isozyme that catalyzes the conversion of ornithine into putrescine, several genes encoding for proteins related to known cell-invasion proteins, and one

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sequence showing homology to a putative hemolysin, all genes widely occurring in lactobacilli and other nonpathogenic bacteria and not implicated in any adverse effects.

- The strain is produced using industry-standard fermentation techniques under a HACCP system. Each lot is tested for enumeration of *L. casei* strain Shirota and subjected to standard plate count and counts of yeasts and mold, coliforms, *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus*. Fermented milk beverages containing the strain have been shown to be stable throughout their shelf lives.
- *L. casei* strain Shirota does not possess high capability for adhesion to intestinal surfaces, ability to degrade mucin, record of infectivity, production of undesirable metabolites, or the ability to transfer antibiotic resistance to other bacteria.
- In toxicity studies of *L. casei* strain Shirota, in which the strain was orally administered for up to 6 months at doses as high as 10^{12} cfu/day to mice, hamsters, rats, and rabbits, no signs of toxicity or infectivity were observed.
- *L. casei* strain Shirota has been given to healthy adults at doses up to 3.6×10^{11} cfu/day for as long as 5 weeks with no apparent adverse effects, and to health-compromised adults at doses up to 8×10^{10} cfu/day for up to 4 years, again with no observed adverse effects.
- Both healthy and health-compromised children, as well as healthy and compromised infants, have received *L. casei* strain Shirota for up to 55 months at doses up to 3×10^{10} cfu/day. No adverse effects were reported in any of these studies, nor in case reports in which severely ill infants or children were treated therapeutically with doses up to 4.5×10^9 cfu *L. casei* strain Shirota/day for as long as 2 years.
- In 1998, the Food and Drug Administration filed without comment a New Dietary Ingredient notification for the use of *L. casei* in dietary supplements. Similarly, in 2007 the European Food Safety Authority placed both *L. casei* and *L. paracasei* on its list of bacteria eligible for Qualified Presumption of Safety (QPS). This opinion was restated in 2008, 2009, 2010, and 2011 when QPS status assignments were reassessed.
- Finally, the safety of the intended use of *L. casei* strain Shirota is supported by its history of safe use with over 30 million bottles of Yakult consumed daily worldwide, including over 100,000 bottles a day in the U.S.

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Conclusion

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

All available evidence demonstrates clearly that there is no reason to suspect harm to individuals consuming fermented milk products containing *Lactobacillus casei* strain Shirota under its intended conditions of use, resulting in an estimated daily intake not exceeding 8×10^{10} cfu *L. casei* strain Shirota/day.

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

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Conclusion

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Conclusion

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Version 1.0

SUBMISSION END

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