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ORIGINAL SUBMISSION

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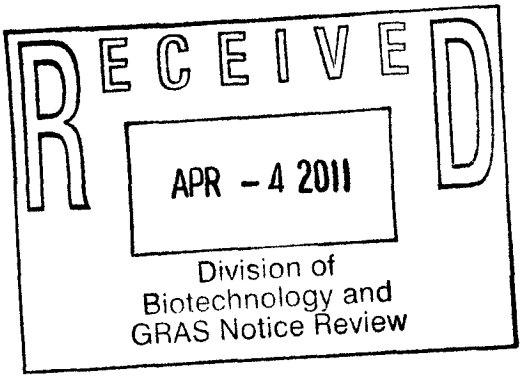
JHeimbach LLC



March 28, 2011

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), Cargill, Inc., through me as its agent, herby provides notice of a claim that the use of *Bifidobacterium animalis* ssp. *lactis* strain Bf-6 in conventional foods as described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because Cargill 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. Each copy includes the signatures of the three members of the GRAS expert panel at the end of the document.

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

Sincerely,

(b) (6)

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

Encl.

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Generally Recognized as Safe (GRAS) Determination for the Use of *Bifidobacterium animalis* ssp. *lactis* Strain Bf-6 in Conventional Foods

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Division of
Biotechnology and
GRAS Notice Review

Prepared for
Cargill, Inc.
Wayzata MN

Prepared by
JHeimbach LLC
Port Royal VA

March 2010

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1. GRAS Exemption Claim

Cargill, Inc., through its agent JHEIMBACH LLC, hereby notifies the Food and Drug Administration that the use of *Bifidobacterium animalis* ssp. *lactis* strain Bf-6 as described below is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because Cargill 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

Date 3/25/2011

1.1. Name and Address of Notifier

Cargill, Incorporated
15407 McGinty Road West
Wayzata MN 55391

Contact: Lore Kolberg, Ph.D.
Senior Manager, Regulatory & Scientific Affairs
Telephone: 952-742-1047
Facsimile:
E-mail: lore_kolberg@cargill.com

1.2. Name of GRAS Organism

The subject of this Generally Recognized as Safe (GRAS) determination is a strain of the probiotic bacterium *Bifidobacterium animalis* ssp. *lactis* designated Bf-6 . The strain was isolated from the feces of a healthy human.

1.3. Intended Use and Consumer Exposure

B. animalis ssp. *lactis* strain Bf-6 is intended to be added to a variety of foods that can sustain viable *B. animalis* ssp. *lactis* for the shelf life of the food. These foods include, but are not necessarily limited to, such dairy foods as fluid milks, yogurt, milk-based desserts and gravies, and cheeses; dry seeds, nuts, and nut butters; grain products such as flour, yeast breads, quick breads, cakes, cookies, pies, pastries, crackers, pancakes, waffles, French toast, and crepes; pasta, cooked and rte cereals, grain mixtures, and meat substitutes; fruits and fruit beverages; dark-green vegetables, olives, pickles, relishes, and vegetable soups; salad dressings; sugars and sugar substitutes, syrups, honey, molasses, jellies, jams preserves, gelatin desserts, ices and popsicles, candies, and chewing gum; and carbonated soft drinks, sports drinks and thirst quenchers, energy drinks, and water. The maximum use level (i.e., the initial addition level, intended to assure that target levels of viable *B. animalis* ssp. *lactis* remain throughout the shelf life) is 10^{11} cfu/serving.

1.4. Basis for GRAS Determination

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Cargill's GRAS determination for the intended use of *B. animalis* ssp. *lactis* strain Bf-6 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 the strain was made through the deliberations of

an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., and Michael W. Pariza, Ph.D., who reviewed a monograph prepared by JHeimbach LLC as well as other information available to them. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients including probiotic microorganisms. They critically reviewed and evaluated the publicly available information and the potential human exposure to *B. animalis* ssp. *lactis* strain Bf-6 resulting from its intended uses and individually and collectively concluded that no evidence exists in the available information on strain Bf-6 or other *B. animalis* strains that demonstrates, or suggests reasonable grounds to suspect, a hazard to adults or children under the intended conditions of use of *B. animalis* ssp. *lactis* strain Bf-6.

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 *B. animalis* ssp. *lactis* strain Bf-6 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

The subject of this Generally Recognized as Safe (GRAS) determination is a strain of the probiotic bacterium *Bifidobacterium animalis* ssp. *lactis* designated Bf-6. This strain has been referred to by many names in the published literature, in some cases due to taxonomic changes. The classification of the genus Bifidobacterium has been controversial since the first strain was isolated from the feces of a breast-fed infant in 1888 and named *Bacillus bifidus* (Velazquez and Feirtag 1997). The fragment "bifid," meaning "cleft in two" and based on the colony morphology of the genus, has been a common part of the name of the bacterium from the outset. After being regarded as a *Lactobacillus* species, bifidobacteria were later named *Bacterium bifidum*. When the Bf-6 strain was first isolated in the early 1990s it was identified as a *Bifidobacterium longum* and consequently was often referenced as *B. longum* B6 or *B. longum*-6. The strain was also referred to as *B. bifidum* and identified as *Bifidobacterium* Bf-6, BIF-6, Bf-12, or Bf-13 or *B. bifidum* Bf-6, Bf-12, or Bf-13. During the period between 1997 and 2004, when *B. lactis* was regarded as a separate species (Meile et al. 1997) rather than a subspecies of *B. animalis*, the strain was known as *B. lactis* Bf-6. It has been designated as *B. animalis* ssp. *lactis* since 2004 when it was decided, following a suggestion by Ventura and Zink (2002), that *lactis* is better regarded as a subspecies of *animalis* (Masco et al. 2004) rather than a separate species. The strain has also been denoted commercially or by university researchers as BA-61, CB111, SAN-94, and BG9.

2.2. Source

B. animalis ssp. *lactis* Bf-6 was isolated in the early 1990s from the feces of a healthy human and has been sold commercially since 1993. The strain was deposited with the Belgian Coordinated Collections of Microorganisms/Laboratory for Microbiology of the Faculty of Sciences of the Ghent University (BCCM/LMG) with LMG accession number LMG 24384. It is also maintained in the PROSAFE strain collection (Vankerckhoven et al. 2004) under the number PRSF-B112.

2.3. Description

Bifidobacterium animalis ssp. *lactis* is a Gram-positive, non-spore-forming, anaerobic rod-shaped bacterium measuring about 0.5-1.3 x 1.5-8.0 μm (Holt et al. 1994), and commonly occurs in "V"-shapes. Because of bifidobacteria's similarities in biochemical, physiological, and ecological properties, it is commonly considered to be a member of the broad classification of lactic acid bacteria (LAB) despite being part of the Actinobacteria phylum and family *Actinomycetaceae* (Velazquez and Feirtag 1997; Axelsson 1998; Vankerckhoven et al. 2008). LAB comprise a taxonomically diverse 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 generally aerotolerant, acid-tolerant, and strictly fermentative. 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; Vankerckhoven et al. 2004). LAB have a long history of use in fermented and non-fermented foods and have been noted for their ability to inhibit other microorganisms capable of

causing foodborne illness or food spoilage (Adams, 1999; Donohue and Salminen 1996). Furthermore, some LAB are ubiquitous as minor components in the intestinal epithelium and the gastrointestinal tract of humans of all ages. In humans, bifidobacteria occur predominantly in the colon, but can be commonly found in the mouth and vagina (Velazquez and Feirtag 1997). 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.

Bifidobacteria metabolize glucose exclusively by the fructose-6-phosphate pathway (Rogosa 1974), producing lactic and acetic acids in a theoretical molar ratio of 1.5:1 (Briczinski et al. 2009). This pathway, mediated by fructose-6-phosphate phosphoketolase, is often regarded as a unique identification feature due to the presence of this enzyme in bifidobacteria but not other LAB (Velazquez and Feirtag 1997). Barrangou et al. (2009) observed that the variety of hydrolases present in *B. animalis* ssp. *lactis* strains suggests the ability to utilize a wide range of complex carbohydrates, including milk galactosides and indigestible plant-derived oligosaccharides. Desjardins et al. (1990) determined that all bifidobacteria possess leucine aminopeptidase and α- and β-galactosidase activity, and *in vitro* studies of *B. animalis* ssp. *lactis* Bf-6, *B infantis* CCRC 14633, and *B. longum* CCRC 15708 by Hsu et al. (2005) showed that all3 were capable of producing β-galactosidase with transgalactosylation activity. This activity was highest when lactose, rather than glucose or galactose, was the carbon source.

It has been recognized for some time that *B. animalis* ssp. *lactis* is a subspecies with remarkably little genetic or phenotypic diversity. Two recent genomic analyses reveal just how genetically homogeneous the subspecies is. Barrangou et al. (2009) completed shotgun genome sequencing of 2 strains, DSM 10140 (the type strain) and BI-04. Comparison of the 2 genomes revealed "nearly perfect alignment"; overall, the 2 genomes were 99.975% identical. Optical mapping was also used to analyze the genome layouts of the 2 strains along with 3 other *B. animalis* ssp. *lactis* strains, Bi-07, HN019, and B420 and an experimental optical map of BI-04. The results are shown in Figure 1, reproduced from Barrangou et al. (2009).

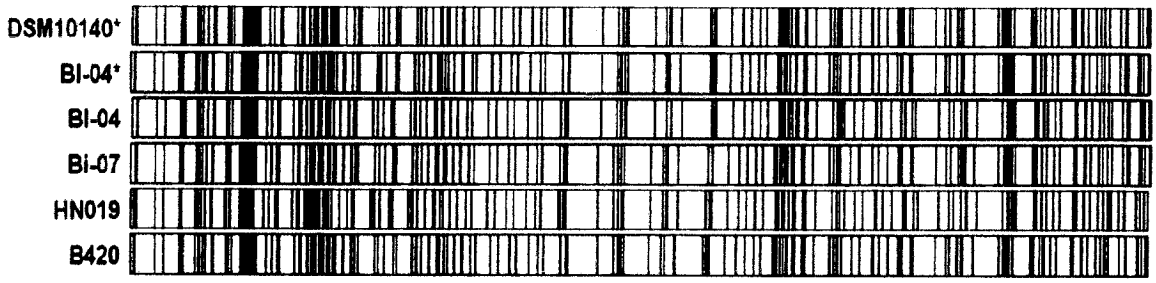

Figure 1. Optical Maps of *B. animalis* ssp. *lactis* Strains (Barrangou et al. 2009).

Barrangou et al. (2009) observed that, "The highly similar optical maps of this strain set … indicate a high degree of genome conservation in terms of size, organization, and sequence. The lack of polymorphism observed is indicative of a genomically monomorphic subspecies."

Briczinski et al. (2009) used randomly amplified polymorphic DNA-PCR (RAPD-PCR) analyses with 7 different primers to analyze 24 strains of *B. animalis* ssp. *lactis*. Although this is one of the most sensitive nucleic acid-based techniques available to differentiate closely related

strains, no differences were observed among the 24 strains analyzed with any of the 7 primers evaluated. A typical RAPD-PCR pattern, resulting from application of one primer and showing 6 of the 24 strains, is shown in Figure 2[1]. (The top and bottom lanes are 100-bp molecular weight markers; 6 *B. animalis* ssp. *lactis* strains are shown in lanes 2-7.)

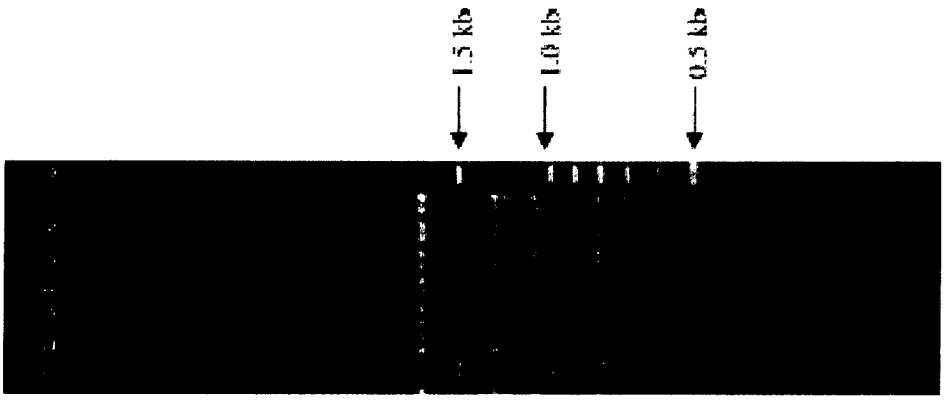

Figure 2. RAPD-PCR Patterns of *B. animalis* ssp. *lactis* Strains (Briczinski et al. 2009).

The high degree of similarity in the RAPD-PCR patterns among the tested strains is evident. Briczinski et al. (2009) concluded that, "Differentiating among a genomically monomorphic group, such as this collection of *B. animalis* subsp. *lactis* strains, was not possible with a variety of conventional phenotypic and nucleic acid-based techniques." (It was found that strains could be distinguished based on nonsynonymous single-nucleotide polymorphisms, which can appear in a small number of generations after separation from a common ancestor.)

The rep-PCR pattern of *B. animalis* ssp. *lactis* strain Bf-6 was compared with those of 2 other strains of the same subspecies, Bb-12 and DN 173010, that are present as probiotic bacteria in products currently sold in the U.S. It was found, as shown in Figure 3, that the 3 strains of *B. animalis* ssp. *lactis* had identical rep-PCR banding patterns. In order to further compare the 3 strains, rep-PCRs were repeated using both BOX and enterobacterial repetitive intergenic consensus (ERIC) primer sets. The BOX primer is generally recommended because it generates robust fingerprints and yields a highly complex fragment pattern, while ERIC generates highly discriminatory patterns (de Bruijn 2010). Even with the use of these primer sets the rep-PCRs for the 3 tested strains of *B. animalis* ssp. *lactis* were indistinguishable (Figure 4).

This same degree of genetic similarity was found in a study of antibiotic susceptibility of bifidobacterial strains in the Japanese market by Xiao et al. (2010), who obtained 7 strains of *B. animalis* subsp. *lactis* from Japanese products and found that they were indistinguishable with RAPD analysis, as well as in a review of the genomic characteristics of *B. animalis* ssp. *lactis* strain V9 (Sun et al. 2010).

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[1] Reproduced from Briczinski et al. (2009) and rotated 90° counterclockwise for convenience.

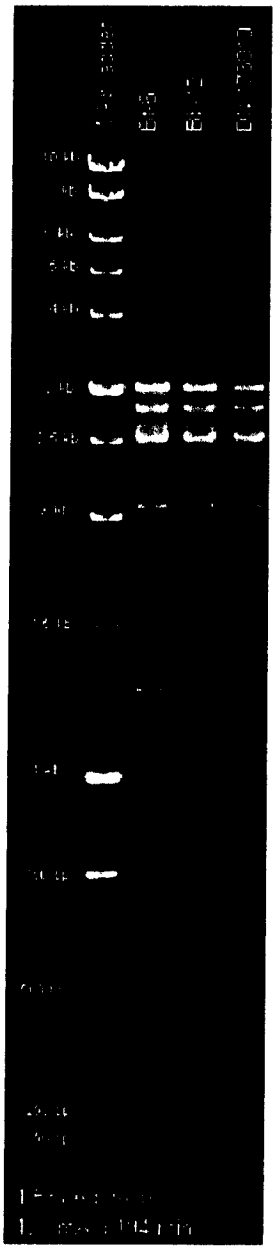

Figure 3. rep-PCRs of Strains Bf-6, Bb-12, and DN 173010.

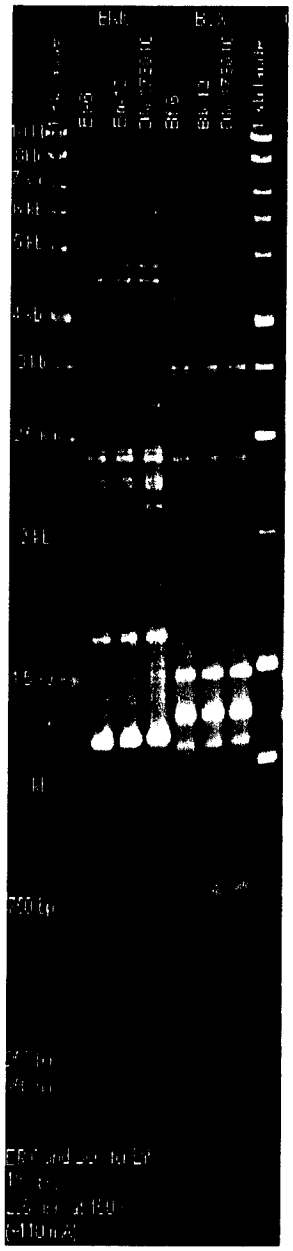

Figure 4. rep-PCRs with ERIC and BOX Primers.

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2.4. Genomic Analysis

The whole genome shotgun sequence of *B. animalis* ssp. *lactis* strain Bf-6 was determined and the resulting sequence was annotated and analyzed for genes that may be possible safety concerns. Bf-6 genomic DNA sequencing resulted in 221,208 reads giving 94,775,473 sequenced base pairs (bp). Assembly of these data produced 24 contigs of 1,916,102 bases (nearly all—1,915,271 bases—in 20 contigs of >500 bp, the remainder in 4 small contigs). The largest single contig contained 531,165 bases and the mean contig size was 95,763 bp.

The size of the Bf-6 chromosome—1,916,102 bp—compares with 1,942,198 and 1,944,050 bp in the recently published genome sequences of strain Bb-12 (Garrigues et al. 2010) and V9 (Sun et al. 2010), respectively. The latter strain has a slighly larger genome than other *B. animalis* ssp. *lactis* strains due to a unique insertion of 4,037 bp.

Barrangou et al. (2009) noted that the relatively small size of the *B. animalis* ssp. *lactis* genome is "typical of the genomic evolution of microbes that live in nutritionally rich environments, such as the human gut." From a safety point of view, this tendency of such bacteria to shed unneeded DNA results in a strain that is unable to survive in any other environment and thus reduces the risk of infection even if translocation were to occur. Lee and O'Sullivan (2010) observed, "The prolonged exposure of this subspecies to dairy fermentation environments is further supported by the reduced size of the genomes of this subspecies, which is a phenomenon that has been found to occur in organisms that are in less-complex environments where fewer features are needed to be successful. This may explain the inability of these bacteria to persist in the intestines of human subjects, as they have been observed to disappear rapidly after stoppage of eating foods containing these bacteria."

2.4.1. Antibiotic Resistance

The *tet*(W) gene was identified, as expected. Analysis of matches to published genomes showed 100% homology to ORF RBIC00158 from *B. animalis* ssp. *lactis* HN019. Further, the neighboring genes were highly homologous between the 2 strains, as well as with another published strain, *B. animalis* ssp. *lactis* AD011, which, however, lacks a nearby DNA-binding gene present in the other 2 strains.

Additionally, one ORF was identified as a possible kanamycin kinase gene, which showed about 60% homology with ORF RBBI01932 of *B. bifidum* NCIMB41171 and about 30% with *L. gasseri* ATCC-33323. This open reading frame and its surrounding contigs are 100% homologous to the published genomes of *B. animalis* ssp. *lactis* AD011, Bl-4, DSM10140, and HN019. It is not clear that this gene is functional; although strain Bf-6 is resistant to kanamycin, as discussed in section 4.3.5, this resistance is one manifestation of a more general nonsusceptibility to aminoglycosides and is more likely due to the absence of a cytochrome-mediated drug transporter (Moubareck et al. 2005).

Partial matches to several other antibiotic resistance genes were also found; these again are fully identical to open reading frames and the neighborhood (300 base pairs up- and downstream) in all published *B. animalis* ssp. *lactis* genomes. These hypothetical resistances, to daunorubicin, methicillin, mycinamicin, polymyxin, 5-nitroimidazole, phosphinothricin, and penicillin, may well not be functional; in the case of penicillin, at least, phenotypic data reveal that strain Bf-6 is susceptible to the drug.

2.4.2. 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 are histidine, tyrosine, hydroxytryptophane, tryptophane, lysine, ornithine, and arginine, which may be catalyzed by specific decarboxylases into histamine, tyramine, serotonin, tryptamine, cadaverine, putrescine, and spermine/spermidine, respectively.

The genome of strain Bf-6 was searched for genes that might encode the production of any of these specific decarboxylases. A total of 7 decarboxylases was identified, but only one that may possibly produce a biogenic amine—lysine decarboxylase, which may form cadaverine. This is identified by NCBI as a hypothetical function that has not been proven. Further searches revealed that this same open reading frame is found in all 4 strains of *B. animalis* ssp. *lactis* with published genomes, AD011, Bl-04, DSM10140, and HN019, and the surrounding contigs are identical in all strains.

2.4.3. Adhesion

Genes putatively encoding for 2 collagen adhesion proteins were identified and, more hypothetically, a gene possibly encoding for production of a fibronectin-binding protein.

2.4.4. Virulence/Infectivity

The genome of strain Bf-6 was examined for genes encoding factors putatively associated with virulence, including enterotoxins, outer-membrane or cytoskeleton-distending proteins, hemolysins, enterohemolysins, cell invasion and modulation proteins, endotoxins, cell-surface proteins, exotoxins, and inclusion proteins. No such genes were found. "Virulence factor MviN" is present in the genome, but since this is a widely distributed gene found in both pathogenic and nonpathogenic bacteria, its relevance to virulence has been questioned (Wassenaar and Gaastra 2001).

2.5. Production Process

All production steps are performed under an approved HACCP plan and consistent with current Good Manufacturing Practice.

2.5.1. Cryo Scale-Up

A solution of 93.9% purified water, 5.0% nonfat dry milk, 0.5% dextrose, and 0.5% yeast extract is sterilized at 121°C for 15 minutes, then lowered to 41°C. Filter-sterilized L-cysteine hydrochloride solution is added (0.05%) and the solution is inoculated with 0.5% *B. animalis* ssp. *lactis* Bf-6 culture. Incubation proceeds until the pH reaches 5.2-5.5, approximately 8 hours.

2.5.2. Preparation of Inoculum

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The growth medium is composed of 93.9% purified water and the following food-grade substances: nonfat dry milk (5.0%,) dextrose (0.5%), yeast extract (0.5%), polysorbate 80 (21 CFR §172.840; 0.1%). The incubator is sterilized by raising the temperature to 121°C for 15 minutes, and then the temperature is lowered to 41°C and filter-sterilized L-cysteine hydrochloride is added at a concentration of 0.05% along with 1.67% cryo scale-up *B. animalis* ssp. *lactis* Bf-6 culture. Incubation proceeds until the pH reaches 5.2-5.5, approximately 8 hours.

2.5.3. Production Fermentation

The fermentation medium comprises food-grade substances as follows: 91.9% purified water, 4.0% nonfat dry milk, 1.5% lactose, 2.0% yeast extract, 0.5% sodium citrate (21 CFR §184.1751), and 0.1% sodium hexametaphosphate (21 CFR §182.6760). The headspace is filled with CO_2.

After the fermentation tank is sterilized by a 6-second exposure at 141°C, the temperature is reduced to 41°C. *B. animalis* ssp. *lactis* strain Bf-6 seed culture is inoculated at a rate of 0.3%. As fermentation proceeds the pH is maintained at 5.5-5.6 by the use of food-grade ammonium hydroxide (21 CFR §184.1139). The medium is agitated as little as possible, but sufficiently to maintain the pH of the solution within the specified range.

The fermentation is regarded as having reached stationary phase when the pH shows no further tendency to decrease and no further addition of ammonium hydroxide is needed for 15 minutes. Ammonium hydroxide is then added to raise the pH to neutrality (7.0). The solution is pumped into a cooling tank in which its temperature is lowered to 12-15°C, after which the solution is centrifuged to increase the bifidobacteria concentration about 25 times. Before packing, the cell concentrate is passed through a rare-earth in-line magnet to detect any metal particles resulting from metal-to-metal abrasion.

2.5.4. Freeze-Drying

B. animalis ssp. *lactis* Bf-6 may be sold in the form of a cell concentrate as described above, or may be further processed to produce a freeze-dried product. If the culture is to be freeze-dried, a cryoprotectant consisting of 37% purified water, 58% sucrose, 2% trisodium citrate, and 3% propyl gallate is added. The product is frozen into small pellets and submerged in liquid nitrogen. The frozen cryoprotected pellets are loaded into a freeze-dryer and dried under vacuum. Once dried, the pellets are packaged into heat-sealed poly/foil bags.

2.6. Microbiological Purity

Each lot of *B. animalis* ssp. *lactis* Bf-6 seed strain is tested to assure that the strain is free of microbiological contaminants as shown in Table 1.

Table 1. Tests of Microbiological Purity for *B. animalis* ssp. *lactis* Bf-6 Seed Strains.

Parameter	Specification	Method
Coliforms (cfu[1]/g)	<1	AOAC 996.02
Coagulase-positive *Staphylococcus* spp. (cfu/g)	<1, absent	AOAC 2003.07 & 08
Enterococci (cfu/g)	<1	IDF[2] Std. 122A:1998
Non-lactics (cfu/g)	<1	IDF Std. 153:1991
Heterofermentative lactobacilli (cfu/g)	<1, absent	Internal method
Citrate utilization (cfu/g)	<1, absent	Internal method
Yeasts and molds (cfu/g)	<1	AOAC 997.02
Low-level cocci (cfu/g)	<1	Internal method
Low-level non-Bf-6 rods (cfu/g)	<1	Internal method

1. cfu = colony-forming units
2. IDF = International Dairy Federation

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Additionally, each fermentation batch of *B. animalis* ssp. *lactis* Bf-6 is screened for microbiological contaminants as shown in Table 2.

Table 2. Microbiological Screening for *B. animalis* ssp. *lactis* Bf-6 Fermentation Batches.

Parameter	Specification	Method
B. animalis ssp. *lactis* Bf-6 (cfu[1]/g)	$>6x10^{10}$	Internal method
Coliforms (cfu/g)	<1	AOAC 996.02
Coagulase-positive *Staphylococcus* spp. (cfu/g)	<1, absent	AOAC 2003.07 & 08
Enterococci (cfu/g)	<10	IDF[2] Std. 122A:1998
Non-lactics (cfu/g)	<100	IDF Std. 153:1991
Heterofermentative lactobacilli (cfu/g)	<1, absent	Internal method
Citrate utilization (cfu/g)	<1, absent	Internal method
Yeasts and molds (cfu/g)	<1	AOAC 997.02
Low-level cocci (cfu/g)	<1000	Internal method
Low-level non-Bf-6 rods (cfu/g)	<1000	Internal method
Salmonella spp. (25 g)	Absent	AOAC 2003.09
Listeria monocytogenes (25 g)	Absent	AOAC 2003.12

1. cfu = colony-forming units
2. IDF = International Dairy Federation

2.7. Stability

Observing that *B. animalis* ssp. *lactis* strains are by far the most widely used probiotic microorganisms, Briczinski et al. (2009) noted that the subspecies is robust with regard to stressful conditions such as acidity and oxygen, and so is able to withstand the adverse conditions of product manufacture and storage and can maintain viability and stability during product shelf life.

2.7.1. In-House Studies

The ability of *B. animalis* ssp. *lactis* to survive in a low-pH environment was tested by suspending cell pellets in phosphate-saline buffer adjusted to pH 3.0 by addition of HCl and held at 37°C for 0, 1, 2, and 3 hours. The results, shown in Figure 5, indicate excellent survival at this pH.

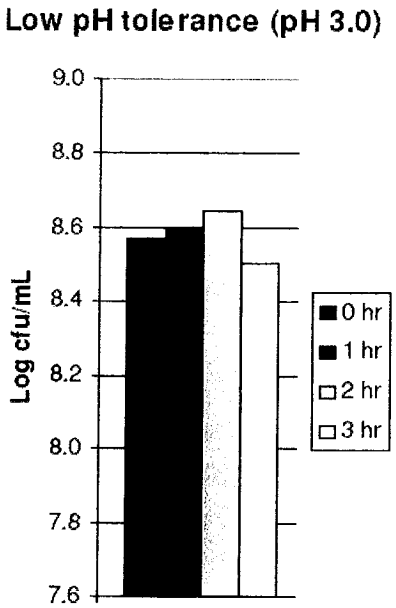

Figure 5. Survival of *B. animalis* ssp. *lactis* Bf-6 at pH 3.0.

Tolerance against bile was evaluated by plating cultures on MRS-cys agar containing oxgall at concentrations of 0, 0.2, 0.4, 0.6, 0.8, or 1.0%. The presence of bile slows bacterial growth but does not kill the cells, and *B. animalis* ssp. *lactis* Bf-6 was very tolerant of the presence of bile at concentrations as high as 1.0% (Figure 6), much higher than mean intestinal bile concentrations of about 0.3% (Gilliland et al. 1984).

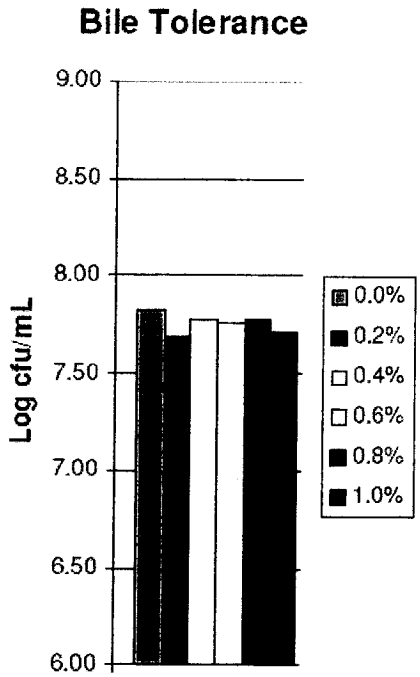

Figure 6. Bile Tolerance of *B. animalis* ssp. *lactis* Bf-6.

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Finally, in order to simulate the passage of ingested bacteria in the human gastrointestinal tract, in which the organisms survive the acid environment of the stomach before reaching the bile-containing environment of the intestine, *B. animalis* ssp. *lactis* Bf-6 cells were first exposed to pH 3.0 for 2 hours and then plated on agar containing 0, 0.5, or 1.0% oxgall; culture optical densities were measured over 12 hours. Proliferation of the strain was not significantly inhibited by this treatment, as shown in Figure 7.

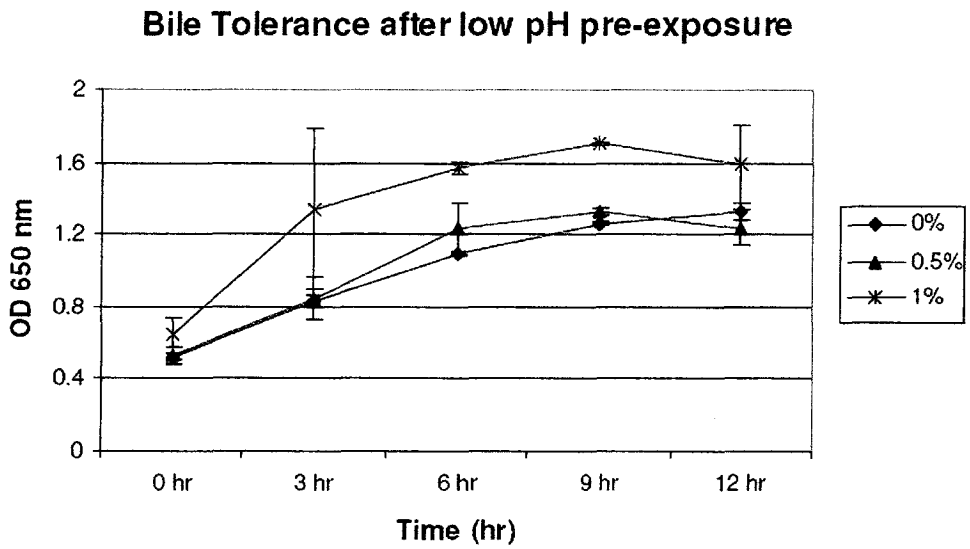

Figure 7. Bile Tolerance of *B. animalis* ssp. lactis Bf-6 after Acid Exposure.

2.7.2. Published Studies

Lian et al. (2002) compared the ability of several strains of bifidobacteria to survive spray drying using a variety of carrier media at different temperatures. The strains included *B. animalis* ssp. *lactis* Bf-6 (here designated as *B. longum*), *B. longum* CCRC 14634 and ATCC 15708, and *B. infantis* CCRC 14633 and CCRC 14661 and the spray-dry media included gelatin, gum arabic, soluble starch, and skim milk. In order to achieve the highest survival rates, all strains were tested while in stationary phase. The highest survival rate was 82.6% survival of strain Bf-6 when sprayed with skim milk at 50°C. This strain also achieved 63.7% survival when sprayed with gelatin and 41.2% with gum arabic, as compared with ranges of 10.8 to 54.3% for the *B. longum* strains and only 0.08 to 15.99% for the *B. infantis* strains. Most loss of viability was attributed to thermal injury.

Wang et al. (2004) also studied the viability in fermented soymilk subjected to freeze-drying or spray-drying, rehydration, and storage for 4 months at either 4°C or 25°C. The study included 4 strains of LAB—*B. animalis* ssp. *lactis* Bf-6 (here designated as *B. longum*), and strains of *B. infantis, L. acidophilus*, and *S. thermophilus*. Spray-drying resulted in a moisture content of the fermented soymilk of 4.2-10.6% while freeze-drying produced a drier product with only 2.9-3.5% moisture and generally resulting in a higher survival percentage of the LAB. Strain Bf-6, as has been found in other experiments with *B. animalis* ssp. *lactis* strains, exhibited greater survival than *B. infantis, S. thermophilus,* or lactobacilli. Storage at 4°C was significantly more conducive to survival than storage at 25°C, and storage in laminated pouches was superior

to storage in glass or PET bottles. The overall highest rate of survival of Bf-6 during 4 months storage of fermented soymilk was when the soymilk was freeze-dried and stored in laminated pouches at 4°C—68.8%. An increase of the temperature to 25°C reduced survival to 60.8%, and for no other condition did survival exceed 60%. The lowest survival—only 23.4%--resulted from spray-drying followed by storage in PET bottles at 25°C.

Adhikari et al. (2000) studied the viability of microencapsulated and non-encapsulated cells of *B. animalis* ssp. *lactis* Bf-6 and *B. longum* ATCC 15708 during 30 days' refrigerated (4.4°C) storage in yogurt. While a decline in viable populations of approximately 75% was seen for the non-encapsulated cells (due primarily to the low pH of yogurt), no loss in viability was seen with microencapsulation in κ-carrageenan.

Adhikari et al. (2003) followed up their previous work with additional study of the viability of microencapsulated (in κ-carrageenan) and non-encapsulated cells of *B. animalis* ssp. *lactis* Bf-6 and *B. longum* ATCC 15708 in yogurt. In this study the metabolic activity of the bifidobacteria was also examined. Encapsulated or non-encapsulated cells of the two strains were introduced into stirred yogurt which was refrigerated at 4.4°C and stored for 30 days. The non-encapsulated cell populations decreased by approximately 90% while no loss in viability was seen in populations of microencapsulated cells. All yogurt samples had similar levels of lactic and acetic acids, which the authors concluded indicates that the tested strains of bifidobacteria have little metabolic activity in yogurt.

Hsiao et al. (2004) further investigated the stability of microencapsulated strains of *B. animalis* ssp. *lactis* Bf-6 (referred to here as *B. longum* B6) and *B. infantis* CCRC 14633. The strains were microencapsulated with skim milk, gum arabic, gelatin, or soluble starch capsules and viability over 42 days of storage was assessed when the capsules were held at either 4°C or 25°C, in either glass or polyester bottles, with or without deoxidant and desiccant. The Bf-6 strain was generally more stable than CCRC 14633. Viability was highest when the capsule walls were composed of skim milk, when the storage temperature was 4°C rather than 25°C, when the capsules were kept in glass bottles, and when antioxidant and desiccant were added. However, addition of antioxidant and desiccant were of little value when storage was at 4°C because loss was already negligible—only 0.15-0.20 log, but it made a significant difference at 25°C.

Chou and Hou (2000) studied growth of *B. animalis* ssp. *lactis* Bf-6 (referred to as *B. longum* B6) and *B. infantis* CCRC 14633 in soymilk, both unsupplemented and with added glucose, lactose, or galactose. Growth of both strains was supported in the unsweetened soymilk and significantly increased by the addition of sugars. During 10-day storage of the fermented soymilk at 5°C, viable populations of both strains—but especially Bf-6—were significantly reduced, CCRC 14633 by 0.44 log and Bf-6 by 3.18 log. Viability loss was significantly greater when the fermented soymilk was stored at 25°C rather than at 5°C. There was less reduction in pH with strain Bf-6 than with CCRC 14633, indicating a lower level of metabolic activity.

Wang et al. (2002) also studied the growth and survival of several bacterial strains during the fermentation and storage of soymilk. In addition to *B. animalis* ssp. *lactis* Bf-6 (referred to as *B. longum* B6), tested strains included *B. infantis* CCRC 14633, *Streptococcus thermophilus* CCRC 14085, *Lactobacillus acidophilus* CCRC 14079, and *L. bulgaricus* CCRC 14009. All strains grew well during fermentation, although the presence of bifidobacteria had a deleterious effect on the growth of *L. bulgaricus*. Metabolic activity of strain Bf-6 was low as evidenced by

little reduction in the pH of the soymilk during fermentation with this strain. Only negligible loss of viability was observed when fermented soymilk was stored for 10 days at 5°C, but survival was low at 25°C with about a 6-log decline.

Chick et al. (2001) investigated survival and metabolic activity of *B. animalis* ssp. *lactis* Bf-6 (referred to as *B bifidum* Bf-13), and strains of *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *L. delbrukeii* ssp. *bulgaricus* in reconstituted nonfat dry milk containing 0 or 5% honey, sucrose, or fructose. Samples were incubated for 24 hours at 37°C. All 4 strains grew equally well with any of the sweeteners or in the control unsweetened nonfat dry milk. Strain Bf-6, but not the other tested strains, produced significantly more lactic acid in the presence of honey than other sweeteners, but production of acetic acid did not differ between samples.

In a study similar to Chick et al. (2001), Ustunol and Gandhi (2001) also studied the growth and metabolic activity of bifidobacteria in reconstituted nonfat dry milk containing 5% honey, sucrose, fructose, or glucose. The test strains were designated as *B. bifidus* Bf-1 and Bf-6; the latter strain is that now known as *B. animalis* ssp. *lactis* Bf-6. After inoculation with the bacterial strains, the skim milk was incubated anaerobically for 48 hours at 37°C with samples taken every 12 hours. After fermentation, the skim milk was stored for 28 days at 4°C with samples taken every 7 days.

Both strains grew best in the skim milk supplemented with honey. During the first 12 hours of fermentation, the doubling time of Bf-6 ranged from 222 minutes in skim milk with honey to 375 minutes in skim milk with fructose; during the last 12 hours the doubling times were 665 minutes and 810 minutes, respectively. During this time the pH of the skim milk decreased from about 6.2 to about 3.7 due to production of both acetic and lactic acids. Added honey also resulted in a significantly higher survival rate—59%--during the first week of cold storage, but little difference between sweeteners was apparent after 3 or more weeks when viability had decreased to only 3-5%.

Davidson et al. (2000) produced yogurt using only the traditional yogurt strains *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, or with the traditional strains plus *B. animalis* ssp. *lactis* Bf-6 (identified here as *B. longum*) and *L. acidophilus*. The yogurt was frozen and stored for 11 weeks at -20°C. None of the strains showed any loss in viable populations over the storage period, and there was no difference between the two yogurts in lactose or protein concentration.

3. Intended Use and Exposure

B. animalis ssp. *lactis* Bf-6 is intended to be added as a probiotic microorganism to conventional foods at concentrations consistent with cGMP needed to provide at least 10^9 cfu/serving throughout the shelf life of the product. This addition level will usually be between $5x10^9$ and $5x10^{10}$ cfu/serving but may reach 10^{11} cfu/serving, which provides for the loss of viability of from 80% to 99% of the bacteria added. The addition level will vary by food category, since the stability of the organism is different in different foods and because different categories of foods have different shelf lives. The foods that are most widely consumed for their probiotic benefits are dairy products such as yogurt, and these are also the foods in which bifidobacteria are most stable. Consequently, these are foods in which less overage in probiotic addition is needed than some other foods.

3.1. Food Categories for Addition of *B. animalis* ssp. *lactis*

B. animalis ssp. *lactis* strain Bf-6 is intended to be added to a variety of foods that can sustain viable *B. animalis* ssp. *lactis* for the shelf life of the food[1]. This includes both foods for which current technology ensures sustainability and also those for which such technology may be developed in the future. These foods include, but are not limited to, such dairy foods as fluid milks, yogurt, milk-based desserts and gravies, and cheeses; dry seeds, nuts, and nut butters; grain products such as flour, yeast breads, quick breads, cakes, cookies, pies, pastries, crackers, pancakes, waffles, French toast, and crepes; pasta, cooked and rte cereals, grain mixtures, and meat substitutes; fruits and fruit beverages; dark-green vegetables, olives, pickles, relishes, and vegetable soups; salad dressings; sugars and sugar substitutes, syrups, honey, molasses, jellies, jams preserves, gelatin desserts, ices and popsicles, candies, and chewing gum; and carbonated soft drinks, sports drinks and thirst quenchers, energy drinks, and water. It is not intended to add *B. animalis* ssp. *lactis* Bf-6 to meat or poultry products or to foods intended for infants.

Because non-fat dry milk is used in the production of the organism, all foods to which *B. animalis* ssp. *lactis* Bf-6 is added will bear a label statement regarding the presence of milk products.

3.2. Estimated Daily Intake of *B. animalis* ssp. *lactis*

B. animalis ssp. *lactis* strain Bf-6 is expected to be present in a limited number of foods at between 10^9 and 10^{11} cfu/serving, usually at less than 10^{10} cfu/serving. It will not proliferate in the foods and beverages to which it is added, but instead will decline over the shelf-life of the food. Its likely maximum ingestion is thus less than 10^{11} cfu/day, well within levels that have been shown to be safe.

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[1] The food categories to which strain Bf-6 may be added correspond to the following food-group codes from the NHANES: 111, 113, 115, 116, 118, 121, 122, 123, 131, 132, 133, 134, 140, 141, 142, 143, 144, 145, 146, 147; 421, 422, 423, 424, 425, 431; 510, 511, 512, 513, 514, 515, 516, 518, 521, 522, 523, 524, 531, 532, 533, 534, 535, 536, 540, 541, 542, 543, 551, 552, 553, 554, 555, 556, 557, 558, 561, 562, 570, 571, 572, 573, 574, 576, 578, 581, 582, 583, 584, 585, 590; 611, 612, 621, 631, 632, 633, 634, 641, 642, 671, 672, 673, 674, 675; 721, 722, 723, 755, 756; 831, 832; 911, 912, 913, 914, 915, 916, 917, 918, 924, 925, 926, 929, 940, 941, 942.

If all foods were consumed late in their shelf life when bacterial count had declined, intake of 10^{11} cfu/day would be achieved only by those who consume 100 servings/day of foods supplemented with bifidobacteria. At more reasonable concentrations of $5x10^9$ to 10^{10} cfu/ serving, between 10 and 20 servings of foods containing the strain are required to ingest 10^{11} cfu. Since the average individual eats only about 20 servings per day of all food combined, and it is most unlikely that half or more of them will include *B. animalis* ssp. *lactis* strain Bf-6, 10^{11} cfu/day is a very conservative estimate of potential daily intake.

4. Safety

4.1. Safety of Lactic Acid Bacteria and Bifidobacterium 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). *Bifidobacterium* is a dominant genus considered beneficial to humans (Barrangou et al. 2009), the population of which varies over time. Following vaginal delivery, bifidobacteria colonize the infant gut during the first days of life and typically remain dominant until weaning.

Probiotic strains are selected to impart beneficial effects on the host and on the composition and/or metabolism of the intestinal microbiota without causing adverse changes (e.g., invasion of the epithelial cells, degradation of the intestinal mucin layer, production of toxins, transference of antibiotic resistance) that would imperil the health or nutritional status of the host. *B. animalis* ssp. *lactis* is the most common *Bifidobacterium* utilized as a probiotic in commercial dairy products in North America and Europe (Barrangou et al. 2009). Briczinski et al. (2009) also noted this widespread use of *B. animalis* ssp. *lactis* strains, pointing out that the subspecies is relatively less sensitive to stressful conditions such as bile, acidity, and oxygen, which might be encountered in the mammalian gastrointestinal tract, and is thereby better able to withstand the adverse conditions of product manufacture and storage and to maintain viability and stability during product shelf life.

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 bifidobacteria 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). 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

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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."

The 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, has caused some to question the safety of probiotics. The subjects in this study were acutely ill with a condition the authors indicated has a 10-30% mortality rate. It was noted that 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. 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. The route of nutrient administration in acute pancreatitis, be it naso-jejunal or parenteral, is highly debated, therefore provoking further controversy regarding the methodology employed in this trial. Further, the chosen formula was a complex formula containing multiple blends of dietary fiber (15 g/L) that may have potentiated fermentation in the small bowel given the impaired digestion and intestinal dysmotility expected in these patients and the concomitant provision of lactic acid-producing bacteria.

Albeit hypothetical, this scenario of intestinal statis, fermentable substrate, and lactic-acid producing bacterial overgrowth is consistent with suspected etiology in non-occlusive small bowel necrosis and would only be applicable to probiotics administered along with a fermentable substrate by intrajejunal tube feeding to patients with severely restricted small intestinal motility. The conditions of use of probiotics in this study differ so radically from the intended use of *B. animalis* ssp. *lactis* Bf-6 that the outcome does not raise any concern regarding the safety of the intended use of *B. animalis* ssp. *lactis* Bf-6.

4.2. History of Consumption of *B. animalis* ssp. *lactis*

Mogensen et al. (2002) observed that LAB (including bifidobacteria) are consumed "in enormous quantities," primarily through consumption of fermented foods. They estimated that the average European ingests about 2.2×10^{12} LAB/year[1]. Mogensen et al. (2002) also reviewed 54 cases of endocarditis in which LAB were isolated; none of these isolates was a *Bifidobacterium*. Although bifidobacteria have been isolated from non-specific bacteremias, the species isolated are not those in use as probiotics (Sanders 2006). As noted earlier, Barrangou et al. (2009) and Briczinski et al. (2009) reported that *B. animalis* ssp. *lactis* is the most common *Bifidobacterium* utilized as a probiotic in commercial dairy products in North America and Europe. Sanders (2006) specifically observed that the history of use of *Bifidobacterium* species, including *B. animalis* ssp. *lactis*, is a long history of *safe* use in that no cases of clinical infection have been reported from such use.

4.3. Safety Parameters

4.3.1. Ability to Adhere to Intestinal Cells

Although adherence of probiotic bacteria to intestinal surfaces is not confirmed to be required for health benefits, it has been hypothesized to be involved in establishing residence, for stimulation of the immune system, and for antagonistic activity against enteropathogens (Gopal et al. 2001). Nevertheless, some concern has been expressed that high adhesion capability—a characteristic of pathogens—may facilitate platelet aggregation and bacterial infectivity (Kirjavainen et al. 1999). *In vitro* assays of the adherence ability of bacterial strains are commonly conducted; however, their ability to predict *in vivo* adherence is uncertain. Similarly, the presence of adhesins in the bacterial genome—as in the case of *B. animalis* ssp. *lactis* strain Bf-6 and nearly all other bacterial strains—is of uncertain significance with regard to in vivo adherence.

The adherence properties of 5 probiotic strains, including *B. animalis* ssp. *lactis* HN019, were evaluated *in vitro* by Gopal et al. (2001). Bacterial strains *B. animalis* ssp. *lactis* HN019, *L. rhamnosus* HN001, *L. rhamnosus* GG, *L acidophilus* LA-1, and *L. acidophilus* HN017 were grown in MRS broth to a concentration of 10^8 cfu/ml, which was then added to monolayers of cell-lines HT-29 (ATCC 38-HTB), Caco-2 (ATCC 2102-CRL), or HT29-MTX (a mucous-secreting cell-line) at 37°C. *L. bulgaricus* LB-1, known not to adhere under these conditions, was used as a negative control. Adherence was evaluated in 20 random microscopic fields and the number of adhering bacteria per 100 cells of epithelial cell-line was determined. Adhesion was also measured indirectly by radiolabeling the probiotic strains with ^{14}C-uracil and ^{14}C-adenine and measuring the radioactivity incorporated into the cell-lines. The ability of probiotic strains *B. animalis* ssp. *lactis* HN019, *L. rhamnosus* HN001, and *L. acidophilus* HN017 to inhibit colonization of the intestinal cell monolayers by a known enterotoxigenic strain of *Escherichia coli*, O157:H7 was also tested. Finally, the effects of 2 proteolytic enzymes (trypsin and proteinase K) and of lactate dehydrogenase on antimicrobial activity were investigated.

There were no significant differences in adhesion ability among the 5 probiotic strains tested; all 5 exhibited strong adhesion properties, especially with the mucous-secreting cell-line HT29-MTX. The negative control, *L. bulgaricus* LB-1, showed a complete absence of adhesion

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[1] Equivalent to 6.0×10^9 LAB/day.

to any cell-line, providing evidence that the adhesion was more than non-specific physical entrapment. Pretreatment of the cell-lines with probiotic strains reduced both the adherence of *E. coli* O157:H7 and its invasiveness. Treatment extracts of the probiotic strains with a proteolytic enzyme significantly reduced their antimicrobial activities and lesser reductions resulted from treatment with lactate dehydrogenase, suggesting that overall inhibition may be due to a synergistic action of proteinaceous substances and lactic acid.

On the other hand, Wagner et al. (1997a), in a study discussed in detail in Section 4.5.1.2, studied probiotics in congenitally immunodeficient gnotobiotic beige-athymic (*bg/bg-nu/nu*) and beige-euthymic (*bg/bg-nu/+*) mice. An unidentified strain of *B. animalis* adhered well, as did the tested strain of *L. acidophilus*, but *L. rhamnosus* strain GG (referred to in the article as *L. casei*) and a strain of *L. reuteri* adhered poorly. Translocation generally correlated with adherence, although the bacteria that did translocate did not result in bacteremia, even in this doubly immunodeficient model. (Indeed, the authors concluded that "*L. acidophilus* and *B. animalis* appear to be innocuous probiotics in immunodeficient mice.") The likelihood that adherence to intestinal mucosa may also increase bacterial translocation was more recently noted by Boyle et al. (2006).

Ouwehand et al. (2003) studied adhesion of 6 selected lactic acid bacteria to resected colonic tissue and mucus in patients with three major intestinal diseases (diverticulitis, rectal carcinoma, and inflammatory bowel disease) and compared this adherence with adherence to healthy control tissue. The strains tested were *B. animalis* ssp. *lactis* strain Bb-12, 3 *L. rhamnosus* strains, *L. breve* PEL1, and *L. reuteri* ING1. Tissue adhesion varied widely; the highest adhesion was 9.7% of LGG to tissue from patients with IBD while the lowest was 1.4% of LC705 to tissue from patients with diverticulitis. All strains were observed to adhere better to immobilized mucus than to whole intestinal tissue; for example, 27.6% of LGG adhered to intestinal mucus from healthy individuals while only 4.9% adhered to healthy intestinal tissue. All strains adhered better to tissue resected from IBD patients than tissue from healthy individuals although this difference was significant only for *L. rhamnosus* strain GG and *L. reuteri*. Similarly, tested strains displayed disease-specific adhesion to intestinal mucus. Adherence was not related to species; overall, the best and worst adhering strains were both *L. rhamnosus*, strain GG (best) and strain LC705 (worst).

4.3.2. Ability to Degrade Mucin

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

Zhou et al. (2001) investigated the ability of *B. animalis* ssp. *lactis* HN019, *L. rhamnosus* HN001, and *L. acidophilus* HN017 to degrade mucin *in vitro*. The mucin source was purified hog gastric mucin. Mucin was suspended with one of the test strains, or with *L. acidophilus* LA-1 (as a negative control) or a fecal flora extract (as a positive control), for 24 hours at 37°C.

Mucin suspensions were prepared both with 1% glucose as an energy source or without glucose, leaving the glycoproteinaceous mucin itself as the only potential source of energy. Bacterial growth was measured under each condition. Three approaches were used to assess potential mucin degradation. First, changes in the carbohydrate and protein concentrations were measured. A decrease in carbohydrate concentration indicates degradation of the oligosaccharide chains while a decrease in the protein concentration indicates degradation of the protein backbone. Second, SDS-PAGE was used to analyze the electrophoretic patterns of mucin samples before and after exposure to bacterial strains in order to detect any changes in the molecular weight of the glycoprotein. Finally, mucin was placed in a Petri dish and inoculated with a bacterial sample; after 24 hours at 37°C the size of the mucin lysis zone was measured.

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

Table 3. Degradation of Mucin.

Treatment	% Degradation	
	Carbohydrate	Protein
B. animalis ssp. *lactis* HN019	4.7	3.9
L. acidophilus NH017	-8.3	5.0
L. acidophilus LA-1	3.3	-44.7
L. rhamnosus HN001	-33.9*	-7.3
Fecal extract	78.1	48.7

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

Abe et al. (2009) evaluated the mucin degradation potential of 3 bifidobacterial strains, *B. longum* ssp. *longum* BB536, *B. breve* M-16V, and *B. longum* ssp. *infantis* M-63, isolated from infants, as well as the corresponding type strains, *B. longum* ATCC 15707, *B. breve* ATCC 15700, and *B. infantis* ATCC 15696. Mucinolytic activity against purified hog gastric mucin was examined using three tests: growth in liquid medium (Fildes solution), SDS-PAGE analysis of degraded mucin residues, and degradation assay in a Petri dish. Each test was performed in triplicate.

None of the bifidobacteria strains were able to grow with mucin as the only carbon source, and there were no differences in pH between basal medium and basal medium with mucin. The mucin residues analyzed by SDS-PAGE showed no indication of degradation, indicating an absence of mucin degradation activity of the tested strains. Finally, the Petri-dish test found that no bifidobacteria strain established colonies around mucin inocula nor did any

form lysis zones. The authors regarded these experiments as demonstrating that the tested bifidobacteria have no mucin degrading activity.

These findings are consistent with the observations of Barrangou et al. (2009), who observed that in complete genomes of several strains of *B. animalis* ssp. *lactis* the genes involved in the degradation and utilization of mucin (endo-α-N-acetylgalactosaminidase) were not identified. These genes have also been found to be absent in *B. animalis* ssp. *lactis* strain Bf-6.

4.3.3. Infectivity

Cases of bacteremia resulting from translocation of probiotic bifidobacteria appear to be exceedingly rare and, indeed, have not been reported in the literature. While translocation of bifidobacteria appears possible, there seems little risk of infection. Indeed, there is considerable evidence that a cecal and colonic bifidobacterial population reduces the risk of translocation and infection by pathogens by improving barrier function (Romond et al. 2008). In mice with induced human intestinal flora, Romond et al. (2008) found that colonization by bifidobacteria was associated with poorer bacterial contamination of blood, liver, and lungs.

Zhou et al. (2005) studied the ability of *B. animalis* ssp. *lactis* HN019 and *L. rhamnosus* strains HN001 and GG to induce human platelet aggregation *in vitro*. The three probiotic strains, along with *Streptococcus sanguis* 133-79, used as a positive control, were cultured, purified, and concentrated into a suspension containing approximately 10^9 cfu/ml. Blood samples were taken from 6 healthy volunteers age 24 to 45, centrifuged, exposed to the bacterial cells at a 1:1 ratio of platelets to cells (the optimum ratio for inducing platelet aggregation), and incubated at room temperature for 30 minutes. Platelet samples were analyzed by flow cytometry for mean fluorescence intensity and the percentage of FITC-CD41a and PE-CD62p (markers for normal and activated platelets, respectively) double-positive cells. Additionally, the following hematologic parameters were measured: total red blood cell and platelet counts, hemoglobin concentration, packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration.

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

As was discussed earlier, the genome of *B. animalis* ssp. *lactis* strain Bf-6 has been found to harbor no open reading frames consistent with the production of known toxins, hemolysins, or cell-invasion proteins.

4.3.4. Undesirable Metabolic Activity

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." Naidu et al. (1999) pointed out that, by producing lactic acid, probiotic LAB reduce intestinal pH, which in turn limits the growth of many potential putrefactive bacteria that produce harmful biogenic amines. *B. animalis* ssp. *lactis* strain Bf-6 contains one gene hypothetically capable of producing a lysine decarboxylase (which would produce cadaverine), but the functionality of this gene is uncertain. No other gene encoding for an amino acid decarboxylase is present.

It has also been suggested by a number of investigators (e.g., Marteau and Gerhardt 1995) that the ability to deconjugate bile salts may be a safety hazard due to the mutagenic effects of high levels of free bile acids. However, positive effects have also been ascribed to bile salt deconjugation activity (e.g., Agerholm-Larsen et al. 2000; Pereira et al. 2003). The PROSAFE committee concluded that many commensal bacteria, including bifidobacteria and many strains of lactobacilli, have this capacity, that introduced probiotic bacteria are present in small numbers in comparison with the community present in the gut, and that bile salt conjugation is irrelevant for safety assessment (Vankerckhoven et al. 2008).

4.3.5. Presence of Antibiotic Resistance Genes and Likelihood of Transference

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

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

Temmerman et al. (2003) found resistance to kanamycin in all strains of *B. longum* and *B. lactis* tested, as well as all tested strains of *L. rhamnosus, L. acidophilus*, *L. reuteri, L. casei, L. johnsonii,* and *L. lactis*; no genetic basis for kanamycin resistance was identifiable and thus it is presumptively intrinsic.

Arthur and Courvalin (1993), in a review of antibiotic resistance of enterococci, noted that plasmid-mediated resistance to the glycopeptide antibiotics vancomycin and teicoplanin was first detected in 1986 (Leclercq et al. 1988; Uttley et al. 1989) and that inducible resistance to high levels of vancomycin and teicoplanin defines the *van(A)* phenotype. They concluded that nucleotide sequences related to the *van(A)* gene have not been detected in Gram-positive organisms with intrinsic resistance to glycopeptides, indicating that the resistance genes are not part of the chromosomes of these species and are not transferable.

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

With regard to the potential for gene transfer, a crucial distinction is between intrinsic and acquired antimicrobial resistance. Resistance is regarded as likely to be intrinsic when it is widely found in all or nearly all strains of a species or subspecies, while it is likely an acquired resistance if the tested strain exhibits significantly greater resistance to a given antibiotic agent than do most other strains (Klare et al. 2007). With respect to a specific antibiotic, a bacterial strain is referred to as "wild-type" if its susceptibility is similar to that of other strains, and "non-wild-type" if its resistance is significantly higher. This is important, because acquired resistance is far more likely to be transferable than intrinsic resistance (Vankerckhoven et al. 2008) since the genes that encode acquired resistance are associated with mobile genetic elements such as plasmids, transposons, or prophages. The European Food Safety Authority's (EFSA) panel on additives and products or substances used in animal feed concluded that intrinsic resistance and resistance due to mutation of chromosomal genes present a low risk of horizontal dissemination, and such strains should be acceptable for food consumption (FEEDAP 2005).

The antibiotic susceptibility of *B. animalis* ssp. *lactis* strain Bf-6 to 22 antimicrobial agents representing 15 different classes of antimicrobials was independently tested by both the EU PROSAFE project and the University of Agricultural Sciences (Universität für Bodenkultur--BOKU) in Vienna, Austria. Both laboratories used the method of microdilution by microtiter plates to determine minimum inhibitory concentrations (MIC) of the strain and to compare the MIC with the epidemiological cut-off (ECOFF) values. Each laboratory performed 3 independent replications of the tests and the reported MIC is the highest value obtained. The results of these two series of tests are shown in Table 4.

Although *B. animalis* ssp. *lactis* Bf-6 was found to exhibit resistance to 9 antibiotic agents, both the PROSAFE and BOKU investigators determined that none of these resistances was unusual, or higher than the normal range of the MIC distribution of the wild-type population of the subspecies, and concluded that the findings did not indicate the presence of any acquired resistances. The resistance to fusidic acid and ciprofloxacin, both gram-negative spectrum antibiotics, is common among all species of bifidobacteria (Moubareck et al 2005; Ammor et al. 2007). Moubareck et al. (2005) also observed the absence of cytochrome-mediated drug transporters among anaerobes, accounting for the intrinsic resistance to the aminoglycosides (kanamycin, gentamicin, neomycin, and streptomycin). A number of mechanisms could be responsible for the intrinsic low susceptibility to cefazolin (a relatively low activity antimicrobial agent; cf. Lim et al. 1993), and the resistance to metronidizole is most likely due to absence of the ferredoxin system needed for metronidizole's activity (Moubareck 2005). Finally, resistance to the tetracyclines is due to the presence of the *tet*(W) gene (see below), which is widely distributed in *B. animalis* ssp. *lactis* (Masco et al. 2006 found it in all strains they tested, as did Aires et al. 2007), is chromosomally located, and is regarded as non-mobile (Kastner et al. 2006; Masco et al. 2006; Matto et al. 2007). Specifically, it is not associated with the conjugative transposon Tn*B1230* found is some other *tet*(W)-positive bacteria (Masco et al. 2006). Aires et al. (2007) reported that attempted parallel conjugation of *tet*(W) among *Bifidobacterium* isolates failed to produce any transconjugants.

Table 4. Antimicrobial Agents Tested and *B. animalis* ssp. *lactis* Bf-6 Resistances.

Antimicrobial Class	Antimicrobial Agent	Proposed ECOFF[1] Values (µg/ml)	Bf-6 MIC[2] (µg/ml)		R/S[3]
			PROSAFE[4]	BOKU[5]	
β-Lactam	Penicillin	≤0.5	0.5	n.p.[6]	S
β-Lactam	Ampicillin	≤0.5	0.25	0.25	S
β-Lactam	Ampicillin/sulbactam	≤0.5	0.25	0.5	S
Glycopeptide	Teichoplanin	n.d.[7]	<0.125	n.p.	S
Glycopeptide	Vancomycin	≤1	0.5	1	S
Steroid	Fusidic acid	≤16	8	8	R
Phenicole	Chloramphenicol	≤4	1	2	S
Rifampycin	Rifampin/Rifampicin	≤2	n.p.	2	S
Tetracycline	Oxytetracycline/Tetracycline	≤2	4	16	R
Aminoglycoside	Kanamycin	≤256	n.p.	256	R
Aminoglycoside	Gentamicin	≤256	64	64	R
Aminoglycoside	Neomycin	≤256	n.p.	64	R
Aminoglycoside	Streptomycin	≤256	32	64	R
Cephalosporin	Cefazolin	≤32	n.p.	32	R
Fluoroquinolone	Ciprofloxacin	n.d.	n.d.	8	R
Lincosamide	Clindamycin	≤0.125	<0.032	0.06	S
Macrolide	Erythromycin	≤1	0.032	0.5	S
Imidazole	Metronidazole	≤16	n.p.	8	R
Oxazolidinone	Linezolid	≤1	n.p.	1	S
Streptogramin	Quinupristin/Dalfopristin	≤0.25	0.063	0.25	S
Sulfonaminde	Sulfamethoxazole	≤0.5	<0.25	0.25	S
Sulfonaminde	Trimethoprim	≤1	<0.25	<0.12	S

1. ECOFF = epidemiological cut-off
2. MIC = minimum inhibitory concentration
3. R/S = resistant/susceptible
4. PROSAFE = testing performed by the EU-funded PROSAFE project
5. BOKU = testing performed by the Universität für Bodenkultur
6. n.p. = not performed
7. n.d. = not defined

The most extensive study of tetracyline resistance in *B. animalis* ssp. *lactis* was conducted by Gueimonde et al. (2010), who screened 26 strains of the subspecies and found *tet*(W) in all of them, with a transposase gene upstream. They further determined that "*tet*(W) is necessary and sufficient for the tetracycline resistance seen in *B. animalis* subsp. *lactis*." Noting the presence of the transposase gene, the authors nevertheless concluded that "there is no evidence that *tet*(W) in *B. animalis* subsp. *lactis* is transmissible."

B. animalis ssp. *lactis* strains Bf-6, Bb-12, and DN 173010 were tested for the presence of both the tet(W) and tet(M) genes, using the class-specific primer sets of Aminov et al. (2001). *E. coli* carriers of the 2 genes were used as control strains. As is evident in Figures 8 and 9, showing the PCR traces resulting from application of the TetW-FW/TetW-RV and TetM-FW/TetM-RV primer sets, respectively, both *tet*(W) and *tet*(M) are present in the control strains,

and *tet*(W) is present in all 3 *B. animalis* ssp. *lactis* strains (represented by the band generated by the 168-bp fragment amplified from the gene) while *tet*(M) is not.

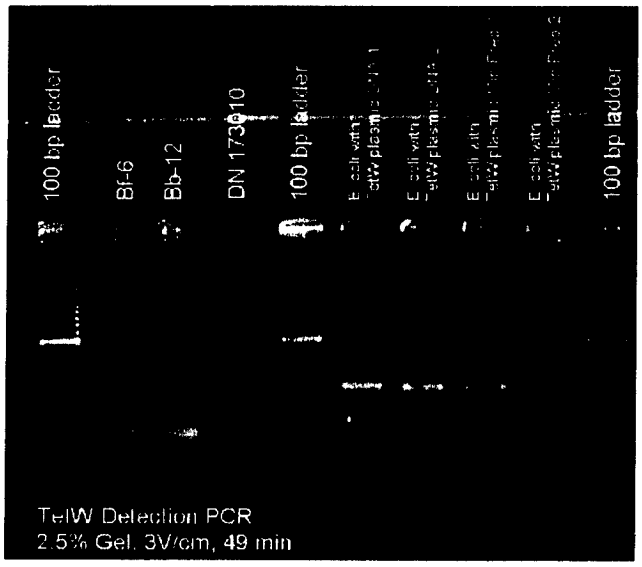
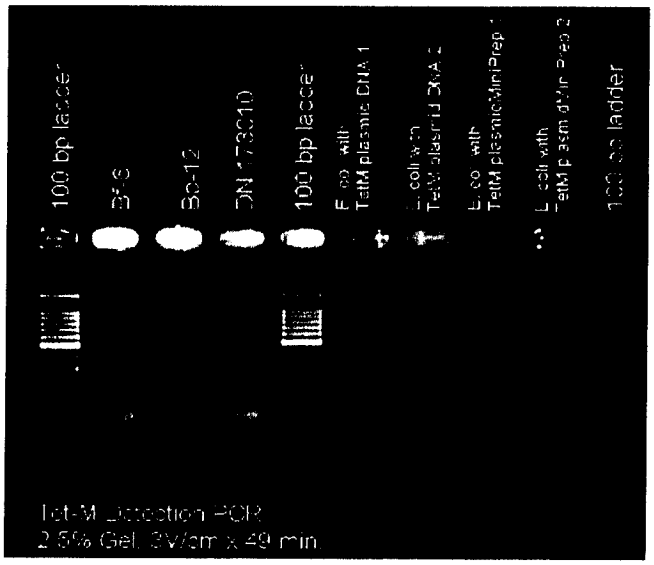


Figure 8. Tet(W) Detection PCR.



000034

Figure 9. Tet(M) Detection PCR.

Saarela et al. (2007) tested tetracyline susceptibility of ingested *L. acidophilus* LaCH-5 and *B. animalis* ssp. *lactis* strain Bb-12 during treatment of 10 patients (mean age = 42 years) suffering from respiratory tract infections with doxycycline, a tetracycline-group antibiotic. They found that the therapy reduced the numbers and diversity of fecal microbiota, including bifidobacteria, and increased the proportion of tetracycline-resistant bacteria. However, concomitant ingestion of tetracycline-resistant *B. animalis* ssp. *lactis* Bb-12 with the antibiotic

"did not generate a safety risk regarding the possible transfer of tetracycline resistance genes to the ingested strains."

Xiao et al. (2010) recovered 7 strains of *B. animalis* ssp. *lactis* from products in the Japanese marketplace and found that all of them harbored *tet*(W) and exhibited resistance to tetracycline; no other tetracycline resistance gene was found in any of the strains. Noting that *tet*(W) is located in a single copy on the chromosome with no linkage to a mobile element, the authors concluded that, "It therefore seems that the risk of *tet*(W) in these strains is not highly alarming."

4.4. *In Vitro* Studies of *B. animalis* ssp. *lactis*

In an *in vitro* study of the ability of bifidobacteria and lactobacilli to inhibit lipid peroxidation, Lin and Yen (1999a) used sonification to disrupt cells of *B. animalis* ssp. *lactis* Bf-6 (here designated as *B. longum*), another *B. longum* strain, and 6 strains of *L. acidophilus*, then removed the cell debris and retained the intracellular cell-free extract. The extract was mixed with linoleic acid and incubated for 12 hours at 37°C. All tested strains demonstrated inhibition of peroxidation ranging from 33 to 46%; the inhibition rate of strain Bf-6 was 43.0%. No tested strain was capable of scavenging *tert*-butyl hydroperoxide, a primary product of lipid peroxidation, but all were able to scavenge another product, malondialdehyde. The authors concluded that consumption of foods containing these probiotics "may be recommended as healthful."

Lin and Yen (1999b) further studied the *in vitro* antioxidative activity of probiotic bacteria with 19 LAB strains, including *B. animalis* ssp. *lactis* Bf-6 (here designated as *B. longum*) and another bifidobacterial strain, 6 strains of *L. acidophilus*, 6 strains of *L. bulgaricus*, and 5 strains of *S. thermophilus*. Intracellular extracts of all tested strains showed antioxidative activity based on chelation of metal ions, scavenging of reactive oxygen species, and reducing activity; however, superoxide dismutase activity was not observed. Strain Bf-6 exhibited the highest level of hydrogen peroxide scavenging ability and greatest reducing activity among the strains tested.

B. animalis ssp. *lactis* Bf-6 (here designated as *B. longum*) was inoculated into soymilk and incubated at 37°C for 48 hours (Hou et al. 2000). The results included slight increases in titrable acidity, crude protein, free amino acids (resulting from protein hydrolysis), riboflavin, and thiamine, and a small decrease in niacin. Acetic and lactic acid (all L-lactic acid) contents increased significantly. The sugars content was affected by Bf-6 fermentation, with decreased content of stachyose, raffinose, and sucrose and increased content of fructose, glucose, and galactose.

Lin and Young 2000 studied folate production by *B. animalis* ssp. *lactis* Bf-6 (here designated as *B. longum*), another *Bifidobacterium* strain, 2 strains of *L. acidophilus*, 2 strains of *L. delbrueckii*, and 2 strains of *S. thermophilus* in reconstituted milk. At 37°C, all strains grew well and reached stationary phase in about 8-12 hours; maximum folate levels were recorded at 6 hours. Strain Bf-6 had the highest level of folate among the strains tested, especially 5-methyltetrahydrofolate and 5-formyltetrahydrofolate. During storage at 4°C, folate decreased monotonically to 61-88% of original levels after 3 weeks (due to the bacteria's own utilization of folate); Bf-6 retained 76% of its original level of folate after 3 weeks.

Lian et al. (2003) compared the ability of microencapsulated *B. animalis* ssp. *lactis* Bf-6 (here designated as *B. longum*) and *B. longum* CCRC 14634 to survive in simulated gastric juice and bile solution. Cells from 12-hour cultures were harvested and suspended in phosphate buffer, then mixed with a carrier solution of gelatin, gum arabic, soluble starch, or skim milk to provide 10^{10}-10^{11} cfu/g dry weight and 10% carrier, and spray dried. Simulated gastric juice consisted of pepsin suspended in saline with HCl added to adjust the pH to either 2.0 or 3.0. The bile solutions included either 0.5% or 2.0% oxgall. Microparticles or free cells were added to each solution, which were maintained at 37°C, and sampled at 0 and 4 hours for the gastric juice and at 0 and 12 hours for the bile solution.

B. animalis ssp. *lactis* Bf-6 survival was not affected by 4-hour exposure to simulated gastric juice at pH 3.0; there was no significant change over time nor any difference between free cells and any of the microcapsule shell materials. At pH 2.0, on the hand, significant loss of viability was seen for the free cells (with 4-hour survival of only 1.32-1.88%), but survival was greatly improved for the cells encased in gum arabic, gelatin, or soluble starch (survival as high as 76.09%). When exposed to the 0.5% bile solution, neither free cells of strain Bf-6 nor microencapsulated cells showed significant loss over 12 hours; survival of the free cells was 94.46% and that of encapsulated cells did not differ significantly. The 2% bile solution, on the other hand, immediately reduced viability of the free cells of Bf-6 by 99.81-99.93% while that of the encapsulated cells was reduced by 67.76-98.23%. Viability further decreased over the 12 hours tested; the best protection was provided by skim-milk shells, which allowed survival of 1.39% of Bf-6 cells after 12 hours in 2.0% bile solution.

Hsieh and Chou (2006) tested the mutagenicity of soymilk fermented by lactic acid bacteria, including *B. animalis* ssp. *lactis* Bf-6 (referred to in this study as *B. longum* B6) using tester strain *Salmonella typhimurium* TA100, as well as its antimutagenic potential against 4-nitroquinoline-*N*-oxide and 3,2'-dimethyl-4-amino-biphenyl. Soymilk was fermented with Bf-6 alone or with either *L. acidophilus* CCRC 14079 or *S. thermophilus* CCRC 14085 for 32 hours at 37°C. No increase in revertants was observed in either the absence or presence of S9 metabolic activation, indicating a lack of mutagenic effect. Soymilk fermented with *B. animalis* ssp. *lactis* Bf-6 had a significant suppressive effect against both tested mutagens, although the effect was significantly less than that resulting from fermenting soymilk with *S. thermophilus*.

Jiang and Savaiano (1997) tested the effect of adding to fecal slurry 4×10^7 cfu *B. animalis* ssp. *lactis* Bf-6 (called *B. longum* B6) upon lactose fermentation. Studies were carried out using 50 g of a fecal slurry obtained from an otherwise healthy male adult lactose maldigester, maintained at 37°C with constant stirring in an anaerobic environment for 3 days. The addition of Bf-6 to the slurry significantly increased fermentation of lactose, resulting in significant decreases in the concentrations of lactose and D-lactose and a significant increase in the concentration of acetate.

4.5. *In Vivo* Studies of *B. animalis* ssp. *lactis*

4.5.1. Animal Studies

A number of animal studies of *B animalis* ssp. *lactis* strain Bf-6 as well as several other *B. animalis* ssp. *lactis* strains were conducted in order to demonstrate safety and to investigate potential beneficial effects, including protection from *Salmonella typhimurium, E. coli* O157:H7, *Candida albicans* and other infective microbes or reduction in rotavirus infection. As discussed below, the evidence for such benefits is generally supportive. Most important, though, is that

these studies—in which *B. animalis* ssp. *lactis* was given orally to mice, rats, and piglets—have consistently found an absence of any observable adverse effects due to administration of *B. animalis* ssp. *lactis* at daily doses as high as 10^{11} cfu/day.

4.5.1.1. Animal Studies of *B. animalis* ssp. *lactis* Bf-6

Kullen et al. (1998) studied the effects of administration of *B. animalis* ssp. *lactis* Bf-6 in conjunction with a nondigestible carbohydrate on cecal populations of bifidobacteria and *Clostridium perfringens* in the rat. Forty weanling male Wistar rats weighing 50-75 g (neither mean weight nor standard deviation were reported) were individually housed, injected with dimethylhydrazine to induce preneoplastic colonic lesions, and assigned to one of 4 treatment groups (n = 10 rats/group). All rats received 10^8 cfu/day of strain Bf-6 by gavage along with free access to water and AIN-76 control diet, AIN-76 with 2% fructooligosaccharide, AIN-76 with 2% wheat bran oligosaccharide, or AIN-76 with 2% soybean oligosaccharide for 4 weeks. Feed intake and body weight was recorded weekly; after sacrifice, the cecum was excised and the contents were analyzed for bifidobacteria and *C. perfringens*.

Treatments had no effects on feed intake or weight gain. Since bifidobacteria were administered to all rats, the effects of this administration could not be determined; the wheat bran and soybean oligosaccharide, but not the fructooligosaccharide, significantly increased cecal populations of both bifidobacteria and *C. perfringens*.

Ha et al. (1999) gave groups of 8-10 nine-week-old female $B6C3F_1$ mice, caged 3 mice/cage, free access to water and one of 10 different yogurts mixed 1:1 with AIN-93G chow for 4 weeks. Two basic yogurts contained the usual starter cultures of *S. salivarius* ssp. *thermophilus* and *L. delbrueckii* ssp. *bulgaricus* obtained from different suppliers, while 3 test yogurts contained those 2 cultures along with *L. acidophilus* and one of 3 *Bifidobacterium* strains—*animalis* ssp. *lactis* Bf-6, *bifidum*, or *infantis*. Each of these 5 yogurts was prepared both with live cultures and in a form that was heat treated to kill the bacteria. One additional group of mice received no yogurt but only nonfat dry milk. Mice were weighed weekly and feed intake was recorded daily. After sacrifice, spleens, mesenteric lymph nodes, and Peyer's patches were removed for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of cytokines gene transcription.

Over the 4 weeks of the study, the bifidobacteria numbers in the yogurts containing bifidobacteria decreased from an average of about $5x10^7$ cfu/g to about $3x10^6$ cfu/g. Intake of *B. animalis* ssp. *lactis* Bf-6 and the other bifidobacterial strains was about $1.5x10^8$ cfu/day. Generally, ingestion of the various yogurts had little effect on mRNA levels in the test organs for the cytokines IF-γ, TNF-α, IL-2, IL-4, or IL-6, or β_2-microglobulin. Small decreases were occasionally noted, especially in TNF-α, and these decreases tended to be larger when the mice had consumed heat-treated yogurt. The presence of bifidobacteria had no observable effect. The mice remained healthy throughout the trial and diarrhea was not observed in any group. There were no differences in feed intake or weight gain across the treatment conditions.

In another study of the possible immune-stimulating effects of *B. animalis* ssp. *lactis* Bf-6, Tejada-Simon et al. (1999) gave yogurt containing the strain (along with *L. acidophilus*) to mice that were also inoculated with cholera toxin. Nine-week-old female $B6C3F_1$ mice (n = 10 mice/group, caged 5 mice/cage) were given free access to water and either skim-milk control or one of 4 different yogurts mixed 1:1 with AIN-93G chow for 4 weeks. One yogurt contained the traditional starter cultures of *S. salivarius* ssp. *thermophilus* and *L. delbrueckii* ssp. *bulgaricus*,

while 3 yogurts contained the starter cultures along with *L. acidophilus* and one of 3 *Bifidobacterium* strains—*animalis* ssp. *lactis* Bf-6, *bifidum*, or *infantis*. The bifidobacteria were present in the yogurt at concentrations of 2.3×10^6 to 2.5×10^8 cfu/ml, resulting in daily intake of about 2.4×10^7 cfu. The mice received these treatments for 3 weeks, during which they were twice inoculated with 10 μg cholera toxin. Cholera-toxin-specific IgA and IgG antibody titers were determined in extracts of feces collected weekly, blood drawn at the beginning and end of the study, and spleens and Peyer's patches removed after sacrifice.

There were no differences in feed intake or weight gain across the treatment conditions. The mice remained healthy throughout the trial and diarrhea was not observed in any group. While the mice receiving conventional yogurt exhibited nonsignificantly lower IgA titer than did the control mice, those receiving yogurt containing Bf-6 or other bifidobacteria had significantly higher titers of IgA. IgG titer was not affected by treatment.

Pestka et al. (2001) studied the effects of ingesting yogurts containing only starter cultures and yogurts with added probiotic bacteria on lymphocyte populations in the spleens and Peyer's patches in female $B6C3F_1$ mice. One yogurt contained only the starter cultures, *S. salivarius* ssp. *thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, and 3 test yogurts contained those 2 cultures along with *L. acidophilus* and $>10^6$ cfu/ml of one of 3 *Bifidobacterium* strains—*animalis* ssp. *lactis* Bf-6, *bifidum*, or *infantis*. Each yogurt (or a skim-milk control) was mixed 1:1 with AIN-93G chow and provided *ad libitum* to 9-week-old female $B6C3F_1$ mice (n = 8-10/group housed 3 mice/cage) for 2 weeks. After sacrifice, spleens and Peyer's patches were removed for enumeration of lymphocytes.

Daily ingestion of each strain of bifidobacteria was about 3×10^6 cfu. Distributions of IgA^+, IgM^+, $B220^+$, $CD4^+$, and $CD8^+$ lymphocytes were not significantly different in the mice fed the skim milk control or the yogurts not containing *B. animalis* ssp. *lactis* Bf-6, but the percentage of $CD4^+$ (T-helper) cells was significantly increased in the mice receiving yogurt containing Bf-6. The authors concluded that "ingestion of conventional or probiotic-supplemented yogurts for 2 weeks had very little effect on lymphocyte distribution in the systemic or mucosal immune compartments."

4.5.1.2. Animal Studies of Other *B. animalis* ssp. *lactis* Strains

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

Adherence to epithelial surfaces was widely different across the various strains tested, with *B. animalis* and *L. acidophilus* showing 82% and 86% adherence, respectively, compared with 2% and 5%, respectively, for *L. reuteri* and *L. rhamnosus*. Translocation closely correlated with adherence — such translocation to internal organs was detected in 50% or more of the mice colonized with *B. animalis* or *L. acidophilus*, 26% of those colonized with *L. rhamnosus*, and

none of the mice colonized with *L. reuteri*. The translocation did not result in any signs of morbidity, mortality, or pathologic changes in the immunodeficient mice, and the probiotic bacteria neither retarded nor enhanced the growth of male or female athymic or euthymic mice.

Although all adult and neonatal beige-euthymic mice survived probiotic colonization, some infant mortality occurred in beige-athymic pups born to mothers colonized with pure cultures of *L. rhamnosus* or *L. reuteri*; there was no mortality among pups born to dams receiving *B. animalis* or *L. acidophilus*. Indeed, no adverse effects at all were observed for these dams or their pups, and the authors concluded that these species appear to be innocuous even to immunodeficient mice.

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

Shu et al. (1999) assessed the safety of *Bifidobacterium animalis* ssp. *lactis* HN019 as well as *L. rhamnosus* HN001 and *L. acidophilus* HN017 in BALB/c mice. Male mice age 6-8 weeks and weighing about 25 g were acclimatized for 14 days on a skim-milk powder-based diet and then randomized into 13 groups with n = 12 mice/group. The 3 probiotic strains, along with commercially obtained *L. acidophilus* LC1 as a probiotic control, were administered orally to mice at $5x10^7$, 10^9, or $5x10^{10}$ cfu/day; a 13th group of mice served as a non-probiotic control. Mice received their assigned treatment for 7 days. Half of the mice in each group were killed immediately at the termination of treatment while the other half remained on the basal diet (without probiotics) for another 7 days before sacrifice. Clinical observations were conducted daily; feed and water intake and body weight were recorded weekly. After sacrifice, spleens and kidneys were removed to be weighed and examined for any indication of bacterial translocation or infection. Any organisms isolated from tissue samples were subjected to randomly amplified polymorphic DNA (RAPD) finger-printing to identify them. Small sections of ileum, cecum, and colon were excised for histological examination. Blood was drawn after euthanasia and analyzed for plasma glucose level as well as several hematological parameters: erythrocyte and platelet numbers, hemoglobin, mean corpuscular volume, hematocrit, and mean corpuscular hemoglobin concentration.

No adverse clinical signs were noted in any animals, and the mean general appearance scores did not differ among the groups. There was no effect of any treatment on feed or water consumption or on body-weight gain. No viable bacteria were detected in the spleens of any mice. Bacteria were isolated from one mouse in the HN001 high-dose group, one mouse in the HN019 low-dose group, and one mouse in the LC1 mid-dose group, but these isolates produced different DNA finger prints than the test strains and likely came from the mice's intestinal

microbiota. The histological analysis found no effects of any treatment on the villi height, mucosal thickness, or epithelial cell height of the intestinal samples. No effects were seen in blood glucose or any hematological measures. Both absolute and relative spleen weights were unaffected, indicating an absence of splenic inflammation such as would result from bacterial infection.

The authors noted that the high dose of $5x10^{10}$ cfu/day shown to be without adverse effect in this study corresponds to a level of $2x10^{12}$ cfu/kg bw/day; equivalent to $1.5x10^{13}$ cfu/day for a 75-kg adult human.

Zhou et al. (2000a) again used 6-8-week-old male BALB/c mice to assess the safety of *Bifidobacterium animalis* ssp. *lactis* HN019, *L. rhamnosus* HN001, and *L. acidophilus* HN017. Mice (average weight = 20 g) were acclimatized for 14 days on a skim-milk powder-based diet and then randomized into 13 groups with n = 6 mice/group. The 3 probiotic strains, along with commercially obtained *L. acidophilus* LA-1 as a probiotic control, were administered orally to mice at $5x10^7$, 10^9, or $5x10^{10}$ cfu/day; a 13th group of mice served as a non-probiotic control. Mice received their assigned treatment for 21 days. Clinical observations were conducted daily; feed and water intake and body weight were recorded weekly. After sacrifice, spleens, livers, and mesenteric lymph nodes were removed to be weighed and examined, along with blood samples, for any indication of bacterial translocation or infection. Any organisms isolated from tissue samples were subjected to randomly amplified polymorphic DNA (RAPD) finger-printing to identify them. Small sections of ileum, cecum, and colon were excised for histological examination. Blood was drawn after euthanasia and analyzed for both hematological and biochemical parameters. Hematology measures included erythrocyte and platelet numbers, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and both total and differential leukocyte counts (lymphocytes, neutrophils, monocytes, and eosinophils). Biochemistry measures were total plasma protein, albumin, plasma glucose, and total plasma cholesterol.

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

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Zhou et al. (2000b) performed a similar study using these same bacterial strains but administered them at far higher doses, 10^{11} cfu/day. *B. animalis* ssp. *lactis* HN019, *L. rhamnosus* HN001 or GG, *L. acidophilus* HN017 or LA-1, or placebo were administered twice a day at

doses of $5x10^{10}$ via oral pipette to 48 6-8-week-old male BALB/c mice and weighing about 20 g (8 mice/group). Mice were acclimatized for 7 days on a skim-milk powder-based diet and then randomized to receive one of the 6 treatments for 8 days. Clinical observations were conducted twice daily and activity level was recorded daily; feed intake and body weight were recorded weekly. After sacrifice, spleens, livers, and mesenteric lymph nodes were removed to be weighed and examined, along with blood samples, for any indication of bacterial translocation or infection. Prior to excision of tissue samples the surfaces of viscera were swiped with sterile swabs that were subsequently cultured to test for bacterial contamination. Any organisms isolated from visceral surfaces or tissue samples were subjected to randomly amplified polymorphic DNA (RAPD) finger-printing to identify them. Small sections of ileum, cecum, and colon were excised for histological examination.

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

In summary, Zhou et al. (2000b) concluded that "feeding mice with potential probiotic strains *Lb. rhamnosus* HN001, *Lb. acidophilus* HN017 or *B. lactis* HN019 at doses of $2.5x10^9$-$2.5x10^{12}$ cfu/kg body weight/day for 4 weeks had no adverse effects on general health status, growth, haematology, blood biochemistry and histology parameters examined in this study. The test strains did not cause infection and did not translocate from the original colonisation site (gut) after feeding for four weeks."

Using a murine model, Shu et al. (2000) assessed the ability of *B. animalis* ssp. *lactis* strain HN019 to enhance resistance to *Salmonella typhimurium*. Two experiments were conducted with 7-9-week-old male BALB/c mice. In the first, 53 mice were randomly assigned to receive a daily skim-milk gavage or a similar gavage with 10^9 cfu HN019 for 7 days, then challenged with 7 days during which they received low doses of *S. typhimurium* orally in addition to their regular gavage, and finally 7 more days on the assigned gavage. In the second experiment, 77 mice received daily gavage of skim milk with 0 or $2x10^9$ cfu HN019 for 7 days, then challenged with a single high dose of *S. typhimurium*, followed by 21 more days on the assigned gavage. Feed intake and body weight were monitored; after sacrifice, blood was taken for assessing bacterial translocation, serum antibody levels, and leukocyte phagocytic activity; peritoneal cells were collected for measurement of phagocytic activity; spleens and livers were removed and evaluated for translocation; Peyer's patches and mesenteric lymph nodes were analyzed for lymphoproliferative responses; and the small intestine was excised and the mucosal antibody response was measured.

In experiment 1, the mice that received HN019 had significantly higher levels of post-infection feed intake, lost less weight, and had higher health scores than control mice. Results in experiment 2 were similar, and the mice receiving bifidobacteria also exhibited significantly

greater survival through the 21-day post-challenge period (76.7% v. 6.9%). The mice receiving the probiotic had a significantly lower incidence of bacterial translocation to visceral tissues, significantly higher levels of phagocytically active cells in both the blood and peritoneal cells, and significantly higher mean titers of lipoteichoic-acid-specific antibodies in blood and mucosa. The authors concluded that the "results of the present study demonstrate that feeding with [*B. animalis* ssp. *lactis*] is able to provide a significant degree of protection against pathogenic *S. typhimurium*." Further, no adverse effects were observed.

Gill et al. (2000) studied the effect of feeding *B. animalis* ssp. *lactis* HN019, *L. rhamnosus* HN001, or *L. acidophilus* HN017 on indices of natural and acquired immunity in mice. In the first experiment, 80 6-7-week-old male BALB/c mice were fed a milk-based diet for 1 week and then randomized into 4 groups (n = 20 mice/group) receiving 10^9 cfu/day of 1 of the 3 probiotic strains in skim milk or skim milk alone for 10 days. On days 0 and 7, mice were orally administered cholera toxin. At termination on day 10, peritoneal macrophages, blood, spleens, and intestinal washings were taken for examination. In the second experiment, 72 mice were treated as in the first experiment (with 18 mice/group) but maintained on the diets for 28 days and inoculated subcutaneously with tetanus vaccine on days 7 and 21. Feed intake and body weights were measured weekly. At termination on day 28, peritoneal macrophages, blood, spleens, and intestinal washings were taken for examination. Spleens were forced into single-cell suspensions and their proliferation measured *in vitro* in the presence or absence of T- and B-cell mitogens (concanavalin A and lipopolysaccharide, respectively). Spleen cells were also cultured and the presence of interleukin (IL)-4 and interferon (IFN)-γ was determined using ELISA. Serum or intestinal fluid samples were exposed to cholera toxin or tetanus toxoid and ELISA was used to determine antibody response. The expression of CD4, CD8, and CD40 antigens on peripheral blood leucocytes was monitored by flow cytometric analysis. The phagocytic capacity of peripheral blood leucocytes and peritoneal macrophages as well as the natural killer (NK)-cell activity of spleen cells were also assessed using flow cytometry-based assays.

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

Shu et al. (2001) investigated the effect of *B. animalis* ssp. *lactis* strain HN019 on the risk of rotavirus and *E. coli* infection in piglets. Seventeen 3-week-old male suckling piglets weighing 5-7 kg (mean weight and standard deviation were not reported) were randomized to receive skim milk containing 0 or 10^9 cfu HN019/day by orogastric tube. Health appearance and diarrhea were monitored and evaluated; feed intake was recorded daily and the animals were weighed on days 0, 6, and 13. Fecal samples were collected on days 0, 7, and 14 and blood was collected on days 0 and 13 for analysis of phagocytic cell activity and lymphocyte proliferation.

The piglets in the experimental group had significantly higher feed conversion efficiency than the control pigs. The piglets in the control group suffered statistically significantly more severe diarrhea than did those receiving bifidobacteria, and one control pig died of severe

diarrhea and dehydration. The piglets receiving HN019 also showed significantly increased phagocytosis and lymphoproliferative response over those not receiving bifidobacteria as well as significantly increased titers of specific IgA, IgM, and IgG for rotavirus and *E. coli*. There were no adverse effects attributable to daily administration of 10^9 cfu *B. animalis* ssp. *lactis* strain HN019.

Shu and Gill (2001) also investigated the ability of *B. animalis* ssp. *lactis* HN019 to reduce the severity of *E. coli* O157:H7 infection in mice. Forty-four BALB/c mice and 42 C57BL/6 mice, all 7-9-week-old males, were randomly assigned to receive diets supplemented with 0 (n = 20 mice of each strain) or $3x10^8$ cfu HN019/g feed for 7 days before being challenged with *E. coli* O157:H7, then maintained on their assigned diets for 7 more days. Health appearance and morbidity were monitored twice daily; after sacrifice, blood was taken for assessing bacterial translocation and leukocyte phagocytic activity, peritoneal cells were collected for measurement of phagocytic activity, spleens and livers were removed and evaluated for translocation, and the small intestine was excised and the mucosal antibody response (IgA and IgG) to *E. coli* was measured.

The mice fed about $9x10^8$ cfu bifidobacteria/day had significantly higher feed intake and lower morbidity rates following infection. Ingestion of strain HN019 significantly reduced the incidence of *E. coli* translocation to the blood, spleen, and liver, significantly increased phagocytic capacity of both blood and peritoneal cells, and significantly increased IgA titer (although IgG titer was not affected). The authors concluded that "dietary supplementation with *B. lactis* can reduce the severity of E. coli O157:H7 infection in mice. After oral challenge with the pathogen, HN019-fed BALB/c and C57 mice exhibited lower cumulative morbidity, but maintained significantly higher feed intake, than their respective control groups."

Because of the possibility that intestinal microbiota could contribute to the pathogenesis of autoimmune diseases, Zhou and Gill (2005) tested the effect of administration of *B. animalis* ssp. *lactis* HN019 or *L. rhamnosus* HN001 on pathological inflammation in a mouse model of experimental autoimmune thyroiditis. Mouse thyroglobulin was obtained through processing of frozen thyroids from 300 inbred BALB/c and CBA/CaH mice and its purity confirmed by SDS-PAGE analysis. Female CBA/CaH (n = 114) mice 4-6 weeks old and possessing the H-2^k haplotype (which renders them more susceptible to the induction of experimental autoimmune thyroiditis) were acclimated to the housing conditions for 2 weeks and then randomized to receive saline solution, *B. animalis* ssp. *lactis* HN019, *L. rhamnosus* HN001, or *L. rhamnosus* GG once a day via pipette. The daily probiotic dose was $6x10^7$ to $1.5x10^8$ cfu. The mice were randomized to be inoculated subcutaneously with thyroglobulin after 7 days and again after 14 days or to be inoculated with thyroglobulin + lipopolysaccharide precipitated from *E. coli* 055:B5, intended to exacerbate the development of autoimmune response; this second treatment also included another booster dose after 42 days. The health status and behavior of the mice was observed daily, body temperatures were taken every other day, and body weights were measured weekly. Animals were euthanized after receiving probiotics or placebo for 5, 8, or 9 weeks and blood, spleen, and thyroid glands were collected. ELISA was used to determine levels of anti-thyroglobulin IgG1 and IgG2a antibodies in serum in order to confirm the establishment of an autoimmune reaction. Spleens were weighed and spleen lymphocyte proliferative response to thyroglobulin was assessed; and the extent of mononuclear cell infiltration in the thyroid tissues was determined.

There was no mortality, and there were no differences in clinical observations, body temperatures, or body weights of the mice receiving saline or probiotics. All mice inoculated with thyroglobulin produced measurable titers of IgG1 and IgG2a antibodies against thyroglobulin, demonstrating that an autoimmune response had been induced. The mice also showed inflammatory responses in increased relative spleen weights and increased splenic lymphocyte proliferation. Probiotic feeding had no effect on these responses. Histological changes in thyroid tissue were mild after inoculation with thyroglobulin alone but greater with the addition of *E. coli* lipopolysaccharide. As shown in Table 5, administration of probiotic bacteria did not affect the incidence of experimental autoimmune thyroiditis.

Table 5. Incidence of Experimental Autoimmune Thyroiditis.

Group	Thyroglobulin Alone		Thyroglobulin + Lipopolysaccharide	
	N	% EAT*	N	% EAT
Control (no treatment)	12	0	12	0
Saline	12	17	12	50
HN001	12	17	12	50
HN019	12	25	12	42
LGG	11	18	6	50

Source: Zhou and Gill 2005
% EAT = the percentage of mice that exhibited symptoms of experimental autoimmune thyroiditis

Zhou and Gill (2005) concluded that long-term daily ingestion of the tested probiotics had no adverse effect on the induction or progression of thyroglobulin-induced experimental autoimmune thyroiditis in CBA/CaH mice. Because this murine model mimics the cellular and pathological manifestations of Hashimoto's thyroiditis (a human autoimmune disease), it is widely used as a model to study human autoimmunity. Thus, the findings suggest that immunostimulatory probiotic *B. animalis* ssp. *lactis* HN019 (as well as the 2 other strains tested) does not induce or enhance autoimmune response in humans.

Starter cultures used in Chinese fermented milk products were tested for acute toxicity in an unreported number of an unidentified strain of mice (Xu et al. 2008). The studied bacteria included *B. animalis* ssp. *lactis* Bb-12, 2 strains of *L. rhamnosus*, and a strain of *L. reuteri*. None showed any toxic effects when administered orally over 3 days and *B. animalis* ssp. *lactis* Bb-12 showed no toxicity even after intraperitoneal injection at a dose of $5x10^9$ cfu/ml.

4.5.1.3. Conclusions from Studies of *B. animalis* ssp. *lactis* in Animals

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In conclusion, several strains of *B. animalis* ssp. *lactis* (Bf-6, Bb-12, and HN019) were administered to mice, rats, and piglets in a total of 17 studies, with dose levels as high as 10^{11} cfu *B. animalis* ssp. *lactis*/day (Table 6). No adverse effects were seen in standard measures of immune, biochemical, or hematological parameters and no retardation of normal growth was reported. Translocation was observed in only 1 study, Wagner et al. (1997a), which used congenitally immunodeficient gnotobiotic athymic mice. However, the EU-PROSAFE project (Vankerckhoven et al. 2008) considered that use of the immunocompromised mouse model is

premature and recommended against it. Indeed, the homozygote *bg/bg nu/nu* mouse is doubly immunodeficient in that it lacks both NK-cells and functional T-cells and has deficient phagocytosis. It is of questionable scientific validity to infer from findings in such immunocompromised animals to immunocompetent humans. In any event, it is notable that no adverse effects were associated with the observed translocation of bifidobacteria in this model. There are no findings from the animal studies that would bring into question the safety of the administration of *B. animalis* ssp. *lactis* strain Bf-6 under the intended conditions of use.

Table 6. Studies of *B. animalis* ssp. *lactis* in Animals.

Reference	Objective	Study Design	Animal Model	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Gill et al. (2000) (2 reported experiments)	Investigate the effect of feeding *B. animalis* ssp. *lactis* HN019, *L. rhamnosus* HN001, or *L. acidophilus* HN017 on indices of natural and acquired immunity in mice.	1. Mice were acclimatized for 1 week and randomized into 4 groups receiving 1 of the 3 probiotic strains or placebo for 10 days. On days 0 and 7, mice were orally administered cholera toxin 2. Same, but maintained on the diets for 28 days and inoculated subcutaneously with tetanus vaccine or days 7 and 21	1. 80 male BALB/c mice age 6-7 weeks 2. 72 male BALB/c mice age 6-7 weeks	1. 10^9 cfu HN019/day 2. 10^9 cfu HN019/day	1. 10 days 2. 28 days	None of the probiotic strains had any effect on feed intake, body-weight gain, production of IL-4, or expression of CD4, CD8, or CD40 cells in the blood, but all 3 produced significant increases in the phagocytic activity of peripheral blood monocytes and polymorphonuclear cells, peritoneal macrophages, and proliferative responses of spleen cells to T- and B-cell mitogens. The spleen cells of mice given *L. rhamnosus* HN001 or *L. acidophilus* HN017 produced higher amounts of IFN-γ in response to stimulation with concanavalin A, indicating an effect on Th1 T-cells but not on Th2 cells. Serum antibody responses to antigens were enhanced by oral administration of all 3 probiotic strains, but NK-cell activity was increased only in the mice given *L. rhamnosus* HN001. No adverse effects were observed.
Ha et al. (1999)	Investigate the effect of feeding *B. animalis* ssp. *lactis* Bf-6, B. *bifidum*, or B. *infantis* on immunity in mice.	Mice were given basic yogurt or yogurt with *L. acidophilus* and one of 3 *Bifidobacterium* strains—*animalis* ssp. *lactis* Bf-6, *bifidum*, or *infantis*.	8-10 9-week-old female $B6C3F_1$ mice	$5x10^7$ cfu Bf-6/ g yogurt to about $3x10^6$ cfu/g yogurt; intake = $1.5x10^8$ cfu/ day to $9x10^6$ cfu/day	4 weeks	Ingestion of yogurt had little effect on cytokines IF-γ, TNF-α, IL-2, IL-4, or IL-6, or β_2-microglobulin; bifidobacteria had no observable effect. The mice remained healthy throughout the trial and diarrhea was not observed in any group. There were no differences in feed intake or weight gain across the treatment conditions.

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Table 6. Studies of *B. animalis* ssp. *lactis* in Animals.

Reference	Objective	Study Design	Animal Model	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Kullen et al. (1998)	Study the effects of *B. animalis* ssp. *lactis* Bf-6 and a nondigestible carbohydrate on cecal populations of bifidobacteria and *Clostridium perfringens*	Rats received dimethylhydrazine to induce preneoplastic colonic lesions, then received 10^8 cfu/day of strain Bf-6 along with a nondigestible carbohydrate	40 weanling male Wistar rats weighing 50-75 g	10^8 cfu Bf-6/ day	4 weeks	Treatments had no effects on feed intake or weight gain. Since bifidobacteria were administered to all rats, the effects of this administration could not be determined; the wheat bran and soybean oligosaccharide, but not the fructooligosaccharide, significantly increased cecal populations of both bifidobacteria and *C. perfringens.*
Pestka et al. (2001)	Study the effects of yogurts containing only starter cultures and yogurts with added probiotic bacteria on lymphocyte populations	Mice were given basic yogurt or yogurt with *L. acidophilus* and one of 3 *Bifidobacterium* strains—*animalis* ssp. *lactis* Bf-6, *bifidum*, or *infantis.*	40 9-week-old female $B6C3F_1$ mice	10^6 cfu Bf-6/ ml; intake = $3x10^6$ cfu/day	2 weeks	Distributions of IgA^+, IgM^+, $B220^+$, $CD4^+$, and $CD8^+$ lymphocytes were not significantly affected, but the percentage of $CD4^+$ (T-helper) cells was significantly increased in the mice receiving yogurt containing Bf-6. The authors concluded that, while some beneficial immune effect was seen, it is not of large magnitude.
Shu et al. (1999)	Assess the safety of *B. animalis* ssp. *lactis* HN019, *L. rhamnosus* HN001, and *L. acidophilus* HN017	The 4 strains were administered to mice at 3 dose levels; a 13th group was a non-probiotic control. Half of the mice were killed at termination of treatment while the rest remained on basal diet for another 7 days	156 male BALB/c mice aged 6-8 weeks and weighing about 25 g	0, $5x10^7$, 10^9, or $5x10^{10}$ cfu HN019/day	7 days	No adverse clinical signs were noted in any animals, and the mean general appearance scores did not differ. There was no effect on feed or water consumption or on body-weight gain. No viable bacteria were detected in the spleens of any mice. There were no effects on villi height, mucosal thickness, epithelial cell height, blood glucose, or any hematological measures. Absolute and relative spleen weights were unaffected, indicating no splenic inflammation.

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Table 6. Studies of *B. animalis* ssp. *lactis* in Animals.

Reference	Objective	Study Design	Animal Model	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Shu et al. (2000) (2 reported experiments)	Assess the ability of *B. animalis* ssp. *lactis* strain HN019 to enhance resistance to *Salmonella typhimurium*.	1. Mice received a daily skim-milk gavage or a gavage with 10^9 cfu HN019 for 7 days, then challenged with low oral doses of *S. typhimurium* for 7 days and 7 more days on the assigned gavage 2. Mice received a daily skim-milk gavage or a gavage with 10^9 cfu HN019 for 7 days, then challenged with a single high dose of *S. typhimurium* and 21 more days on the assigned gavage	150 7-9-week-old male BALB/c mice	10^9 cfu HN019/ day	21 days and 28 days	In experiment 1, the mice that received HN019 had significantly higher levels of post-infection feed intake, lost less weight, and had higher health scores than control mice. Results in experiment 2 were similar, and the mice receiving bifidobacteria also exhibited significantly greater survival through the 21-day post-challenge period (76.7% v. 6.9%). The mice receiving the probiotic had a significantly lower incidence of bacterial translocation to visceral tissues, significantly higher levels of phagocytically active cells in both the blood and peritoneal cells, and significantly higher mean titers of lipoteichoic-acid-specific antibodies in blood and mucosa. No adverse effects due to HN019 were observed in either experiment.
Shu et al. (2001)	Investigate the effect of *B. animalis* ssp. *lactis* strain HN019 on the risk of rotavirus and *E. coli* infection	Piglets received skim milk containing 0 or 10^9 cfu HN019/day by orogastric tube during weaning.	17 3-week-old male suckling piglets weighing 5-7 kg	10^9 cfu HN019/ day	15 days	Piglets receiving HN019 had better feed efficiency and significantly less severe diarrhea than controls, and one control pig died of severe diarrhea and dehydration. The piglets receiving HN019 also showed significantly increased phagocytosis, lymphoproliferative response, and titers of specific IgA, IgM, and IgG for rotavirus and *E. coli*. There were no adverse effects attributable to daily administration of 10^9 cfu *B. animalis* ssp. *lactis* strain HN019.

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Table 6. Studies of *B. animalis* ssp. *lactis* in Animals.

Reference	Objective	Study Design	Animal Model	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Shu and Gill (2001)	Investigate the ability of *B. animalis* ssp. *lactis* HN019 to reduce the severity of *E. coli* O157:H7 infection	Mice received diets with or without HN019 for 7 days before challenge with *E. coli* O157:H7, then maintained on assigned diets for 7 more days.	44 BALB/c mice and 42 C57BL/6 mice, all 7-9-week-old males	$3x10^8$ cfu HN019/g feed; intake = $9x10^8$ cfu/day	14 days	The mice fed bifidobacteria had significantly higher feed intake; lower morbidity rates following infection; reduced incidence of *E. coli* translocation to the blood, spleen, and liver; increased phagocytic capacity of both blood and peritoneal cells; and increased IgA titer (although IgG titer was not affected). Administration of HN019 to mice caused no adverse effects.
Tejada-Simon et al. (1999)	Study immune-stimulating effects of *B. animalis* ssp. *lactis* Bf-6, *B. bifidum*, or *B. infantis* against cholera toxin	Mice received skim-milk or one of 4 yogurts for 4 weeks during which they were twice inoculated with 10 µg cholera toxin	50 9-week-old female $B6C3F_1$ mice	$5\text{-}8x10^8$ cfu Bf-6 /ml; intake = $2.4x10^9$ cfu/ day	4 weeks	There were no differences in feed intake or weight gain across the treatment conditions. The mice remained healthy throughout the trial and diarrhea was not observed in any group. While the mice receiving conventional yogurt exhibited nonsignificantly lower IgA titer than did the control mice, those receiving yogurt containing Bf-6 or other bifidobacteria had significantly higher titers of IgA. IgG titer was not affected by treatment.
Xu et al. (2008)	Test acute toxicity of starter cultures used in Chinese fermented milk products.	Bacteria (*B. animalis* ssp. *lactis* Bb-12, LGG and an unspecified *L. rhamnosus* strain, or a strain of *L. reuteri*) were administered either orally or by i.p. injection.	Mice (number and strain not reported)	$5x10^7$, $5x10^8$, or $5x10^9$ cfu Bb-12/ml administered orally or by i.p. injection	3 days	No bacteria showed toxic effects when administered orally over 3 days. *B. animalis* ssp. *lactis* Bb-12 showed no toxicity after i.p. injection at the highest dose of $5x10^9$ cfu/ml.

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Table 6. Studies of *B. animalis* ssp. *lactis* in Animals.

Reference	Objective	Study Design	Animal Model	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Wagner et al. (1997a)	Assessed the ability of *B. animalis*, LGG, *L. reuteri*, and *L. acidophilus* to colonize, stimulate immune responses in, and affect growth and survival of congenitally immuno-deficient mice	The bacteria were introduced by means of swabs of the oral and rectal cavities of the mice. Adherence to epithelial surfaces was tested.	Congenitally immuno-deficient gnotobiotic beige-athymic (*bg/bg-nu/nu*) and beige-euthymic (*bg/bg-nu/+*) mice	10^8 cfu/ml introduced into the oral and rectal cavities by swabs	12 weeks	The bacteria colonized and persisted at high concentrations in the alimentary tracts of both mouse strains for the entire study period. Adherence to epithelial surfaces by *B. animalis* was 82%. Translocation to internal organs was detected in >50% of the mice colonized with *B. animalis*, but did not result in any signs of morbidity, mortality, pathologic changes, or effects on growth. There were no effects on the pups.
Wagner et al. (1997b)	Study the ability of *B. animalis*, LGG, *L. reuteri*, and *L. acidophilus*, to protect congenitally immuno-deficient mice from mucosal and systemic candidiasis	The bacteria were introduced by means of swabs of the oral and rectal cavities of the mice. Adherence to epithelial surfaces was tested.	Congenitally immuno-deficient gnotobiotic beige-athymic (*bg/bg-nu/nu*) and beige-euthymic (*bg/bg-nu/+*) mice	10^8 cfu/ml introduced into the oral and rectal cavities by swabs	Single ad-ministration	Each bacterial strain and *Candida albicans* colonized the gastrointestinal tracts of both strains of mice. The presence of probiotic bacteria in the gastrointestinal tracts prolonged the survival of adult and neonatal *bg/bg-nu/nu* mice compared to that of isogenic mice colonized with *C. albicans* alone. The incidence of systemic candidiasis in *bg/bg-nu/nu* mice was significantly reduced by each of the four probiotic bacterial strains. No bacterial infection occurred in this doubly immunodeficient animal model.

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Table 6. Studies of *B. animalis* ssp. *lactis* in Animals.

Reference	Objective	Study Design	Animal Model	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Zhou et al. (2000a)	Assess the safety of *B. animalis* ssp. *lactis* HN019, *L. rhamnosus* HN001, and *L. acidophilus* HN017	Mice were acclimatized and then randomized into 13 groups. The 4 strains were administered orally to mice at 3 dose levels; a 13th group of mice served as a non-probiotic control.	78 male BALB/c mice aged 6-8 weeks and weighing about 20 g	0, $5x10^7$, 10^9, or $5x10^{10}$ cfu HN019/day	21 days	There was no mortality and no adverse clinical signs were noted in any animals. There was no effect of any treatment on feed or water consumption, body-weight gain, any of the hematological or biochemical parameters assessed, or on histological parameters. Both absolute and relative spleen weights were unaffected. Mucosal examination found no indication of inflammation, degeneration, or necrosis or any effect on the villi height, mucosal thickness, or epithelial cell height of the intestinal samples. No viable test-strain bacteria were detected in the spleens, kidneys, or blood of any mice.
Zhou et al. (2000b)	Assess the safety of *B. animalis* ssp. *lactis* HN019, *L. rhamnosus* HN001, and *L. acidophilus* HN017, with *L. rhamnosus* GG and *L. acidophilus* LA-1 as comparison strains	Mice were acclimatized for 7 days and randomized to receive one of the 5 treatments or placebo	48 male BALB/c mice aged 6-8 weeks and weighing about 20 g	10^{11} cfu HN019/day (twice a day at doses of $5x10^{10}$ via oral pipette)	8 days	There was no mortality and no effects of any of the treatments were observed in the animals' behavior, hair luster, activity level, feed intake, or body-weight gain. Macro- and microscopic examination of the excised organs revealed no effect or any sign of inflammation, degeneration, or necrosis of intestinal mucosa; no difference was seen in villus height, epithelial cell height, crypt depth, or mucosa thickness. No hepatomegaly, splenomegaly, or cecal enlargement was seen. Neither absolute nor relative spleen weight was affected. There was no bacterial growth in the visceral swab cultures or in the blood of any animal. No test-strain bacteria were detected in excised tissues. The tested probiotic strains had very low acute toxicity, no effect on mucosa, little likelihood of translocation, and no infectivity.

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Table 6. Studies of *B. animalis* ssp. *lactis* in Animals.

Reference	Objective	Study Design	Animal Model	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Zhou and Gill (2005)	Investigate the effect of administration of *B. animalis* ssp. *lactis* HN019 and *L. rhamnosus* HN001 on pathological inflammation in a mouse model of experimental autoimmune thyroiditis	Mice were acclimated for 2 weeks and randomized to receive one of the test strains, LGG, or placebo. The mice were also randomized to be inoculated subcutaneously with thyroglobulin after 7 days and again after 14 days or to be inoculated with thyroglobulin + lipopolysaccharide with a booster at 42 days	114 female CBA/CaH mice 4-6 weeks old possessing the H-2^k haplotype for susceptibility to experimental autoimmune thyroiditis	$6x10^7$ to $1.5x10^8$ cfu HN019/day via pipette	5, 8, or 9 weeks	There was no mortality and no differences in clinical observations, body temperatures, or body weights of the mice receiving saline or probiotics. All mice inoculated with thyroglobulin produced measurable titers of IgG1 and IgG2a antibodies against thyroglobulin and inflammation was shown in increased relative spleen weights and increased splenic lymphocyte proliferation. Administration of probiotic bacteria did not affect the incidence of experimental autoimmune thyroiditis. Long-term daily ingestion of the tested probiotics had no adverse effect on the induction or progression of thyroglobulin-induced experimental autoimmune thyroiditis in CBA/CaH mice. The findings suggest that B. *animalis* ssp. *lactis* HN019 (as well as the 2 other strains tested) does not induce or enhance autoimmune response in humans.

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4.5.2. Human Studies

4.5.2.1. Studies in Adults

4.5.2.1.1. Studies of B. animalis *ssp.* lactis *Bf-6 in Adults*

Jiang et al. (1996) studied the role of *B. animalis* ssp. *lactis* strain Bf-6 in improving digestion of lactose by 15 healthy adults (7 men and 8 women aged 24-42 years; mean = 29.7 years) identified as lactose maldigesters by a rise in breath H_2 after milk consumption. The subjects consumed in random order 400 ml 2% reduced-fat milk with or without $5x10^8$ cfu of Bf-6. and recorded symptoms of lactose intolerance hourly, as well as all bowel movements and fecal characteristics. Breath hydrogen was measured hourly for 8 hours after ingestion of milk. The level of breath H_2 peaked between 2 and 4 hours after milk ingestion, and both the peak and the total area under the curve were significantly lower when Bf-6 was ingested with the milk. Bf-6 ingestion also significantly lowered the frequency and severity of reported symptoms of lactose intolerance. No adverse effects were reported from ingestion of $5x10^8$ cfu of Bf-6. The authors concluded that "these organisms have significant potential for use in products designed to improve lactose digestion."

Kullen et al. (1997) studied the survival of *B. animalis* ssp. *lactis* strain Bf-6 (identified only as "commercially available bifidobacteria") in the gastrointestinal tracts of 5 healthy 18-35-year-old males (average age = 28.1 years). The subjects consumed 750 ml skim milk containing 10^{10} cfu of Bf-6 daily for 8 days, followed by an additional 8 days of consumption of skim milk without added bacteria. Fecal samples were taken prior to feeding and at 4-day intervals and analyzed for total bifidobacteria and for 16S rRNA matching that of Bf-6. No matches were found prior to ingestion of Bf-6, but following ingestion the concentration of Bf-6 rose to 67.2% of total bifidobacteria. After cessation of feeding, the fecal population of Bf-6 rapidly diminished and was undetectable by the 8th day after feeding. Total bifidobacteria counts increased slightly but significantly during Bf-6 feeding, but "after feeding stopped, the ingested bifidobacteria diminished and became undetectable."

The effect of consumption of *B. animalis* ssp. *lactis* strain Bf-6 (identified only as "commercially available bifidobacteria") on the total colonic bifidobacteria population was evaluated in healthy adults, 5 men and 8 women with a mean age of 24.7 years (Amann et al. 1998). Lactose digestion and non-methane production was demonstrated in all volunteers prior to the initiation of the randomized, double-blind crossover study. During each 12-day feeding period, separated by a 12-day washout, subjects consumed 2-3 servings/day totaling 750 ml skim milk with or without 10^{10} cfu of bifidobacteria. A total of 14-20 stool samples were collected from each participant for bifidobacteria enumeration; randomly selected samples of bifidobacteria colonies were subjected to 16S rRNA gene sequencing for strain identification. Breath samples were taken at the beginning and end of each feeding period and analyzed for H_2, CH_4, and CO_2.

Ingestion of 10^{10} cfu/day of bifidobacteria had no significant effect on the bifidobacteria or total anaerobe populations isolated in feces. During the bifidobacteria feeding period, an average of 46% of the isolated bifidobacteria were the ingested strain, Bf-6; this strain was not identified in any fecal samples taken prior to feeding. Gas concentrations in the breath tests were highly variable and no significant differences were apparent during consumption of skim milk

with or without bifidobacteria. No adverse effects were reported due to ingestion of 10^{10} cfu of strain Bf-6 per day for 12 days. The authors concluded that "these data suggest that it is difficult to permanently alter total colonic bifidobacteria and affect physiologic function by feeding bifidobacteria."

4.5.2.1.2. Studies of Other B. animalis *ssp.* lactis *Strains in Adults*

Schiffrin et al. (1995) investigated immunomodulatory effects of ingestion of *B. animalis* ssp. *lactis* strain Bb-12 (referred to as *B. bifidum*) or *L. acidophilus* strain La1. In a randomized, double-blind, parallel-group design, 16 males and 12 females aged 23-62 years (mean = 36.9 years) were randomly assigned to receive 3 servings/day of fermented milk providing either 10^{10} cfu Bb-12 or $7x10^{10}$ cfu La1/day for 3 weeks, followed by a 6-week washout period. Blood samples were taken at baseline and at 0, 3, and 6 weeks after termination of probiotic feeding for assessment of leukocyte subsets $CD3^-$, $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$, $CD19^+$, $CD56^+$, and HLA-DR^+, as well as phagocytic activity. Fecal samples were taken at baseline, immediately after 3 weeks of probiotic ingestion, and 12 days after cessation, and analyzed for bifidobacteria and lactobacilli.

Fecal counts of bifidobacteria and lactobacilli increased significantly in the groups receiving each of these genera, but levels returned to baseline within 12 days of feeding cessation. No significant changes were seen in either group in the proportions of blood lymphocyte subsets or the degree of T-cell activation, but activity of blood phagocytes was significantly increased by ingestion of both probiotics—significantly more so by bifidobacteria than by lactobacilli. This effect decreased significantly over the 6 weeks after ingestion ceased but still remained significantly higher than at baseline. The authors suggested that these findings indicate that "nonspecific, anti-infective mechanisms of defense can be enhanced by the ingestion of specific lactic acid bacteria strains." There were no reported adverse effects due to the ingestion of either probiotic strain.

In a double-blind intervention trial of the effect of *B. animalis* ssp. *lactis* strain HN019 on cellular immunity (Chiang et al. 2000), 50 healthy adults aged 41-81 (15 males, 35 females; median age = 60 years) consumed lowfat milk for 3 weeks, after which 27 consumed $2.5x10^{10}$ cfu/day HN019 in 200 ml lowfat milk for 3 weeks while 23 others consumed the same dose of probiotic in 200 ml lactose-hydrolyzed lowfat milk for the same period. All subjects then consumed unsupplemented lowfat milk for a 3-week washout period. Blood was taken on days 0, 21, 42, and 63 and separated into mononuclear cells, which were assayed for NK cell activity, and polymorphonuclear cells, in which phagocytic activity was measured.

Both groups showed significant increases in cellular immune response during and after ingestion of *B. animalis* ssp. *lactis* strain HN019; there were no significant differences between vehicles (lowfat milk and lactose-hydrolyzed lowfat milk) in phagocytic activity, but the hydrolyzed milk carrier was more effective in increasing NK cell activity. No adverse effects were seen, and the authors concluded that the probiotic could be incorporated into dairy-based food products offering immunity-promoting benefits.

Arunachalam et al. (2000) also studied the effect of ingestion of *B. animalis* ssp. *lactis* strain HN019 on immune function in a prospective, randomized, double-blind, placebo-controlled study. Twenty-five volunteers aged 60-83 years, 7 males and 18 females with median age = 69 years, consumed 2 daily servings of either nonfat milk (n = 12) or the same milk containing $3x10^{11}$ cfu HN019/day for 6 weeks. Blood was taken at baseline and at 3, 6, and 12

weeks and separated into mononuclear cells, which were assayed for interferon-α (IFNα) production, and polymorphonuclear cells, in which phagocytic and bacteriocidal activity were measured.

There were no issues of tolerance or adverse effects during the study. IFNα production, phagocytic capacity, and bactericidal activity were significantly higher after 6-weeks consumption of HN019 than at baseline or among the placebo group. These increased immune responses showed little decline during the 6 weeks following termination of probiotic ingestion, and the authors concluded that "the results of the present study have clearly demonstrated that dietary *B. lactis* can impart measurable benefits on natural immune function among healthy elderly subjects."

Using PCR amplification of 16S rRNA sequences extracted from fecal bifidobacterial populations, Satokari et al. (2001) studied the effect of oral administration of $3x10^{10}$ cfu/day of *B. animalis* ssp. *lactis* Bb-12 to 10 healthy adults (2 males and 8 females; mean age = 32 years) for 2 weeks. Fecal samples were collected at the beginning and end of the feeding period, after 1 week, and after 2 weeks, and analyzed using PCR[1].

There were no significant changes in bifidobacteria populations as a result of ingestion of $3x10^{10}$ cfu/day of *B. animalis* ssp. *lactis* Bb-12. Strain Bb-12 was not detected with the original forward primer, but was detected in the post-feeding fecal samples of all 10 subjects using the modified primer. It was not found in the prefeeding samples or in those collected 1 or 2 weeks after feeding cessation. This finding is consistent with those of other studies indicating that *B. animalis* ssp *lactis* does not colonize the gastrointestinal system but simply exerts its probiotic effect during passage.

Gill et al. (2001a) investigated the ability of LAB to enhance declining immune function in the elderly. In a comparative study, 27 healthy individuals age 60-84 years (11 males, 16 females; median age 69.5 years) consumed milk for 3 weeks as a run-in, after which 14 participants were randomly assigned to consume $5x10^9$ cfu/day *B. animalis* ssp. *lactis* strain HN019 for 3 weeks while 13 others consumed $5x10^{10}$ cfu/day *L. rhamnosus* HN001 for the same period. All subjects then consumed unsupplemented milk for a 3-week washout period. Peripheral blood samples were taken by venipuncture on day 0, day 21 (after the run-in but before probiotic supplementation), day 42 (after supplementation), and day 63 (after the washout). Mononuclear cells were separated from the blood samples and the proportions staining positive for CD56 or CD3/CD56 were determined. Their tumoricidal (natural killer [NK]-cell) activity was assessed *in vitro* by lysis of target K562 cells, an NK-sensitive erythroleukemic cell line.

The run-in diet had no effect on immune parameters, but enhancement of NK-cell activity was seen during administration of both probiotics. Additionally, the proportion of peripheral blood cells staining for CD56 increased. Levels of both immune-system parameters returned to baseline levels by the end of the washout period. Nevertheless, the authors concluded that "*B. lactis* HN019 could be an effective probiotic dietary supplement for enhancing some aspects of cellular immunity in the elderly." No adverse effects were reported.

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[1] The forward primer Bif164-f, GGGTGGTAATGCCGGATG, has a single nucleotide mismatch with *B. animalis* ssp. *lactis*, and so was modified by one nucleotide to GGGTGGTAAT<u>A</u>CCGGATG in order to amplify the 16S rRNA fragment from this subspecies more efficiently.

Gill et al. (2001b), in another single-blind study in a population of individuals aged 65 to 79 years, investigated the effect on cellular immune (NK cell) function of *B. animalis* ssp. *lactis* HN019 delivered in a lowfat milk matrix. Fourteen healthy individuals (6 males and 8 females) consumed unsupplemented milk for 3 weeks, followed by consumption of milk providing $5x10^9$ cfu HN019/day for 3 weeks and a 3-week washout. Blood was drawn at the end of weeks 0, 3, 6, and 9 and analyzed for $CD3^+$, $CD56^+$, and tumoricidal activity. Significant increases were seen in $CD56^+$ and tumoricidal activity, especially in patients over 70 years of age, but these measures returned to baseline after ingestion of *B. animalis* ssp. *lactis* HN019 ceased. No adverse effects were reported and the authors concluded that dietary intervention using probiotics may be "a safe and effective means of improving geriatric health."

Because nearly all studies of *B. animalis* ssp. *lactis* and other probiotic strains employ dairy products as carriers, Ouwehand et al. (2004) tested the ability of a dry food matrix—an oat-based cereal bar—to deliver viable *B. animalis* ssp. *lactis* Bb-12 cells to healthy adults. Ten volunteers, 3 males and 7 females aged 24-49 years, consumed 1 cereal bar daily for 7 days. Each bar provided $5x10^9$ cfu Bb-12 and had water activity of only 0.25. Fecal samples were collected before and after feeding and again 4 and 7 days later.

One volunteer was eliminated from analysis because she tested positive for Bb-12 before the experiment began. After 7 days of ingestion, 4 of the 9 remaining subjects had detectible Bb-12 in their feces, and these individuals were still excreting Bb-12 at the same level 4 and 7 days after feeding was stopped. There was no effect on total fecal bacterial counts. The authors concluded that dry foods are potential vehicles for probiotic delivery.

Larsen et al. (2006), in a randomized, double-blind, placebo-controlled, parallel dose-response study, administered *B. animalis* ssp. *lactis* Bb-12 and *L. paracasei* ssp. *paracasei* CRL-431 to 71 volunteers (25 men and 46 women aged 18-40 years; mean age = 25.6 years) in doses of 0, 10^8, 10^9, 10^{10}, or 10^{11} cfu/day for 3 weeks, followed by a 2-week washout. The participants maintained a diary recording bowel habits and gastrointestinal signs; blood lipids and fecal microbiota were evaluated before, immediately following, and 2 week after the intervention.

Fecal recovery of Bb-12 increased significantly with increasing dose, but CRL-431 was not recovered in any fecal samples. A significant change toward looser stools was observed with increasing probiotic dose, but there was no effect on blood lipids or overall fecal bacterial composition. The probiotic administration was well tolerated at all doses and no adverse effects were observed.

In an investigation of the effects of 3 probiotics on immune parameters, Kekkonen et al. (2008) conducted a prospective, randomized, double-blind, placebo-controlled parallel-group intervention study with 62 healthy adults (17 males and 45 females aged 23-58 years; mean age = 44 years). After a 3-week run-in, participants were randomly assigned to 1 of 4 groups to receive the specified intervention (in a milk-based fruit drink) for 3 weeks: placebo (n = 16), 1 $3.5x10^{10}$ cfu *B. animalis* ssp. *lactis* Bb-12/day (n = 16), $6x10^{10}$ cfu LGG/day (n = 13), or $3.3x10^{10}$ cfu *Propionibacterium freudenreichii* ssp. *shermanii* JS/day (n = 17). Venous blood samples were taken at baseline, on days 1, 7, and 21, and after a 3-week washout, and analyzed for leukocytes, monocytes, neutrophils, basophils, eosinophils, and lymphocytes; IgA, IgG, and IgM; C-reactive protein; cytokines (TNF-α, IL-6, IFN-γ, and IL-10). An unstimulated saliva sample was taken at each time point for analysis of secretory IgA. Fecal samples were also collected at baseline and on day 21 and probiotic strains were identified and enumerated.

Participants maintained a diary of any respiratory, GI, or other symptoms and the use of any medication.

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

Matsumoto et al. (2009) compared the effects on the fecal microbiota of consumption of yogurt with or without added *B. animalis* ssp. *lactis* strain LKM512 ($5.2x10^7$ cfu/g) by 6 hospitalized elderly patients. In a nonrandomized crossover design, the 3 men and 3 women (mean age = 80.3 years), who suffered from a variety of non-gastrointestinal maladies, consumed 100 g yogurt with LKM512 for 2 weeks followed by yogurt without the strain, separated by a 2-week washout. Fecal samples collected at baseline and every 2 weeks thereafter were analyzed for bacterial loads with species identification based on PCR amplification of the 16S rRNA gene.

Ingestion of $5.2x10^9$ cfu LKM512/day significantly increased fecal counts of *B. animalis* ssp. *lactis* (by 2 to 6 log) and decreased lactobacilli as compared to consumption of the yogurt without bifidobacteria. No adverse effects were reported in this sensitive population.

In a randomized, double-blind, placebo-controlled study of the efficacy of probiotics in the management of evacuation disorders, Del Piano et al. (2010) enrolled 300 otherwise healthy adults (151 males and 149 females) aged 24-71 years (mean age was not reported) with evacuation disorders and hard stools. The 110 participants in the first experimental group received $5x10^{10}$ cfu/day of *B. animalis* ssp. *lactis* strain BS01, the 110 participants in the other experimental grup received $2.55x10^{10}$ cfu/day of each of *L. plantarum* LP01 and *B. breve* BR03, while the 80 controls received placebo for 30 days. All participants were evaluated at enrollment and at 15 and 30 days with a physical and ultrasound study of the abdomen; participants also reported the frequency and consistency of bowel movements, the ease of expulsion and sensation of complete emptying, and side effects such as abdominal bloating or anal itching, burning, or pain during or after defecation using a numerical scaling system.

The overall dropout rate was about 10%--7 members of the *B. animalis* ssp. *lactis* group, 10 from the *L. plantarum/B. breve* group, and 6 from the placebo group. Both probiotic groups reported significant improvement in bowel movement ease and frequency as well as stool hardness as well as a significant reduction in itching, burning, or pain at defecation. The authors stated that "No volunteer manifested any disorder, adverse, or side effects throughout the study."

Dotterud et al. (2010) studied the ability of a blend of three probiotics ($5x10^{10}$ cfu/day each of *B. animalis* ssp. *lactis* Bb-12 and *L. rhamnosus* GG, and $5x10^9$ cfu/day *L. acidophilus* La-5) administered to pregnant women to reduce the incidence of atopic sensitization or allergic disease in their babies. A total of 415 pregnant women who were planning to breastfeed were enrolled at their 36th week of gestation in a prospective, randomized, double-blind, placebo-controlled study and assigned to receive 250 ml fermented milk/day, either with (n = 211) or without (n = 204) probiotics. The women continued to receive the intervention until 3 months postpartum. Stool samples, cord blood, and venous blood were collected from the infants at age 10 days, 3 months, 1 year, and 2 years. Samples were also taken of maternal breast milk and vaginal mucosa and from the infants' oral mucosa. Infants were examined for atopic disease if they developed an itchy rash during the study period; at the end of 2 years all children were

assessed for atopic disease, asthma, allergic rhinoconjunctivitis, and atopic sensitization (based on a positive skin-prick test or elevated specific IgE.

A total of 138 infants in the probiotic group and 140 controls completed the full 2-year study; most drop-outs were due to failure to breastfeed for at least 3 months, maternal noncompliance with consumption of the fermented milk, or moving from the study area. The administration of the probiotic bacteria blend to women for 4 months statistically significantly reduced the incidence of atopic disease among their 2-year-old children, although no effect was seen in atopic sensitization, asthma, or allergic rhinoconjunctivitis. The authors claimed that no adverse events were reported.

4.5.2.1.3. Conclusions from Studies of B. animalis *ssp.* lactis *in Adults*

In 15 studies in which *B. animalis* ssp. *lactis* strains Bf-6, Bb-12, BS01, HN019, and LKM512 were administered to both healthy adults (including pregnant women) and hospitalized and elderly individuals at daily doses as high as $3x10^{11}$ cfu for periods as long as 4 months, no adverse effects were reported (Table 7). This consistent finding of no harm provides strong demonstration of the safety of oral administration of *B. animalis* ssp. *lactis* strain Bf-6.

Table 7. Studies of *B. animalis* ssp. *lactis* in Adults.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Dura-tion	Safety-Related Results
Amann et al. (1998)	Evaluate the bifidogenic effect of consumption of *B. animalis* ssp. *lactis* strain Bf-6	Prospective, randomized, double-blind crossover study	5 healthy men and 8 women with mean age 24.7 years	10^{10} cfu Bf-6/ day	12 days	Ingestion of 10^{10} cfu/day of bifidobacteria had no effect on fecal bifidobacteria or total anaerobe populations. During bifidobacteria feeding, 46% of isolated bifidobacteria were the ingested strain, Bf-6; this strain was not identified in any fecal samples prior to feeding. No adverse effects were reported due to ingestion of 10^{10} cfu of strain Bf-6 per day for 12 days.
Arunachalam et al. (2000)	Evaluate the effect of *B. animalis* ssp. *lactis* strain HN019 on immune function	Prospective, randomized, double-blind, placebo-controlled study	7 healthy males and 18 females aged 60-83 years (median age = 69 years)	$3x10^{11}$ cfu HN019/day	6 weeks	There were no tolerance issues or adverse effects. IFNα production, phagocytic capacity, and bactericidal activity were significantly higher after 6-weeks consumption of HN019 than at baseline or among the placebo group. These increased immune responses showed little decline during the 6 weeks following termination of probiotic ingestion.
Chiang et al. (2000)	Study the effect of *B. animalis* ssp. *lactis* strain HN019 on cellular immunity	Double-blind parallel-group intervention trial	50 healthy adults aged 41-81 (15 males, 35 females; median age = 60 years)	$2.5x10^{10}$ cfu HN019/day	3 weeks	Significant increases were seen in cellular immune response (phagocytic and NK cell activity) during and after ingestion of *B. animalis* ssp. *lactis* strain HN019 both in lowfat milk and lactose-hydrolyzed lowfat milk. No adverse effects were seen, and the authors concluded that the probiotic could be incorporated into dairy-based food products offering immunity-promoting benefits.
Del Piano et al. (2010)	Study the effect of probiotics on evacuation disorders	Prospective, randomized, double-blind, placebo-controlled study	300 otherwise healthy adults (151 males and 149 females) aged 24-71 years with evacuation disorders	$5x10^{10}$ cfu/ BS01/day	30 days	Participants reported significant improvement in bowel movement ease and frequency as well as stool hardness. No gastrointestinal signs or other adverse effects were reported.

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Table 7. Studies of *B. animalis* ssp. *lactis* in Adults.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Dura-tion	Safety-Related Results
Dotterud et al. (2010)	Study whether a blend of *B. animalis* ssp. *lactis* Bb-12, *L. rhamnosus* GG, and *L. acidophilus* La-5 administered to pregnant women reduces the incidence of atopia or allergy in their babies	Prospective, randomized, double-blind, placebo-controlled study	415 pregnant women planning to breastfeed	$5x10^{10}$ cfu Bb-12/day (+ $5x10^{10}$ cfu *L. rhamnosus* GG and $5x10^{9}$ cfu *L. acidophilus* La-5)	4 months	The administration of the probiotic bacteria blend to women for 4 months significantly reduced the incidence of atopic disease among their 2-year-old children, although no effect was seen in atopic sensitization, asthma, or allergic rhinoconjunctivitis. The authors claimed that no adverse events were reported.
Gill et al. (2001a)	Investigate the ability of LAB (including B. *animalis* ssp. *lactis* HN019) to enhance immune function in the elderly	Double-blind comparative study	27 healthy individuals aged 60-84 years, 11 males and 16 females	$5x10^{10}$ cfu HN019/day	3 weeks	The run-in diet had no effect on immune parameters, but enhancement of NK-cell activity was seen during administration of both probiotics. Additionally, the proportion of peripheral blood cells staining for CD56 increased. Levels of both immune-system parameters returned to baseline levels by the end of the washout period. No adverse effects were reported.
Gill et al. (2001b)	Investigated the effect on cellular immune (NK cell) function of *B. animalis* ssp. *lactis* HN019	Single-blind crossover-design study	6 healthy males and 8 females aged 65 to 79 years	$5x10^{9}$ cfu HN019/day	3 weeks	Significant increases were seen in $CD56^{+}$ and tumoricidal activity, especially in patients over 70 years of age, but these measures returned to baseline after ingestion of *B. animalis* ssp. *lactis* HN019 ceased. No adverse effects were reported and the authors concluded that dietary intervention using probiotics may be a safe and effect means of improving geriatric health.

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Table 7. Studies of *B. animalis* ssp. *lactis* in Adults.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Dura-tion	Safety-Related Results
Jiang et al. (1996)	Study the role of *B. animalis* ssp. *lactis* strain Bf-6 in improving digestion of lactose	Randomized single-blinded crossover study	15 healthy adults (7 men and 8 women aged 24-42 years; mean = 29.7 years) identified as lactose maldigesters	$5x10^8$ cfu Bf-6	single dose	Breath H_2 peaked between 2 and 4 hours after milk ingestion, and both the peak and the total area under the curve were significantly lower when Bf-6 was ingested with the milk. Bf-6 ingestion also significantly lowered the frequency and severity of reported symptoms of lactose intolerance. No adverse effects were reported from ingestion of $5x10^8$ cfu of Bf-6.
Kekkonen et al. (2008)	Investigate the effects of 3 probiotics including *B. animalis* ssp. *lactis* Bb-12 on immune parameters	Prospective, randomized, double-blind, placebo-controlled parallel-group intervention study	62 healthy adults (17 males and 45 females aged 23-58 years; mean age = 44 years).	$3.5x10^{10}$ cfu Bb-12/day	3 weeks	LGG, but not *B. animalis* ssp. *lactis* Bb-12, significantly reduced C-reactive protein. There were no significant differences between the groups in white blood cell counts, immunoglobulins, or cytokine levels. No adverse effects were reported.
Kullen et al. (1997)	Study the survival of *B. animalis* ssp. *lactis* strain Bf-6 in the GI tract	Open-label study	5 healthy 18-35-year-old males (average age = 28.1 years)	10^{10} cfu Bf-6/ day	8 days	Following ingestion the concentration of Bf-6 rose to 67.2% of total bifidobacteria. After cessation of feeding, the fecal population of Bf-6 rapidly diminished and was undetectable by the 8th day after feeding. Total bifidobacteria counts increased slightly but significantly during Bf-6 feeding, but quickly returned to baseline levels after cessation.

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Table 7. Studies of *B. animalis* ssp. *lactis* in Adults.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Dura-tion	Safety-Related Results
Larsen et al. (2006)	Evaluate the effect of ingestion of *B. animalis* ssp. *lactis* Bb-12 on blood lipids and fecal microbiota	Randomized double-blind, placebo-controlled, parallel dose-response study	25 healthy men and 46 women aged 18-40 years; mean age = 25.6 years	0, 10^8, 10^9, 10^{10}, or 10^{11} cfu Bb-12/day	3 weeks	Fecal recovery of Bb-12 increased significantly with increasing dose. A significant change toward looser stools was observed with increasing probiotic dose, but there was no effect on blood lipids or overall fecal bacterial composition. The probiotic administration was well tolerated at all doses and no adverse effects were observed.
Matsumoto et al. (2009)	Compare the effect on the fecal microbiota of consumption of yogurt with or without added *B. animalis* ssp. *lactis* strain LKM512	Non-randomized single-blind crossover design	6 hospitalized elderly patients (3 men and 3 women with mean age = 80.3 years)	5.2×10^9 cfu LKM512/day	2 weeks	Ingestion of LKM512 significantly increased fecal counts of *B. animalis* ssp. *lactis* and decreased lactobacilli as compared to consumption of the yogurt without bifidobacteria. No adverse effects were reported in this sensitive population.
Ouwehand et al. (2004)	Tested the ability of a dry food matrix—an oat-based cereal bar—to deliver viable *B. animalis* ssp. *lactis* Bb-12 cells	Open-label study	3 healthy males and 6 females aged 24-49 years	5×10^9 cfu Bb-12/day	7 days	After 7 days of ingestion, 4 of the 9 subjects had detectible Bb-12 in their feces, and these individuals were still excreting Bb-12 at the same level 4 and 7 days after feeding was stopped. There was no effect on total fecal bacterial counts. The authors concluded that dry foods are potential vehicles for probiotic delivery.

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Table 7. Studies of *B. animalis* ssp. *lactis* in Adults.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Dura-tion	Safety-Related Results
Satokari et al. (2001)	Studied the effect of $3x10^{10}$ cfu/day of *B. animalis* ssp. *lactis* Bb-12 on fecal bifidobacterial populations	Open-label study	2 healthy males and 8 females; mean age = 32 years	$3x10^{10}$ cfu Bb-12/day	2 weeks	There were no significant changes in bifidobacteria populations as a result of ingestion of $3x10^{10}$ cfu/day of *B. animalis* ssp. *lactis* Bb-12. Strain Bb-12 was detected in the post-feeding fecal samples of all 10 subjects. It was not found in the prefeeding samples or in those collected 1 or 2 weeks after feeding cessation.
Schiffrin et al. (1995)	Investigate immuno-modulatory effects of ingestion of *B. animalis* ssp. *lactis* strain Bb-12	Randomized double-blind, parallel-group design	16 healthy males and 12 females aged 23-62 years (mean = 36.9 years)	10^{10} cfu Bb-12/ day	3 weeks	Fecal counts of bifidobacteria increased significantly, but levels returned to baseline within 12 days of feeding cessation. No significant changes were seen in the proportions of blood lymphocyte subsets or the degree of T-cell activation, but activity of blood phagocytes was significantly increased. This effect decreased significantly over the 6 weeks after ingestion ceased but still remained significantly higher than at baseline. There were no reported adverse effects due to the ingestion of the probiotic strain.

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4.5.2.2. Studies in Children

Fukushima et al. (1998) studied the effect of ingestion of a milk-based formula providing 10^9 cfu *B. animalis* ssp. *lactis* Bb-12/day on the intestinal microbiota and IgA production in 7 healthy Japanese children aged 15-31 months. The children (4 boys and 3 girls) received the formula for 21 days and fecal samples were taken prior to feeding and on days 4, 9, 21, and 28 (1 week after cessation).

The formula was well tolerated and there were no compliance problems. Prior to the study, bifidobacteria constituted an average of 23% of the fecal biota and strain Bb-12 was not detected. The preponderance of bifidobacteria increased monotonically during feeding, reaching a maximum of 35% of the fecal biota on day 21; Bb-12 was found in the fecal samples from 5 of the 7 children, constituting 27% of the bifidobacteria. By 1 week after cessation of supplementation, strain Bb-12 was no longer found in feces and bifidobacterial prevalence had begun to decline, although it was still significantly higher than before feeding. There was no significant change in total bacteria counts. Fecal IgA also increased significantly during the first week of bifidobacteria feeding, peaking on day 9 and then decreasing to about double its starting level by day 21 and continuing to decrease a week later. The authors concluded that administering *B. animalis* ssp. *lactis* Bb-12 to healthy children has no adverse effects and may "have great potential for preventing infections in children during and after weaning by enhancing the mucosal resistance to infections by active stimulation of local IgA production."

Nopchinda et al. (2002) studied the effect of probiotic-supplemented infant formula on growth in 148 healthy but undernourished children (91 boys and 57 girls) aged 6-36 months. In a randomized, double-blind, placebo-controlled trial, 51 children received formula containing $3x10^7$ cfu *B. animalis* ssp. *lactis* Bb-12/ml, 54 received formula with $3x10^7$ cfu each of Bb-12 and *Streptococcus thermophilus*/ml, and 43 received unsupplemented formula for 6 months. Weight and height were measured monthly.

The children consumed 400-600 ml formula/day, resulting in a daily intake of Bb-12 of $1.2\text{-}1.8x10^{10}$ cfu. Both groups of children receiving probiotic-supplemented formula showed statistically significantly improved growth in height and weight as compared to the placebo group with no apparent adverse effects.

In a prospective, randomized, double-blind, placebo-controlled trial, Sistek et al. (2006) studied the effect of administration of daily doses of $2x10^{10}$ cfu of both *B. animalis* ssp. *lactis* strain HN019 and *L. rhamnosus* strain HN001 on children with atopic dermatitis. A total of 60 children ranging in age from 1 to 11 years (median = 4 years), 32 males and 28 females, previously diagnosed with eczema and with stable SCORADs of 10 or more, were randomized to receive either probiotic or placebo. The probiotic was administered once daily as a powder mixed with drink or food, as was the microcrystalline cellulose placebo. Two weeks before beginning treatment, the study participants' parents completed questionnaires about their children's health and the family history of allergic disease. The parents were also interviewed when treatment began (week 0), after 2 weeks, at the termination of treatment at week 12, and 4 weeks after treatment ceased (week 16). During the 12-week treatment period and the 4-week washout period the parents maintained a diary of any health problems, medication use, and the severity of the atopic dermatitis.

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

Bettler et al. (2006) studied the safety of administration of *B. animalis* ssp. *lactis* strain Bl-07 and Raftilose® P95 fructooligosaccharide (FOS) to healthy male and female toddlers aged 12-34 months (mean = 22.1 months). After a 7-day washout period, 318 children were randomly assigned to receive control formula (n = 105), probiotic formula with 2.6×10^8 cfu bifidobacteria/100 ml (n = 108), or synbiotic formula with bifidobacteria and FOS (n = 105) for 28 days, with parental instructions to feed the children 200 to 400 ml formula/day, resulting in a daily intake of strain Bl-07 of 5.2×10^8 to 1.0×10^9 cfu. Stool samples were collected on days 0, 7, 28, and 43, and interviews were scheduled on days 7, 14, 21, and 28 to learn about any tolerance issues or adverse effects.

A total of 170 toddlers (54%) completed the study. Both numbers of and reasons for withdrawal were similar in all feeding groups, and none of the reasons were related to the study formulas. A total of 186 toddlers reported at least one adverse event, but there were no differences between groups in the frequency, severity, or nature of the adverse events. The amount of formula consumed was similar in all groups and the gains in body weight did not differ between the groups. The experimental strain of bifidobacteria, *B. animalis* ssp. *lactis* Bl-07, was not detected in any fecal samples prior to the study, and in 1% of control toddlers and 38% of toddlers in the probiotic and synbiotic groups on day 7. By day 28 the proportion of toddlers with detectible fecal Bl-07 had risen to 41% of those fed the probiotic formula and 50% of those receiving the synbiotic formula. By day 43—2 weeks after feeding cessation—strain Bl-07 was not recovered in feces. However, the groups did not differ significantly in total fecal bacteria or bifidobacteria counts. At day 28 the probiotic group had a small but statistically significant reduction in clostridia compared to the other 2 groups and the probiotic and synbiotic groups had significantly increased counts of lactobacilli relative to the control group.

With regard to safety, the authors concluded that "the control, probiotic, and synbiotic formulas were well tolerated and appeared to be equally safe as assessed by body weights and in terms of the number and nature of adverse events. Further, the formulas were equally acceptable over the 28-day feeding period."

A total of 182 healthy male and female children aged 1-3 years and attending daycare or school at least 3 days/week were enrolled in a prospective, randomized, double-blind, placebo-controlled study of the effect of *B. animalis* ssp. *lactis* strain Bb-12 on absences due to illness (Merenstein et al. 2010). The probiotic was administered in a strawberry yogurt-based drink at a daily dose of 10^{10} cfu/day for 30 days while control children received an identical drink without the bacteria.

No effects, either beneficial or adverse, were observed from the probiotic intervention.

4.5.2.2.1. Conclusions from Studies of B. animalis *ssp.* lactis *in Children*

As was the case in studies in which *B. animalis* ssp. *lactis* strains were administered to adults, 5 studies in which strains Bb-12, Bl-07, or HN019 were given to children at levels as high as 2×10^{10} cfu/day for up to 6 months uniformly found an absence of adverse effects (Table 8).

The administration of this subspecies of bifidobacteria is generally beneficial, although this has not been demonstrated in all studies, and not harmful, and this body of data supports the safety of the intended use of *B. animalis* ssp. *lactis* Bf-6.

Table 8. Studies of *B. animalis* ssp. *lactis* in Children.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Bettler et al. (2006)	Study the safety of administration of *B. animalis* ssp. *lactis* strain BI-07 and FOS	Prospective randomized, double-blind, placebo-controlled parallel-group design	318 healthy toddlers aged 12-34 months (mean = 22.1 months)	5.2×10^8 to 1.0×10^9 cfu BI-07/day	28 days	Numbers of and reasons for withdrawal were similar in all feeding groups, and none of the reasons were related to the study formulas. There were no differences between groups in the frequency, severity, or nature of adverse events. The amount of formula consumed was similar in all groups and the gains in body weight did not differ between the groups. *B. animalis* ssp. *lactis* BI-07 was detected in 1% of control toddlers and 38% of toddlers in the probiotic and synbiotic groups on day 7. By day 28 toddlers with fecal BI-07 had risen to 41% of those fed the probiotic formula and 50% of those receiving the synbiotic formula. By day 43—2 weeks after feeding cessation—strain BI-07 was not recovered in feces. The groups did not differ significantly in total fecal bacteria or bifidobacteria counts. At day 28 the probiotic group had a small but statistically significant reduction in clostridia compared to the other 2 groups and the probiotic and synbiotic groups had significantly increased counts of lactobacilli relative to the control group. The authors concluded that the control, probiotic, and synbiotic formulas were equally tolerable, equally safe, and equally supportive of normal growth over the 28-day feeding period.

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Table 8. Studies of *B. animalis* ssp. *lactis* in Children.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Fukushima et al. (1998)	Assess the effect of *B. animalis* ssp. *lactis* Bb-12 on the intestinal microbiota and IgA production	Open-label study	7 healthy Japanese children (4 boys and 3 girls) aged 15-31 months	10^9 cfu Bb-12/day	21 days	The formula was well tolerated. Prior to the study, bifidobacteria were 23% of the fecal biota. Bifidobacteria increased during feeding to 35% of fecal biota on day 21; Bb-12 was found in samples from 5 of the 7 children, constituting 27% of the bifidobacteria. Strain Bb-12 was not found in feces 1 week later and bifidobacterial prevalence had begun to decline, but still higher than before feeding. There was no change in total bacteria counts. Fecal IgA also increased during the first week of bifidobacteria feeding, peaking on day 9. The authors concluded that administering *B. animalis* ssp. *lactis* Bb-12 to healthy children has no adverse effects.
Merenstein et al. (2010)	Study the effect of strain Bb-12 on absences of daycare or school children due to illness	Prospective, randomized, double-blind, placebo-controlled study	182 healthy children aged 1-3 years attending daycare or school at least 3 days/week	10^{10} cfu/ Bb-12/day	30 days	No effects, either beneficial or adverse, were observed from the probiotic intervention.
Nopchinda et al. (2002)	Study the effect of probiotic-supplemented infant formula on growth of under-nourished children	Randomized, double-blind, placebo-controlled trial	148 healthy but undernourished children (91 boys and 57 girls) aged 6-36 months	$1.2\text{-}1.8 \times 10^{10}$ cfu Bb-12/day	6 months	Children receiving probiotic-supplemented formula showed significantly improved growth in height and weight as compared to the placebo group with no apparent adverse effects.

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Table 8. Studies of *B. animalis* ssp. *lactis* in Children.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Sistek et al. (2006)	Studied the effect of *B. animalis* ssp. *lactis* strain HN019 and *L. rhamnosus* strain HN001 on children with atopic dermatitis	Prospective, randomized, double-blind, placebo-controlled trial	32 males and 28 females aged 1 to 11 years (median = 4 years) diagnosed with eczema	$2x10^{10}$ cfu HN019/day	12 weeks	Five children withdrew from the probiotic group and 6 from the placebo group, none related to treatment. The probiotic treatment slightly reduced SCORAD scores relative to the placebo group among children with food-related allergies, but not among those with environmental allergies. No adverse effects were reported from the probiotic treatment.

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4.5.2.3. Studies in Infants

Although *B. animalis* ssp. *lactis* strain Bf-6 is not intended for use in infant formula, nevertheless studies in which *B. animalis* ssp. *lactis* strains were ingested by infants are of value in establishing safety. This is because of the close genetic similarity of all strains in the subspecies combined with the fact that infants are a particularly sensitive population with generally lower tolerance for gastrointestinal disturbances, more permeable mucous membranes, and immature immune systems. Four strains have been studied in infants: Bb-12, CUL34, HN019, and CNCM I-3446. The absence of adverse effects from ingestion of any of these strains of *B. animalis* ssp. *lactis* by infants is strong evidence that the subspecies is neither toxigenic nor pathogenic.

A prospective, randomized, double-blind, placebo-controlled study designed to assess the ability of *B. animalis* ssp. *lactis* strain Bb-12 or *L. rhamnosus* strain GG to control allergic inflammation in infants with atopic eczema was conducted by Isolauri et al. (2000). Infants with a mean age of 4.6 months who had developed atopic eczema during breast feeding and had not been exposed to infant formula prior to enrollment were randomly assigned (n = 9 per group) to extensively hydrolyzed whey formulas with or without supplementation with 10^9 cfu Bb-12 or $3x10^8$ cfu LGG/g formula for at least 6 months. The exposure to the 2 probiotics was about 3-$8x10^{10}$ cfu/day. The severity of the atopic eczema was assessed prior to introduction of the whey formulas and at 2 and 6 months, and body measurements and samples of stool and blood were taken at the same times. The primary endpoints were the severity of atopic eczema and the growth of the infants. The mean SCORAD at the beginning of the study was 16, and the infants given Bb-12 or LLG showed significant improvement as compared to the starting levels and to the controls; after 2 months of feeding the Bb-12 group's SCORAD was 0.0 and the LGG group's was 1.0, while the mean SCORAD of the controls was 13.4. Soluble CD4 in blood and eosinophilic protein X in urine were also significantly reduced in the probiotic groups. The authors suggested that these results "constitute the first clinically documented demonstration of a possible role for specific microbes in controlling allergic inflammation." There was no effect on growth, which was normal in all infants studied. No adverse events were reported.

Kankaanpää et al. (2002) performed a pilot study for a randomized, double-blind, placebo-controlled study to investigate the effect of *B. animalis* ssp. *lactis* strain Bb-12 or *L. rhamnosus* strain GG on the composition of plasma lipids in atopic infants. Fifteen breastfed infants (mean age 5.2 months) referred to a pediatric clinic for treatment of atopic eczema were given an extensively hydrolyzed infant formula alone or supplemented with either 10^9 cfu Bb-12 or $3x10^8$ cfu LGG/g formula. The average duration of feeding was 5.5 months. Blood samples were taken at outset and 2 months later. The average formula intake was 75 ml/kg bw/day, thus providing daily doses of $7.5x10^{10}$ cfu Bb-12 or $2.25x10^{10}$ cfu LGG/kg bw, approximately equivalent to $6x10^{11}$ cfu Bb-12 or $1.8x10^{11}$ cfu LGG per infant. Bb-12 and LGG administration both reduced the proportion of α-linolenic acid in plasma neutral lipids, but LGG had no effect on phospholipids. The authors noted that all formulas were well tolerated by the infants, and no adverse effects were reported.

Twenty-one infants (mean age = 5.2 months) with early onset atopic eczema and failure to thrive were transitioned to extensively hydrolyzed whey formula (Kirjavainen et al. 2002). Eight infants were intolerant of the formula while 13 were able to tolerate it; of these 13, 6 were

assigned to receive placebo formula while 7 were exposed to formula with 10^9 cfu *B. animalis* ssp. *lactis* Bb-12/g powder providing an average of $8x10^{10}$ cfu Bb-12/kg bw/day. Feeding continued to a mean age of 9.1 months, an average of about 4 months. Fecal specimens were analyzed for microbiota.

The infants unable to tolerate extensively hydrolyzed whey formula had greater concentrations of lactobacilli and enterococci than did those who could tolerate the formula, and serum total IgE activity correlated with *E. coli* and bacteroides counts; the authors inferred that this indicates an association between these bacteria and the extent of atopic sensitization. Exposure to Bb-12 had no reported adverse effects but resulted in a significant decrease in the counts of *E. coli* and bacteroides. The authors concluded that "bifidobacterial supplementation appears to modify the gut microbiota in a manner that may alleviate allergic inflammation."

Saavedra et al. (2004) studied tolerance and safety of long-term consumption of infant formula containing 10^6-10^7 cfu/g of each of *B. animalis* ssp. *lactis* Bb-12 and *Streptococcus thermophilus* in a prospective, randomized, double-blind, placebo-controlled trial. A total of 118 healthy infants aged 3-24 months (mean age = 6.7 months; 58 males and 60 females) was enrolled and infants were randomly assigned to receive control milk-based powdered formula (n = 40) or formula containing either 10^6 or 10^7 cfu/g of each of the 2 bacteria (n = 39 each) and to remain in the study as long as their formula consumption was at least 240 ml/day. Data were collected weekly on general health status, gastrointestinal signs or symptoms, dietary habits, and defecation patterns. Each month the infants' length and weight were measured.

The infants remained in the study for an average of 30 weeks, with no significant difference between groups. Average daily ingestion of each strain of bacteria was $9.7x10^7$ cfu.kg bw in the low-dose group and $1.3x10^9$ cfu/kg bw in the high-dose group. There were no differences in number of or reasons for withdrawals, in formula consumption, in tolerance issues, or in gains in weight and length. However, the frequency of reporting of colic or irritability and the reported use of antibiotics was significantly lower in both supplemented groups than in the placebo group. The authors concluded that, "Probiotic supplementation resulted in adequate growth. The supplemented formulas were well accepted and tolerated and resulted in a significantly lower reported frequency of colic or irritability. Supplementation also resulted in a trend toward less frequent seeking of health care attention and a significantly lower frequency of antibiotic use."

In a multicenter, double-blind, placebo-controlled study, Chouraqui et al. (2004) studied the effect of a milk formula supplemented with *B. animalis* ssp. *lactis* Bb-12 on reducing the incidence of acute infectious diarrhea. Ninety healthy infants with an average age of 3.7 months, living in residential nurseries, were randomized to receive either formula alone (n = 44) or formula with $1.5x10^7$ cfu Bb-12/100 ml formula (n = 46). The infants were evaluated daily for frequency of defecation and consistency of stools, formula intake, and body weight. Episodes of vomiting or regurgitation were recorded and any changes in clinical status were noted. Length was recorded monthly. Feeding continued until the infant left the center, an average of 137 days.

Non-statistically significant reductions in both the incidence and severity of diarrhea were observed in the group receiving probiotic supplementation, who had an average intake of about $1.2x10^8$ cfu Bb-12/day. There was no effect on growth, no tolerance issues were observed, and adverse effects such as spitting or regurgitation were observed equally frequently in both groups. The authors observed that, "These results provide some evidence that viable

Bifidobacterium lactis strain Bb-12, added to an acidified infant formula, has some protective effect against acute diarrhea in healthy children."

Bakker-Zieriksee et al. (2005) assigned 57 bottle-fed infants (n = 19 infants/group) to receive standard formula, formula with $6x10^9$ cfu *B. animalis* ssp. *lactis* Bb-12/100 ml, or formula with 600 mg galactooligosaccharides (GOS) and fructooligosaccharides (FOS)/100 ml beginning on the first day of life and continuing to age 16 weeks. Infants' food intake, formula tolerance, stool characteristics, health, and anthropometric data were obtained at postnatal days 5, 10, 28, 56, 84, and 112, and fecal samples were collected on the same days. Stool samples were subjected to bacterial enumeration, analysis of short-chain fatty acids and lactic acid, and pH measurement.

The average intake of strain Bb-12 was about $4x10^{10}$ cfu/day. Fecal counts of bifidobacteria did not differ between the groups, nor did SCFA levels. While GOS/FOS lowered fecal pH to the level seen in breast-fed infants, Bb-12 had no effect. All formulas were well tolerated and differences were not observed in adverse effects. In later analyses (Bakker-Zieriksee et al. 2006), it was found that, while prebiotics raised levels of sIgA (an immunoglobulin predominant in mucosal surfaces), the probiotic intervention had no effect on this parameter. As a result, the authors concluded that "it is possible to stimulate the development of the mucosal immune response with a prebiotic mixture of 90% GOS and 10% FOS. The probiotic strain Bb-12 was found to be less effective."

Weizman and Alsheikh (2006) evaluated the safety and tolerance of infant formula containing either of two probiotic bacteria, *B. animalis* ssp. *lactis* Bb-12 or *L. reuteri* ATCC 55730, in a prospective, randomized, double-blind, placebo-controlled trial. A total of 59 full-term healthy infants aged 3-65 days whose parents had elected not to breastfeed were randomized and 49 completed the study. By group, the number of infants beginning and completing the 4-week study were: *B. animalis* ssp. *lactis*—20 and 16; *L. reuteri*—20 and 17; control—19 and 16. The primary outcome measures were clinical adverse effects, including deviations of growth parameters and the experiment was powered to have 85% certainty of detecting at 95% confidence a 20% difference in the targeted growth parameters. Infants received physical examinations at baseline and completion of the trial. The parents completed a questionnaire daily during weeks 1 and 4, including questions on feeding, behavior, and stooling characteristics as described in Weizman et al. (2005). The probiotic bacteria were added at 10^7 cfu/g formula powder or $2.2x10^8$ cfu/180 ml prepared formula; the mean daily formula consumption was about 660 ml, and thus the estimated mean daily ingested dose of probiotic microorganisms was approximately10^9 cfu/day.

There were no withdrawals based on formula-related complaints or adverse effects in the *B. animalis* ssp. *lactis*, the *L. reuteri*, or the control group. The mean daily formula volume did not differ significantly between the groups, nor did food compliance, daily number of meals, or daily number of regurgitation and vomiting episodes. There were no significant differences in stooling scores (effort, fecal consistency, gas), number of bowel movements, crying or restlessness scores, number of severe crying episodes, number of night awakenings, or episodes of acute illness. No adverse effects were noticed throughout the study. There were no significant differences in growth parameters—weight, length, or head circumference. In summary, the probiotic bacteria, at an estimated daily ingestion level of about 10^9 cfu/day *B. animalis* ssp. *lactis* Bb-12 or *L. reuteri* ATCC 55730, "was safe, well tolerated and did not adversely affect growth, stooling habits or infant behavior."

Rautava et al. (2006) studied the effects of probiotics in reducing the risk of infectious disease in non-breast-fed infants. A total of 81 non-breast-fed infants were randomized to receive infant formula providing 0 (n = 43) or 10^{10} cfu of each of the 2 strains *B. animalis* ssp. *lactis* strain Bb-12 and *L. rhamnosus* strain GG (n = 38) to age 1 year. The infants received clinical examinations at study initiation and at 3, 7, and 12 months. Skin-prick tests at 7 and 12 months tested for allergic responses to banana, potato, carrot, apple, wheat, rice, milk, egg, cod, soybean, and gliadin. Blood was drawn at 3, 7, and 12 months for analysis of TGF-β2 and soluble CD14.

The infants receiving probiotics had non-significant reductions in the incidences of eczema and cow's milk allergy. There were no differences in IgA or TGF-β2, but sCD14 was significantly increased by probiotic treatment. The authors suggested that the findings indicate that probiotic supplementation "may promote immunologic maturation in infancy." No adverse effects due to probiotic treatment were reported.

In a prospective, randomized, double-blind, placebo-controlled study, Mohan et al. (2006, 2008) investigated the effect of *B. animalis* ssp. *lactis* Bb-12 supplementation beginning on the first day of life on the gut microbiota of preterm infants. Sixty-nine infants with a gestational age <37 weeks, but otherwise healthy, were randomized to receive placebo (n = 32) or Bb-12 (n = 37) in water at doses of 1.6×10^9 cfu/day on days 1-3 and 4.8×10^9 cfu/day from day 4 to day 21. Routine clinical data were collected on all infants and their mothers; fecal samples were collected weekly and cultured to identify and enumerate bacterial groups.

Administration of Bb-12 significantly increased fecal counts of bifidobacteria and reduced counts of *Enterobacteriaceae* and *Clostridium* spp. Gut ecology of infants receiving the probiotic was more similar to breastfed infants, including significantly increased concentrations of acetate and lactate and lowered pH. *B. animalis* ssp. *lactis* administration reduced fecal pH and improved weight gain in infants receiving antibiotic therapy. Treated infants had lower fecal calprotectin, indicating significantly lowered levels of GI inflammation, and increased IgA. Bacteria resistant to one or more antibiotic agents were identified; the presence of antibiotic-resistant bacteria was dependent on the date the infant was born and tended to occur in clusters, reflecting prevalence in the ICU. Probiotic supplementation "did not reduce the colonization by antibiotic-resistant organisms in the study population. However, the probiotic supplementation increased the cell counts of bifidobacteria and reduced the cell counts of enterobacteria and clostridia." The authors reported that no adverse effects were observed in any of the infants supplemented with Bb-12.

Gibson et al. (2009) studied the safety of supplementing infant formula with 3.85×10^8 cfu *B. animalis* ssp. *lactis* strain CNCM I-3446 /100 kcal in a single-center, randomized, double-blind, controlled, parallel-group trial. A total of 142 healthy term infants was enrolled, with n = 72 assigned to receive the test formula, which also contained LC-PUFA (DHA and AA), while n = 70 received control formula. Feeding began within the first 10 days of life and continued to the age of 7 months. The daily intake of probiotic bacteria was about 2.3×10^9 cfu. Home visits were scheduled when the infants were aged 14, 28, 42, and 91 days old, and hospital visit at ages 56, 119, 182, and 212 days. Parents recorded diet, stooling, symptoms of digestive intolerance, and behavior; infants' anthropometric measures were checked during visits and blood was drawn on days 119 and 212.

There was no difference in weight gain, length, or head circumference, or in blood biochemistries (hemoglobin, albumin, prealbumin, total protein, urea, creatinine, ferritin, calcium,

sodium, potassium, chloride, phosphate, and glucose) between the controls and the group receiving strain CNCM I-3446. No immunological effects were seen in titers of antibodies against diphtheria, *H. influenza* type b , hepatitis B, pertussis filamentous hemaglutinin, pertussis pertactin, pertussis toxin, or tetanus. A total of 64 adverse events were reported among controls and 60 in the experimental group. The only adverse events considered likely to be formula-related were 1 GI problem in each group and 1 respiratory problem in the control group. The authors concluded that feeding a formula containing LC-PUFA and *B. animalis* ssp. *lactis* strain CNCM I-3446 is safe and "promotes normal growth of term infants."

In a multi-center, prospective, randomized, placebo-controlled, double-blind trial, Dekker et al. (2009) investigated the ability of *B. animalis* ssp. *lactis* HN019, as well as *L. rhamnosus* HN001, to reduce the risk of infant eczema and atopy. A number of endpoints related to safety were assessed and these data were published separately from the report of the efficacy measures. A total of 512 pregnant women with a familial history of diagnosed asthma, hay fever, or eczema were enrolled and randomized to receive $9x10^9$ cfu HN019/day (n = 171), $6x10^9$ cfu HN001/day (n = 170), or placebo (n = 171) beginning at gestational week 35 and continuing for up to 6 months postnatally, while their infants received the same regimen from birth to 2 years of age.

The probiotics and placebo were provided in capsules. Breastfeeding mothers continued to take capsules until postnatal month 6. Infants received one capsule per day starting at birth with the contents dissolved in breast milk or water; after weaning, the babies received the contents of the capsule sprinkled on food. Each study participant received 7 nurses' visits: mother's recruitment, infant's birth, and postnatal months 3, 6, 12, 18, and 24. The nurses completed standardized questionnaires that addressed illnesses, hospitalizations, medications including antibiotics, and gastrointestinal events such as loose or watery stools, stooling patterns, reflux, vomiting, or abdominal pain. Head circumference was measured immediately after birth and at 3, 12, and 24 months; length and weight were measured after birth and at 12 and 24 months.

At the end of the two-year period, there were 152 infants in the HN019 group, 144 in the HN001 group, and 150 in the placebo group. There were no statistically-significant differences between the treatment groups in incidence of or reasons for study withdrawal, incidence of adverse events, or antibiotic use over the 2-year feeding period, nor were any effects observed on height, weight, or head circumference. The authors concluded that *B. animalis* ssp. *lactis* HN019 and *L. rhamnosus* HN001 are "safe and well-tolerated when given to infants from birth." In a companion publication (Wickens et al. 2008), it was reported that "supplementation with *L. rhamnosus*, but not *B. animalis* ssp. *lactis*, substantially reduced the cumulative prevalence of eczema, but not atopy, by 2 years."

Vlieger et al. (2009) studied the tolerance and safety of infant formula with added pre- and probiotics. A total of 133 pregnant women and their healthy term infants was enrolled; the infants were given formula containing 240 mg galactooligosaccharides/100 ml and either 0 (n =64) or 10^7 cfu each of *B. animalis* ssp. *lactis* strain Bb-12 and *L. paracasei* ssp. *paracasei* strain CRL-431 (n = 69)/100 ml for 6 months. Solid foods were introduced at 4 months. Parents used a diary to record crying, stooling, use of antibiotics, respiratory or GI infections, and other adverse events such as vomiting, diarrhea, constipation, colic, or eczema. Anthropometric measures were taken monthly and fecal samples were taken at 1, 2, 3, and 6 months for analyses of bacteria.

Intake of each of the 2 strains of bacteria was about $7x10^7$ cfu/day. All infants grew normally and there were no differences in weight, length, or head circumference at 3 or 6 months. The probiotic group had significantly fewer rashes and more frequent and softer stools in the first trimester, but not in the second. No differences were seen in GI or upper respiratory infections or in adverse events. The authors concluded that this safety study demonstrates that the addition of the 2 probiotics to infant formula "is safe, well tolerated, does not adversely affect growth and infant behavior in the short term and can result in softer and more frequent stools."

In a prospective, randomized, double-blind, placebo-controlled study, Taipale et al. (2010) investigated the effect of administration of *B. animalis* ssp. *lactis* strain Bb-12 to healthy term infants on the risk of acute infection. Thirty-eight 1-month-old infants received two doses/day of xylitol powder with probiotic totaling 10^{10} cfu by means of either a slow-release pacifier or a spoon while 37 control infants received a placebo powder (xylitol alone) by the same routes. The infants received physical examinations at enrollment and at 8 months of age; at that time infectious and non-infectious diseases in the infant were reported as well as diseases such as asthma, allergies, atopic dermatitis, diabetes, rheumatoid diseases, and GI disorders in relatives. The 8-month visit included assessment of protocol compliance, use of complementary foods, symptoms of colic, and any adverse effects as well as collection of stool samples.

Four infants from the probiotic group and 2 from the control group failed to complete the study, none for reasons apparently related to xylitol or *B. animalis* ssp. *lactis*. Ingestion of Bb-12 statistically significantly reduced the incidence of respiratory infections reported by the parents, although no difference was seen in acute otitis media (which occurred with low frequency in both groups). The groups did not differ in the incidence of gastrointestinal infections. No serious adverse effects were observed during the study.

In one of the few studies in infants having probiotic safety as the primary endpoint, Allen et al. (2010) enrolled pregnant women and their infants in a prospective, randomized, double-blind, placebo-controlled study of a probiotic supplement in the prevention of atopy. The test article was a 10^{10}-cfu/day blend of *B. animalis* ssp. *lactis* CUL34, *B. bifidum* CUL20, *L. salivarius* CUL61, and *L. paracasei* CUL08. A total of 454 women in their last month of pregnancy were enrolled, 413 carrying a fetus at increased risk of atopy based on family history; 220 women were randomly assigned to receive a daily capsule composed of hydroxypropyl methylcellulose with the probiotic while 234 women received a placebo capsule containing maltodextrin. Mothers took the capsules daily until gestation, after which the infant received the capsule contents mixed with formula or expressed breast milk to the age of 6 months. In addition to spontaeous reporting, parents were questioned about adverse events at weeks 6, 12, 18, and 24 postpartum.

Thirty-six mothers—15 in the probiotic group and 21 in the control group—reported a total of 44 adverse effects on themselves, 18 classified as by the attending physicians as serious adverse events. These included pregnancy-induced hypertension and complications of Caesarean section. Neither the total number nor the individual adverse events differed significantly between the test and control groups. There were 2 intrauterine deaths, both in the placebo group. None of the adverse events were attributed to the interventions and no lactobacilli or bifidobacteria infections were identified.

Birthweights of infants in the 2 groups did not differ, and there were no differences in growth (weight, length, and head circumference) up to 6 months of age. The frequency of

gastrointestinal symptoms did not differ between the 2 groups during the first 8 weeks with the exception of constipation, which was significantly more frequent among infants receiving placebo. Over 6 months, adverse effects were reported in 73 infants receiving probiotics and 75 receiving placebo. One infant receiving placebo died. Two types of adverse events occurred with significantly greater frequency in the probiotic group than in the placebo group. First were unspecified acute lower respiratory infections (11 test infants and 3 control infants); 5 of these (4 among the test infants and 1 control infant) required hospitalization and were therefore classified as serious adverse events. Infectious agents were not identified in any of these infants and the causes were thought to be viral infections. The other adverse effects seen more often in the treatment group were vomiting or diarrhea, experienced by 5 infants receiving probiotic but none receiving placebo. On the other hand, 6 cases of neonatal jaundice were observed, all in the placebo group, and all 6 infants who had serious adverse events involving the gastrointestinal system were in the placebo group. No infections involving lactobacilli or bifidobacteria were identified. The authors concluded that, "There was no evidence that these strains were associated with adverse effects in these women or their infants."

4.5.2.3.1. Conclusions from Studies of B. animalis *ssp.* lactis *in Infants*

Three different strains of *B. animalis* ssp. *lactis* have been added to infant formula and given to both term and preterm infants in 14 prospective, double-blind, parallel-group trials with no indication of any adverse effects (Table 9). This record of safety in a sensitive population confirms the abundant evidence from studies in children and adults and from the history of the subspecies as a probiotic supplement in widespread use that ingestion of doses as high as $6x10^{11}$ cfu *B. animalis* ssp. *lactis*/day (equivalent to $7.5x10^{10}$ cfu/kg bw/day) or higher is safe.

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Table 9. Studies of *B. animalis ssp. lactis* in Infants.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Allen et al. (2010)	Assess the safety of a blend of *B. animalis* ssp. *lactis* CUL34, *B. bifidum* CUL20, *L. salivarius* CUL61, and *L. paracasei* CUL08	Prospective, randomized, double-blind, placebo-controlled study	454 women in their last month of pregnancy and their infants	10^{10} cfu/day of *B. animalis* ssp. *lactis* CUL34, *B. bifidum* CUL20, *L. salivarius* CUL61, and *L. paracasei* CUL08	pregnant women—1 month; infants—6 months	15 women in the probiotic group and 21 in the control group reported 44 adverse effects, 18 classified as serious adverse events. Neither the total number nor the individual adverse events differed significantly between the test and control groups. None of the adverse events were attributed to the interventions and no lactobacilli or bifidobacteria infections were identified. Birthweights of infants in the 2 groups did not differ, and there were no differences in growth. The frequency of GI symptoms did not differ between the 2 groups during the first 8 weeks with the exception of constipation, significantly more frequent among infants receiving placebo. Over 6 months, adverse effects were reported in 73 infants receiving probiotics and 75 receiving placebo. Adverse events occurring with significantly greater frequency in the probiotic group than in the placebo group were unspecified acute lower respiratory infections and vomiting or diarrhea. 6 cases of neonatal jaundice were observed, all in the placebo group, and all 6 infants who had serious adverse events involving the gastrointestinal system were in the placebo group. No infections involving lactobacilli or bifidobacteria were identified. The authors concluded that, "There was no evidence that these strains were associated with adverse effects in these women or their infants."

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Table 9. Studies of *B. animalis ssp. lactis* in Infants.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Bakker-Zierikzee et al. (2005, 2006)	Investigate the effects of pro- and prebiotics on the intestinal microbiota.	Prospective, randomized, placebo-controlled, double-blind trial	120 pregnant women and their healthy term infants	$6x10^9$ cfu Bb-12/100 ml in formula; intake = $4x10^{10}$ cfu Bb-12/day	16 weeks	Fecal counts of bifidobacteria did not differ between the groups, nor did SCFA levels. While GOS/FOS lowered fecal pH to the level seen in breast-fed infants, Bb-12 had no effect. All formulas were well tolerated and differences were no observed in adverse effects. While prebiotics raised levels of SIgA (an immunoglobulin predominant in mucosal surfaces), the probiotic intervention had no effect.
Chouraqui et al. (2004)	Investigate the effect of *B. animalis* ssp. *lactis* Bb-12 on the incidence of acute infectious diarrhea in infants.	Multicenter, double-blind, placebo-controlled study	90 healthy infants (average age 3.7 months) living in residential nurseries	$1.5x10^7$ cfu Bb-12/100 ml milk-based formula; daily intake = $1.2x10^9$ cfu Bb-12/day	average = 137 days	Non-statistically significant reduction in incidence and severity of diarrhea; no effect on growth, no tolerance issues, and no adverse effects.

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Table 9. Studies of *B. animalis ssp. lactis* in Infants.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Dekker et al. (2009) and Wickens et al. (2008)	Investigate the ability of *B. animalis* ssp. *lactis* HN019 or *L. rhamnosus* HN001, to reduce the risk of infant eczema and atopy	Multi-center, prospective, randomized, placebo-controlled, double-blind trial	512 pregnant women with a familial history of diagnosed asthma, hay fever, or eczema and their infants	$9x10^9$ cfu HN019/day	Mothers, from gestational week 35 to post-natal month 6; infants from birth to age 2 years	No statistically significant differences between the groups in reasons for study withdrawal, incidence of adverse events, or antibiotic use over the 2-year feeding period, nor were any effects observed on height, weight, or head circumference. The authors concluded that *B. animalis* ssp. *lactis* HN019 and *L. rhamnosus* HN001 are safe and well-tolerated in an infant population fed from 0 to 24 months of age and have no effect on normal growth.
Gibson et al. (2009)	Evaluate the bioequivalence of formula with or without LC-PUFA and probiotic based on weight gain, immune response, and biochemistry	Single-center, randomized, double-blind, placebo-controlled, parallel-group	142 healthy term infants; test n=72; control n=70; at follow-up test n=62; control n=62	$3.85x10^8$ cfu CNCMI-3446/100 kcal; about $2.3x10^9$ cfu CNCMI-3446/day	7 months	There was no difference in weight gain or in blood biochemistries. No immunological effects were seen in titers of antibodies against diphtheria, *H. influenza* type b , hepatitis B, pertussis filamentous hemaglutinin, pertussis pertactin, pertussis toxin, or tetanus. The only adverse events considered likely to be formula-related were 1 GI problem in each group and 1 respiratory problem in the control group.
Isolauri et al. (2000)	Assess the ability of *B. animalis* ssp. *lactis* strain Bb-12 or LGG to control allergic inflammation in infants with atopic eczema	Prospective, randomized, double-blind, placebo-controlled study	27 infants with a mean age of 4.6 months who had developed atopic eczema during breast feeding and had not been exposed to infant formula	10^9 cfu Bb-12/g formula, approximately equivalent to $8x10^{10}$ cfu Bb-12/day	6 months or longer	SCORAD, soluble CD4 in blood, and eosinophilic protein X in urine were significantly reduced in the probiotic groups. There was no effect on growth, which was normal in all infants in all groups. No adverse events were reported.

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Table 9. Studies of *B. animalis ssp. lactis* in Infants.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Kankaanpää et al. (2002)	Investigate the effect of *B. animalis* ssp. *lactis* strain Bb-12 and LGG on the composition of plasma lipids in atopic infants	Pilot for a randomized, double-blind, placebo-controlled study	15 breastfed infants (mean age = 5.2 months) referred to a pediatric clinic for treatment of atopic eczema	10^9 cfu Bb-12/g formula, approximately equivalent to $6x10^{11}$ cfu Bb-12/day or $7.5x10^{10}$ cfu Bb-12/kg bw/day	average 5.5 months	*B. animalis* ssp. *lactis* strain Bb-12 had no effect on phospholipids. The authors noted that all formulas were well tolerated by the infants, and no adverse effects were reported.
Kirjavainen et al. (2002)	Investigate the ability of Bb-12 to modulate the intestinal biome associated with atopia.	Randomized, double-blind, placebo-controlled study.	13 infants (mean age = 5.2 months) with early onset atopic eczema and failure to thrive but able to tolerate extensively hydrolyzed whey formula	10^9 cfu Bb-12/g powder, providing $8x10^{10}$ cfu Bb-12/kg bw/day	4 months	Bb-12 had no reported adverse effects but resulted in a significant decrease in the counts of *E. coli* and bacteroides, which were associated with the severity of atopic sensitization.
Mohan et al. (2006, 2008)	Study the effect of probiotics on the gut micro-biota in preterm infants.	Randomized, double-blind, placebo-controlled study.	69 preterm infants; test n=37; control n=32	$1.6x10^9$cfu Bb-12/day on days 1-3 and $4.8x10^9$ cfu Bb-12/day from day 4 onward	21 days	Administration of Bb-12 significantly increased fecal counts of bifidobacteria and reduced counts of *Enterobacteriaceae* and *Clostridium* spp. Gut ecology was more similar to breastfed infants. *B. lactis* administration reduced fecal pH and improved weight gain in infants receiving anti-biotic therapy. Treated infants had lower fecal calprotectin, indicating significantly lowered levels of GI inflammation, and increased IgA.

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Table 9. Studies of *B. animalis ssp. lactis* in Infants.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Rautava et al. (2006)**	Study the effects of probiotics in reducing the risk of infectious disease in non-breast-fed infants.	Randomized, double-blind, placebo-controlled study.	81 non-breast-fed infants; test n=38; control n=43; at 12 months, test n=32; control n=40	10^{10} cfu Bb-12/day	To age 1 year	The infants receiving probiotics had non-significant reductions in the incidences of eczema and cow's milk allergy. There were no differences in IgA or TGF-β2, but sCD14 was significantly increased by probiotic treatment. The authors suggested that probiotic supplementation may promote immunologic maturation in infancy.
Saavedra et al. (2004)	Studied tolerance and safety of long-term intake of infant formula containing 10^6-10^7 cfu/g of *B. animalis* ssp. *lactis* Bb-12 or *Streptococcus thermophilus*	Randomized, double-blind, placebo-controlled study.	58 healthy male and 60 female infants aged 3-24 months (mean age = 6.7 months)	9.7×10^7 cfu Bb-12/kg bw/ day in the low-dose group and 1.3×10^9 cfu Bb-12 /kg bw/day in the high-dose group	30 weeks average	There were no differences in number of or reasons for withdrawals, in formula consumption, in tolerance issues, or in gains in weight and length. The frequency of colic or irritability and the use of antibiotics was significantly lower in the Bb-12 group than in the placebo group. The authors concluded that, "Probiotic supplementation resulted in adequate growth. The supplemented formulas were well accepted and tolerated and resulted in a significantly lower reported frequency of colic or irritability. Supplementation also resulted in a trend toward less frequent seeking of health care attention and a significantly lower frequency of antibiotic use."
Taipale et al. (2010)	Study the effect of strain Bb-12 on the risk of acute infection in healthy term infants	Prospective, randomized, double-blind, placebo-controlled study	75 healthy term infants aged 1 month	10^{10} cfu Bb-12/day in 2 doses	7 months	Ingestion of Bb-12 significantly reduced the incidence of respiratory infections. The groups did not differ in the incidence of gastrointestinal infections. No serious adverse effects were observed during the study.

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Table 9. Studies of *B. animalis ssp. lactis* in Infants.

Reference	Objective	Study Design	Subjects	*B. animalis* ssp. *lactis* Strain and Dose	Duration	Safety-Related Results
Vlieger et al. (2009)	Assess the tolerance and safety of infant formula with added pre- and probiotics	Randomized, double-blind study.	133 pregnant women and their healthy term infants; test n=69; control n=64; at 3 and 6 months the test n=53/41 and the control n=47/38	$7x10^7$ cfu Bb-12/day	6 months	All infants grew normally and there were no differences in weight, length, or head circumference at 3 or 6 months. The probiotic group had fewer rashes and more frequent and softer stools in the first trimester, but not in the second. No differences were seen in GI or upper respiratory infections or in adverse events.
Weizman and Alsheikh (2006)	Determine the safety and tolerance of infant formula containing probiotic bacteria (*L. reuteri* ATCC 55730 or *B. animalis* ssp. *lactis* Bb-12)	Prospective, randomized, double-blind, placebo-controlled study.	59 full-term healthy infants age 3-65 days; 49 completed the study. Ns were: *B. animalis* ssp. *lactis*, 20/16 *L. reuteri*, 20/17 Control, 9/16	$2.2x10^8$ cfu Bb-12/180 ml prepared formula; 10^9 cfu Bb-12/day	4 weeks	There were no withdrawals based on formula-related complaints or adverse effects. The mean daily formula volume did not differ significantly between the groups, nor did food compliance, daily number of meals, or daily number of regurgitation and vomiting episodes. There were no significant differences in stooling scores (effort, fecal consistency, gas), number of bowel movements, crying or restlessness scores, number of severe crying episodes, number of night awakenings, or episodes of acute illness. No adverse effects were noticed throughout the study. There were no significant differences in growth parameters—weight, length, or head circumference. In summary, the probiotic bacteria did not affect either the health nor the daily habits of the infants.

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4.6. Review Articles Regarding the Safety of *B. animalis* ssp. *lactis*

A Cochran Collaboration review by Johnston et al. (2007) focused on the use of probiotics for the prevention of antibiotic-associated diarrhea. Ten studies met the inclusion criteria; they included 1986 participants of whom 1015 received probiotic treatment with *Bifidobacterium* spp., *Lactobacillus* spp., *Streptococcus* spp., or *Saccharomyces boulardii*. Five of the 10 trials (including 647 patients) included specific monitoring for adverse events; of these, 3 reported that no adverse events had occurred and 2 reported that observed adverse events were equally prevalent among test and control groups and were not attributable to the probiotic intervention. The authors concluded that, "Probiotics were generally well tolerated and side effects occurred infrequently."

4.7. Safety Evaluations of *B. animalis* ssp. *lactis* by Authoritative Bodies

As noted previously, *B. animalis* ssp. *lactis* strain Bf-6 is deposited in the Belgian Coordinated Collections of Microorganisms (BCCM) under accession number LMG 24384. BCCM has placed the strain into Biohazard group 1, the least restrictive classification, consisting of "organisms that are unlikely to cause human disease. Should be handled under containment level 1. Can be distributed unrestrictedly to any bona fide teaching, research or industrial institution" (BCCM 2010).

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, 2007b). 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 *B. animalis*. In listing *B. animalis* and other species of *Bifidobacterium* as suitable for QPS status, the Committee stated, "Where QPS status is proposed, the Scientific Committee is satisfied that the body of knowledge available is sufficient to provide adequate assurance that any potential to produce adverse effects in humans, livestock or the wider environment is understood and capable of exclusion" (EFSA 2007a, p8) and that the recommendations are "based on a thorough review of the available scientific literature and the knowledge and experience of the scientists involved" (EFSA 2007a, p8).

In December 2008, EFSA's Panel on Biological Hazards released an opinion reassessing the QPS status of bacterial strains. No need for either review or change of the QPS status of *Bifidobacterium animalis* was reported.

In 2002, FDA, responding to a GRAS notice submitted by Nestle USA regarding the use of *B. animalis* ssp. *lactis* strain Bb-12 and *Streptococcus thermophilus* strain Th4 as ingredients

in milk-based infant formula intended for consumption by infants four months and older at levels not to exceed good manufacturing practice, stated that the agency had no questions at that time (FDA 2002). The GRAS use of strain Bb-12 was based on an estimated concentration of 10^6 to 10^8 cfu/g reconstituted formula. Assuming consumption of 800 g formula/day, the exposure of infants to *B. animalis* ssp. *lactis* strain Bb-12 would be as high as 10^{11} cfu/day.

At the time of the GRAS notice and the FDA response, neither Nestle nor the agency was aware that strain Bb-12 harbors the *tet*(W) gene encoding for resistance to tetracycline. Nestle convened an expert panel which concluded, based on consideration of the distribution of *tet*(W) in food and microbes, the potential for gene transfer, antibiotic susceptibility, and clinical consequences of exposure to the *tet*(W) gene, that the presence of the gene in *B. animalis* ssp. *lactis* has no impact on the safety of the bacterium for its intended use. The agency accepted this determination with no questions (FDA 2005).

5. Safety Assessment and GRAS Determination

5.1. Introduction

This section presents an assessment that demonstrates that the addition of *Bifidobacterium animalis* ssp. *lactis* strain Bf-6 to conventional foods as probiotic bacteria under the conditions of use described is safe and is GRAS based on scientific procedures.

This safety assessment and GRAS determination entail two steps. In the first step, the safety of the intended use of *B. animalis* ssp. *lactis* Bf-6 is demonstrated. Safety is established by demonstrating a reasonable certainty that the exposure of adults, children, and infants to *B. animalis* ssp. *lactis* Bf-6 under its intended conditions of use is not harmful. In the second step, the intended use of *B. animalis* ssp. *lactis* Bf-6 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 *B. animalis* ssp. *lactis* Bf-6 to foods is safe and is GRAS.

5.2. Safety Evaluation

The body of evidence supporting the safety of oral administration of *Bifidobacterium* strains in general, and *B. animalis* ssp. *lactis* strains in particular, is large and convincing. Numerous commentators—in addition to authoritative bodies such as EFSA and FDA—have noted the safe history of human ingestion of bifidobacteria over many hundreds of years. Bifidobacteria produce no deleterious metabolites and are 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, this phenomenon has not been observed and, when bifidobacteria 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.

Bifidobacteria are not regarded as pathogens and, despite the widespread use of bifidobacteria as probiotics, there has never been one documented case of bacteremia attributed to an ingested probiotic strain of bifidobacteria. Further, the genomic analysis of *B. animalis* ssp. *lactis* Bf-6 confirms that it contains no genes linked to virulence, toxicity, hemolysis, or infectivity.

The safe history of human exposure to bifidobacteria strains is strongly supported by an extremely large body of published research. Because of the high degree of genetic similarity among strains of *B. animalis* ssp. *lactis*, it is appropriate to pool the findings of research studies conducted with different strains in evaluating the safety of strain Bf-6. In addition to *in vitro* work, the published literature regarding the subspecies *B. animalis* ssp. *lactis* alone includes 17 experimental studies in both normal and immunodeficient mice, rats, and piglets as well as 34 separate studies in humans: 15 in adults, 5 in children, and 14 in infants. These studies have included about 335 adults, 533 children, and 1379 infants, a total of well over 2200 individuals. No adverse effects were seen in either animal or human studies.

The final issue in evaluating the safety of the intended probiotic use of *B. animalis* ssp. *lactis* Bf-6 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 ingestion of LAB. Antibiotic susceptibility studies by 2 bodies, including the EU's PROSAFE, both reached the conclusion that *B. animalis* ssp. *lactis* strain Bf-6 exhibits no antibiotic resistances that appear to be acquired and thus likely to be transferable. While it does harbor the *tet*(W) gene, as do all strains of *B. animalis* ssp. *lactis* that have been subjected to genomic analysis, the gene is not associated with mobile elements and is likely not transferable. It is noteworthy that bifidobacteria that possess *tet*(W) have never been observed to exhibit a conjugation capability for this gene.

All of the available evidence demonstrates clearly that there is no reason to suspect harm to individuals consuming conventional foods supplemented with *B. animalis* ssp. *lactis* Bf-6.

5.3. General Recognition of the Safety of *B. animalis* ssp. *lactis* Strain Bf-6

The intended use of *B. animalis* ssp. *lactis* Bf-6, to be added as a probiotic to a variety of conventional foods, has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b). This safety was shown by establishing the identity and probiotic characteristics of the strain, demonstrating its freedom from pathogenic or other risk factors, and concluding that the expected exposure to *B. animalis* ssp. *lactis* Bf-6 by adults and children is without significant risk of harm. Finally, because this safety assessment satisfies the common knowledge requirement of a GRAS determination, this intended use can be considered GRAS.

Determination of the safety and GRAS status of the addition of *B. animalis* ssp. *lactis* Bf-6 to food has been made through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., and Michael W. Pariza, Ph.D., who reviewed a monograph prepared by JHeimbach LLC as well as other information available to them. These individuals are qualified by scientific training and experience to evaluate the safety of food and

food ingredients, including probiotic bacteria. They independently critically reviewed and evaluated the publicly available information and the potential human exposure to *B. animalis* ssp. *lactis* Bf-6 anticipated to result from its intended uses, and individually and collectively determined that no evidence exists in the available information on *B. animalis* ssp. *lactis* Bf-6 or other *B. animalis* ssp. *lactis* strains, that demonstrates, or suggests reasonable grounds to suspect, a hazard to either adults or children under the intended conditions of use of *B. animalis* ssp. *lactis* Bf-6. 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 *Bifidobacterium animalis* ssp. *lactis* Bf-6 is GRAS by scientific procedures.

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CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF *BIFIDOBACTERIUM ANIMALIS* SSP. *LACTIS* STRAIN BF-6 IN CONVENTIONAL FOODS

We, the members of the expert panel, have individually and collectively critically evaluated the publicly available information on *Bifidobacterium animalis* ssp. *lactis* strain Bf-6 and other *B. animalis* ssp. *lactis* strains summarized in a monograph prepared by JHEIMBACH LLC, as well as other material deemed appropriate or necessary. Our evaluation included review of the identity and characteristic properties of *B. animalis* ssp. *lactis*, including *B. animalis* ssp. *lactis* strain Bf-6, the potential exposure resulting from the intended use of *B. animalis* ssp. *lactis* strain Bf-6, and published research bearing on the safety of *B. animalis* ssp. *lactis* and strain Bf-6. Our summary and conclusion resulting from this critical evaluation are presented below.

Summary

- The probiotic bacterium that is the subject of this generally recognized as safe (GRAS) determination is *Bifidobacterium animalis* ssp. *lactis* strain Bf-6, a Gram-positive, non-spore-forming, catalase-negative, strictly fermentative, and nonpathogenic bacterium

- *B. animalis* ssp. *lactis* strain Bf-6 was isolated from the feces of a healthy human and was deposited with the Belgian Coordinated Collections of Microorganisms/Laboratory for Microbiology of the Faculty of Sciences of the Ghent University (BCCM/LMG) with LMG accession number LMG 24384. It is also maintained in the PROSAFE strain collection under the number PRSF-B112.

- *B. animalis* ssp. *lactis* strain Bf-6 is intended to be added as a probiotic to a variety of foods that can sustain viable *B. animalis* ssp. *lactis* for the shelf life of the food. These foods include, but are not limited to, such dairy foods as fluid milks, yogurt, milk-based desserts and gravies, and cheeses; dry seeds, nuts, and nut butters; grain products such as flour, yeast breads, quick breads, cakes, cookies, pies, pastries, crackers, pancakes, waffles, French toast, and crepes; pasta, cooked and rte cereals, grain mixtures, and meat substitutes; fruits and fruit beverages; dark-green vegetables, olives, pickles, relishes, and vegetable soups; salad dressings; sugars and sugar substitutes, syrups, honey, molasses, jellies, jams preserves, gelatin desserts, ices and popsicles, candies, and chewing gum; and carbonated soft drinks, sports drinks and thirst quenchers, energy drinks, and water.

- The maximum intended use level (i.e., the initial addition level, intended to assure that the target level of 10^9 cfu/serving of viable *B. animalis* ssp. *lactis* remain throughout the shelf life) is 10^{11} cfu/serving. The strain is expected to be present in a limited number of foods, usually at less than 10^{10} cfu/serving. It will not proliferate in the foods and beverages to which it is added, but instead will decline over the shelf-life of the food. Its likely maximum ingestion is thus less than 10^{11} cfu/day, well within levels that have been shown to be safe.

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- *Bifidobacterium* is a non-pathogenic genus ubiquitous in the human colon and vagina, where it metabolizes glucose exclusively by the fructose-6-phosphate pathway, producing lactic and acetic acids. *B. animalis* ssp. *lactis* is a subspecies with remarkably little phenotypic or genetic diversity; published genomes of several strains are over 99.9% identical and can be distinguished only by nonsynonymous single-nucleotide polymorphisms.

- The whole genome shotgun sequence of *B. animalis* ssp. *lactis strain* Bf-6 was determined and the resulting sequence was annotated and analysed for genes that may be possible safety concerns. DNA sequencing resulted in 221,208 reads giving 94,775,473 sequenced base pairs. Assembly of these data produced 24 contigs of 1,916,102 bases

- The *tet*(W) gene was identified, as expected. Analysis of matches to published genomes showed 100% homology to ORF RBIC00158 from *B. animalis* ssp. *lactis* HN019. Partial matches to several other antibiotic resistance genes were also found; these again are fully identical to open reading frames and the neighborhood (300 base pairs up- and downstream) in all published *B. animalis* ssp. *lactis* genomes. These hypothetical resistances, to daunorubicin, methicillin, mycinamicin, polymyxin, 5-nitroimidazole, phosphinothricin, and penicillin, may well not be functional. Although *B. animalis* ssp. *lactis* Bf-6 exhibits resistance to 9 antibiotic agents, none of these resistances lies outside the normal range of the resistance distribution of the wild-type population of *B. animalis* ssp. *lactis*, and they are regarded as innate rather than acquired.

- The genome was searched for genes that might encode the production of decarboxylases that may produce biogenic amines. One gene was found that may encode production of lysine decarboxylase, which may form cadaverine. This is identified by NCBI as a hypothetical function that has not been proven.

- The genomic data confirm phenotypic and history-of-use information that *B. animalis* ssp. *lactis* strains are nonpathogenic and exhibit no adverse metabolic effects. The genome of strain Bf-6 was specifically examined for genes encoding factors putatively associated with virulence, including enterotoxins, outer-membrane or cytoskeleton-distending proteins, hemolysins, enterohemolysins, cell invasion and modulation proteins, endotoxins, cell-surface proteins, exotoxins, and inclusion proteins. No such genes were found. "Virulence factor MviN" is present in the genome, but since this is a widely distributed gene found in both pathogenic and nonpathogenic bacteria, its relevance to virulence has been questioned (Wassenaar and Gaastra 2001).

- Production of *B. animalis* ssp. *lactis* strain Bf-6 is based on standard fermentation techniques, and all fermentation medium components are food-grade materials. Cargill tests each batch of *B. animalis* ssp. *lactis* strain Bf-6 for microbiological purity. Testing has shown the ability of the strain to withstand the adverse conditions of product manufacture and storage and to maintain viability and stability during product shelf life.

- *B. animalis* ssp. *lactis* strains were administered orally to mice, rats, and piglets in 17 studies at doses up to 10^{11} cfu/day. Translocation was observed in one study with congenitally immunodeficient gnotobiotic athymic mice, but no associated adverse effects were seen. No adverse effects were seen in standard measures of immune, biochemical, or hematological parameters and no retardation of normal growth was reported in any study.

- The safety of *B. animalis* ssp. *lactis* was also assessed in 15 studies with human adults, 5 with children, and 14 with infants. Maximum doses were $3x10^{11}$ cfu/day in adults, $2x10^{10}$ cfu/day in children, and $6x10^{11}$ cfu in infants. Study durations were as long as 4 months in adults, 6 months in children, and 24 months in infants. No treatment-related adverse effects were observed in any of these studies.

- In 2007, the European Food Safety Authority (EFSA) classified *Bifidobacterium* as an organism having a Qualified Presumption of Safety (QPS) and thus being "freed from the need for further safety assessment." This status was reconfirmed in 2008.

- In 2002, FDA accepted with no questions a GRAS notice submitted by Nestle USA for the use of *B. animalis* ssp. *lactis* strain Bb-12 at levels not to exceed good manufacturing practice in milk-based infant formula intended for consumption by infants four months and older, with estimated exposure of 10^{11} cfu/day. Three years later FDA accepted with no questions the report of an Expert Panel convened by Nestle that, based on consideration of the distribution of *tet*(W) in food and microbes, the potential for gene transfer, antibiotic susceptibility, and clinical consequences of exposure to the *tet*(W) gene, concluded that the presence of the gene in *B. animalis* ssp. *lactis* has no impact on the safety of the bacterium for its intended use.

Conclusion

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

We recognize that *Bifidobacterium* species have a long history of safe use and are appropriately regarded as non-pathogenic and non-toxicogenic. *B. animalis* ssp. *lactis* strain Bf-6 has been adequately identified and characterized and that both phenotypic and genotypic research confirm that no concerns exist regarding the safety of ingestion of this probiotic bacterium at levels up to 10^{11} cfu/day. Therefore, we conclude that the addition of *B. animalis* ssp. *lactis* strain Bf-6 to conventional foods as described is safe.

We further conclude that the intended use of *B. animalis* ssp. *lactis* strain Bf-6 in conventional foods as described is generally recognized as safe (GRAS) based on scientific procedures and corroborated the long history of safe use of *Bifidobacterium* species.

It is our opinion that other qualified and competent scientists reviewing the same publicly available information would concur with these conclusions.

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SUBMISSION END