

GRAS Notice (GRN) No. 445

<http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/GRASListings/default.htm>

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ORIGINAL SUBMISSION

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September 21, 2012

Dr. Paulette Gaynor
Office of Food Additive Safety, GRAS Notification Program (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notice-Exemption Claim for *Bifidobacterium animalis* ssp. *lactis*
isolates, HN019, Bi-07, BI-04 and B420

Dear Dr Gaynor:

On behalf of my client, Danisco USA, Inc., please accept the attached documentation, in compliance with the GRAS notification procedure set out in the April 17, 1997 Federal Register (62 FR 18937), as submission of notice of a GRAS exemption claim for the above referenced substance, i.e. use in food of *Bifidobacterium animalis* ssp. *lactis* isolates, HN019, Bi-07, BI-04 and B420. As specified in the aforementioned proposed rule, this GRAS notice is submitted in triplicate with each containing: a GRAS notice exemption claim; detailed information on the notified substance; and an appendix containing further referenced and substantiating information on the substance.

Please promptly contact me should you have any question regarding the submitted notice. I look forward to receiving acknowledgment of receipt of this notice and to a timely response regarding the noticed substance. Thank you.

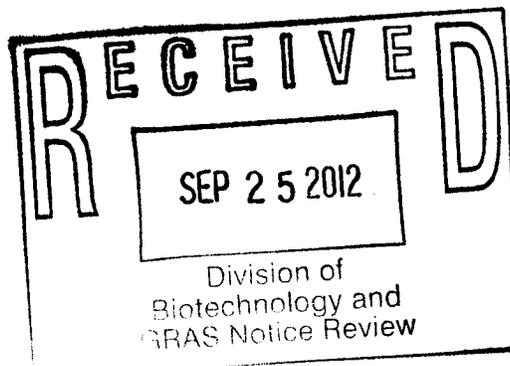
Sincerely,


Robert H. Sindt

Enc.

Cc : Sarah Kraak-Ripple, Danisco USA, Inc.

RHS/bs

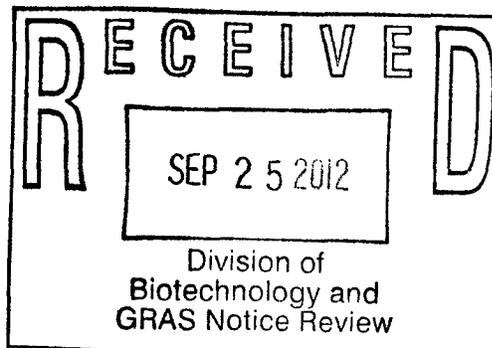


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**GENERALLY RECOGNIZED
AS SAFE NOTICE**

***Bifidobacterium animalis* ssp. *lactis* isolates,
HN019, Bi-07, Bl-04, and B420**

September 2012



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September 19, 2012

Dr. Paulette Gaynor
GRAS Notification Program
Office of Food Additive Safety
Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740

Re: GRAS Notice-Exemption Claim for isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HNO19, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420

Dear Dr. Gaynor:

On behalf of my client, Danisco USA, Inc. (Danisco), and in accordance with FDA's proposed rule of April 17, 1997 (62 FR 18938) relating to the filing of generally recognized as safe (GRAS) notices, please accept this claim and the attached information, submitted in triplicate, for that purpose as it relates to the use of isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HNO19, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420 in certain foods. Specifically, Danisco claims that use of isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HNO19, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420 as ingredients in foods, including ready-to-eat breakfast cereals, bars, cheese, milk drinks and milk products, bottled water and teas, fruit juices, fruit nectars, fruit ades and fruit drinks, chewing gum and confections (as specified in the detailed information submitted herewith) are exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act based on its determination that such uses are GRAS. In conformity with the requirements outlined in the proposed rule, the following information is included with this exemption claim:

- (i) Name and Address of the Notifier:
Danisco USA, Inc.
3329 Agricultural Drive
Madison, WI 53716
- (ii) Common or Usual Name of Notified Substance: isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HNO19, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420

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- (iii) **Applicable Conditions of Use:** Isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HNO19, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420 are manufactured in compliance with current Good Manufacturing Practice as specified in 21 CFR Part 110. Isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HNO19, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420 are manufactured through a specific time and temperature controlled fermentation of suitable food grade ingredients with *Bifidobacterium animalis* ssp. *lactis*. The isolates are used as ingredients in foods, including ready-to-eat breakfast cereals, bars, cheese, milk drinks and milk products, bottled water and teas, fruit juices, fruit nectars, fruit ades and fruit drinks, chewing gum and confections, at levels not to exceed current good manufacturing practice in accordance with 21 CFR 184.1(b). The targeted use level of foods will be to typically contain 5×10^9 cfu/serving of *Bifidobacterium animalis* ssp. *lactis* at consumption. All population age groups, except infants, are expected to consume these foods.
- (iv) **Basis for the GRAS Determination:** Scientific procedures, supported by a history of common use in foods.
- (v) **Availability to FDA of Data and Information that are Basis of Determination:** The data and information forming the basis for Danisco's GRAS determination and the exemption claim asserted herein are available for FDA review and copying during reasonable business hours at the following address, or will be sent to FDA upon request:

Robert H. Sindt, Attorney at Law
Suite 110G
1025 Thomas Jefferson Street, NW
Washington, DC 20007
Phone: (202) 466-4500
rsindt@bobsindtlaw.com

Consequently, on the basis of the above specified information, and the additional requested information as specified in the proposed rule and as attached hereto and submitted with this letter, please accept this as Danisco's GRAS notification and claim of exemption from the statutory premarket approval requirements for the use of isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HNO19, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420 as ingredients in foods, including ready-to-eat breakfast cereals, bars, cheese, milk drinks and milk products, bottled water and teas, fruit juices, fruit nectars, fruit ades and fruit drinks, chewing gum and confections.

Should you have any questions regarding the submission of this notice, please contact me at the above number. Thank you for your prompt consideration of, and response to, this notice.

Dr. Paulette Gaynor, OFAS-FDA
September 19, 2012

Sincerely, / s /



Robert H. Sindt

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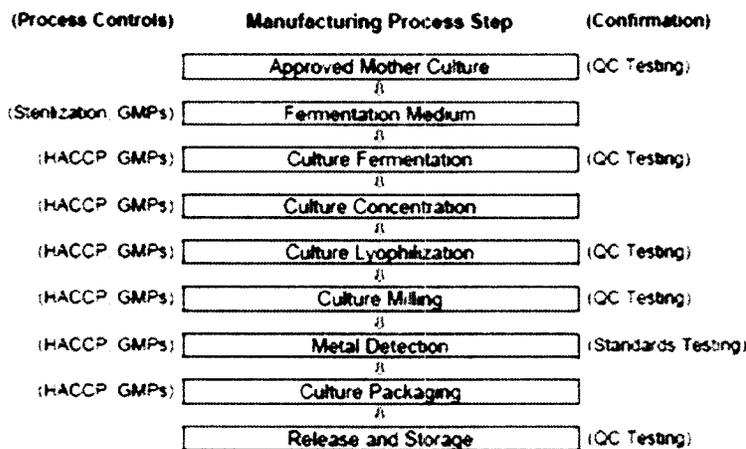
Attachments

***Bifidobacterium animalis* ssp. *lactis*--GRAS Notice Information**

(2) DETAILED INFORMATION ABOUT THE IDENTITY OF THE NOTIFIED SUBSTANCE

- Common and Usual Name of the Food Grade Substance: *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420
- Chemical Name for *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420: None
- Chemical Abstract Service (CAS) Registry Number for *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420: None
- Empirical Formula for *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420: None
- Structural Formula for *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420: None
- Quantitative Composition for *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420: *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420 are commercially available food ingredients produced by culture fermentation utilizing *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* Bl-04, or *B. lactis* B420, respectively, as the source organism. Use in foods will be targeted to typically contain 5×10^9 cfu/serving of *B. lactis* at consumption.

- Method of Manufacture for *Bifidobacterium animalis ssp. lactis* (*B. lactis*), including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* BI-04, and *B. lactis* B420: *B. lactis* isolates HN019, Bi-07, BI-04, and B420 are manufactured in compliance with the U.S. Food and Drug Administration's current Good Manufacturing Practice guidelines, as specified in FDA regulations (21 CFR, part 110), and in an FDA regulated and inspected facility. All ingredients utilized are food grade or approved for use by the FDA. The manufacturing process is summarized below:



The source organism used is *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* BI-04, or *B. lactis* B420, respectively. The cultures are maintained in the culture bank of Danisco USA, Inc. (Danisco) as frozen 1 ml. vials at -80°C. Danisco independently verifies the identity of each organism. Each seed lot in the culture bank is fully characterized to insure the identity of the seed strains. From the seed vials, Danisco produces concentrated starter for the industrial fermentation.

Each product is manufactured through a specific time and temperature controlled

fermentation of suitable food grade ingredients with *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* BI-04, or *B. lactis* B420, respectively. Prior to addition of *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* BI-04, or *B. lactis* B420, respectively, the mixture is sterilized and cooled to an incubation temperature of 37° C. The mixture is then inoculated with *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* BI-04, or *B. lactis* B420, respectively, and allowed to incubate to the fermentation endpoint under constant temperature.

After the required incubation period, the pH is adjusted with ammonium hydroxide, and concentrated via centrifugation. To the concentrated bacterial slurry, food-grade cryoprotectants are added; the material is frozen; and subsequently freeze-dried. The dried cultured product is then packaged and stored in a cool, dry environment.

Release of product for sale according to established specifications is under the responsibility of Danisco Quality Control. Final product testing methods comply with standard Methods for the Examination of Dairy Products of the American Public Health Association.

- Source Information for *Bifidobacterium animalis ssp.lactis*: *Bifidobacterium* spp. are Gram-positive, non-spore forming, anaerobic, pleomorphic bacilli, which are dominant microbial residents of the colonic microbiota. The *Bifidobacterium* group does not contain spp. that are considered pathogenic to man(1, 2, 8, 9). *Bifidobacteria* were first discovered in 1899 in the feces of breast-fed infants. This was of particular interest to scientists as these bacteria are often the most abundantly found in the intestine of breast-fed infants and regarded as one of the primary reasons for the greater resistance of breast-fed infants to disease. *Bifidobacterium* spp. are prevalent members of the intestinal colonic

microbiota, and although species distribution can change through the influence of age and other factors, it is well accepted that *Bifidobacteria* play a key role in the intestinal microbiota of humans throughout life.

Bifidobacterium lactis is a well-characterized, non-pathogenic, non-toxicogenic, homogeneous subspecies grouping, which was originally described by Meile et. al (3). Taxonomic differentiation of *B. lactis* and *B. animalis* strains has been difficult and *B. lactis* was recently regrouped with *B. animalis* as *Bifidobacterium animalis* ssp. *lactis* based on molecular techniques and phenotypic characteristics (4). This grouping contains many, if not all, *Bifidobacterium* strains that are used in dairy products where growth of the strain is required.

All *Bifidobacterium* species are listed as Biosafety Level 1 organisms by the American Type Culture Collection, indicating that they are not known to cause disease in healthy human adults. (<http://www.atcc.org/common/catalog/numSearch/numResults.cfm?atccNum=25527>).

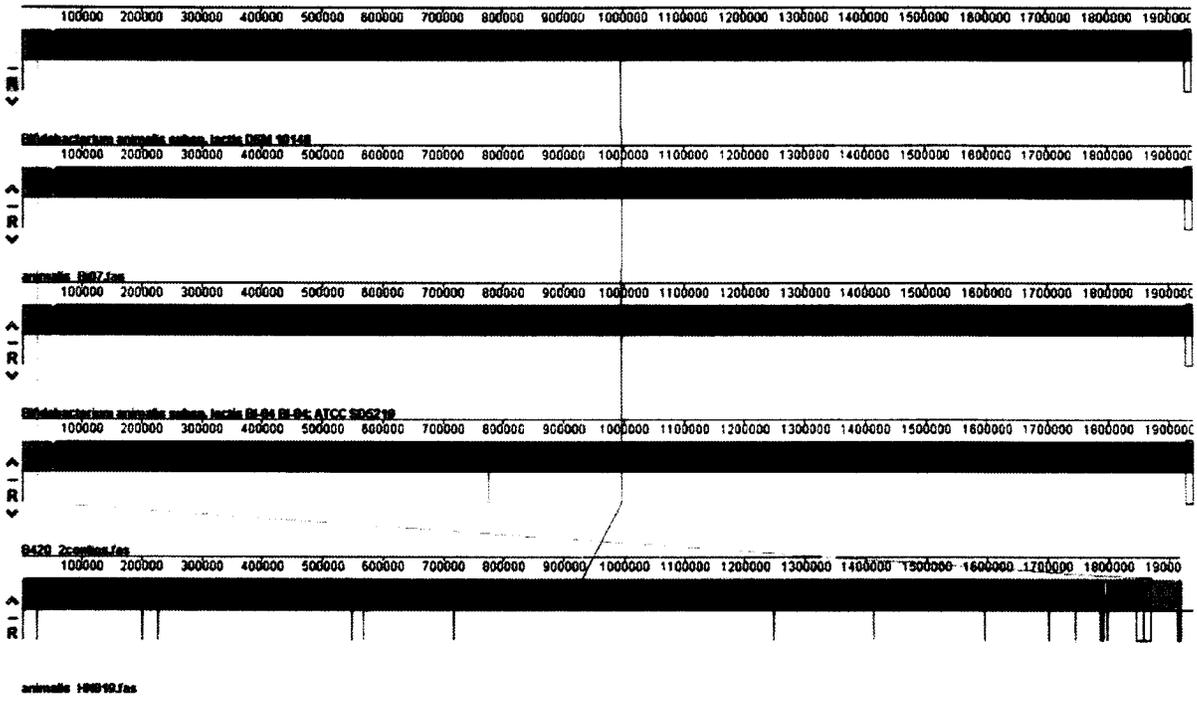
Because of the recent changes in classification within the *Bifidobacterium* group, *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* BI-04, and *B. lactis* B420 have been characterized and properly classified as *B. animalis* ssp. *lactis* by modern genotypic methods including 16S rRNA gene sequencing, PCR using species-specific primers(5), and optical mapping, as well as their demonstrated ability to grow in milk.

Genomic information of all the *Bifidoacterium animalis* ssp. *lactis* strains that Danisco manufactures has been gathered using whole genome sequencing of BI-04 and B420, and Bi-07. HN0019 was sequenced by Fonterra Research Group and deposited publicly at NCBI. Comparative genomics of the sequenced strains has been completed and many of the differences have been published(5a). Since the publication of the BI-04 and HN019

genomes, some additional single nucleotide polymorphisms (SNP) have been identified in B420 and Bi-07 strains. These differences have increased the power to discriminate between the strains. However, among all the sequenced strains of *B. animalis* ssp. *lactis* published both publically and held privately, there are very few differences. Overall, the sequencing has revealed that the genome architecture of this species is highly conserved among different strains, with over 99% of the genome conserved across all strains sequenced.

Much of the genetic differences between the *B. animalis* ssp. *lactis* strains have previously been published, with the non-synonymous mutations in protein regions also undergoing phenotypic analysis. Overall, with the high degree of genetic relatedness, only one phenotypic difference was observed. Glucose uptake is thought to be affected by a Single SNP in the *glcU* gene in a subset of the strains analyzed(5b). This functional difference may be attributed to drift due to commercialism of these strains in the dairy industry, but should not have an influence on safety.

An alignment of the whole genomes of all four commercial strains, along with the type strain for the species (DSM 10140) has been done in order to demonstrate the overall genomic similarity in both genetic content and genome arrangement. The HN019 draft genome was deposited by Fonterra Research Center to NCBI. Red lines show the contig boundaries of the genomes. Significant differences are visualized by white lines, similarity in DNA content is shown by shading nearly identical regions the same color. Overall, the genomes are highly collinear and nearly identical at the sequence level, without any large insertions, deletions or inversions.

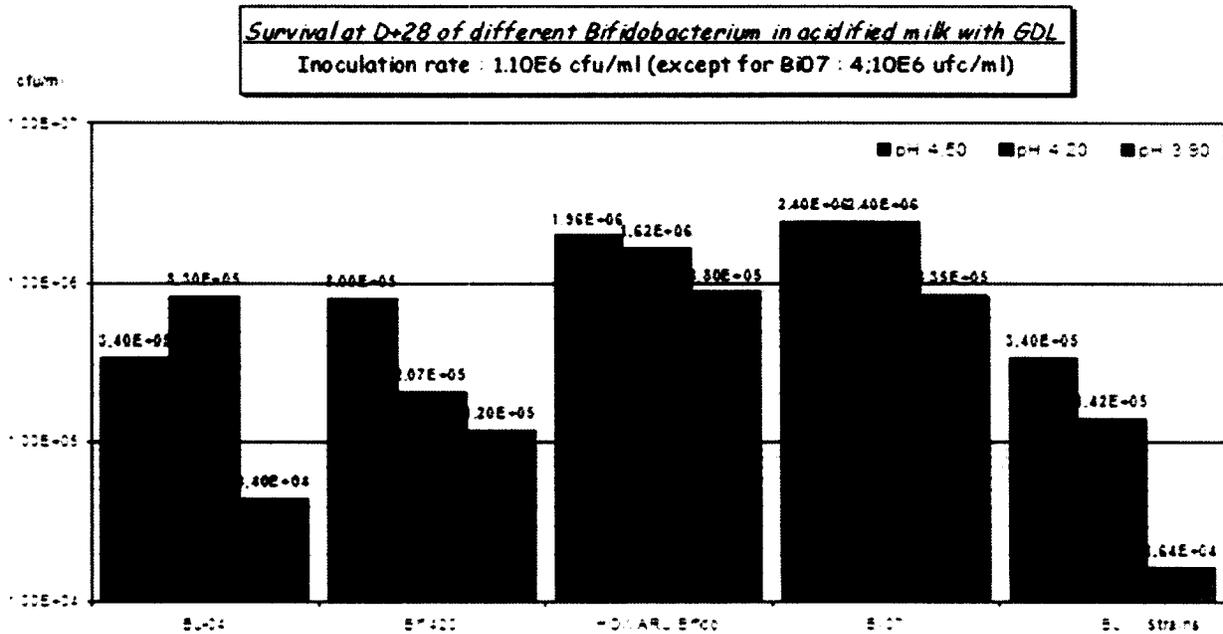


Functional Differences between the *B. lactis* strains HN019, Bi-07, Bi-04, and B420 have been identified. It has been shown that the four *B. lactis* strains are genetically very similar, but not identical. The minor genetic differences appear to result in functional differences. In order to understand whether these minor genetic differences result in a phenotypic functional difference, they have been evaluated using Fermentation Yield Evaluation, and Functional Application Comparison.

Fermentation Yield Evaluation: Direct fermentation yield comparison between Bi-07 and Bi-04 demonstrates that strain Bi-04 provides approximately a 30-35% increase in the amount of cells per ml in the fermenter, as well as enhanced stability in the freeze-dried state. In comparing Bi-04 and HN019, very similar fermentation yields and stability results are obtained. In comparing B420 and Bi-07, very similar fermentation yields and

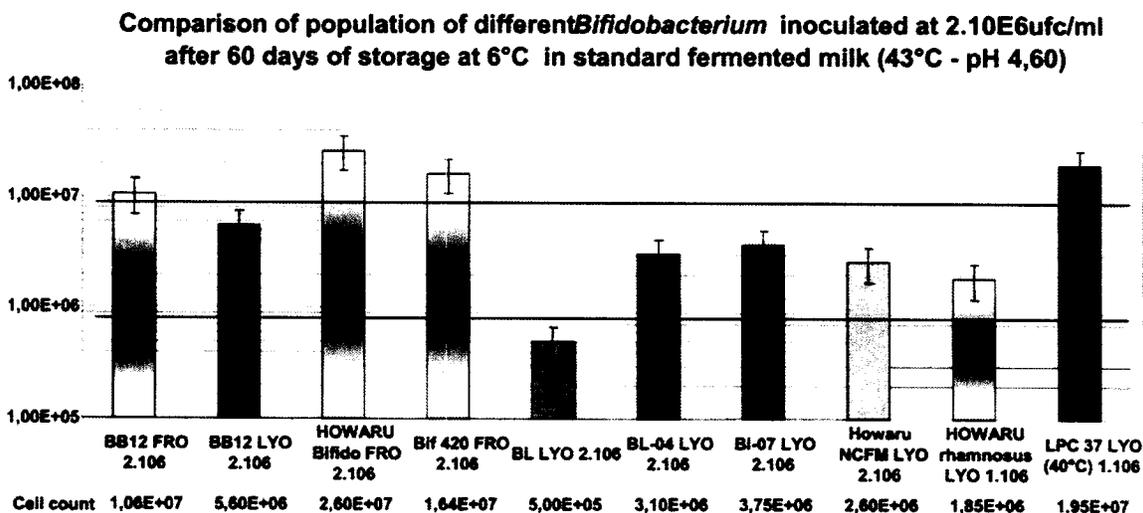
stability results are obtained. Based on fermentation yields alone, phenotypic differences are clearly apparent for Bi-07 or B420 compared with either BI-04 or HN019.

Functional Application Comparison: An additional way to demonstrate functional differences between the strains is to compare how the strains behave in specific end-product applications, for example in the production of fermented milks or yogurts. In the example below, one can see that the 28-day survival of the four strains in acidified milk is different between the strains when tested under identical conditions.



In a fermented yogurt example, phenotypic differences can be observed after a 60-day storage test. Although starting at equivalent cell counts at the start of the stability evaluation, the four strains give different stability profiles after shelf-life storage, an indication of clear functional and phenotypic differences between the four strains. It is these functional and phenotypic differences that lead customers to prefer one strain over

the others, depending on their application. (Note HOWARU Bifido in below chart is HN019 isolate.)



- Characteristic Properties of *B. lactis* isolates: *B. lactis* isolates HN019, Bi-07, BI-04, and B420, respectively, are harmless lactic acid producing bacterium. Commercially, *B. lactis* isolates HN019, Bi-07, BI-04, and B420 are produced by fermentation utilizing *B. lactis* isolates HN019, Bi-07, BI-04, and B420, respectively, all safe and suitable bacterium. *B. lactis* isolates HN019, Bi-07, BI-04, and B420, respectively, in powdered form exhibit a cream to white color and are typically stored at or below 4°C.

- Content of Potential Human Toxicants for *B. lactis* isolates HN019, Bi-07, BI-04, and B420: None

- Specifications for Food Grade *B. lactis* isolates HN019, Bi-07, Bl-04, and B420: *B. lactis* isolates HN019, Bi-07, and Bl-04 are white to cream colored freeze dried powders, while B420 is a concentrated, deep-frozen culture in pellet form. All are produced by culture fermentation utilizing *B. lactis* isolates HN019, Bi-07, Bl-04, and B420, respectively. Microbiological specifications/kg (/100 DCU for B420) for the *B. lactis* isolates are:

B. lactis Bi-07

Cell Count	>2.5E+10/g
Non-Lactic Count	<5000/g
Enterococci	<100/g
Coliforms	<10/g
E. coli	neg. by test (<0.3/g)
Staphylococcus (coag. pos.)	neg. by test (<10/g)
Salmonella	neg. (40 g enrichment)
Listeria	neg. (25 g enrichment)
Aerobic MRS Count	<10,000/g

B. lactis Bl-04

Cell Count	>4.5E+11/g
Non-Lactic Count	<5000/g
Enterococci	<100/g
Coliforms	<10/g
E. coli	neg. by test (<0.3/g)
Staphylococcus (coag. pos.)	neg. by test (<10/g)
Salmonella	neg. (40 g enrichment)
Listeria	neg. (25 g enrichment)
Aerobic MRS Count	<10,000/g

B. lactis HN019

Cell Count	>3.00E+11/g
Non-Lactic Count	<5000/g
Enterococci	<100/g
Coliforms	<10/g
E. coli	neg. by test (<0.3/g)
Staphylococcus (coag. pos.)	neg. by test (<10/g)
Salmonella	neg. (40 g enrichment)
Listeria	neg. (25 g enrichment)

<i>B. lactis</i> B420	
Cell Count	>1.0E+10/DCU
Non-Lactic count	<100/ml
Enterococci	<1/ml
Yeast and molds	<10/ml
E. coli	neg. by test (<0.3/g)
Staphylococcus aureus	<1/ml
Salmonella	neg. /25 ml
Listeria	neg. /25 ml
Bacillus cereus	<10/ml

***Bifidobacterium animalis ssp. lactis*-GRAS Notice
Information**

**(3) INFORMATION ON SELF-LIMITING LEVELS OF USE, IF
ANY**

- Uses are self-limited to those foods that can sustain living *B. lactis* isolates
HN019, Bi-07, BI-04, and B420 for the shelf life of the food.

***Bifidobacterium animalis ssp.lactis*-GRAS Notice Information**

(4) DETAILED SUMMARY OF THE BASIS FOR GRAS DETERMINATION

- (i) Danisco's determination, that the notified uses of *B.lactis* isolates HN019, Bi-07, Bl-04, and B420 (as ingredients in foods, including ready-to-eat breakfast cereals, bars, cheese, milk drinks and milk products, bottled water and teas, fruit juices, fruit nectars, fruit ades and fruit drinks, chewing gum and confections) are exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act based on its determination that such uses are GRAS, is based on scientific procedures as supported by a history of experience based on common use in food. The determination has been confirmed by an independent panel of scientific experts convened by Danisco to conduct such a critical review. Each member of the independent expert panel was qualified by extensive scientific training and experience to evaluate the safety of substances used in food. The independent expert panel's report and determinations, updated to September 2012, is included in its entirety in the Appendix attached hereto. Danisco's analysis follows:

- (A) Safety of *Bifidobacterium animalis ssp. lactis* (*B. lactis*), including *B. lactis* HN019, *B.lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420: The *B. lactis* isolates are produced by a fermentation process utilizing seed strains of independently identified *Bifidobacterium animalis ssp. lactis* (*B. lactis*), including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420, all safe and suitable lactic acid

producing bacterium.

(1) **Safety and Suitability of Organism.** In conducting its assessment and making its determination, Danisco reviewed the existing regulatory status, animal studies, human use information, and other published and unpublished studies and information relating to *B.lactis*.

FDA, EU and scientific consensus on B. lactis

When considering the safety of cultures, the issues that need to be assessed are pathogenicity, toxicity, and the presence of transferable antibiotic resistance genes.

Data from animal and human studies were considered.

Species of the genus *Bifidobacterium* are considered to be non-pathogenic, non-toxicogenic and have generally been considered safe for food use (EFSA, Appendix).

Boriello, et al. (14) reviewed data pertinent to safety concerns for these bacteria and concluded that "current evidence suggests that the risk of infection with probiotic *lactobacilli* or *bifidobacteria* is similar to that of infection with commensal strains, and that consumption of such products presents a negligible risk to consumers...". This opinion is echoed in other publications (8, 19). Additionally, the species *B. lactis* is proposed for inclusion on the EU QPS list (See Appendix).

Animal studies

Strain *B. lactis* HN019 was assessed in several studies using in vitro and mouse model systems for traits deemed important to safety and tolerance. Zhou et al.(10) conducted an unblinded study on healthy, conventionally colonized, male BALBc mice, 6-8 weeks of age. Groups of eight mice were fed 10^{11} cfu/d HN019 in skim milk for eight consecutive days. The following parameters were assessed: general health status, behavior, activity

level, feed intake, body weight, intestinal mucosal morphology (villus height, crypt depth, epithelial cell height and mucosal thickness) and the presence of bacteria in both blood and tissue (mesenteric lymph nodes, liver and spleen). Controls were fed other strains of *Bifidobacterium* with skim milk or skim milk without cultures. There were no significant differences detected between the controls and the HN019-fed mice in any of the parameters tested. No microbes were cultured from the blood of any of the mice. Of the microbes cultured on MRS agar from other organs, none tested were identified as HN019 using a strain-specific, randomly amplified polymorphic DNA (RAPD) fingerprinting approach. This study demonstrates the absence of infectivity, acute oral toxicity, translocation and disruption of intestinal mucosal integrity by short-term (eight day) consumption of HN019 at levels of 5×10^{12} /kg body weight per day. The authors concluded that “This [study] suggests that the probiotic strains HN019, HN001, and HN017 are non-pathogens and likely to be safe for human consumption.”

A similar study was conducted documenting safety of consumption by healthy, colonized, male BALBc mice (6-8 weeks of age) of HN019 at a range of doses (2.5×10^9 cfu, 5×10^{10} , 2.5×10^{12} cfu/kg body weight/d) for 4 weeks(11). Measured parameters included various indicators of general health status, hematology and blood chemistry, translocation and gut mucosal histology. No microbes with HN019’s RAPD pattern were isolated from any tissue. No adverse effects on any measured parameters were detected at any of the doses tested. The authors concluded “The results obtained in this study suggests that the potentially probiotic LAB strains HN001, HN017 and HN019 are non-toxic for mice and therefore likely to be safe for human use.” The findings from these studies by Zhou et al.(10, 11) confirm the conclusions from a previous 7-day feeding study by Shu et al.(12), which found no adverse reactions when fed to healthy male BALBc mice at a rate of 5×10^7 , 10^9 , or 5×10^{10} cfu/mouse/day.

Zhou and Gill(13) tested HN019 for pro-inflammatory activity in a mouse model of

experimental thyroiditis. This was conducted to determine if the immunostimulating properties of this microbe might exacerbate the symptoms of individuals with overactive immune responses as occurs with autoimmune disorders. Results indicated that HN019-fed mice were indistinguishable from control mice in the induction or progression of the autoimmune disorder suggesting that HN019 should not be expected to intensify an autoimmune response. The authors concluded that “The results of this study suggest that immunostimulatory probiotic HN001 and HN019 do not induce or enhance autoimmune responses in animals which have the genetic potential to develop autoimmunity.

In an immunodeficient mouse model of candidiasis(16) *B. lactis* Bi-07 was found to protect both adult and neonatal mice against the lethal effects of *Candida* infection. This was shown to be through a variety of immunologic and non-immunologic mechanisms. Bi-07 was found to be especially beneficial against the incidence and severity of mucosal candidiasis. Although this study didn’t look specifically at safety, Bi-07 was found to be the most biotherapeutic in comparison to *L. acidophilus*, *L. reuteri*, and LGG. Bi-07 provided the best overall protection against mucosal and systemic candidiasis. In this very immuno-compromised model system, Bi-07 posed no safety risk and instead, provided protection against a lethal challenge of *Candida*.

In a follow-on study evaluating the capacity of four probiotic bacterial to colonize, infect, stimulate immune responses in, and affect the growth and survival of congenitally immunodeficient gnotobiotic mice(17), *B. lactis* Bi-07 was found to be innocuous for the adult mice and neonatal mice. In evaluating the pathogenic potential of the strains, a congenitally immunodeficient host model, two probiotic strains, *L. reuteri* and *L. rhamnosus* GG, produced some infant mortality in the model system. The conclusion from the authors was that “*L. acidophilus* and *B. animalis* appear to be innocuous probiotics in immunodeficient mice. Overall, probiotic bacteria are likely to be safe for

immunocompetent and immunodeficient adults but they should be tested for immunodeficient neonates.”

Additional *in vitro* analyses were conducted on safety biomarkers of mucin degradation and platelet aggregation. HN019 was not able to degrade gastric mucin *in vitro*(14). This supports the non-invasive nature of HN019 observed in the mouse studies. HN019 was also unable to aggregate platelets *in vitro* (15.) Strains unable to aggregate platelets would be expected to be less able to participate in the pathogenesis of infective endocarditis.

In another *in vitro* study on characteristics related to safety of microbes(17a), clinical isolates *B. lactis* B420, *B. lactis* Bb-12, and *B. lactis* 1100 were used to identify their properties in order to understand why they were involved in bacteraemia and to assess potential risk factors of *bifidobacteria* by comparing clinical and fecal isolates. In this study, none of the tested potential risk factors, based on fecal, clinical and diary bifido isolates for properties that are known virulence factors in “true” pathogens, were found to be particularly associated with the clinical strains and no risk factors could be identified. Although this study did not include human subjects consuming the B420 strain, with the applied *in vitro* assays, the authors suggest *Bifidobacterium* to be safe for human consumption.

Human studies

In human trials of clinical efficacy where adverse effects are monitored in human test populations (individual studies involving children, adult, or elderly, healthy, mildly-ill, to critically-ill subjects), strain HN019 has been consumed at doses ranging from 1.9×10^7 to 3×10^{11} cfu/d for periods of 7 days to two years, with no adverse events reported(22-27c).

Additionally, in a recent trial where 9×10^9 cfu/day of strain HN019 was fed to 152

infants with a family history of eczema or allergies. These infants were fed for two years, from birth to two years of age with no adverse events having been noted(28). Importantly, there were no differences in any morphometric analyses.

In a recent study by Bettler et. al.(29), safety of toddler formulas containing *B. lactis* Bi-07 ($5e8 - 1e9$ CFU/day), *B. lactis* Bi-07 ($5e8 - 1e9$ CFU/day) with fructooligosaccharides (0.3 – 0.6 g/day), or a formula control, were evaluated. In this multicenter, prospective, randomized, double-blind, parallel study, healthy toddlers 12 to 34 months of age received 200 – 400 ml per day of the formulas and were evaluated for fecal microbiology, the ability of *B. lactis* Bi-07 to colonize, and general tolerance indicators. A total of 318 toddlers entered the study, with 170 completing the 28 days of formula consumption and stool sample submission. The number of toddlers, who discontinued the study, and the reasons for discontinuation, were similar across formula groups. The presence of *B. lactis* was detected in fecal samples from the probiotic and symbiotic group during the active feeding period, with numbers declining during the washout period. There were no statistically significant differences among the three formula groups for the number of toddlers with any adverse event or withdrawals related to adverse events. As the authors' concluded,

“Adverse events, acceptance and tolerance to the formulas were similar across the groups.”

Fisberg et al.(30) evaluated a symbiotic formulation consisting of *B. lactis* Bi-07, *L. acidophilus* NCFM™ and fructooligosaccharides (at 0.5 g/L after reconstitution). The incidence and duration of illness, and anthropometrics, were determined in children who received this symbiotic formulation along with a nutritional supplement, versus children who received the nutritional supplement alone. In this double-blind, randomized study of 616 children aged 1-6 years old, both study feedings were well tolerated and the overall

incidence of adverse events was very low, with none of the adverse events considered as study-related. The probiotic dose in this study was not well communicated in the manuscript but is estimated to be greater than one billion organisms per day. The conclusion from the authors on this study was that “Oral supplementation with a nutritionally complete product at an average intake of 40 ml/kg/day, can improve the nutritional status of underweight preschool children as demonstrated by 1) Catch up and 2) improvement in immune functioning.”

B. lactis Bi-07 was also tested in another symbiotic study where the combination of *B. lactis* Bi-07, *Lactobacillus acidophilus* NCFM™, and fructooligosaccharides was used as the symbiotic arm(31). In this study, 129 children aged 1-6 who were acutely ill and receiving antibiotic therapy were randomized to receive a nutritional supplement with or without the symbiotics or a fruit-flavored drink. The probiotic dose was the same as in the aforementioned study (Fisberg et al.(30). The authors determined “Data from present study suggest that the use of nutritional supplements in the dietary management of children affected with upper respiratory infections receiving antibiotics is beneficial and safe. “

A subgroup in a large placebo-controlled double-blind study conducted by Leyer et al.(30a) involved 112 healthy children aged 3-5 yrs who were fed a mixture of *B. lactis* Bi-07 and *L. acidophilus* NCFM at a rate of 1×10^{10} daily for 6 months. The investigators reported “Daily probiotic dietary supplementation during the winter months was a safe and effective way to reduce episodes of fever, rhino rhea, and cough, the cumulative duration of those symptoms, the incidence of antibiotic prescriptions and the number of missed school days attributed to illness.

B. lactis BI-04 was the subject of seven human clinical studies(32-32b) on potential

efficacy, with no adverse events being recorded(32-33). These clinical studies include children with pollen allergies, healthy adults to adults with mild to critical labeling, with dosage rates of up to 2×10^{10} cfu/day.

Nestle reported in GRAS Notice GRN00049 that clinical trials have been performed using five (5) *Bifidobacteria* species without a single adverse event report(34). Also, *Bifidobacterium lactis* species have been studied extensively in a cross-section of infants, children, healthy adults, and elderly, with doses up to 10^{11} cfu/day, with no associated adverse effects noted35-68. These clinical studies include infants, children, adults, and elderly with the highest dosage of 10^{11} cfu/day.

Non-transferable antibiotic resistance of *Bifidobacterium animalis* subsp. *lactis* strains HN019, Bi-07, BI-04, and B420

Introduction:

Antimicrobial resistance in bacteria can be mediated by many different mechanisms that range from unknown and non-specific to fully understood and well-studied. In order to address the question of transferability of antibiotic resistance, it is best to define the two types of resistance. Intrinsic resistance reflects an organism's ability to thrive in the presence of an antimicrobial agent, is not horizontally transferable, and is typical of the strains of a given species (1). In contrast, when a strain is resistant to a drug that the species is typically sensitive to, it may be considered acquired resistance. Acquired resistance can be mediated by mutation of indigenous genes or by added genes (14). The primary concern of acquired resistance is not the acquisition of a gene or mutation that provides resistance, but rather the ability of that resistance to be horizontally transferred.

Therefore, the focus has been on acquired resistance genes with the belief that they present a greater risk of transfer of resistance via horizontal gene transfer within and between species (1). LAB have been reported to have both intrinsic and acquired resistances to many classes of antibiotics, only some of which are known to be transferable (2,9). There are three identified mechanisms of horizontal gene transfer (HGT) in bacteria; natural transformation, conjugation and transduction. Some LAB species have these abilities and some do not, in fact strain level differences need to be evaluated in order to determine if HGT is possible (3,8). Three types of HGT were evaluated in this investigation, conjugative plasmids, transposases, and prophage/bacteriophage elements. Antibiotic resistance has been previously documented to be transferable on plasmids, transposases and phage (6,7,8,10). Therefore, the highest risk of an antibiotic gene being mobilized to another strain/species comes from these mechanisms of HGT, all of which have previously been reported in LAB in both in vitro and in vivo studies (1).

Methods:

In each case, a whole genome sequence of the manufactured strain was obtained and analyzed for the mechanisms of HGT. Using the sequence, comparisons to known drug resistance markers was done in order to determine their presence. When the mechanism of resistance was well documented and genomically located in the sequence, an evaluation of the flanking regions as well as the sequence identity was done. When a mechanism of resistance was not well understood, examination of all the known HGT mechanisms in that strain was completed to rule out a possibility of a resistance gene located in the vicinity. Note that not all

drug resistances were evaluated. Only the genes responsible for the drug resistance over the EFSA breakpoint were investigated.

Analysis of *Bifidobacterium animalis* subsp. *lactis* HN019, DGCC 2013:

Antibiogram of DGCC 2013 was established using ISO 10932 IDF223 method and VetMIC Lact-1 and 2 micro-dilution plates that include all antibiotics that are recommended by the FEEDAP. Recorded MICs are displayed in the table below. MIC values are below or equal to the Microbial Break Points (MBPs) defined for *Bifidobacterium* the EFSA Journal 2012 (14). One antibiotic resistance profile (MIC) exceeds the epidemiological breakpoint published by EFSA 2012; Tetracycline. (27).

APPENDIX : Antibiotic Susceptibility Profile

Method used : ISO 10932 IDF 223 with VetMIC Lact 1 and 2 microdilution plates

	Gentamycin	Kanamycin	Streptomycin	Tetracycline	Erythromycin	Clindamycin	Chloramphenicol	Ampicillin	Vancomycin	Virginamycin*
	Gm	Km	Sm	Tc	Em	Cl	Ch	Amp	Va	Vi*
DGCC 2013	MIC µg/ml									
<i>Bifidobacterium animalis</i>	Max. 64	Max. 256	Max. 64	Max. 32	Max. 0,06	Max. 0,06	Max. 2	Max. 0,12	Max. 0,5	Max. 0,25
MBP for <i>Bifidobacterium</i>**	64	NR***	128	8	0,5	0,25	4	2	2	1

* Virginamycin instead of Synercid

** The EFSA Journal (2008) 732 : 5-15

NR***: not required

Analysis of *Bifidobacterium animalis* subsp. *lactis* Bi-07, DGCC 2907:

Antibiogram of DGCC 2907 was established using ISO 10932 IDF223 method and VetMIC Lact-1 and 2 micro-dilution plates that include all antibiotics that are recommended by the FEEDAP. Recorded MICs are displayed in the table below. MIC values are below or equal to the Microbial Break Points (MBPs) defined for *Bifidobacterium* the EFSA Journal 2012(14).

APPENDIX : Antibiotic Susceptibility Profile

Method used : ISO 10932 IDF 223 with VetMIC Lact 1 and 2 microdilution plates

	Gentamycin	Kanamycin	Streptomycin	Tetracycline	Erythromycin	Clindamycin	Chloramphenicol	Ampicillin	Vancomycin	Virginamycin*
	Gm	Km	Sm	Tc	Em	Cl	Ch	Amp	Va	Vi*
DGCC 2907	MIC µg/ml									
<i>Bifidobacterium animalis</i>	Max. 64	Max. 256	Max. 64	Max. 8	Max. 0,12	Max. < 0,03	Max. 2	Max. 0,25	Max. 0,5	Max. 0,25
MBP for <i>Bifidobacterium</i>**	64	NR***	128	8	1	1	4	2	2	1

* Virginamycin instead of Synercid

** The EFSA Journal (2008) 732 : 5-15

NR***: not required

Analysis of *Bifidobacterium animalis* subsp. *lactis* BI-04, DGCC 2908:

Antibiogram of DGCC 2908 was established using ISO 10932 IDF223 method and VetMIC Lact-1 and 2 micro-dilution plates that include all antibiotics that are recommended by the FEEDAP. Recorded MICs are displayed in the table below. MIC values are below or equal to the Microbial Break Points (MBPs) defined for *Bifidobacterium* the EFSA Journal 2012(14). One antibiotic resistance profile (MIC) exceeds the epidemiological breakpoint published by EFSA 2012; Tetracycline(27).

APPENDIX : Antibiotic Susceptibility Profile

Method used : ISO 10932 IDF 223 with VetMIC Lact 1 and 2 microdilution plates

	Gentamycin	Kanamycin	Streptomycin	Tetracycline	Erythromycin	Clindamycin	Chloramphenicol	Ampicillin	Vancomycin	Virginamycin*
	Gm	Km	Sm	Tc	Em	Cl	Ch	Amp	Va	Vi*
DGCC 2908	MIC µg/ml									
<i>Bifidobacterium animalis</i>	Max. 64	Max. 512	Max. 64	Max. 16	Max. 0,06	Max. < 0,03	Max. 2	Max. 0,5	Max. 1	Max. 0,25
MBP for <i>Bifidobacterium</i>**	64	NR***	128	8	0,5	0,25	4	2	2	1

* Virginamycin instead of Synercid

** The EFSA Journal (2008) 732 : 5-15

NR***: not required

Analysis of *Bifidobacterium animalis* subsp. *lactis* B420, DGCC 420:

Antibiogram of DGCC 420 was established using ISO 10932 IDF223 method and VetMIC Lact-1 and 2 micro-dilution plates that include all antibiotics that are recommended by the FEEDAP. Recorded MICs are displayed in the table below. MIC values are below or equal to the Microbial Break Points (MBPs) defined for *Bifidobacterium* the EFSA Journal 2012(14). One antibiotic resistance profile (MIC) exceeds the epidemiological breakpoint published by EFSA 2012; Tetracycline(27).

	Gentamycin	Kanamycin	Streptomycin	Tetracycline	Erythromycin	Clindamycin	Chloramphenicol	Ampicillin	Vancomycin	Virginamycin*
	Gm	Km	Sm	Tc	Em	Cl	Ch	Amp	Va	Vi*
DGCC 420	MIC µg/ml									
<i>Bifidobacterium animalis</i>	Max. 64	Max. 256	Max. 64	Max. 16	Max. 0,06	Max. 0,06	Max. 2	Max. 0,25	Max. 0,5	Max. 0,25
MBP for <i>Bifidobacterium</i>**	64	NR***	128	8	0,5	0,25	4	2	2	1

* Virginamycin instead of Synercid

** The EFSA Journal (2008) 732 : 5-15

NR***: not required

Genome summary:

A complete genome sequence of *B. animalis* subsp. *lactis* HN019 was deposited by Fonterra Research Centre publicly to NCBI ([NZ_ABOT00000000](https://www.ncbi.nlm.nih.gov/nuclot/NZ_ABOT00000000)). The draft genome contains 28 contigs. A comparative analysis of the sequence to other bifidobacteria ([NC_012814.1](https://www.ncbi.nlm.nih.gov/nuclot/NC_012814.1)) was reported and findings indicate a very genomically conserved subspecies, with high identity to type strain DSM 10140 as well as others(12).

A complete genome sequence of *B. animalis* subsp. *lactis* BI-04 was obtained using published methods. The resulting genome was advanced to a single closed, circular chromosome with 1,938,709 total basepairs in length. The genome was deposited publicly

at NCBI along with comparative analysis of the sequence to other bifidobacteria. The reported findings indicate a very genomically conserved subspecies, with 99.975% identity of BI-04 to type strain DSM 10140 (12).

A complete genome sequence of *B. animalis* subsp. *lactis* Bi-07 was obtained using published methods. The resulting genome was advanced to a single closed, circular chromosome with 1,938,822 total basepairs in length. The genome was deposited publicly at NCBI along with comparative analysis of the sequence to other bifidobacteria (NC_017867.1). The reported findings indicate a very genomically conserved subspecies, with 99.975% identity of BBI to type strain DSM 10140 (Stahl, Jbac In Press).

A complete genome sequence of *B. animalis* subsp. *lactis* B420 was obtained using published methods. The resulting genome was advanced to a single closed, circular chromosome with 1,938,595 total basepairs in length. The genome was deposited publicly at NCBI along with comparative analysis of the sequence to other bifidobacteria (NC_017866.1). The reported findings indicate a very genomically conserved subspecies, with >99.9% identity of B420 to type strain DSM 10140 (In Press Jbac).

Tetracycline Resistance in *B. animalis* subsp. *lactis*:

Tetracycline resistance in *B. animalis* subsp. *lactis* has previously been shown to correlate directly with the presence of a single gene, *tetW* (13).

Plasmid analysis of HN019, Bi-07, BI-04, and B420:

No plasmid was detected in these strains.

Insertion elements (HN019, Bi-07, BI-04, and B420):

Nine transposases were identified within the genome of each of these strains, one putative transposase, *trp*, has been identified immediately upstream of the *tetW* gene.

Gene Mining (HN019, Bi-07, BI-04, and B420):

The presence of a *tetW* gene that is immediately downstream of a transposon (*trp*) has been

identified in these strains. This *tetW* gene sequence is identical to the previously reported in *B. animalis* subsp. *lactis* that has demonstrated the genes ability to confer the resistance to tetracycline (13).

Conclusion:

B. animalis subsp. *lactis* strains HN019, Bi-07, BI-04, and B420 have the same structure of transposon *trp* and *tetW* genetically as the strains evaluated in the study by Gueimonde et al, 2010. The ability of the strains to transfer the tetracycline resistance was evaluated and the authors found that they could not demonstrate any transfer of resistance to other *B. animalis* subsps. *lactis* or any of the 3 other species they evaluated in the in vivo experiment. As of date, there has not been any evidence that the *tetW* gene that is co-transcribed in tandem with this transposase has any ability to transfer resistance, and therefore poses no known risk of transfer. Additionally, through comparative genomics of 5 total proprietary and public genomes of *B. animalis* subsp. *lactis*, analysis finds that the overall genomic plasticity of the species is extremely stable. In fact, a genome wide comparison of all the strains that have currently been sequenced reveals little diversity—47 confirmed Single Nucleotide Polymorphisms (SNPs) and four insertion/deletion (INDELs) events (12). From this analysis, it is clear that there has not been an observed incidence of transposition between current *B. animalis* subsp. *lactis* genomes to date, else there would be some evidence of polymorphism between the strains as it relates to transposon insertion. Additionally, the individual sequence composition of the *tetW* gene was analyzed, and no sharp distinction can be made between the overall GC content of the genome and the GC content of the *tetW* gene. This further highlights the likelihood that the gene is intrinsic to *B. animalis* subsp. *lactis*, because horizontal gene transfer is often marked with different

GC content of the genetic material received than the host genetic material. To conclude, the implied risk of *tetW* transfer is deemed to be insignificant, as transposition has not been demonstrated experimentally, nor has it been observed naturally.

Antibiotic Resistance References:

1. Shalini Mathur, Rameshwar Singh. 2005. Antibiotic resistance in food lactic acid bacteria-a review. *International Journal of Food Microbiology*. 105; 281-295.
2. Muhammand Nawaz, Juan Wang, Aiping Zhou, Chaofeng Ma, Xiaokang Wu, John E. Moore, B. Cherie Millar, and Jiru Xu. 2011. Characterization and transfer of Antibiotic Resistance in Lactic Acid Bacteria from Fermented Food Products. *Curr. Microbiol*; 62: 1081-1089.
3. Ouoba LI, Lei V, Jensen LB. 2008. Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: determination and transferability of the resistance genes to other bacteria. *Int J Food Microbiol*. Jan 31;121(2):217-24.
4. Eric Altermann,* W. Michael Russell,*†‡ M. Andrea Azcarate-Peril,* Rodolphe Barrangou,*§ B. Logan Buck,*¶ Olivia McAuliffe,* Nicole Souther,* Alleson Dobson,* Tri Duong,*§ Michael Callanan,* Sonja Lick,*** Alice Hamrick,†† Raul Cano,†† and Todd R. Klaenhammer. 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *Proc Natl Acad Sci U S A*. 2005 March 15; 102(11): 3906–3912
5. Morten Danielsen, Anette Wind. 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *International Journal of Food Microbiology* Volume 82, Issue 1, 15 April 2003, Pages 1-11.
6. J. Aires,* F. Doucet-Populaire, and M. J. Butel. 2007. Tetracycline Resistance Mediated by *tet(W)*, *tet(M)*, and *tet(O)* Genes of *Bifidobacterium* Isolates from Humans. *Appl Environ Microbiol*. 2007 April; 73(8): 2751–2754.
7. Wang HH, Manuzon M, Lehman M, Wan K, Luo H, Wittum TE, Yousef A, Bakaletz LO. 2006. Food commensal microbes as a potentially important avenue in transmitting antibiotic resistance genes. *FEMS Microbiol Lett*. Jan;254(2):226-31.
8. Bonnie M. Marshall, Dorothy J. Ochieng, and Stuart B. Levy. 2009. Commensals: Underappreciated Reservoir of Antibiotic Resistance. *Microbe*. Vol4, No.5, 231-238.
9. Zhang L, Kinkelaar D, Huang Y, Li Y, Li X, Wang HH. 2011. Acquired antibiotic resistance: are we born with it? *Appl Environ Microbiol*. Oct;77(20):7134-41. Epub 2011

Aug 5.

10. Colomer-Lluch M, Imamovic L, Jofre J, Muniesa M. 2011. Bacteriophages carrying antibiotic resistance genes in fecal waste from cattle, pigs, and poultry. *Antimicrob Agents Chemother.* 2011 Oct;55(10):4908-11.
11. Chopra, I., and M. Roberts. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* 65:232-260.
12. Barrangou R, Briczinski EP, Traeger LL, Loquasto JR, Richards M, Horvath P, Coûté-Monvoisin AC, Leyer G, Rendulic S, Steele JL, Broadbent JR, Oberg T, Dudley EG, Schuster S, Romero DA, Roberts RF. 2009. Comparison of the complete genome sequences of *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and BI-04. *J Bacteriol.* Jul;191(13):4144-51.
13. Genetic basis of tetracycline resistance in *Bifidobacterium animalis* subsp. *lactis*. Gueimonde M, Flórez AB, van Hoek AH, Stuer-Lauridsen B, Strøman P, de los Reyes-Gavilán CG, Margolles A. *Appl Environ Microbiol.* 2010 May;76(10):3364-9.
14. European Food Safety Authority. 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). *EFSA Journal* 2012;10(6):2740.

(2) Supporting Recent Safe History of Use in Food .

Although basing its GRAS determination on scientific procedures, Danisco notes that *Bifidobacterium* species have a supporting recent history of safe food use when consumed as part of dairy food and supplement products. There are eight (8) species (*longum*, *infantis*, *breve*, *bifidum*, *adolescents*, *pseudolongum*, and *animalis* subspecies *lactis* and *animalis*) listed in IDF Bulletin No. 377: Inventory of Microorganisms with a Documented History of Use in Food(6).

No cases of clinical infection have been reported from such use.

Bifidobacterium lactis has been added to human food since at least 1980 and is very common in dairy products worldwide including the US where the organism is the most common *Bifidobacterium* in yogurt products(6). In particular *B. lactis* HN019 has been safely added to foods globally in dairy products and dietary supplements for at least five years, *B. lactis* Bi-07 and *B. lactis* BI-04 for at least 15 years, and *B. lactis* B420 for more than 20 years, all without a report of adverse effect on consumers.

On rare occasions, *Bifidobacterium* has been associated with some cases of clinical infection (7,8), but the species isolated are distinct from the species in general use. Since patients were not consuming *Bifidobacterium*-containing products at the time of infection, the source of the infective bacterium is presumed to be indigenous *Bifidobacterium* strains in a compromised host. In this regard, Borriello et al.(9) stated that “current evidence suggests that the risk of infection with probiotic *lactobacilli* or *bifidobacteria* is similar to that of

infection with commensal strains, and that the consumption of such products presents a negligible risk to consumers, including immunocompromised hosts.”

(3) Probable Consumption/Exposure of *B. lactis* isolates in Diet.

Uses are limited to those foods that can sustain living *B. lactis* for the shelf life of the food. These are currently envisioned to include ready-to-eat breakfast cereals; bars; cheeses, milk drinks, and milk products; bottled water and teas; fruit juices, fruit nectars, fruit “ades”, and fruit drinks; chewing gum; and confections. Danisco estimates that relatively few foods and beverages within each category will be developed with *B. lactis*, and that consumption will be for the express purpose of ingesting the proper amount of the organisms to achieve the claimed benefit--generally in a single serving per day.

The individual *B. lactis* isolates will be added to the targeted foods at concentrations needed to provide at least 5×10^9 cfu/250 g serving throughout the shelf life of the product. The initial addition level may be as high as 2×10^{11} cfu/250 g serving in order to insure at least 5×10^9 cfu/250 g serving remains over the product shelf life.

Danisco projects that there will be limited types of foods that will be available containing these isolates, thus the safety margin developed above is believed to be highly conservative. Consumers are likely to only consume the food to achieve the daily benefit for products containing *B. lactis*. For instance, in the beverage category, it is not envisioned that *B. lactis* containing products will compete with the myriad of functional food beverages on the market today, due

to either product incompatibilities or cost. And, few products in any given category will likely contain one of these strains. Based on these assumptions, consumers will most probably consume a single 250 g serving to achieve the benefit, thus ingesting approximately 2×10^{11} cfu/per day. However, in a maximum exposure scenario, the consumption of 10 servings per day at a level of 2×10^{11} cfu/per serving would result in a total daily consumption of 2×10^{12} cfu/day. Since this level is well below the expected normal level of this organism in the human gut, there is no concern with consumption of this organism at that level.

Because this organism is normally present and growing within the gut, Danisco is unable to calculate the actual amount of this organism that will be in an individual's gastrointestinal tract and can only address what an individual might eat.

(B) Information That May Appear Inconsistent With GRAS Determination:

Danisco is not aware of information that appears to be inconsistent with the determination of safety or general recognition of safety for the present or proposed uses of *B. lactis* isolates HN019, Bi-07, Bl-04, and B420. Danisco does, however, note the previously described sensitivity to relevant antibiotics, while further noting it does not provide a scientific basis to vary the conclusion that the isolates are safe to consume.

(C) Expert Consensus for GRAS Determination for *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420:

To further its internal safety and GRAS determinations of the subject food uses of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* Bl-04, and *B. lactis* B420, Danisco convened a panel of independent scientists ("Expert Panel"), qualified by their relevant national and international experience and scientific training, to evaluate the safety of food and food ingredients, to conduct a critical and comprehensive evaluation of the available pertinent published literature and other information on *B. Lactis* and *B. lactis* isolates HN019, Bi-07, Bl-04, and B420. Danisco asked the Panel to determine, based on its review, the safety and the GRAS status of the intended uses of *B. lactis* and *B. lactis* isolates HN019, Bi-07, Bl-04, and B420 in various foods. The Expert Panel consisted of Professor Emeritus Joseph F. Borzelleca, Ph.D. (Department of Pharmacology and Toxicology, Virginia Commonwealth

University, School of Medicine); Professor Emeritus, Food Science, Michael W. Pariza, Ph.D. (University of Wisconsin- Madison); and Walter H. Glinsmann, M.D. (President, Glinsmann Associates and formerly of the USFDA). Following its critical evaluation of all relevant information, the Expert Panel confirmed Danisco's determination of the safety and general recognition of safety of the present and proposed uses of *B. lactis* and *B. lactis* isolates HN019, Bi-07, Bl-04, and B420. (See Appendix for Expert Panel Report)

Specifically, in making its determination, the Expert Panel stated that it had "independently and collectively, critically evaluated a supporting GRAS dossier (GRAS Dossier, *Bifidobacterium animalis* subsp. *lactis*; August 15, 2012) submitted by Danisco, which included a description of *B. lactis* HN019, Bi-07, Bl-04 and B420; details of the manufacturing process and product specifications; history of use in foods; intended uses and use levels; exposures; safety testing; safety assessment; bibliography and appendix. The Expert Panel also considered other materials deemed appropriate or necessary."

During its review, the Expert Panel described the *Bifidobacterium* as "Gram-positive, non-spore forming, anaerobic, pleomorphic bacilli, and the dominant microbial residents of the colonic microbiota. The *Bifidobacterium* group does not contain species that are considered pathogenic to man. All *Bifidobacterium* species are listed as Biosafety Level 1 organisms by the American Type Culture Collection, indicating that they are not known to cause disease in healthy human adults. Because of the recent changes in classification within the *Bifidobacterium* group, *B. lactis* HN019, *B.*

B. lactis Bi-07, *B. lactis* BI-04, and *B. lactis* B420 have been genetically characterized and properly classified as *B. animalis* subsp. *lactis* using modern genotypic methods including 16S rRNA gene sequencing, PCR using species-specific primers, and optical mapping, as well as their demonstrated ability to grow in milk.

The four *B. lactis* strains are genetically very similar, but not identical. The minor genetic differences appear to result in minor functional differences. For example, the four strains display different stability profiles in a 60-day storage test in fermented yogurt. Such functional and phenotypic differences lead customers to prefer one strain over another, depending on application.”

The Expert Panel noted that the four isolates are produced in accordance with FDA current Good Manufacturing Practices guidelines in FDA regulated and inspected facilities. It also observed that *Bifidobacterium* species have a long history of safe use in dairy foods and supplement products and that no cases of clinical infection have been reported from such use. The Expert Panel further stated that “*Bifidobacterium lactis* has been added to human food since at least 1980 and is very common in dairy products worldwide, including in the US where the organism is the most common *Bifidobacterium* in yogurt products. In particular, *B. lactis* HN019 has been safely added to foods globally in dairy products and dietary supplements for at least 5 years, *B. lactis* Bi-07 and *B. lactis* BI-04 for at least 15 years, and *B. lactis* B420 for more than 20 years, all without a report of adverse health effects on consumers.

Although on rare occasions *Bifidobacterium* has been associated with some cases of clinical infection, Boriello, et al (2003) reported, following a critical and extensive review of the literature, that “current evidence suggests that the risk of infection with probiotic lactobacilli or bifidobacteria is similar to that of infection with commensal strains, and that the consumption of such products presents a negligible risk to consumers, including immunocompromised hosts.”

In reviewing use levels of the organisms and possible consumer exposure the Expert Panel noted that “Intended uses are limited to those foods that can sustain living *B. lactis* for the shelf life of the food and may include ready-to-eat breakfast cereals; bars; cheeses, milk drinks, and milk products; bottled water and teas; fruit juices, fruit nectars, fruit “ades”, and fruit drinks; chewing gum; and confections.” The Expert Panel stated that the strains are intended to be added to the foods “at concentrations needed to provide at least 5×10^9 cfu/250 g serving throughout the shelf life of the product. The initial addition level may be as high as 2×10^{11} cfu/250 g serving in order to insure at least 5×10^9 cfu /250 g serving remains over the product shelf life. In attempting to assess exposure, it is noted that there will be limited types of foods available containing the strain and consumers are very likely only to consume these foods to achieve the daily benefit of products containing *B. lactis*. Foods containing *B. lactis* will not be competing with other functional foods or beverages because of cost or specific health benefits of *B. lactis* strains. Based on these assumptions,

consumers will most probably consume a single 250 g serving to achieve the benefit thus ingesting approximately 2×10^{11} cfu per day.

Although *B. lactis* is normally present and growing within the human gastrointestinal tract, it is extremely difficult to quantify the amount present. Therefore, it is not possible to determine the potential effect on the body burden of *B. lactis* following ingestion of 2×10^{11} cfu/250 g serving/day.”

With regard to safety testing, the Expert Panel first examined the regulatory status of *B. lactis* and observed “Species of the genus *Bifidobacterium* are considered to be non-pathogenic, non-toxicogenic and are considered safe for use in foods (EFSA, Appendix A). FDA, in GRAS Notice No. GRN 000049, had no questions to the assertion that *B. Lactis* strain Bb12 was safe for use in certain milk-based infant formula.”

Second, the Expert Panel reviewed available animal studies and concluded “A comprehensive search of the scientific literature failed to identify classical/standard toxicity tests in animals for *B. lactis* probably because these tests are not appropriate for microorganisms. Strain *B. lactis* HN019 was assessed in several studies using in vitro and mouse model systems for traits important to safety and tolerance including effects in conventional mice (BALBc), immunodeficient mice (e.g., candidiasis model), and in an autoimmune thyroiditis model. Wagner, et al (1997) concluded from their mouse studies that “*L. acidophilus* and *B. animalis* appear to be innocuous

probiotics in immunodeficient mice. Overall, probiotic bacteria are likely to be safe for immunocompetent and immunodeficient adults but they should be tested for immunodeficient neonates.”

Third, the Expert Panel examined human studies and observed “A large number of human studies were analyzed and tabulated in the dossier. A few examples are presented below. It may be concluded from these studies that infants, children, adults, and elderly adults can safely tolerate *Bifidobacteria* species at doses up to 6×10^{11} cfu/day for up to two years.

Strain *B. lactis* HN019 was tested in healthy and ill infants, children and adults at doses from 1.9×10^7 to 3×10^{11} cfu/day for periods of 7 days to two years with no adverse effects reported. Strain *B. lactis* Bi-07 was tested for safety in toddler formulas for 28 days at a level of 5×10^8 - 1×10^9 CFU/day and no adverse effects were reported. A mixture of *B. lactis* Bi-07 and *L. acidophilus* (1×10^{10} cfu/day) was fed to 112 healthy children, 3-5 years of age, daily for 6 months, and the authors concluded that “Daily probiotic supplementation during the winter months was a safe and effective way to reduce episodes of fever, rhinorrhea, and cough, the cumulative duration of those symptoms, the incidence of antibiotic prescriptions and the number of school days attributed to illness.” (See Summary of Human Studies, Appendix)

Lastly, in reviewing safety testing, the Expert Panel scrutinized antibiotic resistance and stated “Because there is increasing concern of antibiotic resistance in pathogenic

micrororganisms, *B. lactis* HN019 and *B. lactis* B420 were tested for antibiotic resistance and for plasmids that might play a role in the transmission of such resistance to pathogenic organisms. HN019 was reported to be resistant to gram-negative specific antibiotics and to lack plasmids that could be implicated in transmission of antibiotic resistance. *B. lactis* was reported to be resistant to tetracycline only.”

On the issue of antibiotic resistance, it was concluded that “the four strains contained in this document [dossier] are sensitive to clinically relevant antibiotics, and given the widespread distribution of tetracycline genes in nature, the fact that *B. animalis* subsp. *lactis* strains in this document also contain the *tetW* gene is neither clinically or environmentally relevant and does not provide a scientific basis for revising the position that they are safe to consume.”

The Expert Panel then turned to safety assessment and stated that “Data from clinical trials and animal studies demonstrate the safety of *Bifidobacteria* in dairy foods and dietary supplements. The highest doses tested failed to induce significant toxicity and may be considered the No Observable Adverse Effect Levels (NOAELs). For example, a No Observable Adverse Effect Level (NOAEL) of 2.5×10^{12} cfu/kg body weight /day has been reported in mouse studies (this was the highest dose tested). The Expert Panel recognizes that this represents a lowest case estimate of the true NOAEL, because the organisms may proliferate in the GI tract after ingestion.

Lowest Observable Adverse Effect Levels (LOAELs) have never been reported for members of the *Bifidobacterium* group in human or animal studies, including the studies summarized above where a dose of 2.5×10^{12} cfu/kg body weight /day dose of HN019 was fed to mice (equivalent to a dose of 1.5×10^{14} cfu/day for a 60 kg human). Human exposure will be limited to those foods that will provide the beneficial effects sought by consumers. Food types may be limited by the cost of including these strains and by the nature of the food. Based on these assumptions, consumers will most probably consume a single 250 g serving to achieve the benefit, thus ingesting approximately 2×10^{11} cfu per day.”

Finally, the Expert Panel reached its conclusions of safety and general recognition of safety for the proposed food uses, stating “We, the Expert Panel, have individually and collectively critically evaluated the information concerning specific isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*) including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* BI-04 and *B. lactis* B420 summarized in the dossier and other information deemed appropriate, and we unanimously conclude that the proposed uses presented in the dossier of specific isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*) including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* BI-04 and *B. lactis* B420, produced consistent with cGMP and meeting appropriate food grade specifications presented in the dossier, are safe (i.e., meets the standard of reasonable certainty of no harm) and suitable.

We further unanimously conclude that the proposed uses presented in the dossier of specific isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*) including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* B1-04 and *B. lactis* B420, produced consistent with cGMP and meeting appropriate food grade specifications presented in the dossier, are safe and “Generally Recognized as Safe” (“GRAS”) based on scientific procedures corroborated by a long history of safe use.

It is our opinion that other experts qualified to assess the safety of food and food ingredients would concur with these conclusions.”

Based on the information contained in the exemption claim, the above additional and supplementary information, and the information contained in the Appendix attached hereto, a clear and ample basis exists to support Danisco's determination, confirmed by the Expert Panel, of general recognition of safety for the food uses, present and proposed herein, of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*) including *B. lactis* HN019, *B. Lactis* Bi-07, *B. lactis* B1-04, and *B. lactis* B420.

Bibliography

1. Scardovi V. Genus *Bifidobacterium*. In: Sneath P, Mair N, Sharpe M, Holt JG, eds. *Bergey's Manual of Systematic Bacteriology*. Vol 2. Baltimore, MD: Williams & Wilkins; 1986:1418-1434.
2. Mitsuoka T. Intestinal flora and human health. *Asia Pacific J. Clin. Nutr.* 1996;5:2-9.
3. Meile L, Ludwig W, Rueger U, et al. *Bifidobacterium lactis* sp. nov., a moderately oxygen tolerant species isolated from fermented milk. *Syst. Appl. Microbiol.* 1997;20:57-64.
4. Masco L, Ventura M, Zink R, Huys G, Swings J. Polyphasic taxonomic analysis of *Bifidobacterium animalis* and *Bifidobacterium lactis* reveals relatedness at the subspecies level: reclassification of *Bifidobacterium animalis* as *Bifidobacterium animalis* subsp. *animalis* subsp. nov. and *Bifidobacterium lactis* as *Bifidobacterium animalis* subsp. *lactis* subsp. nov. *Int J Syst Evol Microbiol.* Jul 2004;54(Pt 4):1137-1143.
5. Ventura M, Zink R. Rapid identification, differentiation, and proposed new taxonomic classification of *B. lactis* GRAS NOTICE INFORMATION

Bifidobacterium lactis. *Appl Environ Microbiol*. Dec 2002;68(12):6429-6434.

- 5a. Barrangou, R. Briczinski, E. Traeger, L. Loquasto, J. Richards, M. Horvath, P. Coûté-Monvoisin, A. Leyer, G. Rendulic, S. Steele, J. Broadbent, J. Oberg, T. Dudley, E. Schuster, S. Romero, D. and Roberts, R. 2009. *Journal of Bacteriology*, July, p. 4144 - 4151.
- 5b. Briczinski, E. Loquasto, J. Barrangou, R. Dudley, E. Roberts, A. and Roberts, R. 2009. Strain-Specific Genotyping of *Bifidobacterium animalis* subsp. *Lactis* by Using Single-Nucleotide Polymorphisms, Insertions, and Deletions. *AEM* Dec. 2009, Vol.75, No. 23, p. 7501-7508.
6. Mogensen G, Salminen S, O'Brien J, et al. Inventory of microorganisms with a documented history of safe use in food. *Bull. Int. Dairy Fed.* 2002;377:10-19.
7. Bourne KA, Beebe JL, Lue YA, Ellner PD. Bacteremia due to *Bifidobacterium*, *Eubacterium* or *Lactobacillus*; twenty-one cases and review of the literature. *Yale J Biol Med*. Sep-Oct 1978;51(5):505-512.
8. Gasser F. Safety of lactic acid bacteria and their occurrence in human clinical infections. *Bull. Inst. Pasteur*. 1994;92:45-67.
9. Borriello SP, Hammes WP, Holzapfel W, et al. Safety of probiotics that contain lactobacilli or bifidobacteria. *Clin Infect Dis*. Mar 15 2003;36(6):775-780.
10. Zhou JS, Shu Q, Rutherford KJ, Prasad J, Gopal PK, Gill HS. Acute oral toxicity and bacterial translocation studies on potentially probiotic strains of lactic acid bacteria. *Food Chem Toxicol*. Feb-Mar 2000;38(2-3):153-161.
11. Zhou JS, Shu Q, Rutherford KJ, et al. Safety assessment of potential probiotic lactic acid bacterial strains *Lactobacillus rhamnosus* HN001, *Lb. acidophilus* HN017, and *Bifidobacterium lactis* HN019 in BALB/c mice. *Int J Food Microbiol*. May 25 2000;56(1):87-96.
12. Shu Q, Zhou JS, Rutherford KJ, et al. Probiotic lactic acid bacteria (*Lactobacillus acidophilus* HN017, *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019) have no adverse effects on the health of mice. *Int Dairy J*. 1999;9:831-836.
13. Zhou JS, Gill HS. Immunostimulatory probiotic *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019 do not induce pathological inflammation in mouse model of experimental autoimmune thyroiditis. *Int J Food Microbiol*. Aug 15 2005;103(1):97-104.
14. Zhou JS, Gopal PK, Gill HS. Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin in vitro. *Int J Food Microbiol*. Jan 22 2001;63(1-2):81-90.
15. Zhou JS, Rutherford KJ, Gill HS. Inability of probiotic bacterial strains *Lactobacillus rhamnosus* HN001 and *Bifidobacterium lactis* HN019 to induce human platelet aggregation in vitro. *J Food Prot*. Nov 2005;68(11):2459-2464.
16. Wagner, R.D., Pierson, C., Warner, T., Dohnalek, M., Farmer, J., Roberts, L., Hilty, M., and E. Balish. 1997. Biotherapeutic effects of probiotic bacteria on candidiasis in immunodeficient mice. *Infect. Immun*. 65:4165-4172.

17. Wagner, R.D., Warner, T., Roberts, L., Farmer, J., and E. Balish. 1997. Colonization of Congenitally Immunodeficient Mice with Probiotic Bacteria. *Infect. Immun.* 65:3345-3351.
- 17a. Ouwehand, A. Saxelin, M. and Salminen, S. 2004. Assessment of potential risk factors and related properties of clinical, fecal and dairy *Bifidobacterium* Isolates. *Bioscience Microflora* Vol. 23(1): 37 - 42.
18. Zhou JS, Pillidge CJ, Gopal PK, Gill HS. Antibiotic susceptibility profiles of new probiotic Lactobacillus and Bifidobacterium strains. *Int J Food Microbiol.* Feb 1 2005;98(2):211-217.
19. Vankerckhoven, V., Huys, G., Vancanneyt, C.V., Klare, I., Romond, M.B., Entenza, J.M., Moreillon, P., Wind, R., Knol, J., Wiertz, E., Pot, B., Vaughan, E., Kahlmeter, G., and H. Goossens. 2008. Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. *Trends Food Sci Technol* 19: 102-114.
20. Chopra, I., and M. Roberts. 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* 65: 232-260.
21. Aires, J., Doucet-Populaire, F., and M.J. Butel. 2007. Tetracycline resistance mediated by *tet(W)*, *tet(M)*, and *tet(O)* genes of *Bifidobacterium* isolates from humans. *Appl Environ Microbiol* 73(8): 2751-2754.
22. Arunachalam K, Gill HS, Chandra RK. Enhancement of natural immune function by dietary consumption of *Bifidobacterium lactis* (HN019). *Eur J Clin Nutr.* Mar 2000;54(3):263-267.
23. Chiang BL, Sheih YH, Wang LH, Liao CK, Gill HS. Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium (*Bifidobacterium lactis* HN019): optimization and definition of cellular immune responses. *Eur J Clin Nutr.* Nov 2000;54(11):849-855.
24. Gopal PK, Prasad J, Gill HS. Effects of consumption of *Bifidobacterium lactis* HN019 (HN019TM) and galacto-oligosaccharides on the gastrointestinal tract in human subjects. *Nutr Res.* 2003;23:1313-1328.
25. Gill HS, Rutherford KJ, Cross ML, Gopal PK. Enhancement of immunity in the elderly by dietary supplementation with the probiotic *Bifidobacterium lactis* HN019. *Am J Clin Nutr.* Dec 2001;74(6):833-839.
26. Gill HS, Rutherford KJ, Cross ML. Dietary probiotic supplementation enhances natural killer cell activity in the elderly: an investigation of age-related immunological changes. *J Clin Immunol.* Jul 2001;21(4):264-271.
27. Sitek D, Kelly R, Wickens K, Stanley T, Fitzharris P, Crane J. Is the effect of probiotics on atopic dermatitis confined to food sensitized children? *Clin Exp Allergy.* May 2006;36(5):629-633.
- 27a. Ahmed, M. Prasad, J. Gill, H. Stevenson, L. and Gopal, P. 2007. Impact of consumption of different levles of *Bifidobacterium lactis* HN019 on the intestinal microflora of elderly human subjects. *The Journal of Nutrition, Health & Aging.* Vol 11, Number 1: 26 - 31.
- 27b. Sazawal, S. Dhingra, U. Hiremath, G. Sarkar, A. Dhingra, P. Dutta, A. Verma, P. Menon, V. and Black, R. 2010. Prebiotic and probiotic fortified milk in prevention of morbidities among children:

community-based, randomized, double-blind, controlled trial. PLoS ONE. August volume 5, Issue 8, E12161: 1-8.

- 27c. Waller, P. Gopal, P. Leyer, G. Ouwehand, A. Reifer, C. Stewart, M. and Miller, L. 2011. Dose-response effect of *Bifidobacterium lactis* HN019 on whole gut transit time and functional gastrointestinal symptoms in adults. Scandinavian J. of Gastroenterology, Early Online: 1 - 8.
28. Wickens, K. Black, P., Stanley, T., Mitchell, E., P., Tannock, G., Purdie, G., and J. Crane, 2008. A differential effect of two probiotics in the prevention of eczema and atopy: a double-blind, randomized placebo-controlled trial. J. Allergy Clin Immun. 122(4): 788-794.
29. Bettler, J., D.K. Mitchell, and M.J. Kullen. 2006. Administration of *Bifidobacterium lactis* with fructo-oligosaccharide to toddlers is safe and results in transient colonization. Int. J. Probiotics and Prebiotics. Vol. 1, no. 3/4, pp. 193 - 202.
30. Fisberg, M., et al. 2002. Effect of oral nutritional supplementation with or without synbiotics on sickness and catch-up growth in preschool children. International Pediatrics. 17: 216-222.
- 30a. Leyer, G. Li, S. Mubasher, M. Reifer, C. and Ouwehand, A. 2009. Probiotic effects on cold and influenza-like symptom incidence and duration in children. Pediatrics. 124;e172-e179.
31. Schrezenmeir, J., et al. 2004. Benefits of oral supplementation with and without synbiotics in young children with acute bacterial infections. Clin. Ped. April: 239-249.
32. Bartosch, S., Woodmansey, E.J., Paterson, J.C.M., McMurdo, M.E.T. & Macfarlane, G.T. (2005). Microbiological effects of consuming a symbiotic containing *Bifidobacterium bifidum*, *Bifidobacterium lactis*, and oligofructose in elderly persons, determined by real-time polymerase chain reaction and counting of viable bacteria. Clinical Infectious Diseases. 40:28-37.
- 32a. Ouwehand, A. Nermes, M. Collado, M. Rautonen, N. Salminen, S. and Isolauri, E. 2009. Specific probiotics alleviate allergic rhinitis during the birch pollen season. World J Gastroenterol, July 14:15(26): 3261 - 3268.
- 32b. Paineau, D. Carcano, D. Leyer, G. Darquy, S. Alyanakian, M. Simoneau, G. Bergmann, J. Brassart, D. Bornet, F. and Ouwehand, A. 2008. Effects of seven potential probiotic strains on specific immune responses in health adults: a double-blind, randomized, controlled trial. FEMS Immunol Med Microbiol 53:107 - 113.
33. Engelbrektson, A.L., Korzenik, J.R., Pittler, A., Sanders, M.E., Klaenhammer, T.R. Leyer G., & C.L. Kitts. 2009. Probiotics to minimize the disruption of fecal microbiota in healthy subjects undergoing antibiotic therapy. Journal of Medicinal Microbiology; 58: 663 - 670.
- 33a. Ouwehand, A. Nurminen, P. Mäkiyuokko, H. and Rautonen, N. 2006. Effect of bifidobacterium lactis 420 on microbiota and immune function. Ital. J. Food Sci. N. 1, vol. 18.
- 33b. Klein, A. Friedrich, U. Vogelsang, H. and Jahreis, G. 2008. Lactobacillus acidophilus 74-2 and bifidobacterium animalis subsp lactis DGCC 420 modulated unspecific cellular immune response in healthy adults. European Journal of Clinical Nutrition 62,584-593.
- 33c. Roessler, A. Friedrich, U. Vogelsang, H. Bauer, A. Kaatz, M. Hipler, U. C. Schmidt, I. and Jahreis, G.

B. lactis GRAS NOTICE INFORMATION

2008. The immune system in healthy adults and patients with atopic dermatitis seems to be affected differently by a probiotic intervention. *Clinical and Experimental Allergy*, 38, 93-102.

- 33d. Lamiki, P. Tsuchiya, J. Pathak, S. Okura, R. Solimene, U. Jain, S. Kawakita, S. and Marotta, F. 2010. Probiotics in diverticular disease of the colon: an open label study. *J. Gastrointestin Liver Dis.* Mar;19(1):31-6.
34. GRAS Notice GRN0049.
<http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=49>
35. Anderson AD, McNaught CE, Jain PK, MacFie J. Randomised clinical trial of synbiotic therapy in elective surgical patients. *Gut.* Feb 2004;53(2):241-245.
36. Bakker-Zierikzee AM, Alles MS, Knol J, Kok FJ, Tolboom JJ, Bindels JG. Effects of infant formula containing a mixture of galacto- and fructo-oligosaccharides or viable *Bifidobacterium animalis* on the intestinal microflora during the first 4 months of life. *Br J Nutr.* Nov 2005;94(5):783-790.
37. Bakker-Zierikzee AM, Tol EA, Kroes H, Alles MS, Kok FJ, Bindels JG. Faecal SIgA secretion in infants fed on pre- or probiotic infant formula. *Pediatr Allergy Immunol.* Mar 2006;17(2):134-140.
38. Black F, Einarsson K, Lidbeck A, Orrhage K, Nord CE. Effect of lactic acid producing bacteria on the human intestinal microflora during ampicillin treatment. *Scand J Infect Dis.* 1991;23(2):247-254.
39. Chouraqui JP, Van Egroo LD, Fichot MC. Acidified milk formula supplemented with bifidobacterium lactis: impact on infant diarrhea in residential care settings. *J Pediatr Gastroenterol Nutr.* Mar 2004;38(3):288-292.
40. Christensen HR, Larsen CN, Kaestel P, et al. Immunomodulating potential of supplementation with probiotics: a dose-response study in healthy young adults. *FEMS Immunol Med Microbiol.* Aug 2006;47(3):380-390.
41. Fukushima Y, Kawata Y, Hara H, Terada A, Mitsuoka T. Effect of a probiotic formula on intestinal immunoglobulin A production in healthy children. *Int J Food Microbiol.* Jun 30 1998;42(1-2):39-44.
42. Hove H, Nordgaard-Andersen I, Mortensen PB. Effect of lactic acid bacteria on the intestinal production of lactate and short-chain fatty acids, and the absorption of lactose. *Am J Clin Nutr.* Jan 1994;59(1):74-79.
43. Isolauri E, Arvola T, Sutas Y, Moilanen E, Salminen S. Probiotics in the management of atopic eczema. *Clin Exp Allergy.* Nov 2000;30(11):1604-1610.
44. Jain PK, McNaught CE, Anderson AD, MacFie J, Mitchell CJ. Influence of synbiotic containing *Lactobacillus acidophilus* La5, *Bifidobacterium lactis* Bb 12, *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and oligofructose on gut barrier function and sepsis in critically ill patients: a randomised controlled trial. *Clin Nutr.* Aug 2004;23(4):467-475.
45. Kankaanpaa PE, Yang B, Kallio HP, Isolauri E, Salminen SJ. Influence of probiotic supplemented infant formula on composition of plasma lipids in atopic infants. *J Nutr Biochem.* Jun 2002;13(6):364-369.

46. Kirjavainen PV, Arvola T, Salminen SJ, Isolauri E. Aberrant composition of gut microbiota of allergic infants: a target of bifidobacterial therapy at weaning? *Gut*. Jul 2002;51(1):51-55.
47. Laake KO, Bjorneklett A, Aamodt G, et al. Outcome of four weeks' intervention with probiotics on symptoms and endoscopic appearance after surgical reconstruction with a J-configured ileal-pouch-anal-anastomosis in ulcerative colitis. *Scand J Gastroenterol*. Jan 2005;40(1):43-51.
48. Laake KO, Line PD, Aabakken L, et al. Assessment of mucosal inflammation and circulation in response to probiotics in patients operated with ileal pouch anal anastomosis for ulcerative colitis. *Scand J Gastroenterol*. Apr 2003;38(4):409-414.
49. Laake KO, Line PD, Grzyb K, et al. Assessment of mucosal inflammation and blood flow in response to four weeks' intervention with probiotics in patients operated with a J-configured ileal-pouch-anal-anastomosis (IPAA). *Scand J Gastroenterol*. Dec 2004;39(12):1228-1235.
50. Langhendries JP, Detry J, Van Hees J, et al. Effect of a fermented infant formula containing viable bifidobacteria on the fecal flora composition and pH of healthy full-term infants. *J Pediatr Gastroenterol Nutr*. Aug 1995;21(2):177-181.
51. Larsen CN, Nielsen S, Kaestel P, et al. Dose-response study of probiotic bacteria *Bifidobacterium animalis* subsp *lactis* BB-12 and *Lactobacillus paracasei* subsp *paracasei* CRL-341 in healthy young adults. *Eur J Clin Nutr*. Nov 2006;60(11):1284-1293.
52. Link-Amster H, Rochat F, Saudan KY, Mignot O, Aeschlimann JM. Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunol Med Microbiol*. Nov 1994;10(1):55-63.
53. Malinen E, Matto J, Salmitie M, Alander M, Saarela M, Palva A. PCR-ELISA II: Analysis of *Bifidobacterium* populations in human faecal samples from a consumption trial with *Bifidobacterium lactis* Bb-12 and a galacto-oligosaccharide preparation. *Syst Appl Microbiol*. Aug 2002;25(2):249-258.
54. Nopchinda S, Varavithya W, Phuapradit P, et al. Effect of *Bifidobacterium* Bb12 with or without *Streptococcus thermophilus* supplemented formula on nutritional status. *J Med Assoc Thai*. Nov 2002;85 Suppl 4:S1225-1231.
55. Nord CE, Lidbeck A, Orrhage K, Sjostedt S. Oral supplementation with lactic acid-producing bacteria during intake of clindamycin. *Clin Microbiol Infect*. Feb 1997;3(1):124-132.
56. Ouwehand AC, Kurvinen T, Rissanen P. Use of a probiotic *Bifidobacterium* in a dry food matrix, an in vivo study. *Int J Food Microbiol*. Aug 15 2004;95(1):103-106.
57. Rautava S, Arvilommi H, Isolauri E. Specific probiotics in enhancing maturation of IgA responses in formula-fed infants. *Pediatr Res*. Aug 2006;60(2):221-224.
58. Rinne MM, Gueimonde M, Kalliomaki M, Hoppu U, Salminen SJ, Isolauri E. Similar bifidogenic effects of prebiotic-supplemented partially hydrolyzed infant formula and breastfeeding on infant gut microbiota. *FEMS Immunol Med Microbiol*. Jan 1 2005;43(1):59-65.
59. Saarela M, Maukonen J, von Wright A, et al. Tetracycline susceptibility of the ingested *Lactobacillus B. lactis*

acidophilus LaCH-5 and *Bifidobacterium animalis* subsp. *lactis* Bb-12 strains during antibiotic/probiotic intervention. *Int J Antimicrob Agents*. Mar 2007;29(3):271-280.

60. Saavedra JM, Bauman NA, Oung I, Perman JA, Yolken RH. Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. *Lancet*. Oct 15 1994;344(8929):1046-1049.
61. Satokari RM, Vaughan EE, Akkermans AD, Saarela M, De Vos WM. Polymerase chain reaction and denaturing gradient gel electrophoresis monitoring of fecal bifidobacterium populations in a prebiotic and probiotic feeding trial. *Syst Appl Microbiol*. Jul 2001;24(2):227-231.
62. Schiffrin EJ, Rochat F, Link-Amster H, Aeschlimann JM, Donnet-Hughes A. Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. *J Dairy Sci*. Mar 1995;78(3):491-497.
63. Sheu BS, Cheng HC, Kao AW, et al. Pretreatment with *Lactobacillus*- and *Bifidobacterium*-containing yogurt can improve the efficacy of quadruple therapy in eradicating residual *Helicobacter pylori* infection after failed triple therapy. *Am J Clin Nutr*. Apr 2006;83(4):864-869.
64. Sullivan A, Barkholt L, Nord CE. *Lactobacillus acidophilus*, *Bifidobacterium lactis* and *Lactobacillus* F19 prevent antibiotic-associated ecological disturbances of *Bacteroides fragilis* in the intestine. *J Antimicrob Chemother*. Aug 2003;52(2):308-311.
65. Weizman Z, Alsheikh A. Safety and tolerance of a probiotic formula in early infancy comparing two probiotic agents: a pilot study. *J Am Coll Nutr*. Oct 2006;25(5):415-419.
66. Weizman Z, Asli G, Alsheikh A. Effect of a probiotic infant formula on infections in child care centers: comparison of two probiotic agents. *Pediatrics*. Jan 2005;115(1):5-9.
67. Wenus C, Goll R, Loken EB, Biong AS, Halvorsen DS, Florholmen J. Prevention of antibiotic-associated diarrhoea by a fermented probiotic milk drink. *Eur J Clin Nutr*. Mar 14 2007.
68. Wildt S, Munck LK, Vinter-Jensen L, et al. Probiotic treatment of collagenous colitis: a randomized, double-blind, placebo-controlled trial with *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis*. *Inflamm Bowel Dis*. May 2006;12(5):395-401.

***Bifidobacterium animalis* ssp. *lactis*--GRAS Notice Appendix**

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**Expert Panel Report on the Generally Recognized as Safe Status of the Proposed Uses
of *Bifidobacterium animalis* subsp. *lactis***

Introduction

Danisco proposes to utilize specific isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*), including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* Bl-04 and *B. lactis* B420 in a variety of foods that have not historically contained the organisms including ready-to-eat breakfast cereals; bars; cheese, milk drinks, and milk products; bottled water and teas; fruit juices, fruit nectars, fruit “ades”, and fruit drinks; chewing gum; and confections.

In making this determination, Danisco critically reviewed (1) the safe history of use of *Bifidobacterium* in food; (2) the safe history of use of *B. lactis* isolates in food; (3) the safety of use of *B. lactis* isolates in clinical trials; and (4) strain safety testing.

Danisco convened an Expert Panel (“The Panel”) of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients and foods, to conduct an independent, critical and comprehensive evaluation of the available information on the safety of *B. animalis* ssp. *lactis* and the four specific isolates, and to determine whether the proposed uses of the isolates are safe and suitable, and are Generally Recognized as Safe (GRAS) based on scientific procedures. The members of the Expert Panel included Professor Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), Walter H. Glinsmann, M.D. (Glinsmann Associates), and Professor Michael W. Pariza (University of Wisconsin-Madison). *Curricula vitae* of the members of the Expert Panel member are included in Appendix A.

The Expert Panel, independently and collectively, critically evaluated a supporting GRAS dossier (**GRAS Dossier, *Bifidobacterium animalis* subsp. *lactis*; August 15, 2012**) submitted by Danisco, which included a description of *B. lactis* HN019, Bi-07, Bl-04, and B420; details of the manufacturing process and product specifications; history of use in foods; intended uses and use levels; exposures; safety testing; safety assessment; bibliography and appendix. The Expert Panel also considered other materials deemed appropriate or necessary.

Following its independent and collective critical evaluation of the available information, the Expert Panel unanimously concluded “the proposed uses presented in the dossier of specific isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*) including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* Bl-04 and *B. lactis* B420, produced consistent with cGMP and meeting appropriate food grade specifications presented in the dossier, are safe and “Generally Recognized as Safe” (“GRAS”) based on scientific procedures corroborated by a long history of safe use.”

A summary of the basis for the conclusions of the Expert Panel is presented below.



Description of *Bifidobacterium animalis* subsp. *lactis* HN019, Bi-07, BI-04 and B420.

Bifidobacteria are Gram-positive, non-spore forming, anaerobic, pleomorphic bacilli, and the dominant microbial residents of the colonic microbiota. The *Bifidobacterium* group does not contain species that are considered pathogenic to man. All *Bifidobacterium* species are listed as Biosafety Level 1 organisms by the American Type Culture Collection, indicating that they are not known to cause disease in healthy human adults. Because of the recent changes in classification within the *Bifidobacterium* group, *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* BI-04, and *B. lactis* B420 have been genetically characterized and properly classified as *B. animalis* subsp. *lactis* using modern genotypic methods including 16S rRNA gene sequencing, PCR using species-specific primers, and optical mapping, as well as their demonstrated ability to grow in milk.

The four *B. lactis* strains are genetically very similar, but not identical. The minor genetic differences appear to result in minor functional differences. For example, the four strains display different stability profiles in a 60-day storage test in fermented yogurt. Such functional and phenotypic differences lead customers to prefer one strain over another, depending on application.

Manufacturing Process

HN019, Bi-07, BI-04, and B420 are manufactured in accordance with the U.S. Food & Drug Administration's current Good Manufacturing Practices guidelines in an FDA regulated and inspected facility. A summary of the manufacturing process is presented below.

(Process Controls)	Manufacturing Process Step	(Confirmation)
	Approved Mother Culture	(QC Testing)
	↓	
(Sterilization, GMPs)	Fermentation Medium	
	↓	
(HACCP, GMPs)	Culture Fermentation	(QC Testing)
	↓	
(HACCP, GMPs)	Culture Concentration	
	↓	
(HACCP, GMPs)	Culture Lyophilization	(QC Testing)
	↓	
(HACCP, GMPs)	Culture Milling	(QC Testing)
	↓	
(HACCP, GMPs)	Metal Detection	(Standards Testing)
	↓	
(HACCP, GMPs)	Culture Packaging	
	↓	
	Release and Storage	(QC Testing)

All ingredients are food grade or approved for use by the U.S. FDA.

Batch analyses demonstrate reproducibility of the manufacturing process (compliance with specifications).

Stability testing of freeze-dried samples (in sachets) from production lots of HN019, Bi-07, BI-04 and B420 demonstrate that the test substances are stable under experimental conditions (4° C



and 25° C) for up to 24 months. Freeze-dried probiotic cultures not metabolically active and are not affected by pH.

History of Use in Food

Bifidobacterium species have a long history of safe use when consumed as part of dairy food and supplement products. There are eight (8) species (*longum*, *infantis*, *breve*, *bifidum*, *adolescentis* and *animalis* subspecies *lactis* and *animalis*) listed in IDF Bulletin No. 377: *Inventory of Microorganisms with a Documented History of Use in Food*. No cases of clinical infection have been reported from such use.

Bifidobacterium lactis has been added to human food since at least 1980 and is very common in dairy products worldwide, including in the US where the organism is the most common *Bifidobacterium* in yogurt products. In particular, *B. lactis* HN019 has been safely added to foods globally in dairy products and dietary supplements for at least 5 years, *B. lactis* Bi-07 and *B. lactis* Bi-04 for at least 15 years, and *B. lactis* B420 for more than 20 years, all without a report of adverse health effects on consumers.

Although on rare occasions *Bifidobacterium* has been associated with some cases of clinical infection, Boriello, et al (2003) reported, following a critical and extensive review of the literature, that “current evidence suggests that the risk of infection with probiotic lactobacilli or bifidobacteria is similar to that of infection with commensal strains, and that the consumption of such products presents a negligible risk to consumers, including immunocompromised hosts.”

Intended Uses/Use Levels

Intended uses are limited to those foods that can sustain living *B. lactis* for the shelf life of the food and may include ready-to-eat breakfast cereals; bars; cheeses, milk drinks, and milk products; bottled water and teas; fruit juices, fruit nectars, fruit “ades”, and fruit drinks; chewing gum; and confections. Danisco estimates that relatively few foods and beverages within each category will be developed with *B. lactis* because these cultures have a relatively high cost. Danisco also believes it is reasonable to assume that consumption will be for the express purpose of ingesting the proper amount of the organisms to achieve the claimed benefit – generally in a single serving per day.

Foods will be targeted to typically contain a minimum of 5×10^9 cfu/serving of *B. lactis*, a concentration reported in the published literature to promote gut and immune health. Delivery of the proper dose is then dependent on storage temperature and shelf life. A proposed use level of up to about 2×10^{11} cfu/serving may be utilized for products likely to experience a decrease in cell count during the shelf life of the food (e.g., fruit juices with a low pH).

Exposure

Bifidobacterium lactis strains are intended to be added to a variety of foods at concentrations needed to provide at least 5×10^9 cfu/250 g serving throughout the shelf life of the product. The



initial addition level may be as high as 2×10^{11} cfu/250 g serving in order to insure at least 5×10^9 cfu/250 g serving remains over the product shelf life.

In attempting to assess exposure, it is noted that there will be limited types of foods available containing the strain and consumers are very likely only to consume these food to achieve the daily benefit of products containing *B. lactis*. Foods containing *B. lactis* will not be competing with other functional foods or beverages because of cost or specific health benefits of *B. lactis* strains. Based on these assumptions, consumers will most probably consume a single 250 g serving to achieve the benefit thus ingesting approximately 2×10^{11} cfu per day.

Although *B. lactis* is normally present and growing within the human gastrointestinal tract, it is extremely difficult to quantify the amount present. Therefore, it is not possible to determine the potential effect on the body burden of *B. lactis* following ingestion of 2×10^{11} cfu/250 g serving/day.

Safety Testing

Regulatory status of *B. lactis*

Species of the genus *Bifidobacterium* are considered to be non-pathogenic, non-toxicogenic and are considered safe for use in foods (EFSA, Appendix A). FDA, in GRAS Notice No. GRN 000049, had no questions to the assertion that *B. Lactis* strain Bb12 was safe for use in certain milk-based infant formula. Boriello, et al. (2003) reviewed data pertinent to safety concerns for these bacteria and concluded that “current evidence suggests that the risk of infection with probiotic lactobacilli or bifidobacteria is similar to that of infection with commensal strains, and that consumption of such products presents a negligible risk to consumers...”. Other publications support this opinion (e.g., Glasser, 1994 and Vankerckhoven, et al, 2008). *B. lactis* is proposed for inclusion on the EU QPS list (Appendix).

Animal Studies

A comprehensive search of the scientific literature failed to identify classical/standard toxicity tests in animals for *B. lactis* probably because these tests are not appropriate for microorganisms. Strain *B. lactis* HN019 was assessed in several studies using *in vitro* and mouse model systems for traits important to safety and tolerance including effects in conventional mice (BALBc), immunodeficient mice (e.g., candidiasis model), and in an autoimmune thyroiditis model. Wagner, et al (1997) concluded from their mouse studies that “*L. acidophilus* and *B. animalis* appear to be innocuous probiotics in immunodeficient mice. Overall, probiotic bacteria are likely to be safe for immunocompetent and immunodeficient adults but they should be tested for immunodeficient neonates.”

Human Studies

A large number of human studies were analyzed and tabulated in the dossier. A few examples are presented below. It may be concluded from these studies that infants, children, adults, and elderly adults can safely tolerate *Bifidobacteria* species at doses up to 6×10^{11} cfu/day for up to two years.



Strain *B. lactis* HN019 was tested in healthy and ill infants, children and adults at doses from 1.9×10^7 to 3×10^{11} cfu/day for periods of 7 days to two years with no adverse effects reported. Strain *B. lactis* Bi-07 was tested for safety in toddler formulas for 28 days at a level of $5e8$ - $1e9$ CFU/day and no adverse effects were reported. A mixture of *B. lactis* Bi-07 and *L. acidophilus* (1×10^{10} cfu/day) was fed to 112 healthy children, 3-5 years of age, daily for 6 months, and the authors concluded that "Daily probiotic supplementation during the winter months was a safe and effective way to reduce episodes of fever, rhinorrhea, and cough, the cumulative duration of those symptoms, the incidence of antibiotic prescriptions and the number of school days attributed to illness."

Antibiotic Resistance

Because there is increasing concern of antibiotic resistance in pathogenic micororganisms, *B. lactis* HN019 and *B. lactis* B420 were tested for antibiotic resistance and for plasmids that might play a role in the transmission of such resistance to pathogenic organisms. HN019 was reported to be resistant to gram-negative specific antibiotics and to lack plasmids that could be implicated in transmission of antibiotic resistance. *B. lactis* was reported to be resistant to tetracycline only. On the issue of antibiotic resistance, it was concluded that "the four strains contained in this document [dossier] are sensitive to clinically relevant antibiotics, and given the widespread distribution of tetracycline genes in nature, the fact that *B. animalis* subsp. *lactis* strains in this document also contain the *tetW* gene is neither clinically or environmentally relevant and does not provide a scientific basis for revising the position that they are safe to consume."

Safety Assessment

Data from clinical trials and animal studies demonstrate the safety of *Bifidobacteria* in dairy foods and dietary supplements. The highest doses tested failed to induce significant toxicity and may be considered the No Observable Adverse Effect Levels (NOAELs). For example, a No Observable Adverse Effect Level (NOAEL) of 2.5×10^{12} cfu/kg body weight /day has been reported in mouse studies (this was the highest dose tested). The Expert Panel recognizes that this represents a lowest case estimate of the true NOAEL, because the organisms may proliferate in the GI tract after ingestion.

Lowest Observable Adverse Effect Levels (LOAELs) have never been reported for members of the *Bifidobacterium* group in human or animal studies, including the studies summarized above where a dose of 2.5×10^{12} cfu/kg body weight /day dose of HN019 was fed to mice (equivalent to a dose of 1.5×10^{14} cfu/day for a 60 kg human).

Human exposure will be limited to those foods that will provide the beneficial effects sought by consumers. Food types may be limited by the cost of including these strains and by the nature of the food. Based on these assumptions, consumers will most probably consume a single 250 g serving to achieve the benefit, thus ingesting approximately 2×10^{11} cfu per day.



Conclusion

We, the Expert Panel, have individually and collectively critically evaluated the information concerning specific isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*) including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* B1-04 and *B. lactis* B420 summarized in the dossier and other information deemed appropriate and we unanimously conclude that the proposed uses presented in the dossier of specific isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*) including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* B1-04 and *B. lactis* B420, produced consistent with cGMP and meeting appropriate food grade specifications presented in the dossier, are safe (i.e., meets the standard of reasonable certainty of no harm) and suitable.

We further unanimously conclude that the proposed uses presented in the dossier of specific isolates of *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*) including *B. lactis* HN019, *B. lactis* Bi-07, *B. lactis* B1-04 and *B. lactis* B420, produced consistent with cGMP and meeting appropriate food grade specifications presented in the dossier, are safe and "Generally Recognized as Safe" ("GRAS") based on scientific procedures corroborated by a long history of safe use.

It is our opinion that other experts qualified to assess the safety of food and food ingredients would concur with these conclusions.

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Summary of Human Studies	Type of Study	Strain(s) Studied	Description and number of subjects	Delivery Format	Size of Dose	Conclusions
Arunachalam, K. et al. Eur J Clin Nutr. Mar 2000;54(3):263-267.	Randomized, double-blind, placebo-controlled	HN001	50 children, 1-10 years of age	Reconstituted in water	10 ¹⁰ cfu/day, 4-week consumption	Reduction in incidence of acute diarrhea
Chiang, B.L. et al. Eur J Clin Nutr. Nov 2000;54(11):849-855.	Randomized, double-blind, placebo-controlled	HN001	100 children, 1-10 years of age	Reconstituted in water	5x10 ¹⁰ cfu/day, 4-week consumption	Reduction in incidence of acute diarrhea
Gopal, P.K. et al. Nutr Res. 2003;23:1313-1328.	Randomized, double-blind, placebo-controlled	HN001	100 children, 1-10 years of age	Reconstituted in water	5x10 ¹⁰ cfu/day, 4-week consumption	Reduction in incidence of acute diarrhea
Gill, H.S. et al. Am J Clin Nutr. Dec 2001;74(6):833-839.	Randomized, double-blind, placebo-controlled	HN001	50 children, 1-10 years of age	Reconstituted in water	5x10 ¹⁰ cfu/day and 5x10 ¹⁰ cfu/day, 4-week consumption	Reduction in incidence of acute diarrhea
Gill, H.S. et al. J Clin Immunol. Jul 2001;21(4):264-271.	Randomized, double-blind, placebo-controlled	HN001, HN002	50 children, 1-10 years of age	Reconstituted in water	5x10 ¹⁰ cfu/day (HN001) or 5x10 ¹⁰ cfu/day (HN002), 4-week consumption	Reduction in incidence of acute diarrhea
Sistek, D. et al. Clin Exp Allergy. May 2006;36(5):629-633.	Randomized, double-blind, placebo-controlled	HN001, HN002	50 children, 1-10 years of age	Probiotic powder in a sachet mixed in a drink or food at appointment	2x10 ¹⁰ cfu/day (1x10 ¹⁰ HN001 and 1x10 ¹⁰ HN002), 12-week consumption	Reduction in SCORAD scores, improvement in atopic dermatitis in food sensitive children
Ahmed, M. et al. The Journal of Nutrition, Health & Aging. 2007; Vol 11, Number 1: 26 - 31.	Randomized, double-blind, placebo-controlled	HN001	50 children, 1-10 years of age	Reconstituted in water	5x10 ¹⁰ cfu/day or 1x10 ¹⁰ cfu/day or 5x10 ¹⁰ cfu/day, 4-week consumption	All dose cohorts achieved bifidobacteria levels. Other positive microbial changes were noted
Sazawal, S. et al. PLoS ONE. August 2010; volume 5, Issue 8, E12161: 1-8.	Randomized, double-blind, placebo-controlled	HN001	600 children, 1-3 years of age	Reconstituted in water	10 ¹⁰ cfu/day plus 2.8x10 ⁸ CFU, 1 year consumption	Reduction of incidence and prevalence of dysentery, of days of severe illness, fever and middle ear infections. Also improved growth and iron status

	Type of Study	Strain(s) Studied	Description and number of subjects	Delivery Format	Size of Dose	Conclusions
Waller, P. et al. <i>Scandinavian J. of Gastroenterology</i> . 2011;Early Online: 1 - 8.	Randomized, controlled, double-blind, parallel study	BB-116	100 patients with irritable bowel syndrome (IBS) (50% female)	Tablet	0.7 billion CFU BB-116 in 1x10 ¹⁰ cfm daily consumption	Reduction in IBS symptoms, including abdominal discomfort, bloating, and constipation
Wickens, K. et al. <i>J. Allergy Clin Immunol</i> . 2008;122(4): 788-794.	Randomized, double-blind, controlled, parallel study	BB-116	100 patients with allergic rhinitis (AR) (50% female) (100% IBS) (50% female)	Probiotic capsules (BB-116) in 1x10 ¹⁰ cfm daily consumption	9.0819 ¹⁰ cfm of BB-116 in 1x10 ¹⁰ cfm daily (100% IBS) (2 years)	Significant improvement effect on the development of allergic rhinitis symptoms in IBS patients
Bettler, J. et al. <i>Int. J. Probiotics and Prebiotics</i> . 2006;Vol. 1, no. 3/4, pp. 193 - 202.	Randomized, double-blind, controlled, parallel study	BB-116	100 patients with IBS (50% female)	Tablet (BB-116) in 1x10 ¹⁰ cfm daily consumption	1x10 ¹⁰ cfm of BB-116 in 1x10 ¹⁰ cfm daily (28 day consumption)	No significant effect on weight, comfort, or well-being and apparent in the study (e.g., assessed by body weights)
Fisberg, M. et al. <i>International Pediatrics</i> . 2002;17: 216-222.	Randomized, double-blind, controlled study	BB-116 and BB-116 + FOS	60 children (age 6-12 years) (50% female) per BB-116 and BB-116 + FOS	Tablet	0.5L FOS + BB-116 and 1L acidophilus BB-116 in 1x10 ¹⁰ cfm (375 + 750 mg) (4-month consumption)	Enhancement of immune function
Leyer, G. et al. <i>Pediatrics</i> . 2009;124:e172-e179.	Double-blind, placebo-controlled study	NCFM, NCFM and BB-07	326 healthy children, 3-5 years of age	Powder mixed into 1% fruit drink	1x10 ¹⁰ cfm day (NCFM) or 1x10 ¹⁰ cfm day (NCFM BB-07 blend)	Enhancement of immune function
Schrezenmeir, J. et al. <i>Clin. Ped.</i> April 2004; 239-249.	Phase II, multi-center, open, randomized, comparative study	FOS, Lactobacillus and Bifidobacterium	146 healthy (0 German) children receiving an antibiotic therapy, 1-6 years of age	Formula	3.5g L-FOS and 1x10 ⁹ cfm L. acidophilus and Bifidum, 300 + 180 AU/day, 7-day consumption	Well tolerated
Bartosch, S. et al. <i>Clinical Infectious Diseases</i> . 2005;40:28-37.	Randomized, double-blind, controlled study	BB-01, BB-01 and one bifidobacterium	18 healthy elderly subjects, 60-90 years of age	Powder mixed into a cold drink and capsule	7x10 ¹⁰ cfm BB-01, 7x10 ¹⁰ cfm BB-01 and one bifidobacterium per day, 3-week consumption	Modification of fecal Bifidobacterial communities
Ouwehand, A. et al. <i>World J Gastroenterol</i> . July 2009;14:15(26): 3261 - 3268.	Randomized, double-blind, placebo-controlled study	NCFM and BB-01	17 children with birch pollen allergy, 4-13 years of age	Capsule	5x10 ¹⁰ cfm day (blend 125% NCFM, 75% BB-01), 4-month consumption	Positively impact markers of respiratory allergies and trend for reduced nasal symptoms

	Type of Study	Strain(s) Studied	Description and number of subjects	Delivery Format	Size of Dose	Conclusions
Paineau, D. et al. FEMS Immunol Med Microbiol 2008;53:107 - 113.	Randomized, placebo-controlled, double-blind study	<i>Lactobacillus casei</i> ATCC 334 (Lc334)	80 healthy adults (20-70 years)	Yogurt	2.5 x 10 ¹⁰ CFU per 100 ml	Increased the number of <i>Lactobacillus</i> spp. in the gut
Engelbrektson, A.L. et al. Journal of Medicinal Microbiology: 2009;58: 663 - 670.	Randomized, placebo-controlled, double-blind study	<i>Lactobacillus plantarum</i> 299 (Lp299)	100 patients with IBS (40-70 years)	Yogurt	1.5 x 10 ¹⁰ CFU per 100 ml	Improved IBS symptoms, increased the number of beneficial bacteria in the gut, decreased the number of pathogenic bacteria
Ouweland, A. et al. Ital. J. Food Sci. 2006;N. 1, vol. 18.	Cross-over study	B420 and <i>Lactobacillus</i>	Healthy adults (20-40 years of age)	Sweet	1.5 x 10 ¹⁰ CFU per 100 ml, 3-week consumption	Increase the metabolic activity of the intestinal bacteria
Klein, A. et al. European Journal of Clinical Nutrition 2008;62:584-593.	Placebo-controlled, randomized, crossover study	<i>Lactobacillus</i> 74-2 and B420	20 healthy adults (22-44 years of age)	Yogurt containing probiotic bacteria	3x10 ¹⁰ CFU B420 and 5x10 ¹⁰ CFU 74-2/100 g yogurt drink, 3-week consumption	Modulation of intestinal immune cell populations is linked to increased phagocytic activity
Roessler, A. et al. Clinical and Experimental Allergy, 2008;38: 93-102.	Double-blind, placebo-controlled, randomized crossover study	<i>L. acidophilus</i> 74-2, B420, and Lpc-37	15 healthy adults, 21-27 years of age, and 15 patients with atopic dermatitis, 20-26 years of age	Probiotic yogurt drink	3.9x10 ¹⁰ cfu/g Lpc-37, 2.9x10 ¹⁰ cfu/g 74-2, and 5.9x10 ¹⁰ cfu/g B420, 200 ml/day, 8-week consumption	Possible therapy of mild to moderate atopic dermatitis, with possible prevention of acute flare-ups
Lamiki, P. et al. J. Gastrointestin Liver Dis. Mar2010;19(1):31-6.	Randomized, open-label study	<i>L. acidophilus</i> 115, 1, <i>Helveticus</i> , and B420	45 patients previously affected by symptomatic uncomplicated diverticular disease of the colon, 39-77 years of age	Probiotic supplement (Nicolforma E)	1.25x10 ¹⁰ cfu/g L115, 1.3x10 ¹⁰ cfu/g 1, 9.5x10 ⁹ cfu/g B420 per 100 ml, 30 ml, per day	Effective in preventing recurrences of symptomatic uncomplicated diverticular disease of the colon

	Type of Study	Strain(s) Studied	Description and number of subjects	Delivery Format	Size of Dose	Conclusions
Anderson, A.D. et al. Gut. Feb 2004;53(2):241-245.	Randomized, double-blind, placebo-controlled study	Bifidobacterium infantis 35619	20 healthy adults, 20-40 years of age	Reconstituted formula	10 ¹⁰ c.f.u./day, 10 ¹⁰ c.f.u./day	Significantly increased immune response to oral supplements were noted
Bakker-Zierikzee, A.M. et al. Br J Nutr. Nov 2005;94(5):783-790.	Randomized, double-blind, placebo-controlled study	Bifidobacterium infantis 35619	20 healthy adults, 20-40 years of age	Reconstituted formula	10 ¹⁰ c.f.u./day, 10 ¹⁰ c.f.u./day	All topics will accept oral probiotic formulae, but have activity with oral probiotic
Bakker-Zierikzee, A.M. et al. Pediatr Allergy Immunol. Mar 2006;17(2):134-140.	Randomized, double-blind, placebo-controlled study	Bifidobacterium infantis 35619	20 healthy adults, 20-40 years of age	Reconstituted formula	10 ¹⁰ c.f.u./day, 10 ¹⁰ c.f.u./day	All topics will accept oral probiotic formulae, but have activity with oral probiotic
Black, F. et al. Scand J Infect Dis. 1991;23(2):247-254.	Randomized, double-blind, placebo-controlled study	Bifidobacterium infantis 35619	20 healthy adults, 20-40 years of age	Reconstituted formula	10 ¹⁰ c.f.u./day, 10 ¹⁰ c.f.u./day	Partial restoration of intestinal microflora after antibiotic therapy
Chouraqui, J.P. et al. J Pediatr Gastroenterol Nutr. Mar 2004;38(3):288-292.	Multi-center, double-blind, placebo-controlled study	Bifidobacterium infantis 35619	90 healthy infants, 14-61 months of age	Reconstituted formula with probiotic bacteria added	1 x 10 ¹⁰ c.f.u./day	Protective impact on infantile acute gastroenteritis and nosocomial infections
Christensen, H.R. et al. FEMS Immunol Med Microbiol. Aug 2006;47(3):380-390.	Double-blind, placebo-controlled, parallel, cross-response study	Bifidobacterium infantis 35619 and Bifidobacterium lactis 35620	78 healthy adults, 18-40 years of age	Tablets	10 ¹⁰ c.f.u./day, 10 ¹⁰ c.f.u./day	No significant outcome
Fukushima, Y. et al. Int J Food Microbiol. Jun 30 1998;42(1-2):39-44.	no information	Bifidobacterium infantis 35619	7 healthy Japanese children, 15-31 months of age	Follow up formula containing probiotic bacteria	10 ¹⁰ c.f.u./day	Possible contribution to enhancement of mucosa resistance against gastrointestinal infections

	Type of Study	Strain(s) Studied	Description and number of subjects	Delivery Format	Size of Dose	Conclusions
Hove, H. et al. Am J Clin Nutr. Jan 1994;59(1):74-79.	Randomized, double-blind, placebo-controlled study	BB-1230	20 patients with allergic rhinitis	Formula	2x10 ¹⁰ c.f.u./day for 4 weeks	Decreased symptoms
Isolaari, E. et al. Clin Exp Allergy. Nov 2000;30(11):1604-1610.	Randomized, double-blind, placebo-controlled study	BB-1230	20 patients with allergic rhinitis	Formula containing probiotic bacteria	2x10 ¹⁰ c.f.u./day for 4 weeks	Modulation of the immune response
Jain, P.K. et al. Clin Nutr. Aug 2004;23(4):467-475.	Randomized, double-blind, placebo-controlled study	BB-1230	20 patients with allergic rhinitis	Caprylic and potassium oligoacrylate formula	1.2 x 10 ¹⁰ c.f.u./day and 15 g lactulose	Modulation of gut microbiota
Kankaanpaa, P.E. et al. J Nutr Biochem. Jun 2002;13(6):364-369.	Randomized, double-blind, placebo-controlled study	BB-1230	15 children with atopic eczema, 3-7 months of age	Formula containing probiotic bacteria	1.4 x 10 ¹⁰ BB-1230 or 3x10 ¹⁰ c.f.u./day lactulose consumption	Baseline physiological effects associated with physiological interventions with polyunsaturated fatty acid
Kirjavainen, P.V. et al. Gut. Jul 2002;51(1):51-55.	Randomized, double-blind study	BB-12	21 infants diagnosed with early-onset atopic eczema, 1-6 months of age	Formula containing probiotic bacteria	6-13x10 ¹⁰ c.f.u./kg bodyweight	Modification of gut microbiota
Laake, K.O. et al. Scand J Gastroenterol. Jan 2005;40(1):43-51.	No information	BB-1230	67 adult patients with ulcerative colitis (UC) or terminal ileitis (TI) or terminal ileitis with proctitis (IP)	Fermented milk product with probiotic bacteria	10 ¹⁰ c.f.u./g La-5 and 10 ¹⁰ c.f.u./g BB-12, 4-week consumption	Positive effect on symptoms and microscopic inflammation of UC and TI
Laake, K.O. et al. Scand J Gastroenterol. Apr 2003;38(4):409-414.	Open, non-randomized, interventional study	BB-1230	10 adult patients operated with ileal-pouch-anal anastomosis (IPAA) for ulcerative colitis (UC)	Fermented milk product with probiotic bacteria	10 ¹⁰ c.f.u./g La-5 and 10 ¹⁰ c.f.u./g BB-12, 4-week consumption	Change of gross appearance of mucosa

	Type of Study	Strain(s) Studied	Description and number of subjects	Delivery Format	Size of Dose	Conclusions
Laake, K.O. et al. Scand J Gastroenterol. Dec 2004;39(12):1228-1235.	Randomized, double-blind, placebo-controlled study	Bifidobacterium infantis	20 healthy adults, 10 in each group	Capsules	10 ¹⁰ CFU/day for 28 days	Significant effect on proinflammatory cytokines and markers of gut barrier
Langhendries, J.P. et al. J Pediatr Gastroenterol Nutr. Aug 1995;21(2):177-181.	Randomized, double-blind, placebo-controlled study	Bifidobacterium infantis	20 healthy adults	Yogurt containing 10 ¹⁰ CFU of B. infantis	10 ¹⁰ CFU of B. infantis in yogurt 2x daily for 2 weeks	Reduction of total bacterial concentration. Well tolerated
Larsen, C.N. et al. Eur J Clin Nutr. Nov 2006;60(11):1284-1293.	Randomized, double-blind, placebo-controlled, crossover study	Bifidobacterium infantis (Bif-1)	20 healthy adults, 10 in each group	Capsules	10 ¹⁰ CFU of Bif-1 in capsules 2x daily for 4 weeks	Increase in fecal consistency observed. Well tolerated
Link-Amster, H. et al. FEMS Immunol Med Microbiol. Nov 1994;10(1):55-63.	Randomized, double-blind, placebo-controlled study	Bifidobacterium infantis	No information	Fermented milk product with <i>Bifidobacterium infantis</i>	10 ¹⁰ CFU of Bif-1 in yogurt 2x daily for 4 weeks	Possible action as adjuvant to the humoral immune response
Malinen, E. et al. Syst Appl Microbiol. Aug 2002;25(2):249-258.	No information	Bif-12 and GOS	30 healthy adult subjects	Yogurt containing probiotic bacteria	2-week consumption	Suggests that Bif-12 is able to transiently replace <i>B. longum</i>
Nopchinda, S. et al. J Med Assoc Thai. Nov 2002;85 Suppl 4:S1225-1231.	Randomized, double-blind, placebo-controlled study	Bif-12 and <i>S. thermophilus</i>	118 children (0-36 months of age)	Formula containing probiotic bacteria	3x 10 ¹⁰ cfu g Bif-12, 3x10 ¹⁰ cfu g Bif-12 S12 (200 ml) for 3600 ml/day (6-month consumption)	Better growth
Nord, C.E. et al. Clin Microbiol Infect. Feb 1997;3(1):124-132.	Randomized, double-blind, placebo-controlled study	10 ⁵ Bif-12, 10 ⁵ Lys 27, and 10 ⁵ S12	23 healthy subjects, 21 (54 years of age)	Capsules	2x10 ¹⁰ CFU mixed strains daily (14-day consumption)	Faster recolonization of the intestine
Ouwehand, A. et al. Int J Food Microbiol. Aug 15 2004;95(1):103-106.	No information	Bif-12	10 healthy adults, 24-49 years of age	Cereal bar	5x10 ¹⁰ cfu bar 7-day consumption	New modes for delivery of probiotics

	Type of Study	Strain(s) Studied	Description and number of subjects	Delivery Format	Size of Dose	Conclusions
Rautava, S. et al. <i>Pediatr Res.</i> Aug 2006;60(2):221-224.	Randomized, double-blind, placebo-controlled trial	BB-1158	20 healthy infants	Preparation: sachet containing 10 ¹⁰ CFU bacteria	1 sachet daily for 14 days	Probiotic sachet preparation is safe and effective
Rinne, M.M. et al. <i>FEMS Immunol Med Microbiol.</i> Jan 1 2005;43(1):59-65.	Randomized, double-blind, placebo-controlled trial	BB-1158 and BB-1216	100 healthy 2-year-old children	Preparation: sachet containing 100 billion bacteria (BB-1158 and BB-1216)	1 sachet 3 times per day for 4 weeks	Early colonization can be modified by specific probiotics
Saarela, M. et al. <i>Int J Antimicrob Agents.</i> Mar 2007;29(3):271-280.	Randomized, double-blind, placebo-controlled trial	BB-1158	100 healthy 12-14 years	Preparation: sachet containing 10 ¹⁰ CFU bacteria	1 sachet 3 times per day for 4 weeks	Non-specific immunomodulatory effects may enhance the immune response
Saavedra, J.M. et al. <i>Lancet.</i> Oct 15 1994;344(8929):1046-1049.	Randomized, double-blind, placebo-controlled trial	BB-1216	15 infants, age 5-21 months	Preparation: sachet containing 10 ¹⁰ CFU bacteria	1 sachet 3 times per day for 8 weeks	Decrease in risk of diarrhea
Satokari, R.M. et al. <i>Syst Appl Microbiol.</i> Jul 2001;24(2):227-231.	Randomized, double-blind, placebo-controlled trial	BB-1216	40 healthy Finnish adults, mean age of 32 years	Preparation: sachet containing 10 ¹⁰ CFU probiotic bacteria	1 sachet 3 times per day for 8 weeks	Well-tolerated, BB-1216 transiently colonized the gut
Schiffrin, E.J. et al. <i>J Dairy Sci.</i> Mar 1995;78(3):491-497.	Randomized, double-blind, placebo-controlled trial	BB-1216	28 healthy adults, 23-62 years of age	Preparation: milk product containing probiotic bacteria	1 sachet 3 times per day for 4 weeks	Non-specific, anti-infective mechanisms of defense can be enhanced
Sheu, B.S. et al. <i>Am J Clin Nutr.</i> Apr 2006;83(4):864-869.	Randomized, double-blind, placebo-controlled trial	BB-1216 and BB-12	138 dyspeptic patients, mean age 47 years	Preparation: yogurt containing probiotic bacteria	4-week consumption	Significant decrease in CO ₂ ml values
Sullivan, A. et al. <i>J Antimicrob Chemother.</i> Aug 2003;52(2):308-311.	Randomized, double-blind, placebo-controlled trial	BB-1216 and BB-1216 blend	24 healthy adults, 21-48 years of age	Preparation: yogurt containing probiotic bacteria	10 ¹⁰ c.f.u./ml or 25% mixing blend 1 sachet daily consumption	Prevention of ecological disturbances in microbiota
Weizman, Z. et al. <i>J Am Coll Nutr.</i> Oct 2006;25(5):415-419.	Prospective, randomized, placebo-controlled trial	BB-1216 or BB-1216 blend	59 full-term, healthy infants, 3-65 days of age	Preparation: formula containing probiotic bacteria	2.2x10 ⁹ c.f.u./180 ml prepared formula BB-1216 or 2.2x10 ⁹ c.f.u./180 ml prepared formula, 2.2x10 ⁹ 4-week consumption	Safe, well tolerated, no adverse reactions or effects

Appendix A: *B. animalis* subsp. *lactis* species is on EFSA's QPS list

From QPS document, EFSA, Appendix A - Assessment of gram-positive non-sporulating bacteria *The EFSA Journal* (2007) 587, Qualified Presumption of Safety

<http://www.efsa.europa.eu/en/efsajournal/doc/587.pdf>

Bifidobacterium

Bifidobacteria are part of the normal gut microbiota of adults and are also one of the first genera to colonise the gut of infants. In addition, they are normal inhabitants of the gut of animals. A limited number of *Bifidobacterium* species have a history of use in dairy products, especially sour milk products like yoghurts and more recently yoghurt and fermented milk drinks.

Taxonomic unit defined

Bifidobacteria belong to the *Actinomycetes* branch of phylum Firmicutes. They are non-motile, non-sporeforming rods of variable appearance, usually curved and clubbed, and are often branched including Y and V forms. They are normally strictly anaerobic, although some species and strains tolerate oxygen. The type species is *Bifidobacterium bifidum*. Bifidobacteria are saccharolytic organisms and they have the ability to ferment glucose, galactose and fructose. Glucose is fermented via the fructose-6-phosphate shunt to acetic and lactic acid. Differences occur between species in their ability to ferment other carbohydrates and alcohols.

The genus consists currently of following species: *Bifidobacterium adolescentis*, *B. angulatum*, *B. animalis* subsp. *Animalis*, *B. animalis* subsp. *lactis*, *B. asteroides*, *B. bifidum*, *B. boum*, *B. breve*, *B. catenulatum*, *B. choerinum*, *B. coryneforme*, *B. cuniculi*, *B. dentium*, *B. gallicum*, *B. gallinarum*, *B. indicum*, *B. longum*, *B. magnum*, *B. merycicum*, *B. minimum*, *B. pseudocatenulatum*, *B. pseudolongum* subsp. *globosum*, *B. pseudolongum* subsp. *pseudolongum*, *B. psychraerophilum*, *B. pullorum*, *B. ruminantium*, *B. saeculare*, *B. scardovii*, *B. subtile*, *B. thermacidophilum* subsp. *porcinum*, *B. thermacidophilum* subsp. *thermacidophilum*, *B. thermophilum*.

Is the body of knowledge sufficient?

The characteristics and habitat of the species of the genus *Bifidobacterium* are well known. The number of established or proposed species has increased only slightly during recent years.

Only a few species have a long history of use in industrial applications. Bifidobacteria are mainly exploited in dairy products like yogurts or yogurt drinks, but also a whole range of sour milk and other milk based products. Occasionally they are also used in feed in combination with other genera. In Europe only a few species are used (*B. animalis*, *B. longum*, *B. breve*, *B. bifidum* and *B. adolescentis*.) and often applied in combination with lactic acid bacteria (Reuter 1990; Reuter 1997; Klein, Pack *et al.* 1998; Reuter 2002).

The genome sequences of *B. longum* (Schell, Karmirantzou *et al.* 2002) and *B. breve* have been determined, while the genome sequencing project of *B. adolescentis* is ongoing.

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Are there safety concerns?

Humans. Safety concerns are so far related mainly only to one species, *B. dentium*, which has been associated with dental caries. It has also been isolated from a case of peritonsillar abscess together with other anaerobes (Civen, Vaisanen *et al.* 1993) and, under its previous designation "*Actinomyces eriksonii*", from pulmonary and subcutaneous abscesses (Slack 1974). Occasionally, other species have been reported to be isolated from human clinical cases, but none of them was the primary cause of disease. Only immunocompromised hosts were infected (Crociani, Biavati *et al.* 1996). These species are not used as food or feed supplements. None of the bifidobacteria used for industrial purposes have been associated with human clinical disease.

Although there are few studies on the antibiotic resistance of bifidobacteria strains, the presence of the acquired tetracycline resistance gene *tet(W)* has been reported in *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium bifidum* (Kastner, Perreten *et al.* 2006; Masco, Van Hoorde *et al.* 2006).

Livestock. No report can be found on safety concerns related to *Bifidobacteria* in animals.

Can the safety concerns be excluded?

There are apparently no specific safety concerns regarding the genus *Bifidobacterium* (especially concerning *B. animalis*; *B. longum*, *B. breve*, *B. adolescentis*, and *B. bifidum*) with the exception of the species associated with dental caries, *B. dentium*. Susceptibility to antibiotics should be assessed as defined by the EFSA opinion (EFSA 2005) for each strain.

Units proposed for QPS status

Due to the long history of safe use of *B. adolescentis*, *B. animalis*; *B. longum*, *B. breve* and *B. bifidum*, these species are proposed for QPS status. Other species could be included subsequent to their industrial application with the exception of the species associated with dental caries (*B. dentium*).

QPS BIBLIOGRAPHY

- Apostolou, E., P. V. Kirjavainen, et al. (2001). "Good adhesion properties of probiotics: a potential risk for bacteremia?" *FEMS Immunol Med Microbiol* **31**(1): 35-9.
- Axelsson, L. T. (2004). Lactic acid bacteria: Classification and physiology. *Lactic Acid Bacteria. Microbiological and Functional Aspects*. S. Salminen, Ouwehand, A., von Wright, A. New York, Marcel Dekker Inc.: 1-66.
- Crociani, F., B. Biavati, et al. (1996). "Bifidobacterium inopinatum sp. nov. and Bifidobacterium denticolens sp. nov., two new species isolated from human dental caries." *Int J Syst Bacteriol* **46**(2): 564-71.
- EFSA (2005). "Opinion of the Scientific Committee on a request from EFSA related to a generic approach to the safety assessment by EFSA of microorganisms used in food/feed and the production of food/feed additives." *The EFSA Journal* **226**: 1-12.
- EFSA (2005). "Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human or veterinary importance." *The EFSA Journal* **223**: 1-12.

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- Gasser, F. 1994. Safety of lactic-acid bacteria and their occurrence in human clinical infections. *Bulletin de L'Institut Pasteur* 92:45-67.
- Kastner, S., V. Perreten, et al. (2006). "Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food." *Syst Appl Microbiol* 29(2): 145-55.
- Klein, G., A. Pack, et al. (1998). "Taxonomy and physiology of probiotic lactic acid bacteria." *Int J Food Microbiol* 41(2): 103-25.
- Martel, A., V. Meulenaere, et al. (2003). "Macrolide and lincosamide resistance in the gram-positive nasal and tonsillar flora of pigs." *Microb Drug Resist* 9(3): 293-7.
- Masco, L., K. Van Hoorde, et al. (2006). "Antimicrobial susceptibility of Bifidobacterium strains from humans, animals and probiotic products." *J Antimicrob Chemother* 58(1): 85-94.
- Morelli, L., Vogensen, F. and von Wright, A. (2004). Genetics of lactic acid bacteria. *Lactic Acid Bacteria. Microbiological and Functional Aspects*. S. Salminen, Ouwehand, A., von Wright, A. New York, Marcel Dekker Inc.: 249-293.
- Perreten, V., F. Schwarz, et al. (1997). "Antibiotic resistance spread in food." *Nature* 389(6653): 801-2.
- Reuter, G. (1990). "Bifidobacteria cultures as components of yoghurt-like products." *Bifidobacteria Microflora* 9: 107-118.
- Reuter, G. (1997). "Present and future of probiotics in Germany and central Europe." *Biosc. Microfl.* 16: 43-51.
- Reuter, G., Klein, G. and Goldberg, M. (2002). "Identification of probiotic cultures in food samples." *Food Research International* 35: 117-124.
- Ringó, E. (2004). Lactic acid bacteria in fish and in fish farming. *Lactic Acid Bacteria. Microbiological and Functional Aspects*. S. Salminen, Ouwehand, A., von Wright, A. New York, Marcel Dekker Inc.: 581-610.
- Schell, M. A., M. Karmirantzou, et al. (2002). "The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract." *Proc Natl Acad Sci U S A* 99(22): 14422-7.

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SUBMISSION END

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