JHeimbach LLC

April 3, 2019

#855

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

Dear Dr. Gaynor:

Pursuant to 21 CFR Part 170, Subpart E, Lallemand Health Solutions (Lallemand), through me as its agent, hereby provides notice of a claim that the addition to non-exempt milk-based term infant formula of *Bifidobacterium animalis* ssp. *lactis* strain R0421 is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because Lallemand has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

As required, one copy of the GRAS monograph and one signed copy of the statement of the Expert Panel are provided. Additionally, I have enclosed a virus-free CD-ROM with the GRAS monograph and the signed statement of the Expert Panel.

If you have any questions regarding this notification, please feel free to contact me at 804-742-5543 or jh@jheimbach.com.

Sincerely,

[redacted]

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

Encl.

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Generally Recognized as Safe (GRAS) Determination for the Use of *Bifidobacterium animalis* subsp. *lactis* R0421 (LAFTI® B94)

Prepared by:
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PART 1. SIGNED STATEMENTS AND CERTIFICATION

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1.1. GRAS Notice Submission

Lallemand Health Solutions of Mirabel, Québec, Canada (Lallemand) submits this GRAS notification through its agent James T. Heimbach, president of the consulting firm JHeimbach LLC, in accordance with the requirements of 21 CFR Part 170, Subpart E.

1.2. Name and Address of Notifier

Lallemand Health Solutions
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Tel (450) 433-9139

Notifier Contact:
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Agent Contact:
James T. Heimbach, Ph.D., F.A.C.N.
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1.3. Name of Notified Organism

The subject of this Generally Recognized as Safe (GRAS) notification is *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94), which is deposited at Centraalbureau voor Schimmel-cultures, Utrecht (Nederlands), under the identification number CBS118529.

1.4. Intended Conditions of Use

A powder of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94), is intended to be added to non-exempt powdered milk-based infant formula intended for healthy term infants. The intended addition level is $5x10^7$ cfu/g powder in formulas with hydration rates of 12.5-13.5 g/100 ml, resulting in an initial load of $5x10^9$ cfu/800 ml hydrated formula, designed to result in intake of at least $5x10^9$ cfu per day throughout the shelf life of the formula, allowing for some loss of viability.

1.5. Statutory Basis for GRAS Status

Lallemand Health Solution's GRAS determination for the intended use of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) is based on scientific procedures as described under 21 CFR §170.30(b).

1.6. Premarket Exempt Status

The intended use of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) is not subject to the premarket approval requirements of the Federal Food Drug and Cosmetic Act based on Lallemand's conclusion that such use is GRAS.

1.7. Availability of Information

The data and information that serve as the basis for the GRAS determination will be sent to the FDA upon request, or are available for the FDA's review and copying at reasonable times at the office of James T. Heimbach, Ph.D., President, JHeimbach LLC, 923 Water Street, P.O. Box 66, Port Royal, Virginia 22535, telephone 804-742-5543 and e-mail jh@jheimbach.com.

1.8. Freedom of Information Act Statement

None of the information in this GRAS notice is exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

1.9. Certification

To the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information as well as favorable information known to me

and pertinent to the evaluation of the safety and GRAS status of the intended use of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94).

1.10. FSIS Statement

Not applicable.

1.11. Name and Title of Signer

James T. Heimbach, Ph.D., F.A.C.N.
President
JHeimbach LLC
Agent to Lallemand Health Solutions

PART 2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND TECHNICAL EFFECT

Sections:

2.1. Name of the GRAS Organism

The subject of this GRAS notification is:

- *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94)

2.2. Source of the GRAS Organism

Bifidobacterium animalis subsp. *lactis* Rosell®-421 (LAFTI® B94) was isolated from a dairy source by DSM and was deposited by DSM at the Centraalbureau voor Schimmelcultures, Utretch, Netherlands, under the number CBS-118529. The strain has since been acquired by Lallemand Health Solutions.

2.3. Description of the GRAS Organism *Bifidobacterium animalis* subsp. *lactis* R0421 (LAFTI® B94)

Strains of *Bifidobacterium* are among the most important organisms in the human microbiome and among probiotics (O'Sullivan et al. 1992, Fuller and Gibson 1997). Probiotic *Bifidobacteria* have been used in food products for decades, with a compelling record of safe consumption (Reid 2002, Kocian et al. 1994, and Guidelines FAO/WHO 2002). The organism that is the subject of this GRAS notice is a thoroughly characterized strain belonging to the *Bifidobacterium* genus, and has been sold around the world for a number of years.

Bifidobacteria predominate in the intestinal tract shortly after birth. They are important and normal constituents of the human gastrointestinal microbiota and occur at concentrations of 10^9 to 10^{10} cells/g feces (Tanaka et al. 2000). *Bifidobacterium animalis* is a natural inhabitant of the intestinal tract microbiota and has been used for many years in fermented foods.

Bifidobacterium animalis was first described as a separate species by Scardovi and Trovatelli (Scardovi and Trovatelli 1974) after examining the feces of chickens, rats, and rabbits.

Bifidobacterium lactis was first described by Meile et al. (Meile et al. 1997) as a separate species from *B. animalis* due to increased aerobicity and differences in metabolic and genetic features. However, several years later, Masco et al. (2004) showed that the genetic homology was such that they were better regarded as two subspecies of of the same species.

B. animalis subsp. *lactis* R0421 (LAFTI® B94), which has been used for centuries in fermented foods, has been well studied and is classified as an acetobacter. LAFTI® B94 is a proprietary culture acquired by Lallemand Health Solutions in 2010 from the Dutch company DSM.

The International Dairy Federation (IDF), in collaboration with the European Food and Feed Cultures Association (EFFCA), assembled a list of microorganisms with a documented history of safe use in food (Bourdichon et al. 2012). *Bifidobacterium animalis* subsp. *lactis* is listed in this inventory. Since 2007, *Bifidobacterium animalis* has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA Journal 2017). A strain belonging to a species listed in QPS and meeting the established criteria can freely be used in foods in Europe.

In Canada, the *Natural Health Products Regulations* of 2004 classified probiotics under the definition of Natural Health Products. In its probiotics monograph, the Natural and Non-prescription Health Products Directorate (NNHPD) of Health Canada listed *Bifidobacterium animalis,* including its subspecies *B. animalis* subsp. *lactis,* as eligible to be used for the general support of gastrointestinal health (Probiotics Monograph, Health Canada, May 26, 2015; see Appendix I). The Food Directorate of Health Canada published a list of species eligible for generic structure/function claims in 2009 (Guidance document: The Use of Probiotic Microorganisms in Food, Health Canada, April 2009). This list includes *B. animalis* subsp. *lactis*.

The Australian Therapeutic Goods Administration (TGA) includes *Bifidobacterium animalis* subsp. *lactis* in the "List of approved substances that can be used as active ingredients in 'listed' medicines" (Appendix II).

B. animalis subsp. *lactis* is also included in the list of "Substances that may typically be considered to be a health supplement" in South Africa (Medicines Control Council, 2014). The Food Safety and Standards Authority of India has recognized *B. animalis* and added it to the List of Strains as Probiotics (Schedule –X of the Food safety and Standards regulation - No. 1-4/Nutraceutical/FSSAI-2013). In Korea, *B. animalis* subsp. *lactis* has been referenced in the Health Functional Food Code (2010), to be used in Health Functional Foods.

In China, *B. animalis* subsp. *lactis* is included in the positive list of strains to be used in foods/health foods (Appendix III).

2.3.1. Phenotypic Identification of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94)

2.3.1.1. Morphology

- Irregular V shaped or curved rods (see Figure 2).
- Non-motile.
- Non spore-forming.
- Cell size: 0.6 to 0.9 µm width x 2 to 4 µm length.
- Forms small white colonies on selective media (see Figure 1).

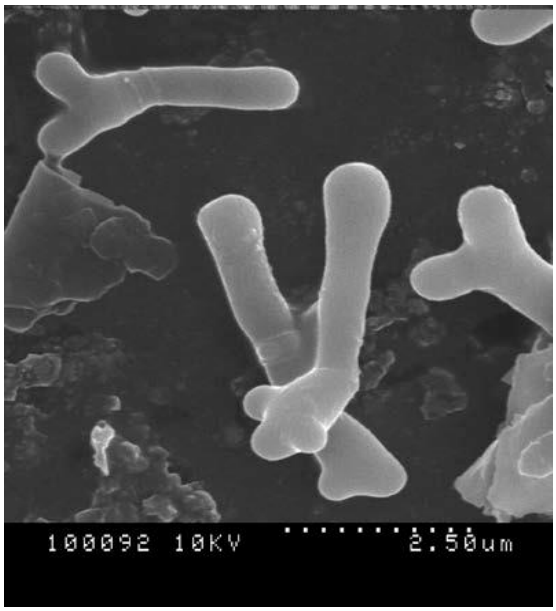

Figure 2. *Bifidobacterium animalis* subsp. *lactis* R0421 (LAFTI B94) (Magnification 15 000x) Scanning Electron Micrograph photo by Dr. A. Smith, U. of Guelph, (Ont), Canada.

Figure 3. Colonies of Strain R0421 on RCM Agar (Lallemand 2018).

2.3.1.2. Gram Stain Reaction

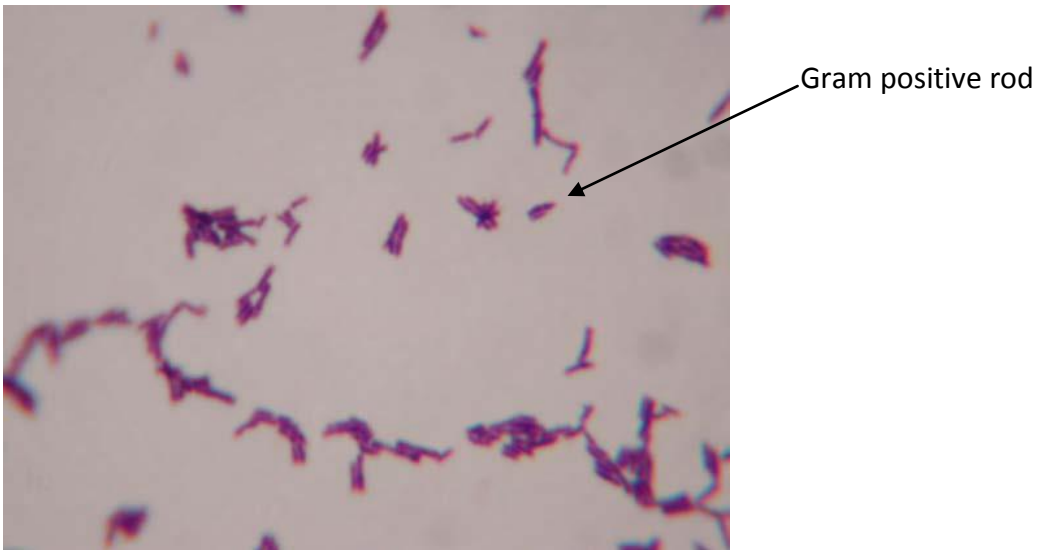

Figure 4. Microscopic Observation of Gram stained *Bifidobacterium animalis* subsp. *lactis* R0421 (Lallemand 2018).

2.3.1.3. Biochemical Testing

Fermentative metabolism	Obligately homofermentative Produces mainly lactic acid during fermentation Trace of acetic acid: 0.28 g/L
Gram Stain	+
Catalase (18-24 h colonies on RCM agar, 37°C, anaerobically)	-
Urease (Christensen's urea agar pH 6.8, 6 days at 37°C anaerobically)	-
Lactic Acid type (D/L-lactic acid Kit M30. broth, 16-18h at 37°C, anaerobically)	L 1.5 g/L
Optimal Growth Temperature	37ºC
Oxygen requirement	Facultative anaerobe

API Analysis (BioMérieux)

Lactobacillus identification is usually performed by standard testing and by API 50 CHL System (BioMérieux, France), according to Bergey's Manual of Systematic Bacteriology, and was performed for the *B. animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) to determine its metabolic activity, but not as a means of identification.

Strain Rosell®-421 (LAFTI® B94) is able to grow on different sugars (see API 50 CHL results).

API 50 CHL (37°C, 48 hours)									
Control	-	Galactose	-	α-methyl-D-mannoside	-	Melibiose	+	D-turanose	-
Glycerol	-	D-glucose	+	α-methyl-D-glucoside	-	Sucrose	+	D-lyxose	-
Erythritol	-	D-fructose	-	N-acetyl-glucosamine	-	Trehalose	-	D-tagatose	-
D-arabinose	-	D-mannose	-	Amygdalin	+	Inulin	-	D-fucose	-
L-arabinose	-	L-sorbose	-	Arbutin	-	Melezitose	-	L-fucose	-
Ribose	+	Rhamnose	-	Esculin	+	D-raffinose	+	D-arabitol	-
D-xylose	+	Dulcitol	-	Salicin	+	Starch	-	L-arabitol	-
L-xylose	-	Inositol	-	Cellobiose	-	Glycogen	-	Gluconate	-
Adonitol	-	Mannitol	-	Maltose	+	Xylitol	-	2-ketogluconate	-
β-methyl-xyloside	-	Sorbitol	-	Lactose	+	β-gentiobiose	+	5-ketogluconate	-

2.3.2. Genotypic identification of *Bifidobacterium animalis* subsp. lactis Rosell®-421 (LAFTI® B94)

Multiple DNA sequencing techniques were performed on *Bifidobacterium animalis* subsp. lactis Rosell®-421 (LAFTI® B94) in order to type it. Techniques included Pulse Field Gel Electrophoresis (PGFE), multi locus sequence typing (MLST), random amplification of polymeric DNA (RAPD), and PATRIC database identification.

2.3.2.1. Pulse Field Gel Electrophoresis (PFGE)

PFGE can be used to determine if there is genetic homology between a known member of the *Bifidobacterium animalis* subsp. *lactis* group and LAFTI® B94. Whole genomes of the reference strain and LAFTI® B94 were restricted with the enzyme *Xba1* and run through an agarose gel with a fluctuating current, which allows gel separation of large amounts of genetic material.

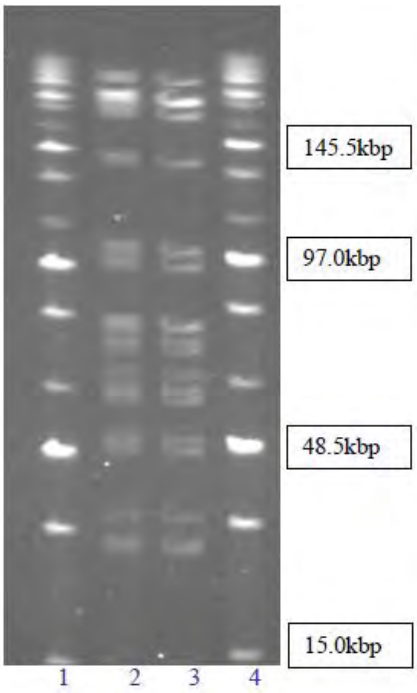

Figure 5. PFGE (CHEF) with Switching Time 0.47s-8.53s for 20.2h at 6V/cm on a 1% Agarose Gel. DNA was previously digested with restriction enzyme *Xba1*. 1) Ladder Concatemer γ (NEB #N3551S); 2) B94 digested with *Xba1*; 3) reference strain digested with *Xba1*; 4) Same as lane 1 (Lallemand 2018).

Both *B. animalis* subsp. *lactis* strains showed the same restriction pattern when digested with *Xba1*. In order to obtain better resolution of some of the larger fragments, a second migration was performed with optimized conditions as shown in Figure 5. Even with a higher resolution at the larger fragment length, the two restriction patterns are nearly identical.

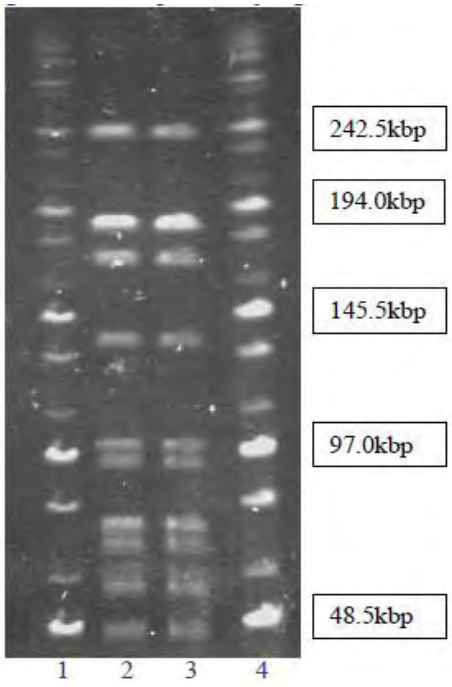

Figure 6. PFGE (CHEF) with Switching 2.3s-17.4s for 27h at 6V/cm on a 1% Agarose Gel. DNA was previously digested with restriction enzyme *Xba1*. 1) Ladder concatemer (NEB #N3551S); 2) B94 digested with *Xba*1; 3) reference strain digested with *Xba1*; 4) Same as lane 1 (Lallemand 2018).

These results show that there is significant genetic homology between strains LAFTI® B94 and the reference strain when investigated using restriction enzymes. Further genetic analysis with comparisons to other strains within the subspecies and those outside the subspecies was conducted to confirm that LAFTI® B94 is properly placed within the *B. animalis* subsp. *lactis* subspecies.

2.3.2.2. Multi-Locus Sequence Typing (MLST)

The MLST analysis was performed on the strain LAFTI® B94, 2 *Bifidobacterium animalis* subsp. *lactis* reference strains, and a *Bifidobacterium animalis* subsp. *animalis* comparison strain (R0417):

The MLST nucleotide sequence analysis was performed using primers that amplify:

- The 16S ribosomal RNA (protocol RM-21),
- Intergenic spacer region (ITS) (protocol RM-34B),
- The gene encoding protease (*clpC*),
- The gene encoding the GTP-binding protein chain elongation factor EF-G (*fusA*),
- The gene encoding the B subunit of DNA gyrase (*gyrB*),
- The gene encoding isoleucyl-tRNA synthetase (*ileS*),
- The gene encoding the beta subunit of RNA polymerase (*rpoB*), and
- Elongation factor EF-Tu (protocol RM-33) (*tuf*).

Each gene was compared to the sequences of the 2 *Bifidobacterium animalis* subsp. *lactis* reference strains and *Bifidobacterium animalis* subsp. *animalis* comparison strain R0417. The analysis was performed with blast-n and the sequences were aligned using ClustalW2.

The genetic similarity between the selected strains and LAFTI® B94's particular genes are shown in Table 1. Given the 100% similarity at the specific loci, LAFTI® B94 is well grouped in with other *Bifidobacterium animalis* subsp. *lactis.*

Table 1. LAFTI® B94 MLST BLAST-n % Identity Nucleotide Comparison (Lallemand 2018).

Strain	LAFTI B94							
	16S	ITS	clpC	fusA	gyrB	ileS	rpoB	tuf
Ssp. *lactis* ref. 1	100%	100%	100%	100%	100%	100%	100%	100%
Ssp. *lactis* ref. 2	100%	100%	100%	100%	100%	100%	100%	100%
Ssp. *animalis* R0417	85%	NA[1]	NA	87%	85%	95%	NA	94%
1. NA = sequence was not available								

2.3.2.3. Random Amplification of Polymeric DNA (RAPD)

RAPD is a type of PCR by which the segments of DNA that are amplified are random. The bacterial DNA is extracted, and then amplified using specific primers. Using selected primers (M-14, OPA-19, and OPL-16) and PCR followed by gel electrophoresis, *Bifidobacterium animalis* subsp. *lactis* R0421 (LAFTI® B94) and a *Bifidobacterium animalis* subsp. *lactis* reference strain were compared.

The results shown in Figure 6 indicate that the 2 strains, LAFTI® B94 and the reference strain, are genetically indistinguishable using RAPD-PCR as an identification technique. The OLP-16 primer shows some variance in the intensity of the produced PCR fragments; however, this is most likely due to the variations in PCR reaction, not the RAPD identification pattern. In fact, the equivalent fragments in the LAFTI® B94 and reference strain lanes can be seen in the LAFTI® B94 lane, but are less-dyed, indicating that fewer cycles of PCR occurred, producing fewer fragments.

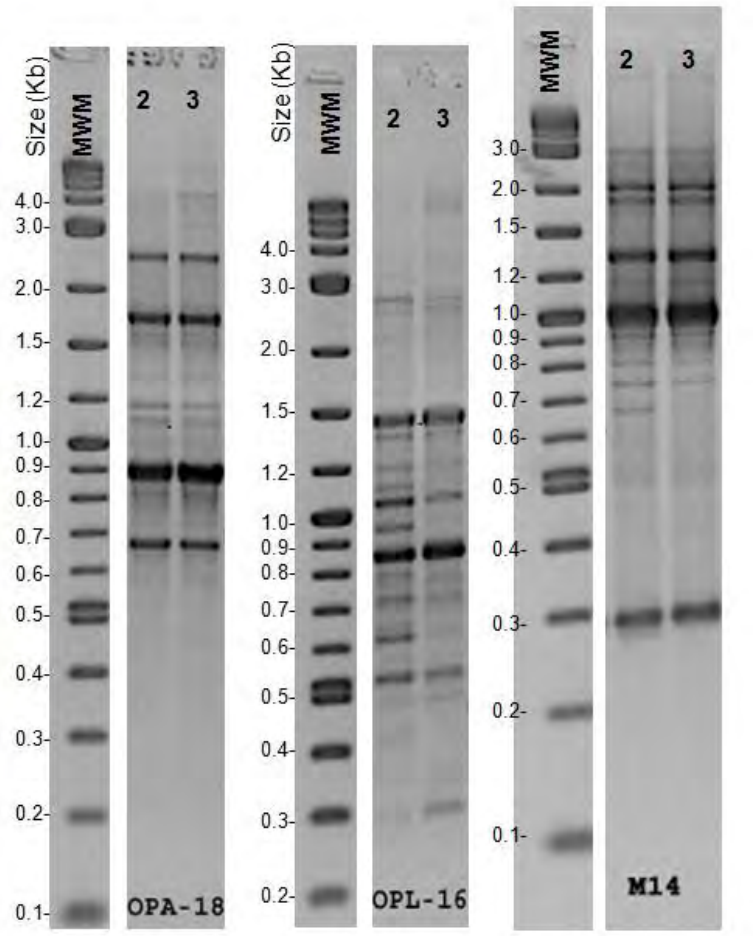

Figure 7. RAPD-PCR profile using primers OPA-18, OPL-16 and M14 of strains *Bifidobacterium animalis* subsp. *lactis* R0421 (LAFTI(R) B94) (lane 2) and reference strain (lane 3). DNA fragment weight maker is in lane MWM (Lallemand 2018).

2.3.2.4. PATRIC database analysis

The PATRIC database contains genomic data of over 80,000 different strains of bacteria (Wattam et al. 2017).

In order to verify that *Bifidobacterium animalis* subsp. *lactis* R0421 (LAFTI® B94) was correctly categorized into its current phylogenetic station, a PATRIC database search was performed to determine genetic homology within the *Bifidobacterium animalis* subspecies, with an outlier of *B. longum* DSM 20219, which is the type strain for *B. longum*. The red arrow in the below image highlights where LAFTI® B94 is located within the *B. animalis* subsp. *lactis* subspecies. The type strains for both *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis* are indicated with the red "T". This tree shows that *Bifidobacterium animalis* subsp. *lactis* R0421 (LAFTI® B94) was included within the *Bifidobacterium animalis* species.

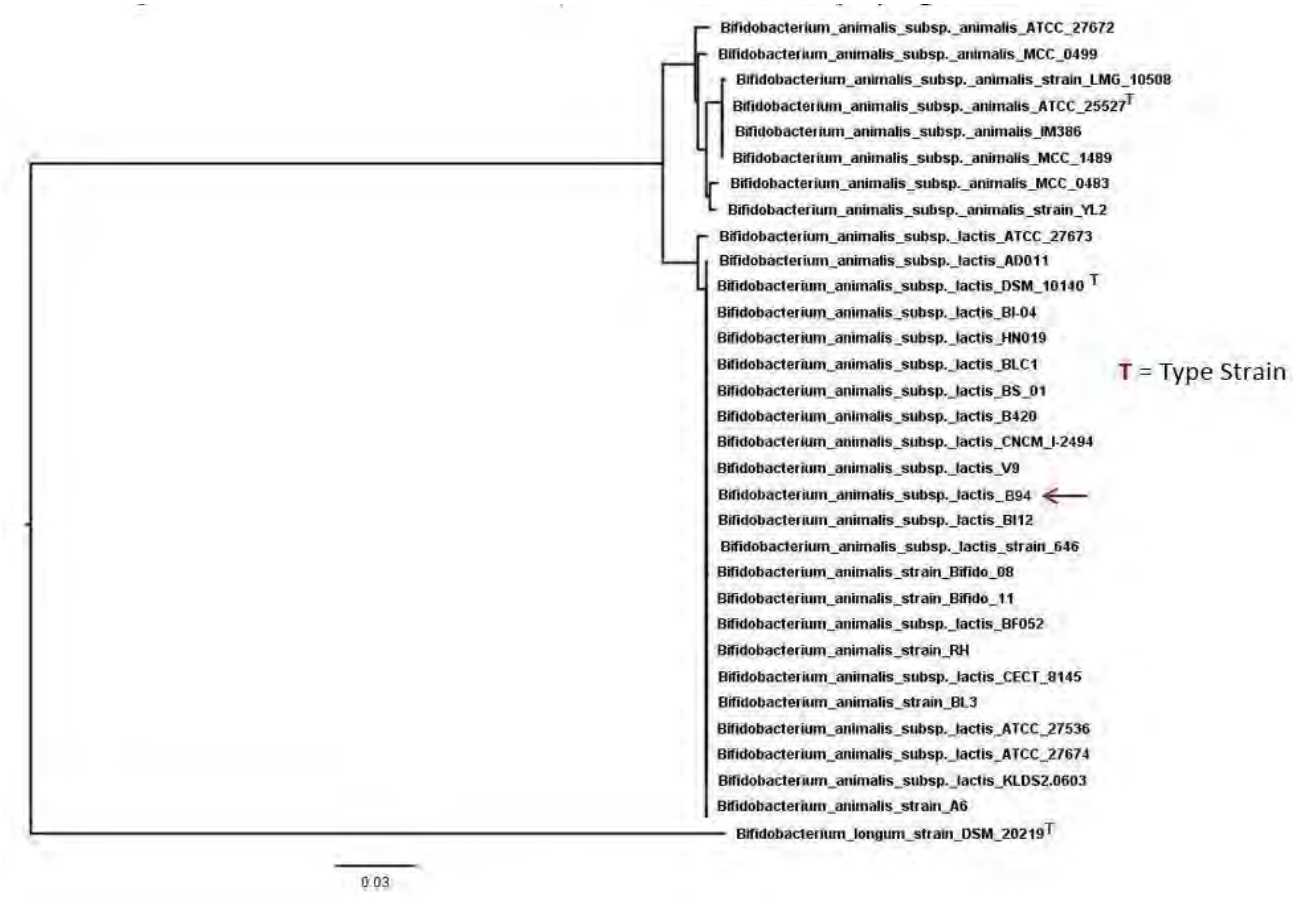

Figure 8. Neighbor-joining tree of LAFTI(R) B94 with *B. animalis* strains and outlier of *B. longum* type strain. Bar, 0.03% divergence (Lallemand 2018).

In Figure 8, the search was performed for homology within the *B. animalis* phylogenetic group, and the results showed that there were two distinct branches of the phylogenetic tree for the species, split between *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis*, and *Bifidobacterium animalis* subsp. *lactis* R0421 (LAFTI® B94) was determined to be correctly within *B. animalis* subsp. *lactis*. The red arrow in Figure 8 highlights where LAFTI® B94 is located within *B. animalis* subsp. *lactis*. The results indicate that LAFTI ® B94, as expected, is closely related to other *B. animalis* subsp. *lactis* strains. The type strains for both *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis* are indicated with the red "T".

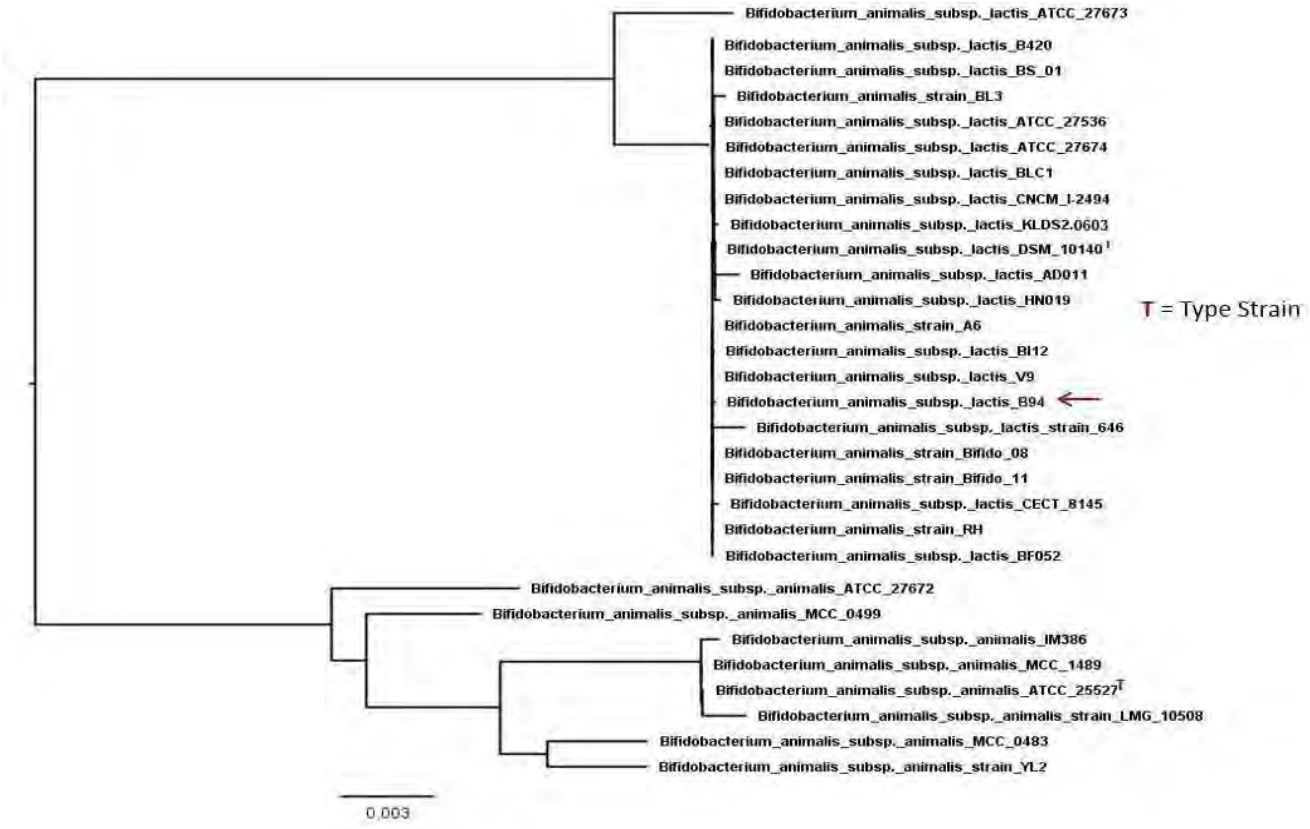

Figure 9. Neighbor-joining tree of *B. animalis* subspecies comparison to LAFTI® B94 generated from the PATRIC database, bar 0.003% divergence (Lallemand 2018).

In addition to the full genetic sequence, partial gene sequences for important individual functional proteins or RNA were analysed in relation to their equivalent sequence in other strains within the *B. animalis* subsp. *lactis* subspecies, as well as other *Bifidobacterium* strains. LAFTI ® B94 is highlighted with a red bar in each phylogenetic tree. Specific gene sequences analyzed were:

- 16S rRNA (Figure 9)
- 16S-23S rDNA intergenic spacer region (Figure 10)
- Partial elongation factor TU (*tuf*) (Figure 11)

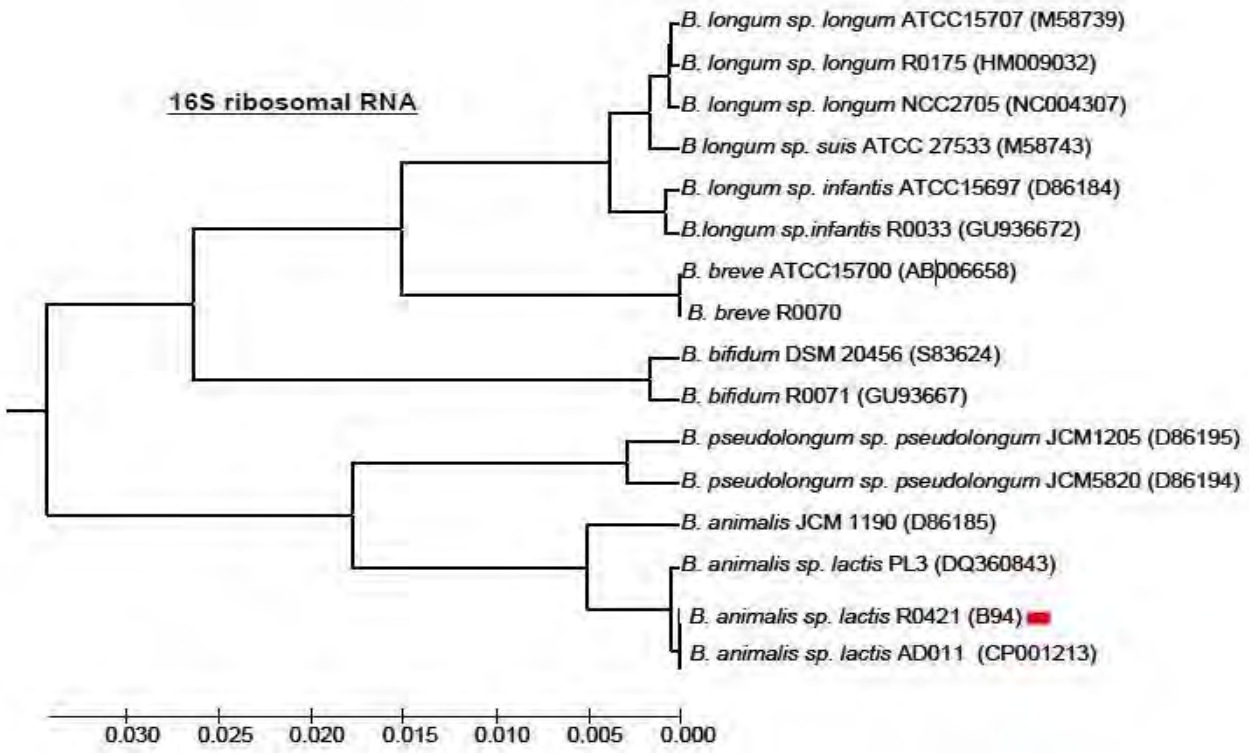

Figure 10. Neighbor-Joining Tree based on the Partial 16S rRNA gene sequences of *B. animalis* subsp. *lactis* R0421 (LAFTI® B94) and other *Bifidobacterium* strains (Lallemand 2018).

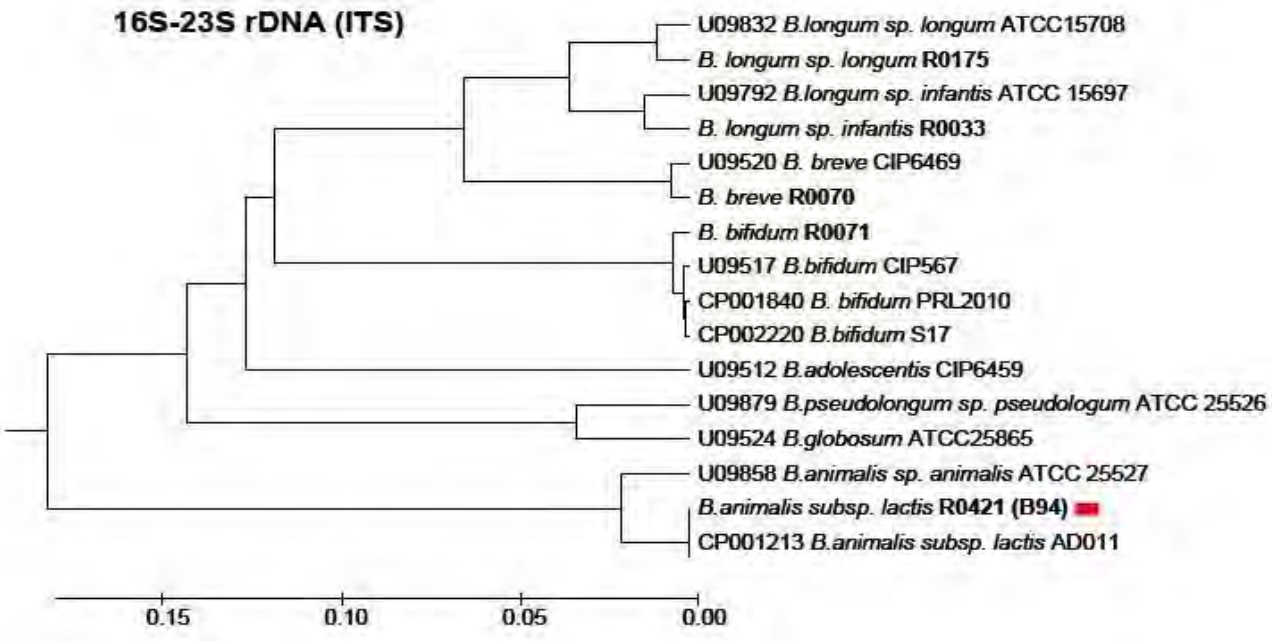

Figure 11. Neighbor-Joining Tree based on the partial 16S-23S rDNA (ITS) gene sequence of *B. animalis* subsp. *lactis* R0421 (LAFTI® B94) and other *Bifidobacterium* strains (Lallemand 2018).

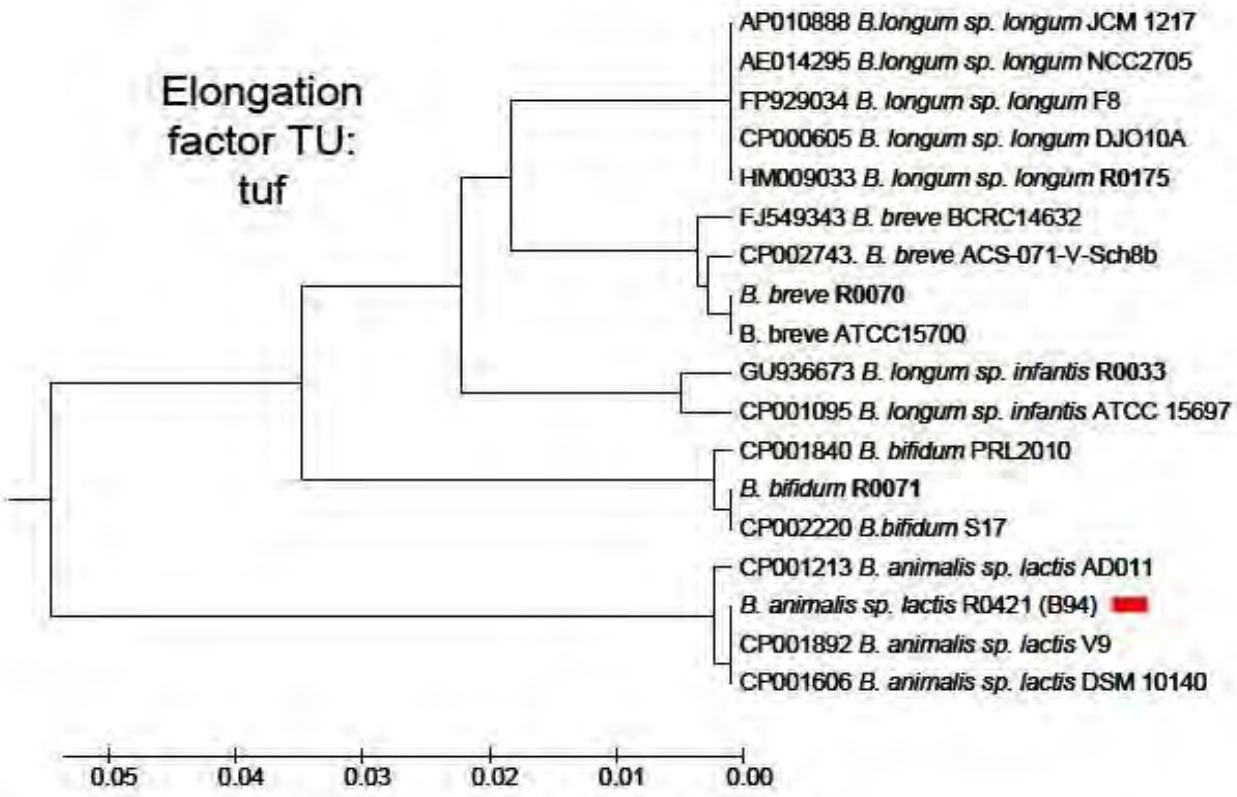

Figure 12. Neighbor-Joining Tree based on the partial *tuf* gene sequence of *B. animalis* subsp. *lactis* (LAFTI(R) B94) and other *Bifidobacterium* strains (Lallemand 2018).

These phylogenetic trees show that there is significant homology between the partial gene sequences of important transcription products of *B. animalis* subsp. *lactis* strains, whereas there is less homogeneity between strains outside the subspecies or species.

2.4. Genomic Analysis of *Bifidobacterium animalis* subsp. *lactis* R0421 (LAFTI® B94)

This bacterial strain has been sequenced and annotated to assure that it does not harbor known virulence genes, potentially transferable antibiotic resistance genes, or the capability to synthesize biogenic amines.

2.4.1. Sequencing

The whole genome sequence (WGS) of *B. animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) was determined and the resulting sequence was annotated and analyzed for genes that may be possible safety concerns. Rosell®-421 genomic DNA (gDNA) was sequenced by the Yale Center for Genome Analyses (YCGA) at Yale University (New Haven, Connecticut). About 5 µg of intact total gDNA were sent to YCGA where a 10 kb library was prepared prior to WGS that was performed by the Pacific Biosciences (PacBio) sequencing technology. A final assembly was conducted with a whole-genome optical map to validate the assembly into a single final contig. See Table 2 for genome sequencing results**.**

Table 2 . Rosell®-421 Genome Sequencing Statistics (Lallemand 2018).

Element	Quantity
Final assembly contigs	1
Genome size (nt)	1,944,140
GC content (%)	60.5

2.4.2. Annotation of the Genome

Annotation of the whole-genome of Rosell®-421 was done online using the RAST pipeline (http://rast.nmpdr.org/rast.cgi) (Aziz et al. 2008, Brettin et al. 2015, Overbeek et al. 2014). The RAST server was developed to annotate microbial genomes. It works by projecting manually curated gene annotations from the SEED database onto newly submitted genomes. The resulting genome included a total of 257 subsystems and other genome annotation statistics are displayed in Table 3.

Table 3RAST predicted that another 6 open-reading frames (ORFs) were “possibly missing.”

Table 3. Rosell®-421 Genome Annotation Statistics (Lallemand 2018).

Element	Quantity
ORFs	1633
RNA coding sequences	64
ORFs in subsystem	705
ORFs not in subsystems	928

2.4.3. Annotation of the Plasmid

The *B. animalis* subsp. *lactis* strain Rosell®-421 (LAFTI® B94) does not contain any plasmids.

2.4.4. Results of the Genomic Analysis

Antibiotic Resistance

The whole genome sequence was used to screen two antibiotic resistance gene databases. First, the ARG-ANNOT ABR gene database is downloadable software that can be used to detect existing and putative new antibiotic resistance in bacterial genomes (Gupta et al. 2014). A total of 1689 antibiotic resistance genes is included in the database. This database uses a BLAST approach for sequence complementary search. ResFinder v2.1 database is a peer-reviewed and published database that is used for screening of acquired antibiotic resistance (Kleinheimz KA et al. 2014). This validated database also uses BLAST to screen the input sequences. ResFinder contains more than 2000 antibiotic resistance genes and is updated periodically.

Screening the Rosell®-421 genome revealed the presence of the *tetW* resistance gene which confers resistance to tetracycline with a 98.16% and 98.95% gene homology in ARG-ANNOT and ResFinder respectively. Tetracycline resistance is common in *Bifidobacterium animalis* subsp *lactis*. Tetracycline resistance is the most common antibiotic resistance in bifidobacteria (Aires et al. 2007, Ammor et al. 2008a, Florez et al. 2006, Masco et al. 2006), and *tetW* is the most common tetracycline resistance gene in bifidobacteria and is associated with the widespread tetracycline resistance among *Bifidobacterium animalis* subsp *lactis* strains (Ammor et al. 2008b, EFSA 2013, Gueimonde et al. 2010). The same resistance gene was detected in the commercially available probiotic strains DSM 10140 and Bb12 (Ashraf et al. 2011). The reports show that there is no evidence that the gene *tetW* in *Bifidobacterium animalis* subsp *lactis* is transmissible (Gueimonde et al. 2010). In light of the recent data on the "medium level" resistance to tetracycline for *Bifidobacterium animalis* subsp *lactis*, EFSA decided to keep this species on their latest list of biological agents recommended for QPS (EFSA 2011).

Synthesis of Biogenic Amines

The Rosell®-421 genome was analyzed for genes encoding amino acid decarboxylases that might catalyze the formation of biogenic amines such as histamine, tyramine, cadaverine, and putrescine. The only decarboxylase that was related to amino acids was diaminopimelate decarboxylase. This enzyme catalyzes a reaction which produces L-lysine. Overall, *Bifidobacterium animalis* subsp *lactis* Rosell®-421 does not harbor any gene responsible for the expression of biogenic amines through amino acid decarboxylation.

Moreover, the Rosell®-421 strain was analyzed by HPLC for biogenic amines in culture supernatants. Results showed the absence of biogenic amines in the Rosell®-421 supernatant.

Adhesion

Specific search in RAST for annotated genes related to adhesins or to collagen binding activities did not reveal any gene in the Rosell®-421 genome. A gene-specific search was conducted by searching for an adhesion gene in the National Center for Biotechnology Information (NCBI) GenBank database; this search was also negative.

Virulence/Infectivity

The whole genome was screened for known virulence factors in *E. coli*, *Enterococcus* spp., *Listeria,* and *S. aureus* with the VirulenceFinder v2.0 database (Joensen et al. 2014). No homologous matches were found in the Rosell®-421 genome.

Table 4. Rosell®-421 Results on VirulenceFinder 2.0 (Lallemand 2018).

Genes	Result
Shiga-toxin genes	No hit found
Virulence genes for *Escherichia coli*	No hit found
Virulence genes for *Listeria*	No hit found
Hostimm genes for *S. aureus*	No hit found
Toxin genes for *S. aureus*	No hit found
Exoenzyme genes for *S. aureus*	No hit found
Virulence genes for *Enterococus*	No hit found

2.5. Production Process of the bacterial powder

The manufacturing process of the bacterial strain powder of *B. animalis* subsp. *lactis* R0421 (LAFTI® B94) is carried at Lallemand Health Solutions (located in Canada) and Lallemand SAS (located in France).

Information regarding the facility involved in the manufacture and testing of *B. animalis* subsp. *lactis* R0421 (LAFTI® B94), including its responsibilities, is provided in Table 5.

Table 5. Facilities and Responsibilities (Lallemand 2018).

Name and Address	Activity
LALLEMAND HEALTH SOLUTIONS INC. (formerly Institut Rosell Inc.) 8480 Saint Laurent Boulevard Montreal, Quebec, H2P 2M6 Canada	Production of dried powder of bacteria: Culture Collection, Fermentation, Concentration, Freeze-Drying, Quality Control, Storage
LALLEMAND S.A.S. 4, Chemin du Bord de l'Eau 15130 Saint Simon France	Production of dried powder of bacteria: Culture Collection, Fermentation, Concentration, Freeze-Drying, Quality Control, Storage

B. animalis subsp. *lactis* R0421 (LAFTI® B94) is produced in compliance with current Good Manufacturing Practices (cGMP).

The facility LALLEMAND HEALTH SOLUTIONS INC, is compliant with the requirements for cGMP set by the local authority (Health Canada) for the manufacturing and handling of the strains under Part 3 of the *Natural Health Products Regulation* of 2004.

The facility LALLEMAND SAS is a contract manufacturer belonging to the group Lallemand. It conforms to Lallemand Health Solutions Quality standards. The site is located in France.

The steps of the manufacturing process of the bacterial strain powder *B. animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) are listed and described schematically in Figure 13. The manufacturing process includes the following steps:

Revivification of the Bacterial Strain

A frozen cryotube from the production cell bank, previously kept at -80°C, is thawed and transferred into a test tube containing sterilized culture medium (previously prepared). All components of the culture medium are food-grade ingredients approved for such use. The culture is incubated according to defined conditions of time and temperature.

Sub Culture

The revivified bacterial strain is transferred to a flask containing sterilized food-grade, approved culture medium. The subculture is then incubated according to defined conditions of time and temperature.

Seed Culture

An aliquot from the sub-culture is transferred to a large flask containing sterilized food-grade, approved culture medium. The seed is incubated according to defined conditions of time and temperature. The following parameters are measured: temperature, pH, optical density, and absence of contamination.

Culture Medium preparation

The raw materials are checked for identity and weighed per culture media recipe. They are then dissolved in water in the fermenter. The pH is adjusted. The culture medium is heat treated *in situ* and cooled to the incubation temperature prior to inoculation with the seed culture. The temperature is continuously monitored during preparation, heat treatment, and cool-down.

Fermentation

The seed culture is transferred from the flask to the heat-treated culture medium for biomass production (“fermentation”). The choice of the fermenter depends on the quantity of biomass required. When larger inoculum volume is required, a pre-fermentation step may be performed in a smaller fermenter prior to the fermentation. During the fermentation, the culture is gently agitated and temperature is controlled. The bacterial strain is grown in the fermenter until the

late exponential phase. Sampling of the culture broth is done periodically during the fermentation to verify the following parameters: pH, and optical density. At the end of the fermentation, a sample is tested by Quality Control for the following specifications: Count of viable cell concentration of the cultured strain and absence of contaminants.

Concentration

The fermentation broth is concentrated by high speed centrifugation or by ultra-filtration.

Cryoprotection and Freeze-Drying

Approved food-grade cryoprotectants are blended with the concentrated bacterial culture until a homogenous solution is obtained. Single-use trays are filled with the blend and introduced into the freeze-dryer. The trays are then freeze-dried. Temperature of the freeze-dryer and of the concentrated culture is monitored throughout the process. The freeze-drying process consists of a primary drying phase under vacuum to sublimate free water and a secondary drying phase under a vacuum to eliminate water linked to the bacteria cells. The cake-like freeze-dried bacterial culture is collected in double bags and stored under refrigerated conditions until grinding. The freeze-dried bacterial powder contains traces of milk products and traces of soy products. Both milk and soy are used in the fermentation; these are the only allergens present.

Grinding and Packaging

The cake-like freeze-dried bacterial culture is ground and collected in laminated foil bags or plastic bags. The bags are closed, weighed, labelled and inventoried. They are then placed in covered bins for storage. A sample of the bacteria powder is brought to Quality Control for control of microbiological quality of the aspect of the powder and enumeration of the bacterial strain.

Storage

The freeze-dried bacterial powder is stored under refrigerated or frozen conditions.

Note: the freeze-dried bacterial powder may be standardized (blended with maltodextrin) before use.

A flow chart of the manufacturing process is provided in Figure 13.

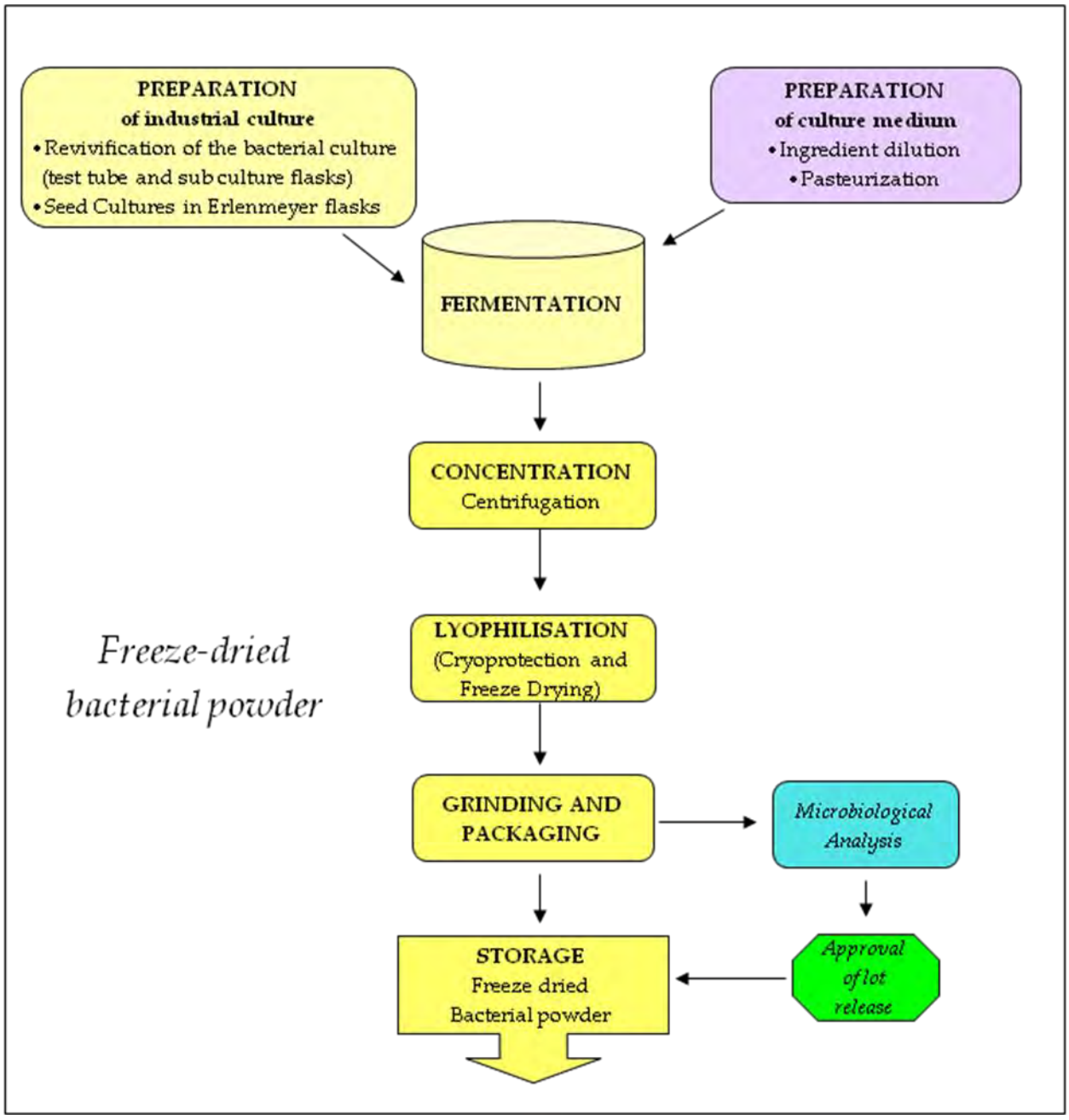

Figure 13. Flow Diagram of Manufacturing Process of the Strains (Lallemand 2018).

2.6. Specifications of the Bacterial Powder

All batches of *B. animalis* subsp. *lactis* R0421 (LAFTI® B94) meet the specifications set forth in Table 6.

Table 6. Specifications for *B. animalis* subsp. *lactis* R0421) Freeze-Dried Powder (Lallemand 2018).

Test	Acceptance Criterion	Methods/Based on
Physical aspect	Fine to granular, ivory to beige powder	Visual observation
B. animalis subsp. *lactis*	NA	Bacteriological enumeration – in-house method
Yeast and Molds	<1000 cfu/g	Enumeration on Sabouraud or PDA culture medium + chloramphenicol, after incubation at 20-25°C for 5 to 7 days Or MFHPB-22 (Enumeration of yeasts and moulds in foods – Government of Canada)
Coliforms	<10 cfu/g	ISO-4831 (Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of coliforms)
Escherichia coli	<10 cfu/g	ISO 7251
Staphylococcus aureus	<10 cfu/g	MFHPB-21
Enterobacter sakazakii (*Cronobacter* spp.)	Negative in 10g in 30 samples*	ISO/TS 22964
Salmonella spp.	Negative in 25g in 60 samples*	MFHPB-20
*Certificate of Analysis follows.		

INSTITUT ROSELL LALLEMAND

CERTIFICAT D'ANALYSE

PRODUIT: *Bifidobacterium animalis* subsp. *lactis* R0421 (LAFTI® B94)
LOT: [redacted]
CODE: 050421SG2
DATE D'ANALYSE: 2019 01 15

Test :	Specifications	Methods	Results
Assay – Total cell count (Enumeration)	NA	QA138	350,9 X 10^9 CFU/g
Microbiological contaminants :			
TYMC/Yeast and Molds	< 1000 CFU/g	Enumeration on SAB or PDA culture medium + Chloramphenicol after incubation at 20-25°C for 5 to 7 days	Complies
Coliforms	< 10 CFU/g	ISO 4831	Complies
Escherichia coli	< 10 CFU/g	ISO 7251	Complies
Staphylococcus aureus	< 10 CFU/g	MFHPB-21	Complies
Enterobacter sakazakii (Cronobacter ssp.) *	Absent/10g (30 samples)	ISO/TS 22964	Complies
Samonella ssp. *	Absent/25g (60 samples)	MFHPB-20	Complies
Physical Aspect :			
Appearance	Fine to granular, ivory to beige powder	Visual observation	Complies

** 21CFR (Code of Federal Regulations) – Part 106 Infant Formula Requirements – Section 106.55 (Controls to prevent adulteration from microorganisms)*

Signed: [redacted] Date: 2019-02-15

Lucie Doyon
Director Quality Control

2.7. Heavy Metals

Heavy metals are potential chemical impurities. To prevent this potential contamination, the raw materials likely to contain these impurities are tested against established specifications and approved for use before they are entered into the manufacturing stream.

Data analysis of the content of heavy metals in samples of *B. animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) meet the specifications set forth in Table 7.

Table 7. Heavy Metals Analysis of *B. animalis* subsp. *lactis* Rosell®-421 (Lallemand 2018).

Test – Heavy Metals	Specifications (mg/kg) (EU regulation 1881/2006)	*B. animalis* subsp. *lactis* Rosell®-421 (sample no)		
		[redacted]	[redacted]	[redacted]
Lead (mg/kg)	3	0.0270	<0.0250	<0.0250
Cadmium (mg/kg)	1	0.03	0.01	0.03
Arsenic (mg/kg)	**	0.0440	0.0430	0.0360
Mercury (mg/kg)	0.10	<0.01	<0.01	<0.01

2.8. Stability of bacterial powder

For *B. animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94), 24 month stability studies have been completed at 4°C and 25° C. The strain, like most live microorganisms, has a higher stability at lower temperature (as demonstrated by the stability data at refrigerated temperature), and as such should be kept refrigerated whenever possible.

The results presented here at 4°C and 25°C show that *B. animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) maintains its minimal guaranteed concentration of $1.3x10^{11}$ cfu/batch for the 24-month shelf life of the product when stored at 4°C.

These data are derived from 6 different stability lots of *B. animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) averaged together.

Table 8. Stability Data for *B. animalis subsp. lactis* Rosell®-421 (LAFTI® B94) at 4°C (Lallemand 2018).

Storage time (months)	0	3	6	12	18	24
Bacterial content (cfu)	$4.77x10^{11}$	$3.83 x10^{11}$	$4.20 x10^{11}$	$4.02 x10^{11}$	$2.78 x10^{11}$	$3.86 x10^{11}$
Survival rate (%)	100	84	91	81	58	74

Table 9. Stability Data for *B. animalis subsp. lactis* Rosell®-421 (LAFTI® B94) at 25°C (Lallemand 2018).

Storage time (months)	0	3	6	9	12	18	24
Bacterial content (cfu)	$4.77x10^{11}$	$2.76 x10^{11}$	$2.53 x10^{11}$	$2.68 x10^{11}$	$2.62 x10^{11}$	$1.63 x10^{11}$	$1.44 x10^{11}$
Survival rate (%)	100	60	56	58	52	34	28

PART 3. DIETARY EXPOSURE (EDI)

The target dietary intake of the strains *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94), is $5x10^9$ cfu/day. The probiotic is intended to be added to nonexempt powdered milk-based infant formula intended for consumption by healthy term infants. In order to provide $5x10^9$ cfu of the probiotic in 800 ml of hydrated formula (an average daily intake), the probiotic must be present in the powder at a concentration of $5x10^7$ cfu/g of powder, assuming a hydration rate of 12.5-13.5 g/100 ml. In order to assure that viable probiotic is present at a concentration of at least $5x10^7$ cfu/g powder through its shelf life, it will be introduced at a concentration of $8x10^7$ cfu/g, leading to a maximum potential daily intake of $8x10^9$ cfu.

If the probiotic is added to a formula with a hydration rate different from 12.5-13.5 g/100 ml, the addition concentration will be adjusted as needed to retain the target intake level of $5x10^9$ cfu/day.

According to tables of daily energy intake by formula-fed infants provided by Fomon (1993), the subpopulation of infants with the highest intake/kg body weight is boys aged 14–27 days. The mean energy intake by this group is 121.1 kcal/kg bw/day with the 90th percentile at 141.3 kcal/kg bw/day. Among girls, the highest energy intake is found in the same age group, 14–27 days, and is nearly as high as boys: the mean and 90th energy intake percentiles are 117.8 and 138.9 kcal/kg bw/day respectively. Most term infant formulas contain 67.6 kcal/100 ml when ready to consume. Therefore, to obtain 141.3 kcal energy/kg BW, an infant boy must consume 209.0 ml formula/kg BW. To reach her 90th percentile of energy consumption, 138.9 kcal/kg bw/day, an infant girl must consume 205.5 ml formula/kg bw/day. The 90th percentile of formula intake for the two sexes combined is about 207 ml/kg bw/day. This would result in a 90^{th} percentile exposure of $8x10^8$ cfu probiotic/kg bw/day, which represents the EDI for the probiotic blend.

The target population is healthy infants and toddlers aged 0-3 years of age. Since it is not expected that these infants and toddlers will have other dietary sources of *B. animalis* subsp. *lactis* Rosell®-421 at age 14-27 days, this figure represents the total EDI.

PART 4: SELF-LIMITING LEVELS OF USE

There is no technological or organoleptic limitation to the concentration of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) which may be added to infant formula or to toddlers' or children's beverages.

PART 5: EXPERIENCE BASED ON COMMON USE IN FOOD

The conclusion that the intended use of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94), is GRAS is based on scientific procedures rather than experience based on common use in food prior to 1958. There is no such prior use.

PART 6: NARRATIVE

6.1. Recognized Safety of *Bifidobacteria*

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

Bifidobacteria are predominant in the intestinal tract shortly after birth. They are important and normal constituents of the human gastrointestinal microbiota and occur at concentrations of 10^{9} to 10^{10} cells/g feces (Tanaka et al. 2000).

The concept that high numbers of *bifidobacteria* in the adult large intestine might be associated with good health and longevity was first proposed by the Russian scientist Eli Metchnikoff at the Institut Pasteur, Paris. Metchnikoff's theories arose from his studies of the intestinal microbiota of adults in France and in certain communities in southern Russia and southeast Europe. Unlike the French populations, these other communities generally had a longer life expectancy and he proposed that the intestinal biota of these communities may have a role in their longevity. *Bifidobacteria* have been part of human nutrition for centuries, and now are more and more being introduced into many fermented food products and dietary supplements. Strains of *Bifidobacterium animalis* have been used for many years in fermented milk products (Biavati et al. 1992).

A Food and Agriculture Organization and World Health Organization expert consultation (FAO/WHO 2001) noted that, "no pathogenic or virulence properties have been found for *bifidobacteria*."

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

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

> "*Lactobacilli* and *bifidobacteria* are ubiquitous in the diet and in the healthy large intestine soon after birth. A classical risk assessment approach, similar to that used for pathogens, is not possible or warranted. Some studies of *lactobacilli* have attempted to define virulence factors. Such classical approaches, although useful for known pathogens, are inherently flawed when applied to normal commensals, *lactobacilli,* or bifidobacteria. In the case of the risk assessment approach for pathogens, pathogenicity is demonstrated and is normally a consequence of several properties, including colonization factors and virulence factors, acting in concert. Frequently, such factors as adhesion are considered to be virulence factors when pathogens are studied. However, mucosal adhesion and other colonization factors are essential features of most commensals. For example, there is a distinct mucosal-associated flora in the gastrointestinal tract. There is little value in screening organisms of low clinical significance and with no proven virulence determinants for such characteristics as potential virulence factors, particularly in the absence of gastrointestinal commensals as comparative controls" (Borriello et al. (2003).

Borriello et al. (2003) argued that the risk of bacteremia from probiotic *lactobacilli* and *bifidobacteria* is well under 1 in a million and concluded that, based on the overall risk from this or other adverse endpoints, "consumption of such products presents a negligible risk to consumers, including immunocompromised hosts." While there are cases of infection due to *lactobacilli* and *bifidobacteria*, they are extremely rare. Boriello et al. (2003) argued that, even though there is reasonable certainty of no harm, any potential concerns should be addressed through testing for acquired (i.e. potentially transferable) antibiotic resistance and virulence factors, as well as testing through human studies. All of these steps have been used to demonstrate the safety of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94).

6.2. History of Consumption of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94)

Bifidobacterium animalis ssp. *lactis* Rosell®-421 (LAFTI® B94) has been sold worldwide since 2010 as powder and in the finished product MAFLOR® sachet since 2011, providing $5x10^9$ cfu/sachet, the same daily intake as is intended from infant formula.

MAFLOR® sachet is used in infants, toddlers, children, and adults. It was first launched in 2011 as a food supplement in Turkey. Additionally, *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) has also been extensively marketed by Lallemand Health Solutions as a combination with other strains in 37 other formulas with no reports of related adverse effects. Among these other formulas, 7 are sold in sachet form, 16 in capsule form, and 14 in bulk powder.

The intake of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) by infants, toddlers, children, and adults has resulted in no actions for safety reasons by any health authority. A

regular review of the published scientific literature detected no reports of adverse events related to the intake of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94).

6.3. Safety Parameters

6.3.1. Ability to Adhere to Intestinal Cells

The ability to adhere to mucosal surfaces is an interesting property for a probiotic. It confers a competitive advantage important for bacterial maintenance and colonization in the human gastrointestinal tract.

Adhesion to gastric epithelial cells has often been suggested as selection criterion for probiotic potency (FAO/WHO 2002). However, there is no scientific evidence to support such a claim. While adhesion may be necessary for some effects, such as direct competition for epithelial cell binding sites with certain adherent forms of pathogenic microbes such as enteropathic and enterohemorrhagic *E. coli* or *H. pylori* (Johnson-Henry et al. 2004), there is no evidence that adhesion is required for other pharmacodynamic properties of a strain as, for example, immune modulation and pathogen inhibition by secreted substances (e.g., lactic acid, hydrogen peroxide, bacteriocins).

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

To date, the available information on the adhesion properties of *Bifidobacteria* is still limited (He et al. 2001). Scientists have developed *in vitro* adherence tests with human cells grown in tissue cultures to measure this adherence.

The capacity of *B. animalis* subsp. *lactis* R0421 (LAFTI® B94) to bind to epithelial cells has been demonstrated. It shows strong adherence to the intestinal epithelial cell line HT-29, but no invasive potential.

B. animalis subsp. *lactis* Rosell®-421 (LAFTI® B94) was added to individual wells of HT-29 cells in triplicate at a concentration of 5.17×10^8 cfu/mL and incubated at 37°C and 5% CO_2 for 3 hours (Shin and Wallace 2005). Following incubation, cells were rinsed gently with PBS to remove unbound bacteria and treated with 1 mL 0.05% Trypsin-EDTA for 30 minutes at 37°C and 5%

CO_2 to detach HT-29 cells/adhesive bacteria. Following centrifugation, spent supernatant was removed and HT-29 cells were lysed by the addition of 100 µL 0.1% bovine albumin. The resulting solution was serially diluted and standard plate counts were performed on MRS agar at 37°C for 48 hours. Control wells containing HT-29 cells alone were treated in a similar manner. Cell counts were performed using a hemocytometer prior to the addition of bovine albumin, allowing for quantitative determination of the binding capacity of the bacterial strain. As illustrated in Figure 14, the different assays demonstrate an average number of 0.88 R0421 adherent cells per HT-29 cell.

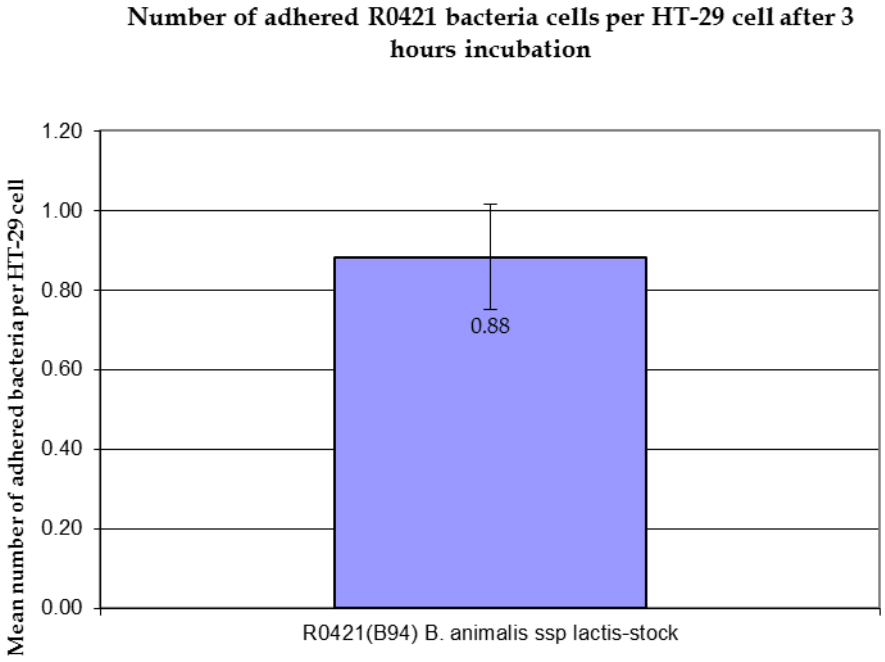

Figure 14. Binding Capacity of *L. helveticus* R0052 to HT-29 Epithelial Cells *in Vitro* (Lallemand 2018).

6.3.2. Infectivity

Cases of infection by *Bifidobacterium* are extremely rare. Reid and Hammond (2005) asserted that, "The safety record of probiotics is remarkable considering that more than 20 billion doses are estimated to be used each year." There have been several reviews attempting to look at the rate of and reasons for bacteremia from *Bifidobacterium* species (Weber et al. 2015, Esaiassen et al. 2017). These reviews indicated that these infections are rare and occur primarily in patients with an underlying medical issue. The most common condition in adults is a co-morbidity of immunocompromization or a gastrointestinal condition which results in increased permeability of the GI tract. Esaiassen et al. (2017) reported on blood culture data in 11 cases of *Bifidobacterium* bacteremia, and found that *Bifidobacterium longum* was the most common infectious agent; 8 of the 11 cases recovered. Boyle et al. (2006) stated firmly, "All cases of

probiotic bacteremia or fungemia have occurred in patients with underlying immune compromise, chronic disease, or debilitation, and no reports have described sepsis related to probiotic use in otherwise healthy persons." In the same vein, a recent study reported that only 7 pediatric cases of *Bifidobacterium* bacteremia had been reported in the PubMed database, and all occurred in preterm infants with additional underlying conditions (Weber et al. 2015). The Esaiassen et al. (2017) review also found 4 cases of *Bifidobacterium* bacteremia in infants, all of whom were of ≤32 weeks gestation. The 3 cases that were ≤24 weeks all recovered, and the fatal case (32 weeks) presented with SIDS at admission to hospital. All infant cases were associated with *B. longum* as the potential etiologic agent.

Bifidobacterium animalis subsp. *lactis* is an organism recognized for its long history of safe use. It is included in an inventory assembled by the International Dairy Federation in collaboration with the European Food and Feed Cultures Association of microorganisms that have a documented history of safe use in food (Bourdichon et al. 2012).

The *Bifidobacterium animalis* subsp. *lactis* taxonomic groups are not known to contain toxin producers or strains that possess virulence factors (Gasser 1994). Therefore, their pathogenic potential is extremely low. Only a limited number of adverse reactions has been published and, overall, consideration should be given to the condition of the consumer or patient. In fact, infection cases reported invariably concern individuals in a fragile state with underlying conditions (Salminen et al. 1998, Weber et al. 2015).

6.3.3. Undesirable Metabolic Activity

6.3.3.1 D-Lactate Production

Bifidobacterium animalis subsp. *lactis* Rosell®-421 (LAFTI B94) does not produce D-lactate, but only L(+)-lactate (1.5 g/L). The UV test kit for the determination of D-/L-lactic acid from Xygen Diagnostics Inc. was used for the quantification. The strain was grown anaerobically in M30 broth for 16-18 hours at 37°C.

6.3.3.2. Bile Salt Deconjugase Activity

Bile salts are steroids with detergent properties which are used to emulsify lipids in foodstuffs passing through the intestine to enable fat digestion and absorption through the intestinal wall. They are secreted from the liver, stored in the gall bladder, and passed through the bile duct into the intestine when food is passing through. Biosynthesis represents the major metabolic fate of cholesterol, accounting for more than half of the 800 mg/day of cholesterol that the average adult uses in metabolic processes. By comparison, steroid hormone biosynthesis consumes only about 50 mg of cholesterol per day. Much more than 400 mg of bile salts is required and secreted into the intestine per day, and this is achieved by re-cycling the bile salts.

Most of the bile salts secreted into the upper region of the small intestine, along with the dietary lipids that they emulsified, are absorbed at the lower end of the small intestine,

separated from the lipids, and returned to the liver for re-use. The most abundant of the bile salts in humans are cholate and deoxycholate, and they are normally conjugated with either glycine or taurine to give glycocholate or taurocholate, respectively. The conjugation is important in identifying the bile salt for re-cycling back to the liver. When these bile salts are deconjugated, that is, glycine or taurine is removed, then the resulting free bile salt forms a precipitate and is not reabsorbed but is excreted with the feces. By increasing the amounts of bile salt excreted, the level of circulating cholesterol can be reduced. The deconjugation of bile salts is achieved through the activity of bile salt hydrolases (BSH), which are produced by intestinal bacteria. *Enterococci* and *Clostridia* contain some of the highest levels of bile salt deconjugase activity (Knarreborg et al. 2002), but BSH activity is also found in many *Bifidobacteria* and some *Lactobacilli*.

An internal study was performed to determine the presence of bile salt deconjugase in *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) and it was shown that *B. animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) does not possess any bile salt deconjugase activity.

Table 10. Bile Salt Deconjugase Activity in *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (Lallemand 2018).

Strain	Growth medium	Incubation conditions	Control	Interpretation	Deconjugase activity
B. animalis subsp. *lactis R0421*	A RCM Agar plate supplemented with 0.5% (w/v) Taurodeoxycholic acid (TDCA)	Plates incubated for 5 days at 37°C under anaerobic conditions	Unsupple-mented RCM agar plate	Bile salt deconjugase activity is shown by the presence of clear precipitate halos around isolated colonies or opaque, granular white colonies com-pared to colonies grown on unsupplemented agar	Positive

6.3.4. Presence of Antibiotic Resistances Genes and Likelihood of transference

6.3.4.1. Minimal Inhibitory Concentrations

The generally recognized method to assess antibiotic susceptibility of microorganisms is by measuring the minimal inhibitory concentration (MIC) and comparing it to standard microbiological breakpoints. Strains with MICs higher than the breakpoints are generally considered resistant. However, this result does not imply that the resistance can be transferred to other microorganisms.

Microbiological breakpoints were suggested by the EFSA/FEEDAP Panel for *Bifidobacterium animalis* subsp. *lactis* in "Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance," published in June 2012. The microbiological breakpoints were set for ten antimicrobial agents, which were chosen to maximize the identification of resistance genotypes to the most commonly used antimicrobials.

The MIC of several antimicrobial agents were determined for *B. animalis* subsp. lactis Rosell®-421 (LAFTI® B94) and compared with FEEDAP 2012 breakpoints. The standard operational protocol (SOP) previously used by Lallemand Health Solutions (formerly known as Institut Rosell) was based on a compilation of various methods available at the time, such as ACE-ART 2005 and CLSI M100-S17 2007. The current SOP is based on the method CLSI M100-S24 2014 on the Performance Standards for Antimicrobial Susceptibility Testing, one of the methodologies recommended by the EFSA/FEEDAP Guidance (FEEDAP 2012). For *B. animalis* subsp. *lactis* Rosell®-421, the MICs were determined by micro-dilution in LSM Broth + Cysteine using the Bio-Rad Plate reader.

Table 11 MIC for R0421 in LSM + Cysteine Broth Using the Recommended ISO/IDF Method (Lallemand 2018).

Antimicrobial Agent	Minimal Inhibitory Concentration (µg/ml)	Microbiological breakpoints (µg/ml) *Bifidobacterium* – (FEEDAP 2012)
Amikacin	64	n.a.
Amoxicillin	0.125	n.a.
Ampicillin	0.5	2
Cefoxitin	2	n.a.
Ceftiofur	0.25	n.a.
Ceftriaxone	>128	n.a.
Cephalothin	8	n.a.
Chloramphenicol	2	4
Ciprofloxacin	4	n.a.
Clindamycin	<0.03125	1
Erythromycin	0.25	1
Gentamicin[1]	32	64
Kanamycin[1]	4	n.a.
Nalidixic acid	16	n.a.
Quinupristin/Dalfopristin	0.5	1
Streptomycin[1]	16	128
Tetracycline	0.5	8
Trimethoprim[1]	<0.03125	n.a.
Vancomycin	0.5	2

[1]possible interference of the growth medium
n.a.: not available

B. animalis subsp. *lactis* Rosell®-421 (LAFTI® B94) is not considered resistant to any of the tested antimicrobial agents.

6.3.4.2. DNA Microarrays

In order to maximize the checking of the safety of the *B. animalis* subsp. *lactis* R0421 (LAFTI® B94), Lallemand Health Solutions obtained access to a microarray developed by Dr. Roland Brousseau, Group Leader of Environmental Genetics, and Dr. Andre Nantel, Research Officer and head of the Microarray laboratory at the Biotechnology Research Institute (National Research Council of Canada, Montreal). This microarray allows detecting 166 known antibiotic resistance genes from each strain. This technique is faster and more reliable than the PCR techniques that were used in the past.

DNA oligonucleotides complementary to the sequence of known antibiotic resistance genes are generated and spotted onto specialized glass slides using specialized robots. Genomic DNA from the bacteria which are to be screened is first labeled with the fluorescent dye Cyanine-5 and then hybridized overnight to allow DNA to bind to complementary oligos. Upon excitation with fluorescent light, Cy5-labelled DNA which has hybridized to specific oligos will illuminate, allowing determination of the identity of the resistance gene.

Several recent studies have demonstrated the efficiency of this approach (Call et al. 2003; Frye et al. 2006; van Hoek et al. 2005), including one array designed specifically for the detection of antibiotic resistance genes in lactic-acid bacteria (Kastner et al. 2006). Recently, an array specific for >300 resistance genes was developed as part of the Assessment and Critical Evaluation of Antibiotic Resistance Transferability in Food Chain (ACE-ART), a European-funded initiative with a mandate to determine the prevalence and risks posed by the presence of antibiotic resistance genes in food-grade microorganisms.

The microarray used by Lallemand Health Solutions contained 182 oligonucleotides corresponding to 166 different acquired antibiotic resistance gene targets (Garneau et al. 2010). EUB338-50 and EUB338-35 were included as positive controls for gram positive and gram negative bacteria, respectively, while shuEUB-50 and shuEUB-35 were included as negative controls.

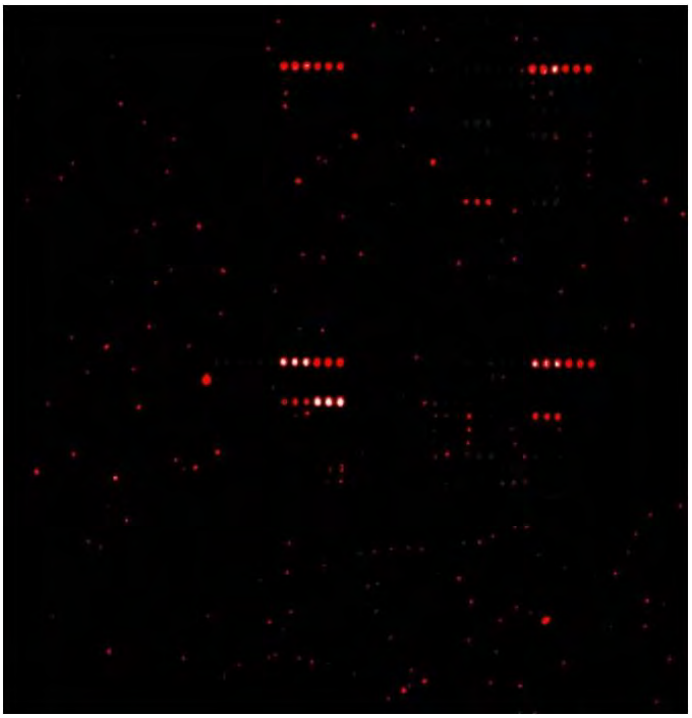

Figure 15. DNA Microarray of *B. animalis* subsp. *lactis* R0421 (LAFTI® B94) for the Detection of Antibiotic Resistance Genes (Lallemand 2018).

The microarray showed that *B. animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) showed positive results to the probes tetW, tetOW, and tetW2, which are all probes used to determine resistance to tetracycline, a common feature among most *Bifidobacterium animalis* subsp. *lactis* strains. In a previous GRAS dossier for a *B. animalis* subsp. *lactis* strain, FDA has agreed that this is not a safety concern[1].

Additionally, tetracycline was one of the antibiotics tested in the antibiotic resistance panel, and the MIC was below the breakpoint for *B. animalis* subsp. *lactis* strains, indicating that while this strain has a tetracycline resistance gene, it does not seem to confer actual resistance. This could be because the gene is nonfunctional due to some mutation, causing a false positive on the microarray.

6.3.4.3. Antibiotic Production

Bifidobacteria are not known to be antibiotic producers. *B. animalis* ssp. *lactis* has not been reported in the literature as able to produce therapeutic antibiotics. Moreover, whole genome sequencing has not revealed any open-reading frames encoding genes for therapeutic antibiotic production.

[1] FDA. Agency Additional Correspondence Lettter GRAS Notice No. GRN 000049. https://wayback.archive-it.org/7993/20171031025206/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm154391.htm

6.3.5. Bioavailability of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94)

Bioavailability of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94), has been demonstrated by the following tests:

- Demonstration of resistance to acidity and bile for the strain (in-house study and Crittenden et al. 2001);
- Demonstration of the persistency of the strain in the gastrointestinal tract (Su et al. 2005 and Su et al. 2007)

6.3.5.1 Resistance to Acidity and Bile

Bifidobacterium animalis subsp. *lactis* Rosell®-421 (LAFTI B94) is resistant in the acidic conditions typically encountered at mealtime (Figure 15). LAFTI B94 is susceptible to very low pH.

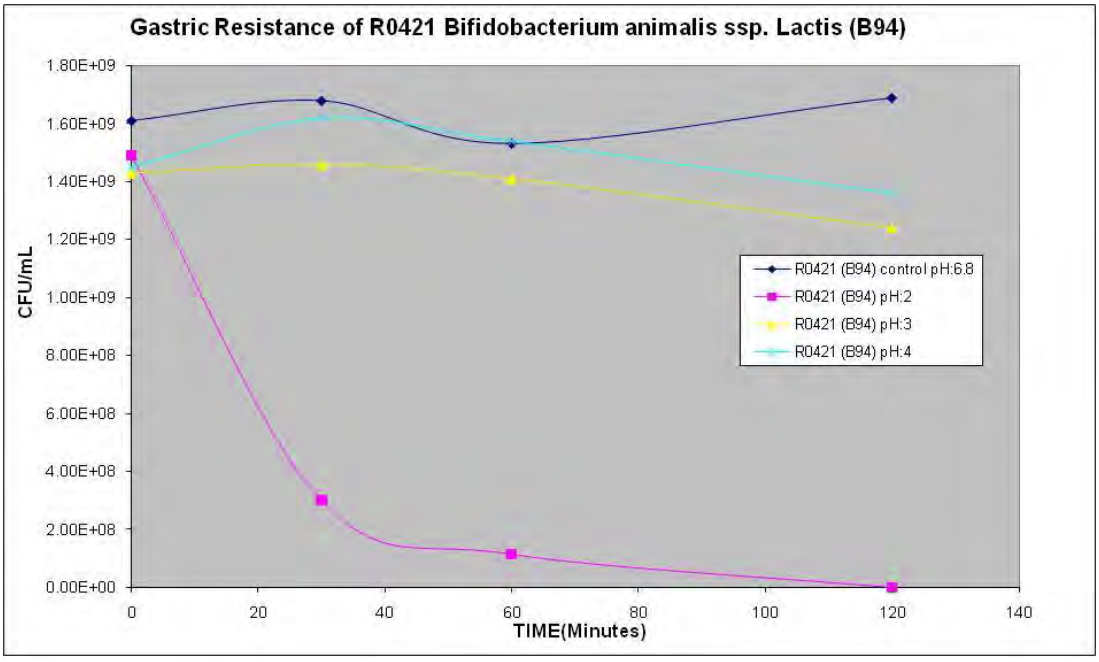

Figure 16. Survival of *Bifidobacterium animalis* subsp. *lactis* LAFTI B94 under different pH over time (Crittenden et al. 2001).

In an *in vitro* model simulating the acidic and protease-rich environment of the human stomach, Crittenden et al. (2001) demonstrated that, of the selection of 18 strains, the strain *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI B94) showed the least loss of viability (Figure 17). It appears that *B. animalis* in general is more acid tolerant than the other species examined.

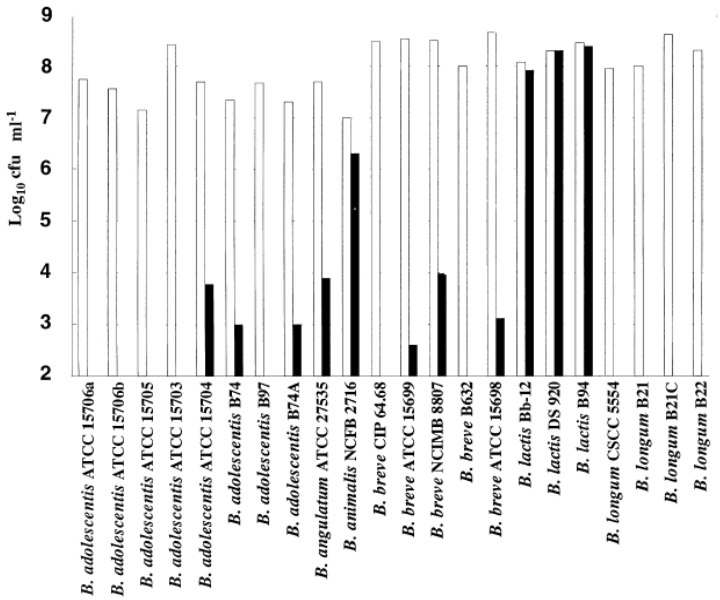

Figure 17. Survival of *Bifidobacterium* isolates in an in vitro model simulating conditions in the human stomach (Crittenden et al. 2001).

Cells were incubated for 105 min at 37 °C in 0-1 mol l^{-1} HCl/KCl buffer, pH 2-0, containing 500 U ml^{-1} pepsin A and 1-0 g l^{-1} bacteriological peptone. □ Viable count of bacteria at t=0; ■ viable count of bacteria at t = 105 min

Additionally, the survival of the *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI B94) was not dramatically affected by exposure to bile, even immediately following acid and protease treatment (Figure 18). *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI B94) is considerably more acid tolerant than the other species examined (Crittenden et al. 2001).

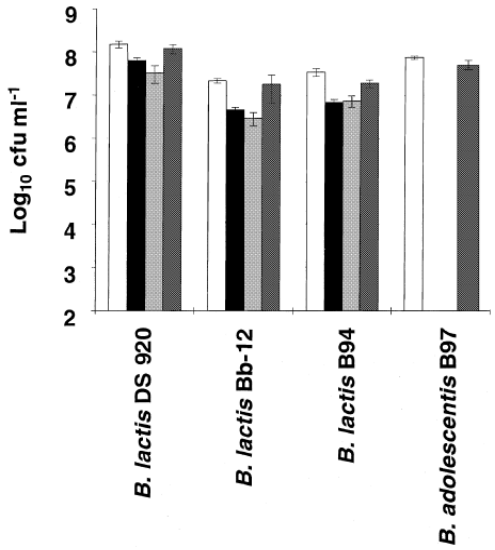

Fig. 2 Survival of bifidobacteria in an *in vitro* model simulating conditions in the human gastrointestinal tract. Error bars represent the standard error. (□), Viable count at time = 0; (■), viable count after passage through an *in vitro* stomach model. (Incubation at 37 °C for 105 min in 0·1 mol l^{-1} HCl/KCl buffer, pH 2·0, containing 500 U ml^{-1} pepsin A and g l^{-1} bacteriological peptone.); (▒), viable count after sequential passage though an *in vitro* stomach model (as above) followed by incubation for 360 min at 37 °C in 3·0 g l^{-1} ox bile in 0·1 mol l^{-1} phosphate buffer, pH 6·5, containing 1·0 g l^{-1} bacteriological peptone; (▓), viable count in the control at time = 465 min. (Controls contained 0·1 mol l^{-1} phosphate buffer, pH 6·5, plus 1·0 g l^{-1} bacteriological peptone and were incubated at 37 °C)

Figure 18. Survival of *bifidobacteria* in an *in vitro* model simulating conditions in the human gastrointestinal tract (Crittenden et al. 2001).

In order to determine the effect of bile on growth of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI B94), the growth curve for the strain was obtained and the log of the optical density was plotted on the y-axis vs time on the x-axis. The growth rate was generated from the exponential growth phase of the bacteria. Bile sensitivity was calculated as a percentage of the exponential growth rate of the control grown in the absence of bile.

Bile exerted an inhibitory effect on growth of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI B94). This was tested by inoculating a tube of 0.3% bacto Oxgall dehydrated fresh bile (Difco Laboratories), incubating at 37°C and comparing the growth with a control tube. 60% less growth was observed in the Oxgall tube for *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI B94) compared to the control without bile.

6.3.5.2 Persistency of the strain in the gastrointestinal tract

The persistency of the strain in the gastrointestinal tract was demonstrated through the following published study.

An observational study was conducted with 5 volunteers (4 female, 1 male), 25-50 years of age, who were fed $5x10^{10}$ CFU *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI B94) cells via capsule twice a day for 7 days (Su et al. 2005). The study objective was to develop a method for detection and quantitative measurement of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI B94) in fecal samples.

The inclusion criterion for the study was no probiotic usage 2 for weeks before the trial and for 4 weeks after the trial. Fecal samples of were taken at day 0 for baseline, and at days 4 and 7 during the feeding period. Fecal levels of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI B94) were measured for 28 days after the feeding was stopped.

Primers were designed in order to detect *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI B94) strains using PCR amplification, and were verified via spiked controls for accuracy. Results showed that the highest levels of Bifidobacterium animalis subsp. lactis Rosell®-421 (LAFTI B94) for all subjects were found on the 7th and last day of feeding. One week into the washout period, 4 of the 5 subjects were still showing detectable levels of Bifidobacterium animalis subsp. lactis LAFTI® B94 (R0421) in the fecal samples. After 2 weeks into the washout period, 2 of the 5 subject's fecal samples contained Bifidobacterium animalis subsp. lactis Rosell®-421 (LAFTI B94). At the end of the 4-week washout period, 1 of the 5 subject's fecal sample was still presenting with detectable levels of Bifidobacterium animalis subsp. lactis Rosell®-421 (LAFTI B94).

In conclusion, the results demonstrated that the Bifidobacterium animalis subsp. lactis Rosell®-421 (LAFTI B94) strain is able to survive and pass through the human gastrointestinal tract (Su et al. 2005).

6.4. Human Studies

6.4.1. Studies in Infants and Children

6.4.1.1. Studies of Maflor® Sachet

Bifidobacterium animalis ssp. *lactis* Rosell®-421 (LAFTI® B94) has been sold for many years as a powder or as a part of the finished product MAFLOR® sachet, in the same proportion as is intended for addition to infant formula powder. While MAFLOR® sachet contains inulin, ascorbic acid, and maltodextrin in addition to the probiotic strain, published studies in which MAFLOR® sachet is consumed at levels providing $5x10^9$ or $10x10^9$ cfu/day of the strain provide evidence of the safety of ingestion of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421.

The research studies of MAFLOR® sachets containing *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) discussed below are summarized in Table 12 at the end of this section.

Erdogan et al. 2012

Erdogan et al. (2012) reported a prospective, randomized, 2-arm, placebo-controlled, double-blind clinical study of the effects of a probiotic product containing *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) in children suffering from acute gastroenteritis caused by Rotavirus. The study was intended to determine which of two probiotics, *Saccharomyces boulardii* or *Bifidobacterium lactis*, provided the better effectiveness in the treatment of acute rotavirus gastroenteritis in combination with oral rehydration therapy and rapid refeeding with a normal diet. The study included 75 children (38 female, 37 male) aged between 5 months and 5 years, with 3 or more incidents of watery diarrhea per day in the last 48 hours and diagnosed as rotavirus gastroenteritis. The patients were randomly allocated to 3 groups of 25 patients each; all patients received oral rehydration therapy and rapid refeeding with a normal diet. In the first group, the rapid refeeding with a normal diet was given with 282.5 mg/day of *Saccharomyces boulardii*, in the second group, with 30 mg/day ($5x10^9$ cfu/day) of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94). The third group was a control group with oral rehydration therapy, rapid refeeding, and a normal diet. Each participant was followed up in hospital until oral rehydration, and then followed post-discharge via telephone for frequency of diarrhea, stool characteristics and consistency, and vomiting episodes per day. The results of the study showed that the mean duration time of diarrhea in the second supplemented with *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) was significantly less than those of the other two groups. There were no reports of adverse events (Erdogan et al. 2012).

Aydin et al. 2012

In a small prospective randomized trial published as an abstract (Aydin et al. 2012), authors in Turkey reported on use of MAFLOR® sachets in very low birth weight infants (n=34). In the

treatment group (n=17), *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 -LAFTI® B94 (in MAFLOR® sachets) was given at $5x10^9$ cfu/day from the first enteral feeding and throughout the hospitalization process. The placebo group (n=17) received the regular enteral feeding.
The treatment group reached the goal of 100 cc/day of enteral feeding significantly faster than the placebo group. There was no difference in early culture or clinically proven sepsis between the two groups, but there was a significant decrease in late sepsis in the treatment group. Necrotizing enterocolitis (NEC) stage ≥2 was significantly higher in the control group, but there were no differences in mortality or in bodyweight during hospitalization and at discharge. No intervention-related adverse effects were reported.

Dilli et al. 2013

Dilli et al. (2013) conducted a prospective, randomized, double-blind, placebo-controlled trial to evaluate the effect of probiotics on nosocomial sepsis, NEC, and mortality in infants with cyanotic congenital heart disease (CCHD).

A total of 100 infants with CCHD were enrolled. The inclusion criteria were infants with CCHD, >35 weeks' of gestational age, fed enterally, and survived beyond the seventh day after admission in neonatal intensive care units (NICUs). The exclusion criteria were congenital anomalies of the intestinal tract and infants who were not fed enterally or died before the seventh day after admission.

Infants aged between 4 to 5 days were randomized into the probiotic or placebo groups (n = 50 infants/group). The study group received Maflor® sachet (containing *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94), 1 sachet per day with breast milk or formula until discharge or death, whichever came first. The placebo (control) group was fed with breast milk or formula without addition of probiotic and received maltodextrin. Feeding was given when the infant had stable vital signs, active bowel sounds without abdominal distension, and no bile or blood from the nasogastric tube. In both groups, feeding was stopped if there was any sign of feeding intolerance. Placebo or probiotics (*Bifidobacterium animalis* ssp. *lactis* Rosell®-421 at the dose of $5x10^9$ cfu/day) were administered for an average of 19 days.

Primary outcomes were nosocomial sepsis and NEC; only infants with confirmed NEC (Bell stage IIa-IIIb) were included. Secondary outcomes were length of NICU stay and death.

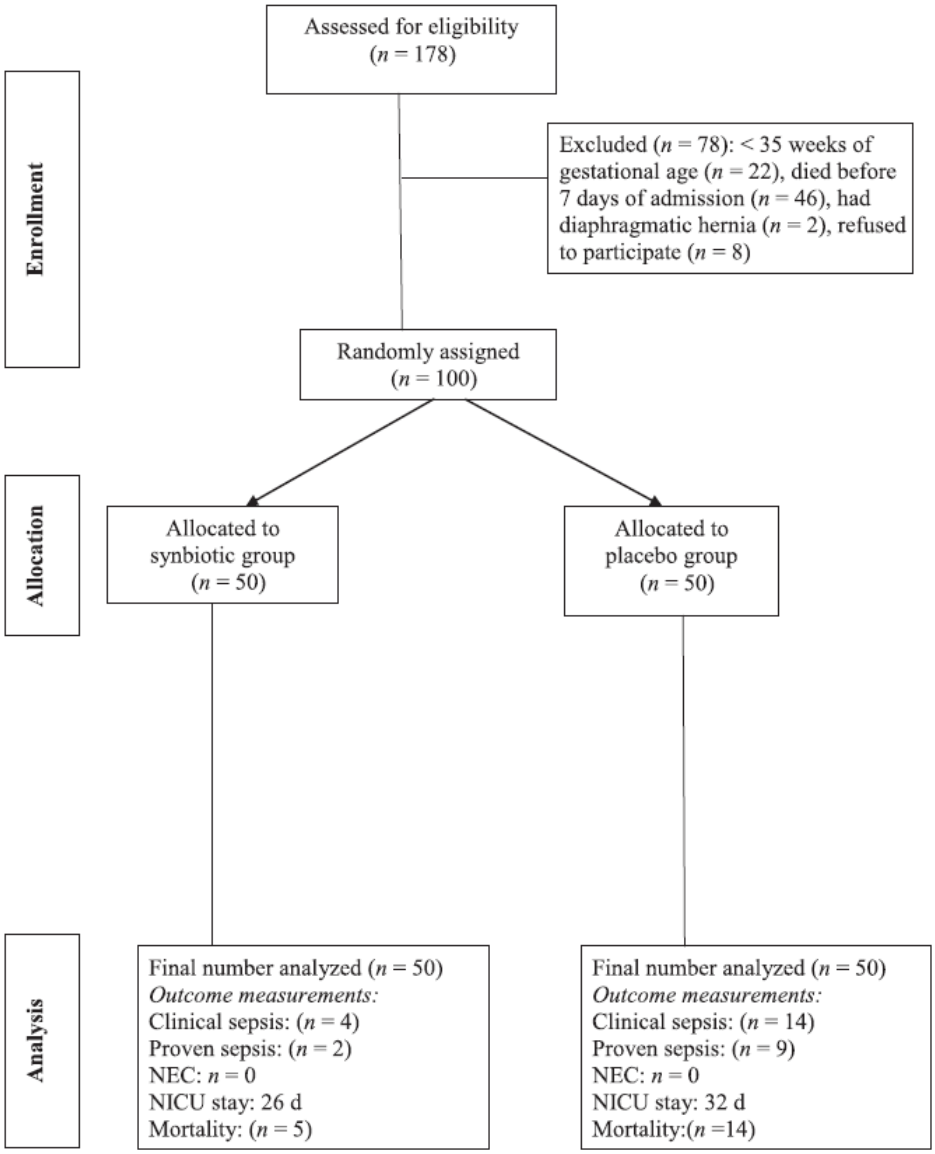

Figure 19. Flow diagram of the randomized trial (Dilli et al. 2013).

The clinical data showed a significantly lower incidence of nosocomial infection in the probiotic group (8% vs 28%). The durations of total parenteral nutrition (7.0 vs 12.5 days) and mechanical ventilation (3 vs 5 days) were significantly shorter in the probiotic group. There were 5 cases of NEC in the placebo group and none in the probiotic group. The length of NICU stay did not differ between the both groups, and the death incidence was 10% in the probiotic group vs 28% in the placebo group. The authors reported no adverse events linked to the Maflor® sachet containing *Bifidobacterium animalis* subsp. *lactis* Rosell®-421.

İşlek et al 2014:

The effects of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) with inulin on the duration of acute gastroenteritis or acute infectious diarrhea in children from 2 months to 5 years old were investigated by İşlek et al. (2014) in a prospective, randomized, double-blind, placebo-controlled trial. The primary endpoint was the duration of diarrhea. Secondary endpoints were the number of stools on the third day of the intervention, percentage of patients with diarrhea on the 5th day, and duration of diarrhea for each etiological agent. A total of 179 children was randomly allocated into two groups; 90 children were assigned to the probiotic group and 89 to the placebo group. The patients in the probiotic group were administered $5x10^9$ cfu of the *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) strain with 900 mg inulin once a day for five days. For the patients in the placebo group, the probiotic preparation was replaced by a maltodextrin-containing placebo with the same appearance as the probiotics.

All patients received routine treatment such as oral and/or intravenous fluid therapy and nutritional support, and breastfeeding was promoted. The parents were telephoned every day for 10 days to verify that their child took the preparations and to answer questions about stool frequency, vomiting frequency (if any), stool consistency, fever, and any dietary problems. During the study period, 11 patients in the probiotic group and 12 patients in the placebo group were excluded from the study as they used antibiotics, did not take the required preparations, or did not communicate. At the beginning of the study, a stool sample of each patient was examined for Rotavirus, Adenovirus, *Entamoeba histolytica*, *Salmonella, Shigella, Campylobacter, Clostridium difficile, Cryptosporidium*, and parasites. In 49.3% of the probiotic group patients and 48% of the placebo-group patients, no specific etiological agents were found. In the probiotic group, the detection rates for Rotavirus, Adenovirus, *Salmonella*, and *E. histolytica* were 33.7%, 12.9%, 2.5%, and 2.5%, respectively, in comparison with 36%, 10%, 2.6%, and 2.6%, respectively, in the placebo group. At the end of the study, the primary endpoint showed that the duration of diarrhea was significantly shorter in the probiotic group than in the placebo group (3.9±1.2 days vs. 5.2±1.3 days)

The frequency of diarrheal stooling on the third day was significantly less in the probiotic group than the placebo group (5.5±2.9 vs. 8.3±3.0). Additionally, on the fifth day, the diarrhea cases were significantly higher in the placebo group than those receiving the probiotic (38.9% vs. 17.7%). No side effects were reported by the parents.

Dilli et al. 2015

Dilli et al. (2015) evaluated the efficacy of the Maflor® sachet and *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI B94) on the prevention of NEC in very low birth weight (VLBW)

infants. The prospective, randomized, double-blind, placebo-controlled, multi-center trial was conducted at 5 NICUs in Turkey.

There were 677 VLBW infants admitted to the 5 NICUs during the 3-year study period. Only VLBW infants with a gestational age of <32 weeks and a birth weight of <1500 g, born at or transferred to the NICU within the first week of life and fed enterally before inclusion, were eligible. Infants with any disease other than those linked to prematurity or congenital anomalies of the intestinal tract, not fed enterally, or who died before the seventh day after birth, whose mothers had taken nondietary probiotic supplements, or whose parents refused to participate were excluded. Four hundred VLBW infants were eligible for the study and were randomly assigned to one of the 4 intervention groups: prebiotic group, probiotic group, symbiotic group, and placebo group (100 in each group). A flow diagram of the randomized trial is presented in Figure 20, and the maternal and infants' demographic characteristics in Figure 21.

The study groups received one sachet per day containing probiotic (*B. animalis* subsp. *lactis* Rosell®-421, $5x10^9$ cfu), prebiotic (inulin, 900 mg), synbiotic (Maflor® sachet (B. *animalis* subsp. *lactis* Rosell®-421, $5x10^9$ cfu + 900 mg inulin) or placebo (maltodextrin). Treatment continued until discharge or death or for a maximum of 8 weeks. Enteral feeding was given when the infant had stable vital signs, active bowel sounds without abdominal distension, and no bile or blood from the nasogastric tube. Continuous feeding was used for a short time only in infants who did not tolerate bolus feeding. The intervention was administered only when an infant was receiving at least 1 ml of milk every 4 hours. The amount of feeding was advanced slowly if tolerated, with no more than a 20 ml/kg bw increment per day. In all groups, feeding was stopped if there was any sign of feeding intolerance. The primary outcome was NEC (Bell stage >2), and the secondary outcomes were time to reach full enteral feeding, late-onset sepsis, length of NICU stay, and death. In addition, many clinical variables and treatment outcomes were recorded during the study, including growth velocity (Figure 22).

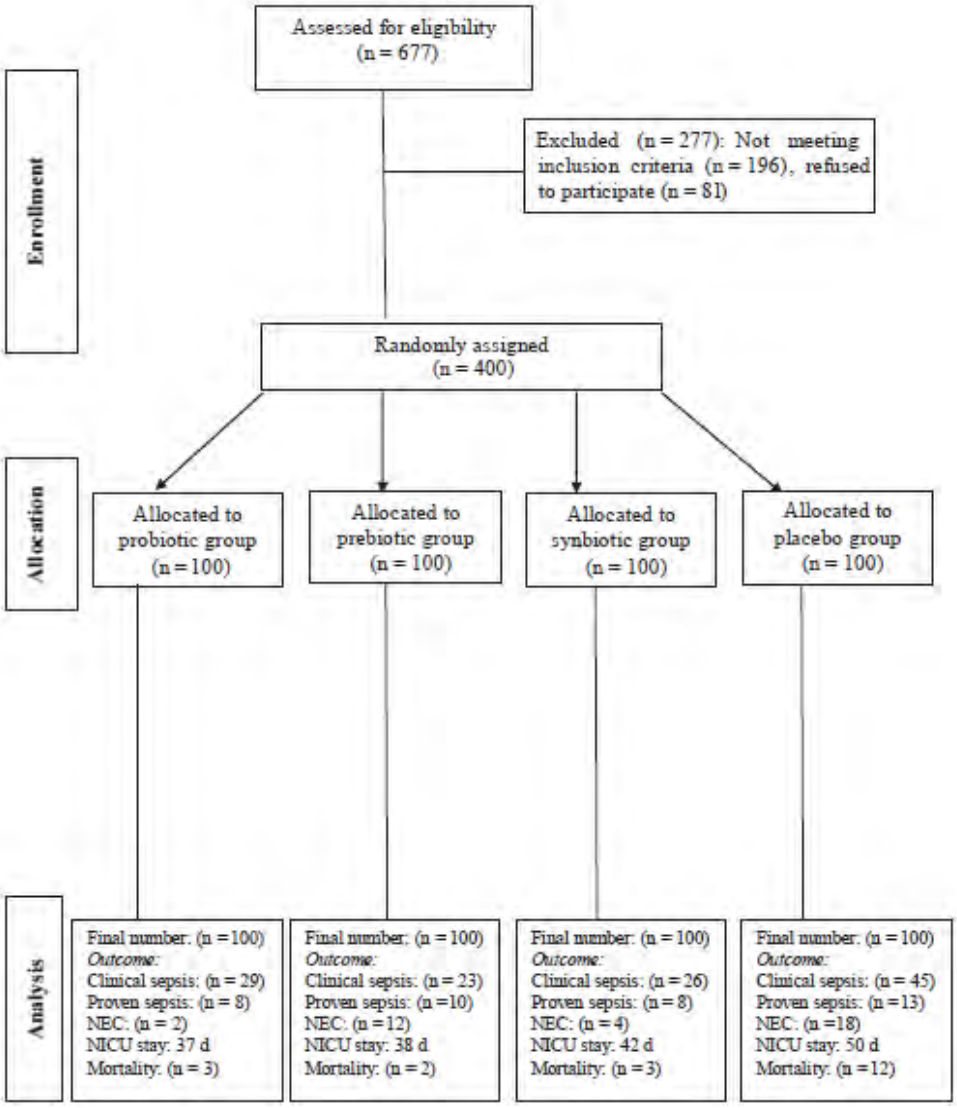

Figure 20. Flow diagram of the randomized trial (Dilli et al. 2015).

Characteristics	Pro group (n = 100)	Pre group (n = 100)	Syn group (n = 100)	Pla group (n = 100)	*P* value
Maternal age, y, mean ± SD	28.0 ± 5.9	26.9 ± 6.1	28.5 ± 6.3	27.1 ± 5.7	.18,* *F: 1.6* Syn-Pla: .34, Syn-Pre: .22, Syn-Pro: .92 Pro-Pla: .72, Pro-Pre: .56, Pre-Pla: .99
Multiple pregnancies, n (%)	13 (13)	11 (11)	8 (8)	9 (9)	.65† Syn-Pla: .80, Syn-Pre: .46, Syn-Pro: .24 Pro-Pla: .36, Pro-Pre: .66, Pre-Pla: .63
Preeclampsia, n (%)	10 (10)	14 (14)	10 (10)	7 (7)	.44† Syn-Pla: .44, Syn-Pre: .38, Syn-Pro: 1.00 Pro-Pla: .44, Pro-Pre: .38, Pre-Pla: .10
Pregnancy-induced hypertension, n (%)	3 (3)	2 (2)	8 (8)	6 (6)	.17† Syn-Pla: .57, Syn-Pre: .05, Syn-Pro: .12 Pro-Pla: .49, Pro-Pre: .65, Pre-Pla: .15
Rupture of membranes >18 h, n (%)	13 (13)	12 (12)	18 (18)	13 (13)	.60† Syn-Pla: .32, Syn-Pre: .23, Syn-Pro: .32 Pro-Pla: 1.00, Pro-Pre: .83, Pre-Pla: .83
Antenatal steroid use, n (%)	57 (57)	62 (62)	47 (47)	53 (53)	.18† Syn-Pla: .39, Syn-Pre: .03, Syn-Pro: .16 Pro-Pla: .57, Pro-Pre: .47, Pre-Pla: .19
Maternal antibiotic exposure, n (%)	3 (3)	8 (8)	9 (9)	7 (7)	.34† Syn-Pla: .60, Syn-Pre: .80, Syn-Pro: .07 Pro-Pla: .19, Pro-Pre: .12, Pre-Pla: .78
Cesarean delivery, n (%)	35 (35)	37 (37)	29 (29)	37 (37)	.18† Syn-Pla: .22, Syn-Pre: .42, Syn-Pro: .36 Pro-Pla: .76, Pro-Pre: .08, Pre-Pla: .05
Sex (male), n (%)	53 (53)	52 (52)	57 (57)	58 (58)	.76† Syn-Pla: 1.00, Syn-Pre: .47, Syn-Pro: .51 Pro-Pla: .42, Pro-Pre: .94, Pre-Pla: .39
Gestation, wk, mean ± SD	28.8 ± 1.9	29.0 ± 1.7	28.9 ± 1.9	28.2 ± 2.2	.05† Syn-Pla: .03, Syn-Pre: .75, Syn-Pro: .62 Pro-Pla: .07, Pro-Pre: .34, Pre-Pla: .01
Birth weight, g, mean ± SD	1236 ± 212	1229 ± 246	1205 ± 240	1147 ± 271	.10† Syn-Pla: .12, Syn-Pre: .46, Syn-Pro: .44 Pro-Pla: .03, Pro-Pre: .98, Pre-Pla: .03
Birth length, cm, mean ± SD	38.1 ± 3.1	38.1 ± 3.5	37.4 ± 3.4	37.1 ± 3.8	.06† Syn-Pla: .41, Syn-Pre: .16, Syn-Pro: .10 Pro-Pla: .03, Pro-Pre: .94, Pre-Pla: .04
Head circumference, cm, mean ± SD	27.3 ± 2.3	27.1 ± 2.5	27.2 ± 2.0	26.3 ± 2.8	.08* Syn-Pla: .04, Syn-Pre: .79, Syn-Pro: .91 Pro-Pla: .03, Pro-Pre: .96, Pre-Pla: .05
Apgar, 5 min, median (IQR)	7 (6-8)	8 (7-8)	7 (7-8)	7 (6-8)	.66† Syn-Pla: .61, Syn-Pre: .96, Syn-Pro: .25 Pro-Pla: .59, Pro-Pre: .27, Pre-Pla: .70
SNAPPE-II score, mean ± SD	16.2 ± 9.5	17.0 ± 13.7	20.4 ± 8.7	23.0 ± 10.9	.06,* *F: 2.5* Syn-Pla: .69, Syn-Pre: .51, Syn-Pro: .54 Pro-Pla: .15, Pro-Pre: .99, Pre-Pla: .09

Pla, placebo; Pre, prebiotic; Pro, probiotic; SNAPPE-II, Score for Neonatal Acute Physiology Perinatal Extension-II; Syn, synbiotic.
*P value for ANOVA test.
†P value for X^2 test.
†‡P value for Kruskal-Wallis test and Mann–Whitney U test.

Figure 21. Maternal and infants' demographic characteristics (Dilli et al. 2015).

	Pro group (n = 100)	Pre group (n = 100)	Syn group (n = 100)	Pla group (n = 100)	*P* value
Clinical variables					
Age at enrollment (d), median (IQR)	3 (3-4)	3 (2-5)	2 (1-4)	2 (2-4)	.10* Syn-Pla: .10, Syn-Pre: .58, Syn-Pro: .09 Pro-Pla: .25, Pro-Pre: .55, Pre-Pla: .09
Umbilical venous catheter (d), median (IQR)	10 (7-15)	10 (7-13)	10 (7-14)	10 (8-14)	.50* Syn-Pla: .31, Syn-Pre: .49, Syn-Pro: .70 Pro-Pla: .72, Pro-Pre: .44, Pre-Pla: .12
Mechanical ventilation (d), median (IQR)	2 (0-5)	1 (0-3)	1 (0-3)	2 (0-10)	.01* Syn-Pla: .01, Syn-Pre: .60, Syn-Pro: .17 Pro-Pla: .21, Pro-Pre: .06, Pre-Pla: .004
Free oxygen therapy (d), median (IQR)	4 (1-14)	3 (1-6)	6 (3-14)	7 (2-20)	.002* Syn-Pla: .89, Syn-Pre: .001, Syn-Pro: .03 Pro-Pla: .12, Pro-Pre: .25, Pre-Pla: .004
Mode of feeding [n (%)]					
Breastmilk alone	53 (53)	46 (46)	45 (45)	48 (48)	.17†
Cow-based formula	23 (23)	18 (18)	18 (18)	28 (28)	Syn-Pla: .23, Syn-Pre: .98, Syn-Pro: .13
Breastmilk and formula (mixed)	24 (24)	36 (36)	37 (37)	24 (24)	Pro-Pla: .09, Pro-Pre: .17, Pre-Pla: .22
The number of stools per wk, median (IQR)	14 (11-21)	21 (14-26)	13 (11-17)	13 (10-18)	<.001* Syn-Pla: .69, Syn-Pre: .001, Syn-Pro: .26 Pro-Pla: .48, Pro-Pre: .008, Pre-Pla: .001
Total parenteral nutrition (d), median (IQR)	16 (10-25)	14 (9-20)	18 (10-28)	21 (12-34)	.003* Syn-Pla: .06, Syn-Pre: .06, Syn-Pro: .93 Pro-Pla: .04, Pro-Pre: .06, Pre-Pla: .001
Apnea, n (%)	59 (59)	57 (57)	58 (58)	64 (64)	.76† Syn-Pla: .43, Syn-Pre: .82, Syn-Pro: .90 Pro-Pla: .46, Pro-Pre: .77, Pre-Pla: .31
Respiratory distress syndrome, n (%)	64 (64)	56 (56)	64 (64)	73 (73)	.09† Syn-Pla: .20, Syn-Pre: .21, Syn-Pro: .92 Pro-Pla: .17, Pro-Pre: .24, Pre-Pla: .01
Patent ductus arteriosus, n (%)	24 (24)	21 (21)	23 (23)	41 (41)	.005† Syn-Pla: .007, Syn-Pre: .70, Syn-Pro: 1.00 Pro-Pla: .01, Pro-Pre: .61, Pre-Pla: .002
Hyperbilirubinemia, n (%)	87 (87)	78 (78)	78 (78)	84 (84)	.29† Syn-Pla: .34, Syn-Pre: .89, Syn-Pro: .12 Pro-Pla: .54, Pro-Pre: .09, Pre-Pla: .27
Intraventricular hemorrhage, n (%)	13 (13)	5 (5)	9 (9)	18 (18)	.02† Syn-Pla: .06, Syn-Pre: .25, Syn-Pro: .37 Pro-Pla: .32, Pro-Pre: .05, Pre-Pla: .004
Overall antibiotic treatment (d), median (IQR)	7 (7-27)	7 (7-27)	7 (7-27)	27 (7-42)	.0001* Syn-Pla: .001, Syn-Pre: .88, Syn-Pro: .60 Pro-Pla: .001, Pro-Pre: .69, Pre-Pla: .001
Cholestasis, n (%)	3 (3)	3 (3)	0 (0)	2 (2)	.39† Syn-Pla: .15, Syn-Pre: .08, Syn-Pro: .08 Pro-Pla: .65, Pro-Pre: 1.00, Pre-Pla: .65
Feeding intolerance, n (%)	1 (1)	3 (3)	4 (4)	9 (9)	.02† Syn-Pla: .008, Syn-Pre: .29, Syn-Pro: .12 Pro-Pla: .001, Pro-Pre: .61, Pre-Pla: .001
Need of transfusions, n (%)					<.001†
Red blood cell, number, median (IQR)	0 (0-2)	0 (0-1)	0 (0-2)	0 (0-4)	Syn-Pla: .05, Syn-Pre: .05, Syn-Pro: .71
Fresh-frozen plasma, number, median (IQR)	0 (0-1)	0 (0-1)	0 (0-2)	0 (0-3)	Pro-Pla: .19, Pro-Pre: .12, Pre-Pla: .001
Bronchopulmonary dysplasia, n (%)	25 (25)	16 (16)	21 (21)	32 (32)	.05† Syn-Pla: .09, Syn-Pre: .32, Syn-Pro: .55 Pro-Pla: .27, Pro-Pre: .11, Pre-Pla: .008
Retinopathy of prematurity, n (%)	0 (0)	2 (2)	2 (2)	3 (3)	.48† Syn-Pla: .11, Syn-Pre: .77, Syn-Pro: .53 Pro-Pla: .32, Pro-Pre: .36, Pre-Pla: .06
Duration of supplementation‡ (d), median (IQR)	34 (24-46)	30 (21-48)	39 (26-56)	36 (20-56)	.06* Syn-Pla: .44, Syn-Pre: .01, Syn-Pro: .05 Pro-Pla: .38, Pro-Pre: .41, Pre-Pla: .18
Treatment outcomes					
Enteral feeding (d), median (IQR)					
The first time of feeding	2 (1-3)	2 (1-3)	2 (2-3)	2 (2-3)	.24* Syn-Pla: .63, Syn-Pre: .37, Syn-Pro: .17 Pro-Pla: .06, Pro-Pre: .66, Pre-Pla: .16
Time to reach 100 mL/kg per day	13 (10-17)	12 (9-18)	15 (10-22)	18 (12-25)	<.001* Syn-Pla: .03, Syn-Pre: .06, Syn-Pro: .21 Pro-Pla: .001, Pro-Pre: .49, Pre-Pla: .001

(*continued*)

	Pro group (n = 100)	Pre group (n = 100)	Syn group (n = 100)	Pla group (n = 100)	*P* value
Time to reach 150 mL/kg per day	18 (14-23)	17 (12-24)	20 (14-30)	25 (15-37)	<.001* Syn-Pla: .05, Syn-Pre: .01, Syn-Pro: .11 Pro-Pla: .001, Pro-Pre: .32, Pre-Pla: .001
Growth velocity, mean ± SD					
Weight gain, g/kg/wk	230 ± 74	241 ± 98.2	229 ± 96	227 ± 100	.90* Syn-Pla: .97, Syn-Pre: .51, Syn-Pro: .73 Pro-Pla: .81, Pro-Pre: .69, Pre-Pla: .55
Length gain, cm/wk	1.3 ± 0.7	1.4 ± 0.6	1.5 ± 0.7	1.2 ± 0.6	.04* Syn-Pla: .01, Syn-Pre: .32, Syn-Pro: .04 Pro-Pla: .53, Pro-Pre: .18, Pre-Pla: .09
Head circumference, cm/wk	1.1 ± 0.5	1.1 ± 0.5	1.2 ± 0.5	1.3 ± 0.7	.06* Syn-Pla: .14, Syn-Pre: .67, Syn-Pro: .57 Pro-Pla: .006, Pro-Pre: .74, Pre-Pla: .03
NEC, n (%)	2 (2)	12 (12)	4 (4)	18 (18)	<.001† Syn-Pla: .002, Syn-Pre: .04, Syn-Pro: .40 Pro-Pla: .001, Pro-Pre: .006, Pre-Pla: .23
Late-onset sepsis, clinical, n (%)	29 (29)	23 (23)	26 (26)	45 (45)	.004† Syn-Pla: .006, Syn-Pre: .59, Syn-Pro: .66 Pro-Pla: .02, Pro-Pre: .33, Pre-Pla: .001
Late-onset sepsis, proven, n (%)	8 (8)	10 (10)	8 (8)	13 (13)	.60† Syn-Pla: .26, Syn-Pre: .63, Syn-Pro: .98 Pro-Pla: .24, Pro-Pre: .62, Pre-Pla: .50
NICU stay (d), median (IQR)	37 (27-50)	38 (27-53)	42 (33-60)	50 (31-70)	.002* Syn-Pla: .26, Syn-Pre: .04, Syn-Pro: .01 Pro-Pla: .001, Pro-Pre: .64, Pre-Pla: .004
Weight at discharge (g), mean ± SD	1979 ± 309	2028 ± 373	2037 ± 297	2081 ± 400	.07* Syn-Pla: .97, Syn-Pre: .07, Syn-Pro: .01 Pro-Pla: .01, Pro-Pre: .82, Pre-Pla: .16
Mortality, n (%)	3 (3)	2 (2)	3 (3)	12 (12)	.003† Syn-Pla: .01, Syn-Pre: .65, Syn-Pro: 1.00 Pro-Pla: .01, Pro-Pre: .65, Pre-Pla: .06

Pla, placebo; Pre, prebiotic; Pro, probiotic; SNAPPE-II, Score for Neonatal Acute Physiology Perinatal Extension-II; Syn, synbiotic.
*P value for Kruskal-Wallis test and Mann–Whitney U test.
†P value for X^2 test.
†‡ Duration of Pro, Pre, Syn, or Pla supplementation.

Figure 22. Clinical variables and treatment outcomes in study infants by group (Dilli et al. 2015).

The incidence of NEC was significantly lower in the probiotic and symbiotic groups compared to the prebiotic and placebo groups. Additionally, the rate of clinical nosocomial sepsis was the greatest, NICU stay was the longest, and mortality rate was the greatest in the placebo group., Of the 20 deaths, 15 were attributed to sepsis and multi-organ failure (12 in the placebo group). Three deaths were attributable to advanced NEC (2 in the synbiotic, 1 in the probiotic group). Regarding safety, no unexpected adverse events were observed during the course of the study. The etiology for culture-positive sepsis was a Gram-positive pathogen in 41% of cases and a Gram-negative pathogen in 59% of cases. No Gram stains were suspicious for probiotic infection, and no cultures were sent to the central laboratory for identification of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421. Infants receiving *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (Lafti® B94) showed similar gains in weight and length to infants not receiving the probiotic.

Baştürk et al. (2016)

Baştürk A et al. (2016) conducted a prospective, randomized, double-blind, 3-arm clinical study to assess the effect of a probiotic product *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) in children suffering from irritable bowel syndrome (IBS). Study aims were to determine the relative efficacy of the probiotic, a synbiotic (LAFTI® B94 + 900 mg inulin), and a prebiotic alone (inulin) in the treatment of IBS. Seventy-six children aged between 4 and 16 years, diagnosed with IBS according to the Rome III criteria were randomized into 3 groups; however, during the study period, 5 patients were excluded from the study because they could not complete their treatment. All groups received 2 sachets daily containing $5x10^9$ cfu *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (probiotic group, n = 24), $5x10^9$ cfu *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 + 900 mg inulin (synbiotic group, n = 23), or 900 mg inulin (prebiotic group, n = 24). Evaluation of the treatment response was performed at the end of 4 weeks. Patients were questioned for changes in initial symptoms (postprandial swelling, belching or abdominal distension, mucoid defecation, difficulty in defecation, feeling of incomplete defecation, or urgent defecation). If there was an improvement of all symptoms, it was considered "fully benefited," and if there was an improvement of at least one symptom, it was "partially benefited." The primary endpoint was complete benefit of the patient with resolution of all present complaints. The secondary endpoint criterion was resolution at the end of the 4-week treatment of one or more of the symptoms.

At the beginning of the study, there was no significant difference between the groups with respect to initial complaints or IBS severity. The most common complaint was sudden urge to defecate (66.2%) followed by bloating after meal (64.8%) and belching (64.8%). The least common complaint was mucus in the stool (42.3 %). At the end of the study, full recovery was observed in 9 patients (39.1%) in the synbiotic group, 7 patients (29.2%) in the probiotic group, and 3 patients (12.5%) in the prebiotic group. In the probiotic group, there was a significant improvement in belching or abdominal fullness, bloating after meals, and difficulty with defecation. In the synbiotic group, the most significant improvement was in belching or abdominal fullness and bloating after meals. No significant improvement in any of the initial complaints was found in the prebiotic group. No side effects were reported by participants of any group from ingestion of 10^{10} cfu of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) per day.

El et al. 2017.

Bifidobacterium animalis ssp. *lactis* Rosell®-421 (LAFTI® B94) was used in a clinical trial, (El et al. 2017) to determine if there was an effect on feeding intolerance and weight gain in 89 preterm infants.

A total of 98 preterm infants were enrolled in this study, with inclusion criteria being admitted to the tertiary NICU with feeding intolerance, along with ≤35 weeks gestational age, and ≤2500g at birth. Exclusion criteria included infants with any disease other than those linked to prematurity or congenital anomalies of the intestinal tract, and children whose parents refused to participate.

The 98 infants were randomized into two groups, the treatment group orally receiving *Bifidobacterium animalis* ssp. *lactis* Rosell®-421-LAFTI® B94 in MAFLOR® sachets 3 times per day. Both groups of infants received total parenteral nutrition feeding, which consisted of 1 g/kg bw/day lipid and 2 g/kg bw/day amino acid infusions from birth. These feedings were increased by 1 g/kg bw/day up to 3.5-4.0 g/kg bw/day for amino acids and by the same amount up to 3 g/kg bw/day for lipid infusions. MAFLOR® sachet was diluted in 10 mL of distilled water, and 1 mL of this was given to the infants 3 times per day. This resulted in a daily dose of $1.5x10^9$ cfu of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94). Dissolved sachets were used within 24 hours of dissolution.

Nine infants were withdrawn from the study (5 from treatment group, 4 from control) due to death for the following reasons: 4 deaths for respiratory complications (2 from control, 2 from treatment), 2 deaths from cardiac causes (1 from each group), and 3 from sepsis (2 from the control group, 1 from the treatment group). These infants were not included in the final result analysis. In the final analysis, 47 infants were in the treatment group and 42 were in the control group.

Infant and maternal characteristics were monitored throughout the study. Duration of total parenteral nutrition feeding, starting time of full enteral feeding, starting time of oral feeding, and daily weight gain were recorded for the infants. The type of feeding was recorded as formula, breast milk, or breast milk and formula.

There was a significant difference in baseline birth weight between the two groups, with the birth weight of the treatment group being lower than the control group.

There was a significant increase in sepsis in the treatment group versus the control group and the duration of total parenteral nutrition was significantly longer in the treatment group versus control group. The treatment group gained weight significantly faster than the control group. Stratifying the groups based on weight at birth into 3 groups of <1000 g, 1001-1500 g, and >1500 g showed that preterm infants who received the treatment gained weight significantly faster than the control group.

In conclusion, MAFLOR® sachets may have had a positive effect in increasing weight gain, but this is uncertain. There were, however, no adverse effects attributable to treatment with MAFLOR® sachets containing *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94).

Table 12. Studies of MAFLOR® Sachets in Infants and Children.

Reference	Objectives	Study Design	Subjects	Dose/Day	Duration	Safety-Related Results
Aydin et al. 2012	Effects of B94 and inulin on morbidity and mortality in preterm infants	Prospective, randomized, placebo controlled with two groups (placebo n=17, probiotic n=17). Treatment = B. *animalis* subsp. *lactis* Rosell®-421, $5x10^9$ cfu.	34 VLBW infants	$1.5x10^9$ cfu/day	duration not specified	Sepsis risk lower in treatment group. NEC (grade ≥2) was higher in control group. Rate of BPD and ROP were lower in treatment group. No difference between groups in mortality, actual weight during hospitalization and at discharge.
Basturk et al. 2016	Efficacy of synbiotic, probiotics, and prebiotic treatments for IBS in children	Double-blinded, Randomized, Prospective, Controlled study, 3 groups: probiotic group (B. lactis B94) n=24 / Synbiotic (B. lactis B94 + 900mg inulin n=23 / Prebiotic group (900 mg inulin) n=24 Dose: twice daily	71 Participants: age 4 to 16 years old with IBS diagnosed by ROME III criteria	$10x10^9$ cfu/day	4 weeks	Primary endpoint: complete benefit for 39.1% of synbiotic, 29.2% of probiotic, and 12.5% of prebiotic. Significant difference between prebiotic and synbiotic group only. In probiotic group, significant improvements in belching–abdominal fullness, bloating after meals, and difficulty with defecation. In synbiotic group, significant improvements in belching–abdominal fullness, bloating after meals, difficulty with defecation, and mucus in the stool. In prebiotic group, no significant improvement for any one symptom. No side effects reported by participants of any group

Reference	Objectives	Study Design	Subjects	Dose/Day	Duration	Safety-Related Results
Dilli et al. 2013	Evaluate the effect of probiotics on nosocomial sepsis, NEC and mortality in infants with cyanotic congenital heart disease (CCHD).	Prospective, blinded, randomized, controlled trial 2 groups (n=50/group): symbiotic group (Maflor® sachet = B. *animalis* subsp. *lactis* Rosell®-421, $5x10^9$ cfu + 900mg inulin), and Placebo group	100 infants with cyanotic congenital heart disease, aged between 4 and 5 days, > 35 weeks of gestational age	$5x10^9$ cfu/day	Average of 19 days	Significantly lower incidence of nosocomial infection in the probiotic group (8% vs 28%). Durations of total parenteral nutrition (7.0 vs 12.5 days) and mechanical ventilation (3 vs 5 days) were longer in the placebo group. Length of NICU stay did not differ between the groups. Death incidence: 10% in the probiotic group vs 28 % in the placebo group. No probiotic-associated adverse events were reported.
Dilli et al. 2015	Evaluate the effect of probiotics on NEC in VLBW infants	Prospective, randomized, controlled trial 4 groups (n=100/group): prebiotic group (900mg inulin), probiotic group (*B. animalis* subsp. *lactis* Rosell®-421, $5x10^9$ cfu), symbiotic group (Maflor® sachet = B. *animalis* subsp. *lactis* Rosell®-421, $5x10^9$ cfu + 900mg inulin), and Placebo group	400 very low birth weight (VLBW) infants, 7 days old, with a gestational age of <32 weeks and a birth weight of <1500g	$5x10^9$ cfu/day	maximum of 8 weeks	Lower incidence of NEC in probiotic and symbiotic groups compared to other groups. Rate of clinical nosocomial sepsis was the greatest, NICU stay was the longest, and mortality rate was the greatest in the placebo group. No unexpected adverse events were observed during the course of the study. The etiology for culture-positive sepsis was a Gram-positive pathogen in 41% of cases and a Gram-negative pathogen in 59% of cases. No Gram stains were suspicious for probiotic infection, and no cultures were sent to the central laboratory for identification of *B. animalis* subsp. *lactis* Rosell®-421 No difference between B94 and placebo in weight gain or length gain.

Reference	Objectives	Study Design	Subjects	Dose/Day	Duration	Safety-Related Results
El et al. 2017	Evaluate the influence of B94 and inulin on feeding intolerance and weight gain in preterm babies	Prospective, randomized, placebo controlled with two groups (placebo n=42; treatment n=47) Treatment = B. *animalis* subsp. *lactis* Rosell®-421, $0.5x10^9$ cfu – 3x/d.	89 preterm infants with feeding intolerance, <35 weeks old, <2500g at birth.	$1.5x10^9$ cfu/day	34 days	Increased incidence of sepsis in treatment group versus control, not mortality rate between groups was similar. Confounded by high risk population with permanent catheter for parenteral feeding, and differences between birth weights (lower in treatment group).
Erdogan et al. 2012	Efficacy of two different probiotics in rotavirus gastroenteritis in children	Randomized, Prospective, Controlled study 3 groups: control group (n=25) / Yeast group (n=25): 282.5 mg/day S. boulardii I-745 / B. Lactis B94 group ($5x10^9$ cfu/day)	75 Participants: age 5 months to 5 years old diagnosed with rotavirus gastroenteritis, last 48 hours	$5x10^9$ cfu/day	duration not specified	Duration of diarrhea was significantly shorter in *B. lactis* group compared to the other 2 groups (4.1 ± 1.3 vs. yeast: 6.6 ± 1.7 and control: 7.0 ± 1.6). No significant differences in vomiting between all 3 groups. There were no reports of intervention-related adverse events
İşlek et al 2014	B94 and acute infectious diarrhea in children	Double-blinded, Randomized, Placebo-controlled study 2 groups: placebo group (n= 77/89)/ Probiotic group (n=77/89)	179 patients age 2-60 months with acute diarrhea which lasted for less than 7 days, presenting at a hospital	$5x10^9$ cfu/day	5 days	Primary endpoint: duration of diarrhea significantly shorter in synbiotic group than placebo (3.9±1.2 days vs. 5.2±1.3 days). Number of diarrheal stool on third day was significantly less in synbiotic group than placebo (5.5±2.9 vs. 8.3±3.0). Cases of diarrhea on 5th day were significantly higher in placebo group than synbiotic (38.9% vs. 1.7%) No significant difference in duration of vomiting or fever. No side effects were reported by parents

6.4.1.2. Studies of other formulations containing *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) in Children

Bifidobacterium animalis ssp. *lactis* Rosell®-421 (LAFTI® B94) has been extensively marketed by Lallemand Health Solutions for use in infants, children, and adults as a part of other formulas, such as Maflor® capsules (not to be confused with Maflor® sachets).

MAFLOR® capsule is a combination of *B. animalis* ssp. *lactis* Rosell®-421 (33%), *L. helveticus* Rosell®-52 (33%), and *L. casei* Rosell®-215 (34%). Each capsule of MAFLOR® capsule contains $7x10^9$ cfu of all strains, $2.3x10^9$ cfu of *B. animalis* ssp. *lactis* Rosell®-421. While studies with this combination product cannot demonstrate efficacy of *B. animalis* ssp. *lactis* Rosell®-421 alone, absence of intervention-associated adverse effects indicates the safety of the probiotic at the level of ingestion of the study. The study discussed below is summarized in Table 13 at the end of this section.

Cakir et al. 2017

A prospective open-label study (Cakir et al. 2017) looked at the efficiency of the probiotic combination (*B. animalis* ssp. *lactis* Rosell®-421 (33%), *L. helveticus* Rosell®-52 (33%), and *L. casei* Rosell®-215 (34%) with 100 mg chicory inulin) in treating children with non-alcoholic fatty liver disease (NAFLD). Study aims were to analyse the efficiency of long-term probiotic treatment with lifestyle changes compared to healthy subjects undergoing the same program. The study duration was 4 months, involving 28 children aged 12.2±2.2 years in the NAFLD group, and 30 children aged 12.2±2.1 years in the healthy group. In addition to the probiotic supplementation over the 4 months of the study, each group received a low caloric diet and followed a moderate exercise program 30-45 minutes/day at least 3 times a week. Compliance to diet and exercise routine was verified by face-to-face questioning at each visit. Two subjects in the NAFLD group were lost during follow up and therefore excluded from the group. Anthropometric data were taken at baseline and 4 months. Blood samples were collected at the beginning of treatment and at the 4-month follow-up for analysis of glucose, liver enzymes, insulin, plasma lipids, C-reactive protein, TNF-α, serum ethanol, total oxidant and anti-oxidant status, and zonulin. An ultrasound of the liver was also performed at entry to the study and at the 4-month follow-up point, and liver steatosis was graded as normal, mild, moderate, or severe depending on the sonographic findings. A response to treatment was determined if there was a decrease in the grade of the liver steatosis of 1 grade or larger at the end of the 4 month period as compared to baseline.

The program of exercise and supplementation was concurrent with a reduction in the severity of the liver steatosis in 67.8% of the children who were in the NAFLD group. Serum ALT and AST, BMI, and total body fat were all significantly decreased from baseline to 4 months. No side effects were reported in the study.

Table 13. Study of MAFLOR® Capsules in Infants and Children.

Reference	Objectives	Study Design	Subjects	Dose/Day	Duration	Safety-Related Results
Cakir et al. 2017	Analyze the efficiency of a long-term synbiotic supplementation, in addition to lifestyle changes in children with non-alcoholic fatty liver disease (NAFLD).	Longitudinal study Two groups: Probiotic groups : 28 children with NAFLD (1 capsule/day) Control group: 30 healthy children	28 children (12 years old) with NAFLD and 30 healthy children (12 years old)	$2.3x10^9$ cfu/day	4 months	In the probiotic group, the grade of fatty liver was decreased (≥1 grade) in 19 of the 28 patients (67.8%). Total cholesterol, LDL levels, TNF-α, CRP, and ethanol were significantly decreased. For both groups: TAS levels were significantly increased at the end of treatment. Median decreases in CRP (-0.16 vs. -0.03 mg/dL) and LDL levels (-17 vs. -3 mg/dL) were higher in patients who responded to the supplementation. No side effects were reported

6.4.1.3. Meta-analysis

Dermyshi et al. (2017) published a systematic review and meta-analysis of randomized controlled trials (RCTs) and observational studies assessing the use of probiotics in very low birth weight (VLBW) preterm infants.

All RCTs and observational studies involving VLBW (<1,500 g) preterm (<34 weeks gestational age) infants with enteral administration of probiotics initiated within 10 days were included. Thirty RCTs and 14 observational studies were selected. The study from Dilli et al. (2015) presented above was selected, with the strain *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94), as well as 2 other studies with other strains of *Bifidobacterium animalis* ssp. *lactis* with a dose of $1x10^9$ cfu/day for 4-6 weeks (Hays et al. 2015) and $2x10^9$ cfu/kg bw/day for 6 weeks (Mihatsch et al. 2010). In 3 other RCTs and 1 observational study included in this meta-analysis, *Bifidobacterium animalis* ssp. *lactis* was also used in combination with other strains (Hays et al. 2015, Jacobs et al. 2013, and Lambæck et al. 2016). See both tables below.

Table 14. Characteristics of the Included RCT Studies (Dermyshi et al. 2017).

Author	Infants on Pro-biotics (n)	Control Infants (n)	Inclusion Criteria (BW/GA)	Strain, Dose, and Duration	NEC probiotics and controls, n	Species probiotics and controls (culture +), n	Mortality probiotics and controls (all cause), n
Dilli 2015	100	100	<32 weeks and <1,500 g	*B. lactis* LAFTI B94 (5×10^9 cfu for 8 weeks)	1/100 and 18/100	8/100 and 13/100	3/100 and 12/100
Mihatsch, 2010	91	89	<30 weeks and <1,500 g	*B. lactis* (2×10^9 cfu/kg/day for 6 weeks)	2/91 and 4/89	28/91 and 29/89	2/91 and 1/89
Hays, 2015	50	52	25 - 31 weeks and 700 – 1,600 g	*B. lactis* (1×10^9 cfu/day for 4 – 6 weeks)	2/50 and 3/52	9/50 and 10/52	1/50 and 1/52
Jacobs 2013	548	551	<32 weeks and <1,500 g	*B. infantis*, *S. thermophilus*, and *B. lactis* (1×10^9 cfu/day until discharge)	11/548 and 24/551	72/548 and 89/551	27/548 and 28/551
Jacobs 2013 (sub-group)	232	239	<1,000 g	*B. infantis*, *S. thermophilus*, and *B. lactis* (1×10^9 cfu/day until discharge)	10/232 and 14/239	53/232 and 58/239	NR and NR
Hays, 2015	47	52	25 – 31 weeks and 700 – 1,600 g	*B. lactis* and *B. longum* (1×10^9 cfu/day for 4 – 6 weeks)	5/47 and 3/52	9/47 and 10/52	1/47 and 1/52

cfu, colony-forming unit; BW, birth weight; GA, gestational age; NR, not reported.

Table 15. Characteristics of the Included Observational Study (Dermyshi et al. 2017).

Author	Infants on pro-biotics (n)	Control Infants (n)	Inclusion Criteria (BW/GA)	Strain, Dose and Duration	NEC probiotics and controls, n	Species probiotics and controls (culture +), n	Mortality probiotics and controls (all cause), n
Lambæk, 2016	333	381	<30 weeks	*B. lactis* BB12/*L. rhamnosus* GG (10^8 and 10^9 cfu)	23/333 and 34/381	NR and NR	54/333 and 66/381

cfu, colony-forming unit; BW, birth weight; GA, gestational age; NR, not reported.

The analysis of these data supports the hypotheses that probiotics potentially prevent severe NEC and late-onset sepsis and reduce mortality in preterm infants. Importantly, no probiotic-associated adverse events were reported in this systematic review and meta-analysis of studies in which probiotics were administered to an especially sensitive population of infants.

6.4.1.4. Conclusions from Studies in Infants and Children

Clinical data for *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) has been obtained from a variety of sources, in a variety of infant populations, from pre-term LBW and VLWB infants, up to full term older infants with gastrointestinal disorders.

In terms of safety, the preterm and LBW infants present strong evidence of safety in an extremely high risk population - preterm birth and low birth weight are the leading risk factors for infant death in the USA, and account for the second largest cause of infant death after congenital malformations, deformations, and chromosomal abnormalities (Mathews and MacDorman 2006; Mathews et al. 2015). In addition to mortality, morbidity rates in preterm infants and low birth weight infants are higher than for full term and normal weight infants, with an inverse correlation between morbidity and age of gestation (McIntire and Leveno, 2008; Glass et al. 2015).

Given the high rate of mortality and morbidity in this population, the fact that *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) actually reduced the rate of mortality in the Dilli et al. (2015) trial, which included in 400 VLBW infants, as well as the morbidities of NEC and other late onset sepsis, offers evidence that this probiotic is safe for this population. Dilli et al. (2015) also evaluated growth parameters (gain in weight, length, and head circumference), and demonstrated that there were no differences between the groups that had ingested the *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) and those that hadn't.

Similar growth results were shown in El et al. 2017, where there was in fact an increase in rate of weight gain for the >1500-g infant group as compared to placebo. One concern in the El et al. 2017 study was an increase in sepsis in the treatment group. This does not present a risk to the normal non-hospitalized, infant population, as this has a high probability of originating from risk factors that would not be evident in the intended population. Specifically, the infants in the treatment group were significantly younger, which led to longer parenteral feeding as compared to the placebo group. Parenteral feeding involves the use of a central venous catheter to deliver nutrients directly to the bloodstream of the neonate, and is one of the largest risks for sepsis in a NICU (van den Hoogen et al. 2006). In addition, the mortality rate for sepsis was similar between both groups, with the control group actually having more deaths from sepsis than the treatment group (2 vs 1, respectively).

This effect was reversed in Dilli et al. (2013), where there was a lower rate of nosocomial infection in the treatment group than in the placebo group. This could be due to the population differences. While the infants in this group are quite sick (cyanotic CHD), they are older (>35 weeks at birth) and were being fed enterally after day 7 as opposed to through a catheter. This study also showed a decrease in mortality in the treatment group versus the placebo group, as

well in morbidities in NEC and intraventricular hemorrhage, sepsis (clinical and proven), antibiotic use, feeding intolerance, and the need for blood transfusions.

Basturk et al. 2016, Erdogan et al. 2012, Islek et al. 2014 reported no adverse events in any of the older populations.

In conclusion, given the safety of intake of $5x10^9$ cfu of the bacterial strain *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) in an extremely at-risk population, and the safe use in older infants and children, it has been shown to be safe for use in children from infancy.

6.4.2. Studies in Adults

6.4.2.1. Studies of other formulation containing Bifidobacterium animalis ssp. lactis Rosell®-421 (LAFTI® B94)

Bifidobacterium animalis ssp. *lactis* Rosell®-421 (LAFTI® B94) has been extensively marketed by Lallemand Health Solutions for use in infants, children, and adults in Maflor® Capsules.

MAFLOR® capsules are combinations of *B. animalis* ssp. *lactis* Rosell®-421 (33%), *L. helveticus* Rosell®-52 (33%), and L. casei Rosell®-215 (34%). Each MAFLOR® capsule contains $7x10^9$ cfu of all strains combined, corresponding to $2.3x10^9$ cfu of *B. animalis* ssp. *lactis* Rosell®-421.

The study discussed below is summarized in Table 16 at the end of this section.

Çekin et al. (2017) reported a prospective, randomized, double-blind, placebo-controlled clinical study to investigate the effect of Maflor® capsules as an adjuvant to sequential *H. pylori* antibiotic therapy. Major study aims were to determine the efficacy of *H. pylori* eradication, as well as determining if the product was able to affect the prevalence of adverse effects in sequential antibiotic therapy. A total of 159 adults with *H. pylori* was recruited to the trial, where the inclusion criterion was diagnosis of *H. pylori* via endoscopic gastric biopsies. Exclusion criteria were previous *H. pylori* eradication therapy, gastric cancer, and known allergic reactions to penicillin therapy. All groups received sequential eradication therapy (ERA) consisted of a 2 week regimen of 1 week of amoxicillin 1000 mg, and PPI 40 mg, and then 1 week of metronidazole 500 mg, clarithromycin 500 mg, and PPI 40 mg (ERA only group, n=54). The probiotic group (ERA+Maflor®capsule, n=52) received the standard ERA program along with a probiotic supplement containing $2.3x10^9$ cfu/day of *Bifidobacterium animalis* subsp. *lactis* LAFTI® B94) in capsule form. The placebo group (ERA+placebo, n=53) received the standard ERA program with 1 capsule of placebo each day. Patient demographics (age, gender), treatment outcome (eradication rate, compliance, reason for treatment discontinuation), and known symptoms of ERA treatment (loss of appetite, nausea, vomiting, taste alteration, dizziness, abdominal pain, diarrhea, headache, and skin rash) were monitored during the trial. Baseline measurements of all parameters were taken before treatment, 1 week into treatment (along with patient surveys of adverse effects), and at the end of the study (2 weeks), at which time the frequency of adverse symptoms and treatment outcomes were recorded.

Treatment outcome was determined by presence of *H. pylori* from endoscopic gastric biopsies and analysed by experienced pathologists blinded to each sample and treatment. Compliance to the treatments was monitored at the end of the study via pill counts, as well as a verbal questionnaire. The rate of eradication comparing the ERA+Maflor®capsule (86.8%) to combined ERA (70.8%) was statistically significant. Data regarding adverse effects indicated that the probiotic helped reduce the frequency of these effects.

Comparing the three groups for Week 1 adverse event reporting showed that the ERA+ Maflor®capsule group had statistically-significant reduced incidence of loss of appetite and

diarrhea. Week 2 analysis of the same groups showed there to be a significant decrease in loss of appetite, nausea, abdominal pain, diarrhea, headache, and skin rash. Only 1 patient in the ERA+Maflor®capsule group dropped out due to diarrhea, versus 13 in the combined ERA group, which was statistically significant. No adverse events following intake of Maflor®capsule was reported by the authors (Çekin et al. 2017).

Table 16. Adult Study of MAFLOR® Capsules.

Reference	Objectives	Study Design	Subjects	Dose/Day	Duration	Safety-Related Results
Cekin et al. 2016	Assess use of probiotics as an adjuvant to sequential *H. pylori* eradication therapy	Prospective, randomized, double-blind, placebo-controlled study; 3 groups of patients: 1. ERA group received ERA treatment n=54 2. ERA+probiotic group received ERA treatment and Maflor® capsule n=52 3. ERA+placebo group received ERA treatment and placebo capsule n=53.	159 participants: mean age 46.8±13.1 years with diagnosed *H. pylori* via endoscopic gastric biopsy	$2.3x10^9$ cfu/ day	2 weeks	No adverse events reported Significantly higher eradication in "ERA+probiotic" group compared to "ERA-only" or "ERA+placebo" group combined. No significant difference in the treatment resistance to antibiotics, overall non-compliance, diarrhea-related non-compliance, and skin-rash-related non-compliance. First week diarrhea related non-compliance was significantly lower in probiotic group compared to other two groups combined. Significantly lower symptoms in probiotic group during first week (loss of appetite, diarrhea, headache) and second week (loss of appetite, nausea, dizziness, abdominal pain, diarrhea, headache, and skin rash).

6.4.2.2. Conclusions from Studies in adults

Data obtained from the clinical study of the MAFLOR® capsule showed that consumption of *B. animalis* ssp. *lactis* Rosell®-421 at a dose of 2.3 2.3x10^9 cfu /day was well tolerated (Çekin et al. 2017). No adverse events were reported, indicating that the strain *B. animalis* ssp. *lactis* Rosell®-421 is safe for use in adults.

6.4.3. Studies in Animals

6.4.3.1. Studies of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94)

The research studies discussed below are summarized in Table 17 at the end of this section.

A 13-week oral toxicity assessment on Wistar rats was performed on *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) by Citoxlab, (under Citoxlab North America GLP Study No. 1015-2021, final report dated July 20, 2018).

Bifidobacterium animalis subsp. *lactis* Rosell®-421 (LAFTI® B94) was administered once daily to 10 male and 10 female Wistar rats, at a dosage of 1000 mg/kg bw/day, equivalent to more than 350 x 10^9 cfu/kg bw/day. The study was conducted in accordance with OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98)17), with the following exceptions:

- The test item was characterized under the Health Canada Natural and Non-prescription Health Products Directorate (NNHPD) GMP regulations.
- The acceptance criteria of the dose formulation results were not defined in the Study Plan before the conduct of the analyses and the analyses of dose formulation samples and remaining test item powder after the treatment period, were not performed according to GLP regulations but in accordance with Health Canada Natural and Non-prescription Health Products Directorate (NNHPD) GMP regulations
- Charles River Kingston, NY, was not qualified at the time of animal arrival; however qualification of this animal source site was completed thereafter during the study. Therefore, this deviation had no impact on the integrity of the study.

The study found that *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) at 1000 mg/kg/day was well tolerated by Wistar rats, and demonstrated no toxicological effects on clinical signs, general behavior in the Fuctional Observation Battery, motor activity, body weight, feed consumption, ophthalmology, clinical pathology, organ weight, or macroscopic or microscopic findings that could be considered to be related to *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94).

Two *in vivo* studies, Mahoney and Henriksson (2003) and Zhang et al. (2008), showed the competition effect of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) against pathogens.

<u>Mahoney and Henriksson 2003</u>

This study was designed to assess competitive activity of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) against a virulent strain of *Listeria* in SPF BALB/c mice. Mice were intragastrically challenged with broth cultures of five strains of *Listeria*. The most virulent strain was selected based on the weight loss of the mice and *Listeria* in fecal samples. The mice were then fed salami batter with no culture, or with a combination of starter cultures (including

Bifidobacterium animalis subsp. *lactis* Rosell®-421 (LAFTI® B94)) added. The amount added to the batter was added to equal 10^6 cfu/g in the final batter, and 30-g aliquots of batter were left for fermentation at the bacteria's stationary phase for 3 days at 25°C. The amount of *Listeria* detected in the fecal samples of the mice after inoculation and batter feeding was determined to be the measure of inhibition, along with an *in vitro* inhibition zone test. The *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) did not show any significant effects inhibiting the *Listeria* strain, but the authors did not report any adverse effects due to the probiotic.

Zhang et al. 2008

Zhang et al. (2008) investigated the immunomodulatory effect of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) and *Lactobacillus casei* LAFTI® L26 on *Helicobacter pylori* –associated gastric inflammation. Forty-eight 8-week-old female C57BL/6 mice were inoculated by oral gavage with *H. pylori* SS1 (150 µl at 10^9 cfu/ml) three times over a period of 1 week.

Two weeks after the *H. pylori* inoculation, mice were randomly divided into 3 groups (n = 16/ group) and fed *L. casei* L26 or *B. lactis* B94 dissolved in milk at a concentration of 10^{10} cfu/ml, or milk alone for 5 weeks. A further 8 mice of the same age, not infected with *H. pylori* and fed a regular mouse diet, were included as normal controls. Gastric histology, protein levels of interleukin (IL)-1β, IL-10, IL-12/23p40, and *H. pylori* colonization density in the gastric tissues, as well as *H. pylori*-specific antibodies, were examined. Protein levels of IL-1β, IL-10, IL-12/23p40, *H. pylori* colonization density, and *H. pylori*-specific antibodies significantly decreased in mice fed with *L. casei* L26 and *B. lactis* B94, reported by the authors to have resulted from a modulation of immune response rather than a decrease of *H. pylori* colonization. Furthermore, *B. lactis* B94 had the intrinsic ability to promote a Th1 immune response through an increase in IL-12/IL-23. The authors did not report any adverse events.

Other animal studies assessed the effects of the strain *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) in other areas such as immune response, cognitive function, metabolic syndrome, and cancer cells.

Peran et al. 2007

Peran et al. (2007) evaluated the intestinal anti-inflammatory effects of three probiotics, *Lactobacillus casei* LAFTI® L26, *Lactobacillus acidophilus* LAFTI® L10, and *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94), in a trinitrobenzenesulphonic acid (TNBS) model of induced rat colitis.

Female Wistar rats weighing 180–200 g were randomly assigned to 5 groups (n = 10); 2 of them (non-colitic and untreated colitis groups) received no probiotic treatment and the remaining 3 groups (treated groups) each received one of the probiotics ($5x10^8$ cfu suspended in 0.5 ml of

skimmed milk) daily for 3 weeks. Both the non-colitic and untreated colitis groups also received skimmed milk without the probiotics.

Two weeks after starting the experiment, the rats were fasted overnight and those from the untreated colitis and –probiotic-treated groups were rendered colitic. Colonic damage was evaluated histologically and biochemically 1 week after TNBS instillation. The administration of probiotics for 2 weeks before colitis induction did not affect weight evolution compared with untreated rats. The intracolonic administration of TNBS resulted in an intestinal inflammatory status in the rats characterized by anorexia, loss of weight, and diarrhea, which gradually increased with time during the 7 days after instillation. These parameters were not significantly modified by any probiotic treatment with the exception of the group treated with *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94), which showed a significantly lower incidence of diarrhea when compared with TNBS control rats. At 1 week after TNBS treatment, the animals that were treated with probiotics had fewer signs of mucosal inflammation. This beneficial effect was evidenced macroscopically by a significant reduction of the colonic weight⁄length ratio in all probiotic treated groups. Biochemically, all probiotics restored colonic glutathione levels, depleted as a consequence of the oxidative stress of the inflammatory process. *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) treatment reduced colonic tumor necrosis factor (TNF)-α production and expression of inducible nitric oxide synthase and cyclo-oxygenase-2 expression. The authors did not report any adverse events.

Goudarzvand et al. 2016

Goudarzvand et al. (2016) investigated the effect of the probiotics *Lactobacillus plantarum* and *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) on the acquisition phase of spatial memory in the local demyelination of rats' hippocampus using ethidium bromide (EtBr). Thirty-two Wistar rats were used, evenly split into 4 groups, one as control (injected with saline), one injected withEtBr, one with EtBr and *Lactobacillus plantarum*, and the last with EtBr and *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94). Each probiotic was administered at $1.5x10^8$ cfu per day for 28 days. Rats were tasked with solving the Morris water maze test, a test used to assess spatial learning and memory using swimming speed and distance travelled to a previously trained hidden podium. No significant effects were seen, and no adverse events were reported.

Le Leu et al. 2005

Le Leu et al. (2005) used *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) in a rat model of colon cancer to assess any protective effects. *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) was given for 4 weeks to Sprague-Dawley rats at $1x10^8$ cfu/g of feed, along with resistant starch in place of cornstarch in their diet. The rats were split into two large groups based on whether or not the diet contained the resistant starch, and then further into 4

subgroups, control (no probiotics), a *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) group, a *Lactobacillus acidophilus* group, and one group receiving both probiotics. These subgroups were mirrored between both diets. A carcinogen test (azoxymethane) was administered to both groups, and a number of end points were measured: bacterial enumeration, fecal and cecal pH, SCFA levels, cell proliferation, and the acute apoptotic response (AARGC). *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) with a high resistant starch diet aided in improving the apoptosis score and removing cancerous cells from the intestinal tract. Other end points were also improved with increased *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) and/or resistant starch. No adverse effects were reported.

Moghadam et al. 2017

This animal study (Moghadam et al. 2017) investigated the effect of *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) and *Lactobacillus plantarum* on blood serum levels of calcium and cholesterol in mice after administration of ethidium bromide. Four groups, each with 10 mice, consisted of a negative control group that did not receive EtBr inoculation, a positive control group that received 3 µl EtBr, and *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) and *Lactobacillus plantarum* groups that both received EtBr inoculations. Both probiotics were administered by gavage to the mice for 28 days at $2x10^8$ cfu/day. Serum levels were measured and no significant effects were seen or adverse events reported.

Table 17. Animal Studies of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94)

Reference	Objectives	Study Design	Subjects	Dose/Day	Duration	Safety-Related results
Mahoney and Henriksson (2003)	Determine effects of strain in combating *Listeria* infectivity in mice	Controlled *in vivo* study	8-10-week-old female BALB/c mice	$3x10^7$ cfu/ day	N/A	No safety related adverse events were reported
Zhang et al. (2008)	Investigation of immunomodulatory effects of B94 in mice during *H. pylori* infection	Controlled *in vivo* study	8-week-old female C57BL/6 mice	10^{10} cfu/ ml	Daily treatment for 5 weeks – 3 weeks lead in with H. pylori infection	In mice with *L. casei* L26 and *B. lactis B94* there was decreased IL-1β, IL-10, IL-12/23p40, *H. pylori* colonization density, and *H. pylori*-specific antibodies. *B. lactis* B94 promoted a Th1 immune response through an increase in IL-12/IL-23. No adverse events were reported.
Peran et al. (2007)	Determining anti-inflammatory effects of B94 in a TBS colitis model in rats.	Controlled *in vivo* study	Female Wistar rats	$5x10^8$ cfu/ day	3 weeks.	Reduction was observed in diarrhea caused by TBS administration, colonic TNF-α production, and inducible nitric oxide synthase and cyclo-oxygenase-2 expression. No weight loss resulted from probiotic administration
Le Leu et al. (2005)	Combination of B94 in combination with resistant starch in reducing carcinogenic damage in rat colon	Controlled *in vivo* study	Male Sprague-Dawley rats	$1x10^8$ cfu/ g of feed	4 weeks	Improvement of the apoptosis score, removal of cancerous cells from the intestinal tract compared to the probiotic and low starch free diet with *B. lactis* B94. No adverse events were reported.
Goudarzvand et al. 2016	Protective effects of probiotics in MS model	Controlled *in vivo* study	Male Wistar rats	$1.5x10^8$ cfu/day	4 weeks	No significant effects were seen, and no adverse events were reported.
Moghadam et al. 2017	Ability of probiotic to improve serum nutrient content	Controlled *in vivo* study	Male Wistar rats	$2x10^8$ cfu/ day	4 weeks	No significant effects were seen, and no adverse events were reported.

6.4.3.2. Meta-analysis

Crittenden et al. (2005) published an overview of the intestinal microbial ecosystem and interactions between gut bacteria, diet, and health of the human host. The review observed that *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) possesses suitable organoleptic properties and does not contain plasmids or unusual antibiotic resistances that might compromise safety. The *in vitro* screening results also provided indications of potential health benefits, including production of vitamin folate in yoghurt (Crittenden et al. 2003), and the inhibition of intestinal pathogens, including *Salmonella typhimurium*. Subsequently, the strain was selected for *in vivo* examination of its ability to protect against *Salmonella* infection. Specific pathogen-free mice were fed for a week with either *B. lactis* LAFTI® B94, another common commercial *B. lactis*, or no probiotic as a control, and then challenged with a single dose of *Salmonella typhimurium* (Henriksson et al. 1999; Henriksson et al. 2001). Even though the mice fed *B. lactis* LAFTI® B94 remained colonised with *Salmonella* to a similar degree as the controls, the probiotic protected the animals against infection and the mice maintained body weight and condition. In contrast, the control mice and those fed the closely related strain of *B. lactis* were severely diseased and rapidly lost body weight. The authors did not report any adverse effects associated with *B. lactis* LAFTI® B94.

6.4.3.3. Conclusions from Studies in Animals

The notified strain *Bifidobacterium animalis* subsp. *lactis* Rosell®-421 (LAFTI® B94) has been widely studied in a variety of rodent animal models. A 13-week high-dosage oral toxicity study in Wistar rats determined that there were no indications of toxicity or pathogenicity at tested doses.

6.5. Safety Evaluations by Authoritative Bodies *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94)

Strains of *Bifidobacterium* are among the most important organisms for human probiotics (O'Sullivan et al. 1992; Fuller and Gibson 1997). Probiotic *Bifidobacteria* have been used in food products and dietary supplements for decades, with a compelling record of safe consumption (Reid 2002; Kocian et al. 1994; Guidelines FAO/WHO 2002). The organism that is the subject of this GRAS notice is a thoroughly characterized strain belonging to the *Bifidobacterium* genus that has been sold world-wide for a number of years.

Bifidobacteria predominate in the intestinal tract shortly after birth. They are important and normal constituents of the human gastrointestinal microbiota and occur at concentrations of 10^9 to 10^{10} cells/g of feces (Tanaka et al. 2000). *Bifidobacterium animalis* is a natural inhabitant of the intestinal tract and has been used for many years in fermented food.

Bifidobacterium animalis was first described as a separate species by Scardovi and Trovatelli (Scardovi and Trovatelli 1974) after examining the feces of chickens, rats, and rabbits. *Bifidobacterium lactis* was first described as a separate species from *B. animalis* due to increased aerobicity and differences in metabolic and genetic features (Meile et al. 1997). However, Masco et al. (2004) showed that the genetic homology between the putative separate species was insignificant enough that they were better regarded as members of the same species, but were sufficiently genetically heterogeneous that two subspecies groupings were necessary.

The International Dairy Federation (IDF), in collaboration with the European Food and Feed Cultures Association (EFFCA), assembled a list of microorganisms with a documented history of safe use in food (Bourdichon et al. 2012). The species *Bifidobacterium animalis* subsp. *lactis* is listed on this inventory. Since 2007, *Bifidobacterium animalis* has been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA 2017). A strain belonging to a species listed on QPS and meeting the established criteria can freely be used in foods in Europe.

In Canada, the *Natural Health Products Regulations* of 2004 classified probiotics under the definition of Natural Health Products. In its probiotics monograph, the Natural and Non-prescription Health Products Directorate (NNHPD) of Health Canada listed *Bifidobacterium animalis*, including the subspecies *B. animalis* subsp. *lactis*, as eligible to be used for the general support of gastrointestinal health (Probiotics Monograph, Health Canada, May 26, 2015; see Appendix I). The Food Directorate of Health Canada published a list of species eligible for generic structure/function claims in 2009 (Guidance document: The Use of Probiotic Microorganisms in Food, Health Canada, April 2009). This list included *B. animalis* subsp. *lactis*.

The Australian Therapeutic Goods Administration (TGA) includes *Bifidobacterium animalis* subsp. *lactis* on the "List of approved substances that can be used as Active ingredients in "Listed" Medicines" (Appendix II).

B. animalis subsp. *lactis* is also included in the list of "Substances that may typically be considered to be a health supplement" in South Africa (Medicines control council. 2014). Food Safety and Standards Authority of India has recognized *B. animalis* and added it in the List of Strains as Probiotics (Schedule –X of the Food safety and Standards regulation - No. 1-4/Nutraceutical/FSSAI-2013). In Korea, *B. animalis* subsp. *lactis* has been referenced in the Health Functional Food Code (2010), to be used in Health Functional Foods.

In China, *B. animalis* subsp. *lactis*, is included in the positive list of strains to be used in foods/health foods (Appendix III).

Futhermore, a review of use of probiotics in infant formula by the ESPGHAN Committee on Nutrition (ESPGHAN 2011) determined that currently evaluated probiotics do not show any safety concerns in terms of growth or adverse effects.

As aforementioned, the *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) strain was obtained by Lallemand Health Solutions (formerly known as Institut Rosell) in 2010. It is a proprietary culture provided to Institut Rosell by the Dutch Company DSM. The strain is deposited under number CBS-118529 at the "Centraalbureau voor Schimmelcultures", Utrecht, Netherlands, which guarantees having an isolate of the strain in a safe and secure place at all times.

Bifidobacterium animalis ssp. *lactis* Rosell®-421 (LAFTI® B94) has been sold worldwide as a powder since 2010, or in the finished product MAFLOR® sachet, providing $5x10^9$ cfu/sachet, the same proportion as is intended for addition to infant formula powder. MAFLOR® sachet is used in infants, young children, children, and adults. It was first launched in 2011 as a food supplement in Turkey.

Bifidobacterium animalis ssp. *lactis* Rosell®-421 (LAFTI® B94) is also a natural product approved after in-depth assessment of safety, quality, and efficacy of the strains and the finished product by the Natural and Non-Prescription Health Product Directorate of Health Canada with the non-traditional health claims cited below (NPN[1] 80021343):

- *Source of probiotics (2 months and older)*
- *Helps support intestinal/gastrointestinal health (2 months and older)*
- *Could promote a favorable gut flora (2 months and older)*
- *Participates in a healthy microflora balance (2 months and older)*
- *Helps to relieve abdominal discomfort, such as bloating and constipation (4 months and older).*
- *Helps children and adolescents with bloating and constipation in irritable bowel syndrome (IBS) (4 months and older).*
- *In adjunct with anti-helicobacter pylori therapy, helps to improve the Helicobacter pylori eradication rate (Adults)*
- *An adjunct to physician-supervised therapy in patients with Helicobacter pylori infections (Adults)*
- *Helps to reduce adverse effects from Helicobacter pylori therapy such as loss of appetite, diarrhea, nausea, headache, dizziness, and abdominal pain (Adults).*

[1]All Natural Product Number (NPN) can be found with relevant details on the publicly accessible database of licensed finished products on Health Canada's website: https://health-products.canada.ca/lnhpd-bdpsnh/index-eng.jsp

Additionally, *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) has been extensively and widely marketed by Lallemand Health Solutions as a combination (with other strains) in 37 other formulas, including the previously mentioned MAFLOR® capsule.

MAFLOR® capsule is a combination of *B. animalis* ssp. *lactis* Rosell®-421 (33%), *L. helveticus* Rosell®-52 (33%), and L. casei Rosell®-215 (34%). Each capsule of MAFLOR® capsule contains $7x10^9$ cfu, corresponding to $2.3x10^9$ cfu of *B. animalis* ssp. *lactis* Rosell®-421.

6.6. Decision-Tree Analysis of the Safety of the Notified Strain

The decision tree published by Pariza et al. (2015) indicates that the notified strain, *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94), "is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption" (Pariza et al. 2015).

The responses to each of the questions asked in the decision tree are as follows:

1. Has the strain been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? **- Yes**

2. Has the strain genome been sequenced? **- Yes**

3. Is the strain genome free of genetic elements encoding virulence factors and/or toxins associated with pathogenicity? **- Yes**

4. Is the strain genome free of functional and transferable antibiotic resistance gene DNA? **- Yes**

5. Does the strain produce antimicrobial substances? **- No**

6. Has the strain been genetically modified using rDNA techniques? **- No**

7. Was the strain isolated from a food that has a history of safe consumption for which the species to which the strain belongs is a substantial and characterizing component (not simply an 'incidental isolate')? **- Yes, it was isolated from a dairy source**

8. Does the strain induce undesirable physiological effects in appropriately designed safety evaluation studies? **- No**

6.7. Safety Assessment and GRAS determination

6.7.1. Introduction

This section presents an assessment that demonstrates that the intended use of the strain *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94), in non-exempt infant formula intended for consumption by healthy term infants is safe and is GRAS.

This safety assessment and GRAS determination involves two steps. In the first step, the safety of the intended use of the strain *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) is demonstrated. Safety is established by demonstrating a reasonable certainty that the exposure of term infants to this strain, under its intended use in infant formula, is not harmful. In the second step, the intended use of this strain is determined to be GRAS by demonstrating that the safety of this probiotic under its intended conditions of use is generally recognized among qualified scientific experts and is based on generally available and accepted information.

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

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

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

The criteria outlined above for a scientific-procedures GRAS determination are applied below in an analysis of whether the addition of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) to non-exempt infant formula intended for consumption by healthy term infants is safe and is GRAS.

6.7.2. Safety Evaluation

Several convergent lines of evidence support the conclusion that the intended use of the strain, *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94), is safe. The strain is member of the genus *Bifidobacterium*, which have long been consumed by humans both as microorganisms used in food processing and as probiotics. The specific species and subspecies, *Bifidobacterium animalis* ssp. *lactis*, also has a long history of safe consumption and the species

has Qualified Presumption of Safety (QPS) status in the European Union as well as equivalent safety recognition in numerous other countries.

The bacterial strain, Rosell®-421, has been widely consumed as a probiotic worldwide for many years, both individually and in other combination products.

The strain has been subjected to tests of minimum inhibitory concentration (MIC) to assess phenotypic resistance to clinically significant antibiotics and has been found not to exhibit resistance above established microbiological breakpoints. It has moderate levels of binding capacity, does not produce biogenic amines, and does not produce antibiotics. The strain Rosell®-421 does not produces D-lactate.

The genome of this strain has been sequenced and fully annotated; the annotations indicate that the strain does not harbor virulence genes, potentially transferable antimicrobial resistance genes, genes encoding decarboxylase capable of forming biogenic amines, or genes encoding production of antibiotics.

Bifidobacterium animalis ssp. *lactis* Rosell®-421- LAFTI® B94 has been studied in preterm infants with very low birth weight and in infants with cyanotic congenital heart disease. This research includes four studies in which MAFLOR® sachet (*Bifidobacterium animalis* ssp. *lactis* Rosell®-421- LAFTI® B94) was given to 623 preterm infants at doses as high as $5x10^9$ cfu/day and for durations as long as 8 weeks. In some of these studies, *Bifidobacterium animalis* ssp. *lactis* Rosell®-421- LAFTI® B94 was able to improve morbidity and mortality in this population.

Additionally, *Bifidobacterium animalis* ssp. *lactis* Rosell®-421- LAFTI® B94 has been studied in term infants and children, both healthy and with conditions such as diarrhea, rotaviral infection, and IBS. The research includes three studies in which MAFLOR® sachet (*Bifidobacterium animalis* ssp. *lactis* Rosell®-421- LAFTI® B94) was given to 325 infants and children at doses as high as $10x10^9$ cfu/day and for durations as long as 4 weeks.

Lastly, another preparation containing *Bifidobacterium animalis* ssp. *lactis* Rosell®-421- LAFTI® B94, MAFLOR® capsule, was given for 4 months to 28 children with non-alcoholic fatty liver disease (NAFLD), with doses up to $2.3x10^9$ cfu/day.

In none of these studies were issues of intolerance or adverse reactions reported differing in nature, frequency, or severity from controls or associated with ingestion of the probiotic.

In addition to the studies in infants and children, which provide the primary clinical evidence for the safety of the intended use of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421- LAFTI® B94 as probiotics to be added in non-exempt infant formula intended for consumption by healthy term infants, there is an extensive body of research in adults and in animals, all of which confirms the safety of this strain.

Finally, a decision-tree analysis based on Pariza et al. (2015) indicated that the strain is "deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption."

6.7.3. General Recognition of Safety

The intended use of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94), to be added as a probiotic to non-exempt infant formula intended for consumption by healthy term infants, has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b). This safety was shown by establishing the identity and probiotic characteristics of the strain, demonstrating its freedom from pathogenic, toxicogenic, or other risk factors, and concluding that the expected exposure to *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) by infants is without significant risk of harm. Finally, because this safety assessment is based on generally available information, and so satisfies the common knowledge requirement of a GRAS determination, this intended use can be considered GRAS.

Determination of the safety and GRAS status of the addition of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) to infant formula has been made through the deliberations of an Expert Panel consisting of Robert J. Nicolosi, Ph.D., Michael W. Pariza, Ph.D., and John A. Thomas, Ph.D., who reviewed this monograph, prepared by Lallemand Health Solutions and edited by JHeimbach LLC, as well as other information available to them. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients, including probiotic bacteria, intended for addition to infant formula. They critically reviewed and evaluated the publicly available information and the potential exposure to *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) anticipated to result from its intended use, and individually and collectively concluded that no evidence exists in the available information on *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) that demonstrates, or suggests reasonable grounds to suspect, a hazard to infants under the intended conditions of use *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94).

It is the Expert Panel's opinion that other qualified scientists reviewing the same publicly available data would reach the same conclusion. Therefore, the intended use of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94), to be added as a probiotic to non-exempt infant formula intended for consumption by healthy term infants, is GRAS by scientific procedures.

6.8. Statement Regarding Information Inconsistent with GRAS

I have reviewed the available data and information and am not aware of any data or information that are, or may appear to be, inconsistent with our conclusion of GRAS status of the intended use of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) .

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

6.9. Conclusion of the Expert Panel

The intended use of *Bifidobacterium animalis ssp. lactis Rosell®-421 (LAFTI® B94)* has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b). This safety was established by first establishing the identity and purity of the material, estimating potential exposure to the strain from its intended use, and demonstrating that this level of exposure is without significant risk of harm. Because this safety assessment satisfies the common knowledge requirement of a GRAS determination, this intended use is considered GRAS.

Determination of the safety and GRAS status of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) under its intended conditions of use has been made through the deliberations of an Expert GRAS Panel consisting of Robert J. Nicolosi, Ph.D., Michael W. Pariza, Ph.D., and John A. Thomas, Ph.D. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. They have critically reviewed and evaluated the publicly available information summarized in this document, including the potential intake resulting from the intended use of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94), and have individually and collectively concluded:

Ingestion of Bifidobacterium animalis *ssp.* lactis *Rosell®-421 (LAFTI® B94) from its intended use results in levels of intake that are within safe limits established by the history of consumption of these probiotics and by published human clinical trials. Therefore, the use of* Bifidobacterium animalis *ssp.* lactis *Rosell®-421 (LAFTI® B94), produced consistent with cGMP and complying with the specifications and use described in this GRAS monograph, at a maximum addition level of $8x10^7$ cfu/g powder of non-exempt infant formula intended for consumption by healthy term infants at a level of $5x10^9$ cfu/day, is safe and GRAS based on scientific procedures.*

It is the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available data would reach the same conclusion.

Robert J. Nicolosi, Ph.D. _____ Date: _____
Professor Emeritus
University of Massachusetts—Lowell
Lowell, Massachusetts

Michael W. Pariza, Ph.D. _____ Date: _____
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin

John A. Thomas, Ph.D. _____ Date: _____
Adjunct Professor
Indiana University School of Medicine
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It is the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available data would reach the same conclusion.

Robert J. Nicolosi, Ph.D. _____ Date: 15 March 2019
Professor Emeritus
University of Massachusetts—Lowell
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Michael W. Pariza, Ph.D. _____ Date: _____
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Robert J. Nicolosi, Ph.D. _____ Date: _____
Professor Emeritus
University of Massachusetts—Lowell
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Determination of the safety and GRAS status of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94) under its intended conditions of use has been made through the deliberations of an Expert GRAS Panel consisting of Robert J. Nicolosi, Ph.D., Michael W. Pariza, Ph.D., and John A. Thomas, Ph.D. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. They have critically reviewed and evaluated the publicly available information summarized in this document, including the potential intake resulting from the intended use of *Bifidobacterium animalis* ssp. *lactis* Rosell®-421 (LAFTI® B94), and have individually and collectively concluded:

Ingestion of Bifidobacterium animalis *ssp.* lactis *Rosell®-421 (LAFTI® B94) from its intended use results in levels of intake that are within safe limits established by the history of consumption of these probiotics and by published human clinical trials. Therefore, the use of* Bifidobacterium animalis *ssp.* lactis *Rosell®-421 (LAFTI® B94), produced consistent with cGMP and complying with the specifications and use described in this GRAS monograph, at a maximum addition level of $8x10^7$ cfu/g powder of non-exempt infant formula intended for consumption by healthy term infants at a level of $5x10^9$ cfu/day, is safe and GRAS based on scientific procedures.*

It is the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available data would reach the same conclusion.

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PART 7. LIST OF SUPPORTING DATA AND INFORMATION

Sections:
7.1. Generally Available (Published) Documents
7.2. Generally Available but Unpublished Government Documents
7.3. Unpublished Documents

7.1. Generally Available (Published) Documents:

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APPENDIX I - HEALTH CANADA

Use(s) or Purpose(s)

Statement to the effect of

Medicinal ingredients from Appendix I, Table 1, 2, and 3

Source of Probiotics.

Medicinal ingredients from Appendix I, Table 1, 2, and 3 except *Lactobacillus crispatus* and *Lactobacillus gallinarum*

Helps support intestinal/gastrointestinal health (Alonso and Guarner 2013; DuPont and DuPont 2011; WGOGG 2011; Rolfe 2000).
Could promote a favorable gut flora (Bezkorovainy 2001; Morelli 2000; Collins et al. 1998).

Table 1: Medicinal Ingredients - BACTERIA

Proper and Common Names	**References**
For "source of probiotics" claim only Return to Table 3 footnote 1 referrer	
Bifidobacterium adolescentis	Masco et al. 2004; Skerman et al. 1980
Bifidobacterium animalis (including *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis*)	Masco et al. 2004; Skerman et al. 1980
Bifidobacterium bifidum	Skerman et al. 1980
Bifidobacterium breve	Skerman et al. 1980
Bifidobacterium longum (including *Bifidobacterium longum* ssp. *infantis, Bifidobacterium longum* ssp. *longum* and *Bifidobacterium longum* ssp. *suis*)	Mattarelli et al. 2008
Lactobacillus acidophilus	Johnson et al. 1980; Skerman et al. 1980
Lactobacillus amylolyticus	Validation List No. 68 1998
Lactobacillus amylovorus	Nakamura 1981
Lactobacillus brevis	Skerman et al. 1980
Lactobacillus buchneri	Skerman et al. 1980
Lactobacillus casei	JCICSB 2008; Skerman et al. 1980
Lactobacillus coryniformis	Skerman et al. 1980
Lactobacillus crispatus Table 3 Footnote1	Skerman et al. 1980
Lactobacillus curvatus	Skerman et al. 1980
Lactobacillus delbrueckii (including *Lactobacillus delbrueckii* ssp. *bulgaricus* & *Lactobacillus delbrueckii* ssp. *delbrueckii*)	Beijerinck 1901; Howey et al. 1990
Lactobacillus farciminis	Validation List no. 11, 1983
Lactobacillus fermentum	Skerman et al. 1980
Lactobacillus gallinarum Table 3 Footnote1	Fujisawa et al. 1992
Lactobacillus gasseri	Validation List No. 4 1980
Lactobacillus helveticus	Skerman et al. 1980
Lactobacillus hilgardii	Skerman et al. 1980
Lactobacillus johnsonii	Fujisawa et al. 1992

Table 1: Medicinal Ingredients - BACTERIA	
Lactobacillus kefiranofaciens	Fujisawa et al. 1988
Lactobacillus kefiri	Validation List no. 11, 1983
Lactobacillus mucosae	Roos et al. 2000
Lactobacillus panis	Wiese et al. 1996
Lactobacillus paracasei	JCICSB 2008; Collins et al. 1989
Lactobacillus paraplantarum	Curk et al. 1996
Lactobacillus plantarum	Skerman et al. 1980
Lactobacillus pontis	Vogel et al. 1994
Lactobacillus reuteri	Validation List No. 8, 1982
Lactobacillus rhamnosus	Collins et al. 1989
Lactobacillus salivarius	Skerman et al. 1980
Lactobacillus sanfranciscensis	Validation List no. 16, 1984b
Lactococcus lactis	Validation List no. 20, 1985
Leuconostoc citreum	Farrow et al. 1989
Leuconostoc pseudomesenteroides	Farrow et al. 1989
Leuconostoc lactis	Skerman et al. 1980
Leuconostoc mesenteroides	Skerman et al. 1980
Oenococcus oeni	Dicks et al. 1995
Pediococcus acidilactici	Skerman et al. 1980
Pediococcus pentosaceus	Skerman et al. 1980
Propionibacterium freudenreichii (including *Propionibacterium freudenreichii* ssp. *shermanii*)	Skerman et al. 1980
Propionibacterium acidipropionici	Skerman et al. 1980

APPENDIX II – TGA AUSTRALIA

Substances that may be used in listed medicines in Australia – Therapeutic Goods Administration Ingredient summary

Australian Government
Department of Health
Therapeutic Goods Administration

Ingredient Summary

Ingredient Name	Bifidobacterium animalis ssp lactis
Ingredient ID	105124
Category	Approved Biological Name
Synonyms	Synonym not held on file
CAS Number	CAS Number not held on file
Availability	Available for use as an Active Ingredient in: Biologicals, Listed Medicines, Prescription Medicines Not available as a Homoeopathic Ingredient in Listed Medicines Available for use as an Excipient Ingredient in: Biologicals, Prescription Medicines Not available as an Equivalent Ingredient in any application *Please note: Only the name and definition of a substance have been reviewed to allow it to be included in the ingredient repository. The approval for use of the ingredient in therapeutic goods is a decision made by the relevant TGA regulatory area. This approval process may require submission of further information, for example safety data for the ingredient or for the finished goods, to meet legislative and regulatory requirements.*
Additional Information	

Naming Reference

Reference	Edition/Year/Volume	Page Number(s)	Accessed Online
International Journal of Systematic & Evoluntionary Microbiology	54/2004/-	1137	No

Restrictions

Restriction	Applies To
Ingredient name approved as an ABN in OOS 1/2005 - dated 27 January 2005 and M 3/2005 - dated 10 February 2005.	Over the Counter

END OF SUMMARY

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APPENDIX III – MOH CHINA

Notice Regarding "the List of Bacterial Species Allowed for Food Application" Issued by the General Administrative Office of
Minister of Health, People's Republic of China

MOH office Notice (2010) No. 65

To Department of Health of all the provinces, autonomous regions, Direct-controlled municipalities, Xinjiang Production and Construction Corps, Chinese Disease Control Center, National Center for Health Inspection and Supervision:
In accordance with "Food Safety Law" and the relevant regulations on its implementation, we organized and established "the list of bacterial species allowed for food application" and issued it herewith. Please Comply with it.

Enclosure: The list of bacterial species allowed for food application

April 22nd, 2011

Bacterial Species Allowed for Food Application

	Name	**Latin Name**
(1)	*Bifidobacterium*	*Bifidobacterium*
1	*Bifidobacterium adolescentis*	*Bifidobacterium adolescentis*
2	*Bifidobacterium animalis (Bifidobacterium lactis)*	*Bifidobacterium animalis (Bifidobacterium lactis)*
3	*Bifidobacterium bifidum*	*Bifidobacterium bifidum*
4	*Bifidobacterium breve*	*Bifidobacterium breve*
5	*Bifidobacterium infantis*	*Bifidobacterium infantis*
6	*Bifidobacterium longum*	*Bifidobacterium longum*
(2)	*Lactobacillus*	*Lactobacillus*
1	*Lactobacillus acidophilus*	*Lactobacillus acidophilus*
2	*Lactobacillus casei*	*Lactobacillus casei*
3	*Lactobacillus crispatus*	*Lactobacillus crispatus*
4	*Lactobacillus delbrueckii* ssp. *Bulgaricus* (*Lactobacillus bulgaricus*)	*Lactobacillus delbrueckii* ssp. *Bulgaricus* (*Lactobacillus bulgaricus*)
5	*Lactobacillus delbrueckii* ssp. *lactis*	*Lactobacillus delbrueckii* ssp. *lactis*
6	*Lactobacillus fermentium*	*Lactobacillus fermentium*
7	*Lactobacillus gasseri*	*Lactobacillus gasseri*
8	*Lactobacillus helveticus*	*Lactobacillus helveticus*
9	*Lactobacillus johnsonii*	*Lactobacillus johnsonii*
10	*Lactobacillus paracasei*	*Lactobacillus paracasei*

11	*Lactobacillus plantarum*	*Lactobacillus plantarum*
12	*Lactobacillus reuteri*	*Lactobacillus reuteri*
13	*Lactobacillus rhamnosus*	*Lactobacillus rhamnosus*
14	*Lactobacillus salivarius*	*Lactobacillus salivarius*
(3)	*Streptococcus*	*Streptococcus*
1	*Streptococcus thermophilus*	*Streptococcus thermophilus*
Note: 1. The bacterial species that have been used in food manufacturing and processing can be used continuously. The new species which are not listed here should comply the "New Resource Food Regulation"; 2. The list of bacterial species allowed for baby food application will be issued separately.		

Bonnette, Richard

From: Jim Heimbach <jh@jheimbach.com>
Sent: Tuesday, April 23, 2019 11:48 AM
To: Bonnette, Richard
Subject: RE: GRAS submission regarding B. animalis ssp. lactis strain R0421 - dated April 3, 2019

Richard—

Thank you so much for asking rather than simply rejecting the notice! You are correct that it is an oversight, for which I apologize. That, and anything else in the submission marked confidential (though hopefully nothing else is), is not intended to be regarded as confidential—everything is disclosable.

Regards,
Jim

James T. Heimbach, Ph.D., F.A.C.N.
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From: Bonnette, Richard [mailto:Richard.Bonnette@fda.hhs.gov]
Sent: Tuesday, April 23, 2019 10:47 AM
To: Jim Heimbach
Subject: GRAS submission regarding B. animalis ssp. lactis strain R0421 - dated April 3, 2019

Good morning Jim,
I have a quick question regarding this submission (*Bifidobacterium animalis* ssp. *lactis* strain R0421 dated April 3, 2019, on behalf of Lallemand Health Solutions. We noticed that page 1 is marked "CONFIDENTIAL" on the bottom of page 1. This looks like an oversight and I just wanted to confirm that, as is noted in Part 1, that none of the information in the submission is exempt from disclosure under FOIA. If that's the case, I'll add your email response to the record for the submission and we'll move forward with the submission as-is.
Thanks,
Richard

Richard E. Bonnette, M.S.
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 240-402-1235
richard.bonnette@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

Questions/Comments Regarding GRN 000855:

1. The subject of the notice is listed as *Bifidobacterium animalis* subsp. *lactis* CBS-118529 for use as an ingredient at up to 8 x 10_7 colony forming units (CFU)/g of non-exempt powdered milk-based infant formula for term infants. However, page 33 of the notice states uses to include "… toddlers' or children's beverages". Please clarify the intended uses.

The intended use is, as stated in Part 3, in "non-exempt powdered milk-based infant formula intended for consumption by healthy term infants." The mention of toddlers' and children's beverages in Part 4 was an error.

While it is true that there is no technological limitation to the concentration of the probiotic that could be added to these beverages, this use is not included in this GRAS notice.

2. The notifier states that some lots maybe standardized with maltodextrin (page 26). Please clarify if this applies to all lots.

Whether maltodextrin is added to the finished product depends on the use of the strain.

Generally, when the strain is sold for use in other applications, such as in capsules, the maltodextrin is added to standardise the dosage for addition into the dosage unit. Such use, of course, is not included in this GRAS notice.

However, as the strain will be sold for addition into infant formula powder, there will be no addition of maltodextrin for this usage. Analyses performed on the product as per specifications were performed on the pure strain, without maltodextrin.

3. Please provide a specification for *B. animalis* CBS-118529.

When *Bifidobacterium animalis* ssp. *lactis* R0421 (also referred to as LAFTI® B94) was deposited in the Centraalbureau von Schimmelcultures in the Netherlands, it was assigned deposit number CBS-118529. Thus, *B. animalis* ssp. *lactis* R0421 and *B. animalis* ssp. *lactis* CBS-118529 are alternative names for the same bacterial strain.

Specifications for the strain were provided in Table 6 in Section 2.6 on page 27 of the GRAS notice.

4. Please provide results from three non-consecutive batches to demonstrate that the manufacturing can meet the provided specifications.

We provided results from one lot of *B. animalis* ssp. *lactis* R0421 (*B. animalis* ssp. *lactis* CBS-118529) tested in accordance with the specifications, and the Certificate of Analysis demonstrating proper compliance with said specifications on page 29 of the GRAS notice. We also provide the results of analyses of heavy metals from three non-consecutive batches of the strain in Table 7 on page 30.

Please note that in order to use the strain in infant formula we are adding additional analyses to ensure the safety of the product (specifically *Cronobacter* and *Salmonella* specifications) that we would not normally perform for use in dietary supplements for the general population. As such, we do not currently have data for three non-consecutive lots, as we have not sold this product for use in infant formula before this GRAS notification.

We began steps for analysing two further lots immediately upon receiving FDA's questions on October 28, but this work is not yet completed. We hope to have results for these lots by the end of November. If we could have an extension to provide these data to FDA within this submission, we would greatly appreciate it.

5. Please indicate if the analytical methods used to analyze the batches for conformance with the stated specifications are validated for that particular purpose.

These methods were not validated for this specific use; however, we chose internationally accepted methods for use in food, such as ISO methods or methods from the Marketed Food and Health Products Directorate of Health Canada (method designations MFHPD-20 and MFHPD-21). For bacterial strength (total count), we are using internal methods designed to quantify *Bifidobacterium* spp., the genus to which this strain belongs. For the total count method, it is an internal procedure that multiple counts are performed per lot, involving testing with replicate plates or different technicians.

6. On page 32 of the notice, 8 x 10_9 CFU is listed as the maximum daily intake, however, the 90th percentile exposure is listed as 8 x 10_8 CFU/kg bw/d. Please provide clarification on the calculated maximum daily intake.

This was clumsily expressed by me. I stated that the target dietary intake of the strain is $5x10^9$ cfu/day and that to achieve this intake level, a concentration of $5x10^7$ cfu/g powder is required. I then wrote that, to assure that this concentration is available throughout the shelf life of the powder, an overage is needed, and thus the strain will be added at $8x10^7$ cfu/g powder. An infant consuming formula prepared with powder containing the original $8x10^7$ cfu/g will have an intake somewhat above the target level, amounting to $8x10^9$ cfu/day. It was in this respect that I misleadingly referred to this as a "maximum potential intake."

From: jheimbach@va.metrocast.net
To: Hice, Stephanie; jh@jheimbach.com
Subject: RE: GRN 000855 - Questions for Notifier
Date: Wednesday, November 20, 2019 11:41:36 AM
Attachments: image001.png
20191119100234349.pdf

Dear Dr. Hice—

This is in response to our telephone conversation earlier today. I've attached the first of the two requested analyses, which my client just received back yesterday. They hope to receive the second batch late next week, probably around the U.S. Thanksgiving, and I'll forward it ASAP. I reminded them that we do need the validation information.

They pointed out that getting yet another batch analyzed will require an additional production run of the probiotic, which is not scheduled. (They made two special runs just to produce batches for the 2 analyses requested by FDA.) They also pointed out that the certificate of analysis for the batch in the GRAS notice was current at the time the notice was submitted; that it is now 10 months old is due to the shut-down last winter and FDA's overload since. They thus suggest that a fourth analysis should not be regarded as essential.

Regards,
Jim

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From: jheimbach@va.metrocast.net <jheimbach@va.metrocast.net>
Sent: Tuesday, November 12, 2019 3:07 PM
To: 'Hice, Stephanie' <Stephanie.Hice@fda.hhs.gov>; jh@jheimbach.com
Subject: RE: GRN 000855 - Questions for Notifier

Dear Dr. Hice—

I have attached our responses to your questions from October 28. These responses are complete with the exception of your request for results of additional analyses of compliance of the strain with specifications. My client immediately set about providing these analyses, but this takes time and the results are not yet available. We hope for them very soon and ask for FDA's indulgence in the delay.

Regards,
Jim

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From: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Sent: Monday, October 28, 2019 11:28 AM
To: jh@jheimbach.com
Subject: GRN 000855 - Questions for Notifier

Dear Dr. Heimbach,

During our review of GRAS Notice No. 000855, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your responses.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD
Staff Fellow (Biologist)
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FDA
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ADMINISTRATION

INSTITUT ROSELL LALLEMAND

CERTIFICAT D'ANALYSE

PRODUIT: BIFIDOBACTERIUM ANIMALIS LAFTI B94 R0421
LOT: U120191676 SEQ. 00127784
CODE: 050421SG2
DATE D'ANALYSE: 2019 10 31

Test :	Specifications	Methods	Results
Assay – Total cell count (Enumeration)	NA	QA138	575,7 X 10^9 CFU/g
Microbiological contaminants :			
TYMC/Yeast and Molds	< 1000 CFU/g	Enumeration on SAB or PDA culture medium + Chloramphenicol after incubation at 20-25^{0}C for 5 to 7 days	Complies
Coliforms	< 10 CFU/g	ISO 4831	Complies
Escherichia coli	< 10 CFU/g	ISO 7251	Complies
Staphylococcus aureus	< 10 CFU/g	MFHPB-21	Complies
Enterobacter sakazakii (Cronobacter ssp.) *	Absent/10g (30 samples)	ISO/TS 22964	Complies
Samonella ssp. *	Absent/25g (60 samples)	MFHPB-20	Complies
Physical Aspect :			
Appearance	Fine to granular, ivory to beige powder	Visual observation	Complies

** 21CFR (Code of Federal Regulations) – Part 106 Infant Formula Requirements – Section 106.55 (Controls to prevent adulteration from microorganisms)*

Signed: _____ Date: 2019-11-19

Eric Guevara
Supervisor Quality Control

From: jheimbach@va.metrocast.net
To: Hice, Stephanie
Cc: "Jim Heimbach"
Subject: RE: GRN 000855 - Questions for Notifier
Date: Friday, November 22, 2019 8:35:10 PM

Dear Dr. Hice—

Following is the response from Lallemand regarding validation of their analytical methods:

While our methods are not validated for this specific strain (*Bifidobacterium animalis* subsp. *lactis* B94), we are using scientifically valid microbiological methods taken from methods published by government or standard setting organizations for contamination testing. Specifically these sources of the methods for each specifications are: total count yeast and mold (MFHPB), *Salmonella* (MFHPB), *S. aureus* (MFHPB), *E. coli* (ISO, *Enterobacter sakazakii (Chronobacter* ssp) (ISO) and coliforms (ISO).

In terms of total count (CFU) we are using an internal scientifically valid method (QA-138) functionally equivalent to a similar method that was internally validated for our strains as described below:

The appropriate method to use for the total cell count in the *Bifidobacterium animalis* Lafti B94 is QA138. The QA138 method has not been validated, but other methods used by Lallemand Health Solutions, such as QA133 and MA-003 have been successfully validated.

The objective of validating methods of analysis is to demonstrate that they are scientifically valid for their intended use.

Method MA-003 for total bacteriological count has already been validated following the ICH guidelines. In order to be considered validated, the method needed to comply to acceptance criteria such as specificity, accuracy, repeatability, linearity, precision and intermediate precision, just to name a few. The following paragraphs present the validation tests characteristics and their definition as well as the ICH recommendations which are accepted by Health Canada.

- The specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the microbiological method of analysis is determined by executing the method of analysis for each bacterial genus produced by LHS. The method is considered specific if it permits the distinction of the targeted microorganism from the others tested.

- The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. There are no available probiotic standard strains at LHS. It is why the accuracy of the dosage methods must be assessed using the results obtained for the linearity, precision and repeatability.

- Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision. The repeatability of the method of analysis is evaluated in conjunction with the linearity. Therefore, each dilution

is performed in triplicate by the same technician in similar conditions and is evaluated with 6 determinations at 100 percent of the test concentration.

- The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The linearity is tested at the extremities of the range being the highest probiotic concentration and the lowest probiotic concentration. The linearity is evaluated in conjunction with the repeatability and the precision, the concentration of each dilution is evaluated with 6 determinations at 100 percent of the test concentration.

- The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Refer to repeatability, intermediate precision and reproducibility. The precision must be expressed as the variance, standard-deviation or coefficient of variation of a series of measurements.

- Intermediate precision expresses within laboratories variations: different days, different analysts, different equipment, etc. Three different technicians proceed with the method of analysis in triplicate. The technicians use different equipment. Three different technicians proceed with the method of analysis in triplicate. The technicians use different equipment. The results obtained for the linearity/reproducibility can be used.

The validation tests were carried out for the MA-003 and met the acceptance criteria such as documentation verification, calibration certificate verification, specificity verification, linearity and repeatability verification, intermediate precision verification and accuracy verification.

MA-003 method of analysis is appropriate for the enumeration of LHS probiotics including *Lactobacillus* spp, *Bifidobacterium* spp, *Lactococcus* spp, *Streptococcus* spp, *Bacillus* spp, *Enterococcus* spp and *Propionibacterium* spp at various concentrations. MA-003 validation results support that RCM is the relevant medium to perform the total bacteriological count of bifidobacteria.

The method used to determine the total bacteriological count of another bacterium, *Bifidobacterium longum* Rosell®-175, is QA133 – this species is part of the same genus as *B. lactis B94*. This method has been validated and the results confirmed the specificity, repeatability, intermediate precision, and linearity for the total bacteriological count of the strain. This method uses similar sample preparation and incubation conditions and the same technique (pour plate)/dilution buffers as the QA138 method used for enumeration of the strain *Bifidobacterium animalis* Lafti B94.

Given that the validated QA133 method of enumeration used for *Bifidobacterium longum* Rosell®-175 is identical to the QA138 used for the *Bifidobacterium animalis* Lafti B94 and that the validated method MA-003 confirmed that RCM is the medium of choice for the total bacteriological count of bifidobacteria, we are confident that the QA138 method is scientifically valid for its intended use.

Regards,

Jim

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

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Email: jh@jheimbach.com

From: jheimbach@va.metrocast.net
To: Hice, Stephanie
Subject: RE: GRN 000855 - Questions for Notifier
Date: Thursday, January 30, 2020 3:42:06 PM
Attachments: image001.png

Dear Stephanie—

Here is my response from Lallemand regarding the absence of a specification for arsenic. (I expected that the answer would be along these lines since I was aware that the EU has an As specification only for rice products.)

Hi Jim,

To answer Stephanie's question, there is not a specification for arsenic for infant formula products, which is why we put **, to indicate no limit. There is only a specification in Europe for rice based infant foods.

In all cases, we use the specifications in Europe, as US FDA has not developed heavy metal limits for infant formulas. We feel that arsenic specification is not a relevant specification for our products in the US and Europe, as our products are not rice-based. What do you think ?

Thanks,
Richard

Regards,
Jim

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Email: jh@jheimbach.com

From: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Sent: Wednesday, January 29, 2020 8:37 AM
To: jheimbach@va.metrocast.net; jh@jheimbach.com
Subject: RE: GRN 000855 - Questions for Notifier

Dear Dr. Heimbach,

Thank you for your email. We have an additional question, as we complete our review:

1. The provided specification for arsenic is listed as "**" (page 30). Please provide a description of this specification.

Thank you again, and please let me know if you have any questions.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD
Staff Fellow (Biologist)
Division of Food Ingredients

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From: jheimbach@va.metrocast.net <jheimbach@va.metrocast.net>
Sent: Wednesday, January 15, 2020 1:58 PM
To: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>; jh@jheimbach.com
Subject: RE: GRN 000855 - Questions for Notifier

Dear Dr. Hice—

The total cell count for *B. animalis* CBS-118529 is >150 x 10^9 CFU/g.

Regards,
Jim

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From: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Sent: Monday, January 6, 2020 2:13 PM

To: jh@jheimbach.com; jheimbach@va.metrocast.net
Subject: RE: GRN 000855 - Questions for Notifier

Dear Dr. Heimbach,

I wanted to take the time to follow-up with you regarding our question/comment on a specification for total cell count (CFU/g). Please know that we require this information in order to complete our review.

Thank you for your attention to our comments. Please let me know if you have any questions.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD
Staff Fellow (Biologist)
Division of Food Ingredients

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stephanie.hice@fda.hhs.gov

From: Hice, Stephanie
Sent: Friday, December 13, 2019 7:33 AM
To: jheimbach@va.metrocast.net; jh@jheimbach.com
Subject: RE: GRN 000855 - Questions for Notifier

Dear Dr. Heimbach,

Thank you for your email.

With regards to the provided response, we would like to draw your attention to our GRAS Notice Inventory, specifically a few recent notices: GRNs 847, 840, 814, 810, 758. We note that a specification for total cell count (CFU/g) is often provided in support of the identity of the ingredient. While we recognize that the total cell count may vary from lot to lot, a minimum cell count specification is an important aspect of ingredient identity and safety.

Thank you, and please let me know if you have any questions.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD
Staff Fellow (Biologist)
Division of Food Ingredients

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stephanie.hice@fda.hhs.gov

From: jheimbach@va.metrocast.net <jheimbach@va.metrocast.net>
Sent: Monday, December 9, 2019 8:09 PM
To: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>; jh@jheimbach.com
Subject: RE: GRN 000855 - Questions for Notifier

Dear Dr. Hice—

Here is the explanation for why the specifications do not include a spec. for total cell count. (BTW—I was pretty sure this was the case. Most producers of probiotics approach it like this, since bacteria don't always cooperate in reaching the same concentration.)

There is not a specification for the total cell count for the pure bacterial strain, as this may vary from lot to lot. To ensure that the finished product meets specifications, it is formulated with the amount of each lot of bacteria, according to each lot`s concentration. The bacteria are then added to the finished product blend that typically contains excipients such as maltodextrin or potato starch. The finished product is then tested to ensure that it meets the stated label claim / concentration.

Please also note that this is not a safety issue, but really one of assuring that the content matches the declaration.

Regards,
Jim

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Email: jh@jheimbach.com

From: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Sent: Wednesday, December 4, 2019 1:25 PM
To: jheimbach@va.metrocast.net; jh@jheimbach.com
Subject: RE: GRN 000855 - Questions for Notifier

Dear Dr. Heimbach,

Thank you for providing us with the additional batch analyses for GRN 000855. The notice is still under review.

We note, that a specification for the total cell count (enumeration) is not provided, and is listed as "NA". Please provide a specification for total cell count in colony forming units (CFU)/g.

Please let me know if you require additional clarification.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD
Staff Fellow (Biologist)
Division of Food Ingredients

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U.S. Food and Drug Administration
stephanie.hice@fda.hhs.gov

From: jheimbach@va.metrocast.net <jheimbach@va.metrocast.net>
Sent: Wednesday, December 4, 2019 12:58 PM
To: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>; jh@jheimbach.com
Subject: RE: GRN 000855 - Questions for Notifier

Dear Dr. Hice—

Here is the final analysis of *B. animalis* Lafti B94 in response to FDA's request. Can you let me know the status of FDA's closure of this notice?

Thank you—
Jim

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Port Royal VA 22535
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Cell: (+1) 202-320-3063
Email: jh@jheimbach.com

From: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Sent: Monday, October 28, 2019 11:28 AM
To: jh@jheimbach.com
Subject: GRN 000855 - Questions for Notifier

Dear Dr. Heimbach,

During our review of GRAS Notice No. 000855, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your responses.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD
Staff Fellow (Biologist)
Division of Food Ingredients

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FDA
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ADMINISTRATION

INSTITUT ROSELL LALLEMAND

CERTIFICAT D'ANALYSE

PRODUIT: BIFIDOBACTERIUM ANIMALIS LAFTI B94 R0421
LOT: 30TH098
CODE: 050421SG2
DATE D'ANALYSE: 2019 11 15

Test :	Specifications	Methods	Results
Assay – Total cell count (Enumeration)	NA	QA138	454,4 X 10^9 CFU/g
Microbiological contaminants :			
TYMC/Yeast and Molds	< 1000 CFU/g	Enumeration on SAB or PDA culture medium + Chloramphenicol after incubation at 20-25^{0}C for 5 to 7 days	Complies
Coliforms	< 10 CFU/g	ISO 4831	Complies
Escherichia coli	< 10 CFU/g	ISO 7251	Complies
Staphylococcus aureus	< 10 CFU/g	MFHPB-21	Complies
Enterobacter sakazakii (*Cronobacter* ssp.) *	Absent/10g (30 samples)	ISO/TS 22964	Complies
Samonella ssp. *	Absent/25g (60 samples)	MFHPB-20	Complies
Physical Aspect :			
Appearance	Fine to granular, ivory to beige powder	Visual observation	Complies

** 21CFR (Code of Federal Regulations) – Part 106 Infant Formula Requirements – Section 106.55 (Controls to prevent adulteration from microorganisms)*

Signed: _____ Date: 2019-12-04

Eric Guevara
Supervisor Quality Control