#722

DuPont Nutrition and Health
3329 Agriculture Drive
Madison, WI 53716
800-255-6837 Tel 608-395-2630 Fax

August 2, 2017

Office of Food Additive Safety
HFS-200
5001 Campus Drive
College Park, MD 20740-3835

Dear Office of Food Additive Safety:

Please accept the enclosed documents as submission for GRAS status of the live microbial culture, *Lactobacillus plantarum* Lp-115. The enclosed documents include the following for your consideration and review:

- Form FDA 3667
- Comprehensive GRAS Assessment
- Appendix documents A-E
- Attachment, unpublished document entitled, *Lactobacillus plantarum Lp-115: Acute Oral Toxicity Study in Rats – Up and Down Procedure*

We truly appreciate the opportunity to present the information within for your consideration of *Lactobacillus plantarum* Lp-115 as Generally Recognized as Safe in accordance with the US Food and Drug Administrations's Final Rule, 81 FR 54959.

Three paper copies of the submission in its entirety are enclosed. Please do hesitate to contact me at any time to discuss details or to request supplemental information as needed.

Thank you for your time and consideration.

(b) (6)

Amy B. Smith, Ph.D.
DuPont Nutrition & Health

Comprehensive GRAS Assessment

of

Lactobacillus plantarum Lp-115

For Usage Conditions for

General Recognition of Safety

for

Danisco USA, Inc

Table of Contents

Part 1 - Signed statements and certification

DuPont Nutrition and Health
3329 Agriculture Drive
Madison, WI 53716
800-255-6837 Tel 608-395-2630 Fax

August 2, 2017

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: GRAS Notice – Exemption claim for the use of *Lactobacillus plantarum* Lp-115

Dear Office of Food Additive Safety:

In accordance with the US Food and Drug Administration's (FDA) Substances Generally Recognized as Safe; Final Rule, (81 FR 54959) relating to the filing of notices for substances that are considered to be generally recognized as safe (GRAS), please accept this claim and the attached information, for that purpose as it relates to the use of *Lactobacillus plantarum* Lp-115 in yogurt and other dairy products, soy products, beverages, chewing gum, confectionary snacks and other foods and also in supplement form including sachets, tablets, and capsules. Specifically, we, DuPont Nutrition & Health (formerly Danisco) claim that the use of *Lactobacillus plantarum* Lp-115 in yogurt and other dairy products, soy products, beverages, chewing gum, confectionary snacks and other foods and also in supplement form including sachets, tablets, and capsules is exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act based on its determination that such uses are GRAS. This conclusion was made in concert with a panel of experts who is qualified by scientific training and experience.

No information used in this part of this notification is trade secret or confidential commercial information. In accordance with the requirements outlined in 21 CFR 170, Subpart E of the final rule, the following information is included with this exemption claim:

(i) Name and address of the Notifier:
Amy B. Smith, Ph.D.
3329 Agriculture Drive
Madison, WI 53716

(ii) Common or Usual Name of the Notified Substance:
Lactobacillus plantarum Lp-115

(iii) Intended Conditions of Use:
Lactobacillus plantarum Lp-115 is manufactured in compliance with current Good Manufacturing Practice as specified in 21 CFR Part 117 *Lactobacillus plantarum* Lp-115 is intended to be used in yogurt, and other dairy products, soy products, beverages, chewing gum, confectionary snacks and other foods and in supplement form including sachets, tablets and capsules. It is intended to be added to conventional foods at initial levels as high as $5x10^{11}$ cfu/250g serving (i.e. $2x10^{9}$ cfu/g) to ensure at least 1×10^{10} CFU/250g serving throughout the shelf life of the product and in dietary supplements to ensure at least 5×10^{10} CFU/serving. The function of *Lactobacillus plantarum* Lp-115 is to serve as a probiotic microorganism to be consumed by the general population.

(iv) Basis for the GRAS Determination:
This GRAS conclusion is based on scientific procedures (21 CFR 170.30 (a) and (b)) as discussed in the detailed description provided below.

(v) Availability to FDA of Data and Information that are the Basis of Determination:
The data and information forming the basis for this GRAS determination and the exemption claim asserted herein are available for FDA review and copying during customary business hours at the following address, or will be sent to FDA either in an electronic format that is accessible for FDA evaluation or on paper, upon request:

Amy B. Smith, Ph.D.
Senior Manager, Regulatory Affairs
DuPont Nutrition & Health
3329 Agriculture Drive
Madison, WI 53716
904-249-7444
Amy.B.Smith@dupont.com

(vi) No data or information contained in parts 2 through 7 of this GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

(vii) If applicable and necessary, as required by §170.270 I authorize FDA to send any trade secrets to the Food Safety Inspection Service (FSIS) of the U. S. Department of Agriculture.

(viii) I certify that, to the best of my knowledge, this GRAS notice for *Lactobacillus plantarum* Lp-115 and its use in yogurt, other dairy products, soy products, beverages, chewing gum, confectionary snacks and other foods and in supplement form, 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 use of the substance.

Should you have any questions regarding the submission of this notice, please contact Dr. Amy Smith of DuPont Nutrition & Health. Thank you for your prompt consideration of, and response to, this notice.

Sincerely,

(b) (6)

Amy B. Smith, Ph.D.
Senior Manager, Regulatory Affairs
DuPont Nutrition & Health

Part 2 – Identity, method of manufacture, specifications, and physical or technical effect

A. Identity:

a. **Name of the GRAS Organisms:** *Lactobacillus plantarum* Lp-115.
The strain is also referred to in the Danisco Global Culture Collection (DGCC) 4715 and has been deposited in the ATCC Culture Collection as SD5209.

b. **Source of the GRAS Organisms:** *L. plantarum* Lp-115 was isolated from plant silage material.

i. **The taxonomic lineage is:**

Kingdom: Bacteria
Phylum: Firmicutes
Class: Lactobacillales
Family: Lactobacilleae
Genus: Lactobacillus
Species: Lactobacillus plantarum
Strain: Lp-115

ii. **Description of the GRAS Organisms:**

In culture, Lp-115 forms Gram positive rods of varied lengths, commonly occurring in short chains.

Lactobacillus plantarum is a member of the lactic acid bacteria (LAB) classification, a group related by the production of lactic acid as the major metabolic end product of carbohydrate metabolism and other physiological traits. LAB are Gram-positive and generally non-spore forming, catalase negative, and devoid of cytochromes. LAB are of nonaerobic habit but are aerotolerant, fastidious, acid-tolerant, and strictly fermentative forming lactic acid as the major end product of sugar fermentation (Holzapfel et al., 2001). LAB is not a defined taxonomic group, rather it is a functional grouping, and thus, the boundaries are controversial. Among the core genera classified LAB are *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus,* and *Streptococcus* (Axelsson, 2004). Most LAB are considered to be non-pathogenic and have a long history of use in fermented and non-fermented foods (Axelsson, 2004; Douillard and De Vos, 2014). Comparative genomics has identified the genes in LAB involved in colonization, persistence, interaction and signaling and helped in the understanding of the response of LAB to their environment and their evolution (Douillard and De Vos, 2014). The long history of safe use in foods, their ubiquitous presence as a minor component in the bowel microflora, and their ability to inhibit

the growth of pathogenic microorganisms leads to the presumption that most LAB are safe for use in foods.

Lactobacillus, the largest of the LAB genera, contains over 80 species. It is a non-pathogenic, rod-shaped, non-motile, and non-sporulating genus that is widespread in nature. Many *Lactobacillus* species have found applications in the food industry. The genus may be categorized into three groups, obligate homofermentative, facultative heterofermentative, and obligate heterofermentative (Axelsson, 2004).

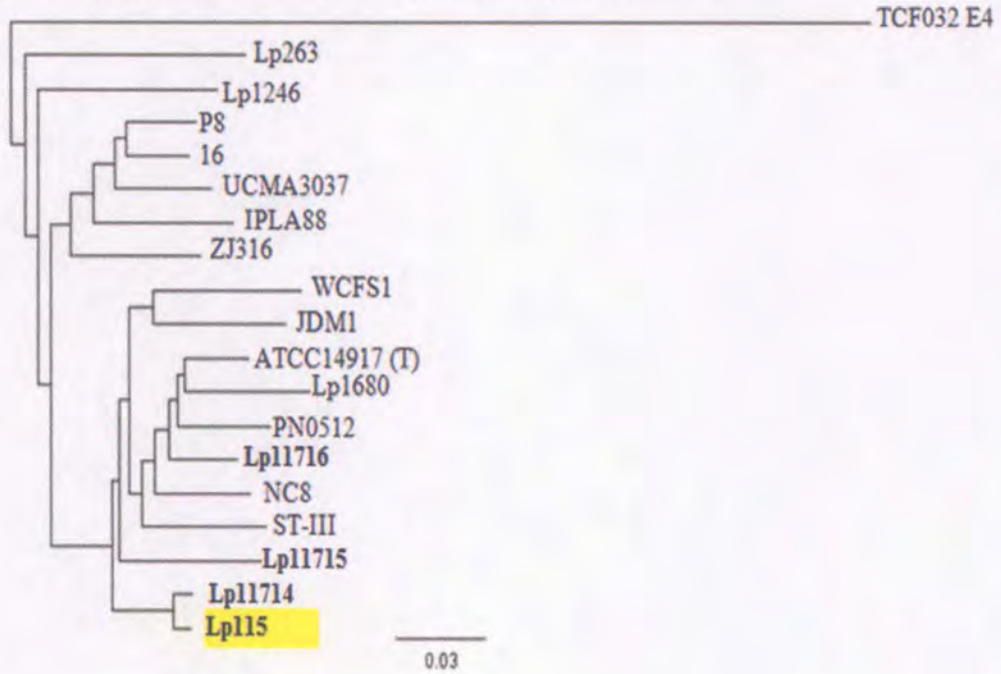
L. plantarum is a facultative, catalase reaction negative, heterofermenter that has been isolated from food products, plant material, oral cavity, vaginal cavity, gastro-intestinal tract, and other body sites (Axelsson, 2004; Douillard and De Vos, 2014; Molin, 2001b, 2008).

iii. **Genomic Analysis**

Sequencing:

A proprietary genome sequence of *L. plantarum* Lp-115 was obtained using published methods. The resulting genome draft yielded 49 contigs with 3,229,044 total base pairs in length and 22X average coverage. Alignment of the resulting draft to whole genome sequences of published *L. plantarum* strains shows good overall genomic synteny and core similarity, with unique regions that can be utilized for strain differentiation. A complete genome sequence of *L. plantarum* type strain ATCC 14917 is not available. However, comparison of strain ATCC 14917 and strain Lp-115 demonstrated 100% identical 16S rRNA sequences, validating the species designation of Lp-115.

Figure 1 ProgressiveMauve guide tree of 19 L. plantarum strain



RiboPrinter® Analysis:

RiboPrinter® analysis targets the 5S, 16S, and 23S regions plus intragenic spacers regions within the genome. This automated southern blot technology provides a genetic fingerprint that allows identification to the Genus and species level, but may also discriminate within a species. The Lp-115 RiboPrinter® pattern matched the RiboPrinter® patterns for *L. plantarum* within the RiboPrinter® database. See attached RiboPrinter® reports in Appendix A.

Antibiotic Resistance

Antimicrobial resistance in lactic acid bacteria (LAB) can be mediated by many different mechanisms that range from unknown and non-specific to fully understood and well-studied. In order to address the question of transferability of antibiotic resistance, it is best to define the two types of resistance. Intrinsic resistance reflects an organism's ability to thrive in the presence of an antimicrobial agent, is not horizontally transferable, and is typical of the strains of a given species (Mathur and Singh, 2005). In contrast, when a strain is resistant to a drug that the species is typically sensitive to, it may be considered acquired resistance. Acquired resistance can be mediated by mutation of indigenous genes or by added genes (EFSA, 2012b). The primary concern of acquired resistance is not the acquisition of a gene or mutation that provides resistance, but rather the ability of that resistance to be horizontally transferred. Therefore, the focus has been on acquired resistance genes with the belief that they present a greater risk of transfer of resistance via horizontal gene transfer within and between species (Mathur and Singh, 2005). LAB have been reported to have both intrinsic and acquired resistances to many classes of antibiotics, only some of which are known to be transferable (Nawaz et al., 2011; Zhang et al., 2011). There are three identified mechanisms of horizontal gene transfer (HGT) in bacteria; natural transformation, conjugation and transduction. Some LAB species have these abilities and some do not, in fact strain level differences need to be evaluated in order to determine if HGT is possible (Marshall et al., 2009; Ouoba et al., 2008). Three types of HGT were evaluated in this investigation, conjugative plasmids, transposases, and prophage/bacteriophage elements. Antibiotic resistance has been previously documented to be transferable on plasmids, transposases and phage (Aires et al., 2007; Colomer-Lluch et al., 2011; Marshall et al., 2009; Wang et al., 2006). Therefore, the highest risk of an antibiotic gene being mobilized to another strain/species comes from these mechanisms of HGT, all of which have previously been reported in LAB in both in vitro and in vivo studies (Mathur and Singh, 2005).

Type of analysis conducted

In each case, a whole genome sequence of the manufactured strain was obtained and analyzed for the mechanisms of HGT. Using the sequence, comparisons to known drug resistance markers could be done in order to determine their presence.

When the mechanism of resistance was well documented and genomically located in the sequence, an evaluation of the flanking regions as well as the sequence identity was done. When a mechanism of resistance was not well understood, examination of all the known HGT mechanisms in that strain was completed to rule out a possibility of a resistance gene located in the vicinity. Note that not all drug resistances were evaluated. Only the genes responsible for the drug resistance over the EFSA breakpoint were investigated.

Analysis of *Lactobacillus plantarum* Lp-115 (DGCC 4715)

An antibiogram of Lp-115 (DGCC 4715) was established using the ISO 10932 IDF223 method and VetMIC Lact-1 and 2 micro-dilution plates that included all antibiotics recommended by the FEEDAP. Recorded MICs are displayed in Table 1. MIC values are below or equal to the Microbial Break Points (MBPs) defined for *Lactobacillus plantarum* (EFSA, 2012a). According to these results, Lp-115 (DGCC4715) does not bear acquired antibiotic resistance.

Genome summary

A proprietary genome sequence of *L. plantarum* Lp-115 was obtained using published methods. The resulting genome draft yielded 49 contigs with 3,229,044 total base pairs in length and 22X average coverage. Alignment of the resulting draft to whole genome sequences of published *L. plantarum* strains shows good overall genomic synteny and core similarity, with unique regions that can be utilized for strain differentiation.

Clindamycin Resistance in *L. plantarum*

Clindamycin is an antibacterial peptide from the family of lincosamides. Resistance to this drug is not fully understood genetically. Recent published studies have reported a possible link in resistance to the erythromycin resistance operon (ermB, ermC, ermT, ermG)(Wang et al., 2006). Intrinsic resistance of this drug has been reported in Lactobacillus species where no (erm) genes were found (Wang et al., 2006). Acquired clindamycin resistance in lactobacilli has been demonstrated to be transferable in previous studies, but always mediated by conjugation of mobile elements encoding the resistance, and only when resistance was encoded within the Erythromycin resistance gene (Wang et al., 2006).

Plasmid analysis of Lp-115

A number of the contigs of Lp-115 show high sequence identity to *L. plantarum* ST-III plasmid pST-III (CP 00223.2). Analysis of the annotated plasmid does not indicate any

known antibiotic resistance genes, but has not been phenotypically tested to determine any actual resistance.

Insertion elements

Several genes that encode known prophage proteins were identified within the genome of Lp-115. However, there did not appear to be any known antibiotic resistance genes associated with prophage encoded within the same operon of genes. Many transposases were identified within the genome of this strain, however no antibiotic genes known to be responsible for Clindamycin resistance (erythromycin genes) were found in the vicinity of the any of the transposons or transposases.

Gene Mining

The public database NCBI was searched for known ermB and ermC genes from *L. plantarum*. DNA sequences and protein sequences of these genes were obtained and BLAST analysis of the *L. plantarum* Lp-115 genome was done to determine if any of the genes were present in the genome. No statistically significant hits were found.

Conclusion

No known resistance mechanism to clindamycin in this strain can be determined at this time. Research has yet to determine all the genes responsible for this drug resistance, but some resistance is thought to be encoded by erythromycin resistance genes. Analysis of the proprietary genome sequence of this strain for known acquired clindamycin resistance genes did not yield any matches. Using the EFSA technical guidance (EFSA, 2012b) to analyze the genome of this strain, we can only conclude that there is no known acquired and/or transferable resistance of clindamycin.

Table 1 Antibiogram of *Lactobacillus plantarum* Lp-115

APPENDIX : Antibiotic Susceptibility Profile

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

	Gentamycin	Kanamycin	Streptomycin	Tetracycline	Erythromycin	Clindamycin	Chloramphenicol	Ampicillin	Vancomycin	Virginamycin*
	Gm	Km	Sm	Tc	Em	Cl	Ch	Amp	Va	Vi*
DGCC 4715	MIC µg/ml									
	Max.	Max.	Max.	Max.	Max.	Max.	Max.	Max.	Max.	Max.
Lactobacillus plantarum	8	64	64	32	0,5	2	8	0,5	> 128	2
MBP for *Lactobacillus plantarum / pentosus***	16	64	NR***	32	1	2	8	2	NR***	4

* Virginamycin is no more included in the FEEDAP recommended list of antibiotics (june 2012) **EFSA Journal 2012;10(6):2740

NR***: not required

Lp-115 was screened for acquired antibiotic resistance as part of the EU-PROSAFE project. The PROSAFE project evaluated over 470 strains of human lactic acid bacteria to assess antibiotic susceptibility, to study horizontal transfer of detected antibiotic resistance genes, and to detect virulence factors. Tentative epidemiological cut-off values were determined for 13 antibiotics (ampicillin, vancomycin, amikacin, gentamicin, kanamycin, netilmycin, streptomycin, erythromycin, clindamycin, dalfopristin-quinup, tetracycline, chloramphenicol, linezolid, and trimethoprim). Species that displayed phenotypic resistance to antibiotics were screened for transmissible resistance genetic determinants (Egervarn, 2009; Sanders et al., 2010; Vankerckhoven et al., 2008). Lp-115 was determined to be free of acquired antibiotic resistance (Danisco, 2014).

iv. **Phenotypic Properties**

Bacteriocins, toxin genes and hemolysin

The genome of *Lactobacillus plantarum* Lp-115 was analyzed for bacteriocins, toxin genes, and genes associated with hemolysin production (DuPont internal study). First, the "Virulence, Disease and Defense" subsystem feature in RAST was mined. Next, the annotations of the genome were mined for key words using the Geneious 6.1.8 viewer. Suspect genes were confirmed using BLAST protein (*blastp*) in NCBI. Finally, local searches were performed using Geneious 6.1.8 with the custom Basic Local Alignment Search Tool (BLAST) function.

The protein sequences of Lp-115 annotations were compared to all of these databases. As noted in the guidelines from the European Food Safety Authority in regard to allergen presence, results that match at least 35% of sequence identities in a sliding 80 amino acid window were considered suspect and analyzed further. Searches from the various collections were refined based on target, as the searches can broadly incorporate elements that are not related to the query (for example, if "bacteriocin" is in the title of the reference organism). Suspect proteins were assessed using *blastp* and UniProt (www.uniprot.org).

- Three bacteriocins were identified that affect bacteria and are not dangerous to hosts.
- No toxin genes were identified to be virulent to hosts.
- Lp-115 is α-hemolytic, meaning that the bacterium produces hydrogen peroxide which partially degrades blood cells, but none of the genes produce hemolysin.

Lactic Acid Production

The overgrowth of commensal microorganisms capable of producing D-lactate during chronic antibiotic exposure in individuals with intestinal failure has been reported to result in D-lactate acidosis (Hudson et al., 1990). However, the consumption of D-lactate producing bacteria has a long history of safe use because D-lactate is readily metabolized in humans (Ewaschuk et al., 2005; Hudson et al., 1990) and toxicity has not been reported in normal individuals with functional small intestines. Thus, ingestion of probiotics that produce a racemic mix of lactate does not pose a significant risk. Lp-115 produces both L- and D- lactic acid in the molar ratio of L/D = 45/55 (Danisco, 2014).

Adhesion and gut stability

In a series of *in vitro* adhesion experiments, Collado et al. (2007) assessed the ability of 12 probiotic bacterial strains to adhere to human intestinal mucous that was previously bound to microtiter plates. The adherence index for the various strains ranged from less than 1% to 20%; Lp-115 was midrange (approximately 7% adhesion). When evaluated in the same adherence assay, Lp-115 was able to effectively inhibit the adherence of 7 different pathogenic bacteria including *Bacteroides, Clostridium, Staphylococcus,* and *Enterobacter.* Moreover, Lp-115 was able to effectively displace the adherent pathogens. The effectiveness of competition depended both on the strain of probiotic and pathogen; Lp-115 was generally midrange among the probiotics tested.

Properties associated with probiotic activity are the ability to auto-aggregate and to co-aggregate with pathogenic bacteria (Collado et al., 2008). These properties may relate to the ability to interact closely with pathogens and interfere with their adhesion to the gut mucosa. The authors evaluated these activities on 12 probiotic bacterial strains. In the auto-aggregation test, Lp-115 was an effective but middle of the range at 20 °C but the most effective of all species evaluated at 37 °C. While Lp-115 co-aggregated with all the pathogenic strains it was most effective co-aggregating with *Staphyloccocus aureus*, *Clostridium hystolyticum*, and *Bacteroides vulgatus*.

Daniel et al. (2006) assessed ability to bind *in vitro* to Caco-2 cells, a human epithelial cell line. The probiotic strains tested included *L. plantarum* Lp-115, *L. plantarum* NCIMB8826, *L. salivarius* Ls-33, and *L. acidophilus* NCFM as well as a non-probiotic *L. paracasei* strain as control. All four probiotic strains bound to the Caco-2 cells after a 90 minute incubation period. Similarly, Lp-115 was found to bind to HT-29, a human colorectal adenocarcinoma cell line (Danisco, 2014).

The survival in the gastrointestinal tract of the lactobacilli was examined *in vitro* by testing resistance to acid, pepsin, pancreatin, and bile salts (Daniel et al., 2006). Simulation of gastric and small intestinal transit tolerance was evaluated by culturing the bacteria in the presence of gastrointestinal components for periods up to 2 hr. Compared to the other strains tested, Lp-115 demonstrated a higher resistance to pepsin, was resistant to bile salts, and a lesser tolerance to pancreatin. Lp-115 was found to be resistant to treatment with hydrochloric acid and 1% pepsin at pH 3 for 20 min and still retained 15.5% viability after 1 hr. Lp-115 retained 87% viability after treatment with 0.1% pancreatin containing medium at pH 8 for 1 hr and was resistant to treatment with 0.3% bile salt containing medium.

Immunomodulation

Foligne et al. (2007) evaluated 13 strains of lactobacilli (including Lp-115) for their ability to stimulate the release of interleukins from peripheral blood mononuclear cells (PBMC). The PBMC were exposed *in vitro* to 1 x 10^9 CFU/mL bacteria and the mice were administered 1 x 10^8 CFU via gavage daily for 6 days. Compared to the other strains tested, Lp-115 treatment induced a moderate level of IL-10 release but a higher level of IL-12 release. This pattern of interleukin expression is consistent with a shift towards Th1 type of response, which has been implicated in an anti-allergenic response.

The immunomodulatory activity of 6 strains of probiotic bacteria was investigated in an *in vitro* assay using cultured $D4^+CD25^-$ T cells to which titrated numbers of T regulatory cells (Tregs) were added (Schmidt et al., 2010). Exposure of antigen presenting cells (APCs) to various enteroantigens and the series of probiotic bacteria did not influence the stimulatory capacity of the APCs, exposure to three of the probiotic strains, but not Lp-115, reduced the suppressive activity of Tregs. Lp-115 had no stimulatory or suppressive activity in this assay. Thus, any Lp-115-induced anti-inflammatory activity is not mediated via Tregs.

Oxalate-degrading Activity

The accumulation of oxalate can result in hyperoxaluria, kidney stones, renal failure, cardiomyopathy, and cardia conductance disorders. Probiotic bacterial strains may decrease the body burden of hyperoxaluria and prevent oxalate-associated disorders. In a study of the oxalate-degrading capacity of 60 Lactobacillus strains, Lp-115 had 40% of the oxalate-degrading activity of the positive control *Oxalobacter formigenes* DSM 4420 while other strains of *L. plantarum* ranged from 0 – 35% (Turroni et al., 2007).

Summary

The strain identity, absence of transferable antibiotic resistance elements, absence of virulence factors, ability to adhere to mucin and intestinal tissue, ability to bind to pathogenic bacteria, and ability to prevent the adherence of biogenic bacteria to intestinal mucus are all consistent with benign probiotic organisms.

B. Method of Manufacture

The DuPont Nutrition & Health (formerly Danisco) Madison plant manufacturing process, for production of cultures, is a batch type fermentation process where a blend of proteins, carbohydrate, and other vitamins and minerals are blended with water, sterilized, and then inoculated with the selected bacteria. Each fermentation product has a defined growth medium and fermentation growth conditions (pH, temperature).

L. plantarum Lp-115 is manufactured in compliance with the U.S. Food and Drug Administration's current Good Manufacturing Practice guidelines[1] in FDA regulated and inspected facilities. All ingredients utilized are food grade or approved for use by the FDA (Appendix B). The manufacturing process is summarized below:

The source organism used is *L. plantarum* Lp-115. The cultures are maintained in the culture bank of Danisco USA Inc. as frozen 1mL vials at -80°C. Danisco USA Inc. independently verifies the identity of each organism. Each seed lot in the culture bank is fully characterized to insure the identity of the seed strains. From the seed vials, Danisco USA Inc. produces concentrated starter for the industrial fermentation.

As the bacteria fermentation products produced by DuPont Nutrition & Health (formerly Danisco) are destined to be either directly consumed or used as starter cultures for food fermentations such as yogurt manufacture, DuPont Nutrition & Health takes great care to ensure the quality of the product. These quality control processes begin with the identification, storage and handling of the bacteria seed stocks.

Genus and species designation for each bacterial species have been determined by 16S rRNA testing. For identification on strain level, a specific DNA-fingerprinting technique is applied that ensures identity of the seed stocks. The fingerprinting technique is applied prior to preservation of every strain.

A Master Seed repository is maintained for each of the bacterial strains at the Danisco Global Culture Collection (DGCC) in Niebull, Germany. The repository is a collection of purified, tested, and qualified Master Seed stocks derived from single strain isolates stored at -180°C in liquid nitrogen to maintain long term cell viability.

The microbiological quality of the Master Seeds is determined by microbiological testing for microbiological contamination at the DGCC.

Testing and release of Master Seed vial lots are performed to insure the Master Seeds meet the specifications listed within are absolute acceptance criteria. If a Master Seed vial lot fails any of the required tests (above), the lot is placed on QC hold to prohibit use and the lot is subsequently destroyed.

---

[1] 21 CFR 117

Working Seeds

Working seeds are prepared under controlled conditions from master seed stock maintaining effective acceptance criteria at DGCC. All Working Seeds are prepared under controlled conditions from Master Seed stock meeting established acceptance criteria (described above) and each new lot of Working Seeds is held in "quarantine" at liquid nitrogen temperature pending QC testing (strain identity and purity as described for the Master Seeds) and release. If the Working Seed vial lot fails any of the required tests, the lot is placed on QC hold and destroyed. Qualified, tested Working Seed stocks are stored at -76°C until use in production fermentation.

The use of tandem Master and Working seed inventories reduces the risk of genetic drift over time due to excessive sub-culturing of strains and insures the integrity of the strain collection.

All steps in the preparation of Master and Working seed are documented in a specified database, allowing traceability of every seed preparation down to each single batch of raw material used.

Fermentation Process

The fermentation begins by withdrawing one of the working seed vials and scaling-up via a series of fermentations until a commercial size batch is complete. The fermentation starts off in a 100mL vessel, then transferred to two intermediate sizes, and finally to a 20,000L - 45,000L fermentation vessel.

As each organism produces organic acids during metabolism, an ammonium hydroxide base must be injected into the medium to maintain pH at the proper set point in order to maintain the optimum pH during growth.

The fermentation production process of each is a closed system with no product exposure from seed inoculation to cell harvest. Prior to each fermentation batch, all mixing tanks, heat exchangers, lines, fermenters and centrifuges are cleaned via automated clean-in-place systems. Systems are then either steamed or chemically sanitized prior to product contact.

At the DuPont Nutrition & Health Madison plant, there are two methods to measure growth in the fermenter. First, flow meters on the ammonium hydroxide feed lines to the fermenters measure the volume of base used to maintain optimum growth pH of the culture. The base addition rate is proportional to the acid developed in the fermentation, which is proportional to cell growth rates.

Second, the pH in the fermenter is monitored on digital display and on recording charts. By consulting these charts, the growth characteristic of a given fermentation can be determined.

Fermenters are normally cooled to stop the fermentation when the pH and base addition data indicate that the fermentation has entered stationary phase. Cooled fermentate is pumped through continuous flow centrifuges and the bacteria are concentrated. Cryoprotectant is added to cooled concentrate and the mixture is then pelletized by immersion of concentrate droplets in liquid nitrogen. These concentrate pellets are then freeze-dried.

Batches of concentrated bacteria are freeze-dried in a qualified facility.

Milling Process

The milling process takes place entirely in the DuPont Nutrition & Health Madison facility. The freeze-dried pellets are milled according to standard procedures utilizing a Fitzpatrick mill fitted with a mesh screen operating at 2000 rpm. Production batch records contain mill charge and appropriate operator sign-off.

Blending Process

The blending process is performed in the Madison, WI facility under 21 CFR 111 cGMPs. Blending can occur by either blending in Marion and/or V-blender mixers, or by utilizing Intermediate Bulk Containers (IBCs). The processes are slightly different, but are used interchangeably due to available resources.

Freeze dried pellets are milled according to standard procedures utilizing a Fitzpatrick mill fitted with a mesh screen. The milled pellets, along with approved excipients are added to the blender. All ingredients added to the blender, both milled pellets and excipients, and are documented on production batch record containing traceability information and appropriate operator sign off. Milling and ingredient addition is performed in a controlled environment.

The blender is allowed to mix for an established amount of time prior to packaging to ensure homogeneity.

Product is dispensed out of blender and through metal detector prior to packaging.

Packaging

Bulk packaging of the product is carried out in a controlled environment within the DuPont Nutrition & Health Madison facility. The HVAC system consists of an air-handling unit with air-cooled direct expansion type condenser including ducted heater for reheating. Pressure relief dampers operate in conjunction with the fresh air intake system maintaining the whole area at a positive pressure to prevent contaminant infiltration to the packaging room. The area design conditions are as follows:

Dry Bulb Temperature	72° F
Relative Humidity	≤ 35% RH

HEPA filter is used in the packaging room for high performance in these demanding operating conditions as the final filter for particulate removal when clean air is required.

Quality Systems

The DuPont Nutrition & Health Madison plant has fully implemented HACCP plans, Standard Operating Procedures and Quality Control programs to ensure the quality of each product. DuPont Nutrition & Health Madison has numerous certifications, including ISO FSSC 22000 food safety certification, ISO 9001 Quality Management System certification, and NSF Dietary Supplements cGMP certification.

A quality control laboratory is maintained on site. Quality control personnel are qualified by training and experience to test products and to release product based on specifications. In addition, a third-party laboratory with ISO 17025 certification, located in Madison WI, performs QC testing for DuPont Nutrition & Health Madison facility under contract.

The Quality Control unit utilizes a SAP computer quality control system for the specification, quality control data entry and product release. No product can be released for use without acceptance by the Quality Control unit according to specified acceptance criteria.

Each bacterial fermentation product must meet specifications and must have a confirmation of identity (compared to the Master Seed) by 16S rDNA sequence analysis or RiboPrinter® analysis for release of the product. Microbiological testing is performed by trained QC microbiologists in the Madison plant laboratory and certified external laboratory using standard methods.

Cleaning and quality testing of the process rooms and equipment are under the control of Manufacturing and Quality Assurance, following the established SOPs. Fermentation rooms are isolated from the freeze- drying processes and access is controlled. Materials cannot enter the milling and blending process areas prior to cleaning, sanitation and subsequent surface testing for cleanliness via ATP testing. Room access is controlled by appropriate signage, and additional protective gowning must be worn in processing rooms where product is potentially exposed. Operator sign-off for clean, sanitation and testing is required on the lot batch ticket. Quality Assurance is responsible for review of completed batch tickets.

Process rooms are segregated from other manufacturing areas with appropriate closures. Room air quality is controlled via HEPA air filtration of incoming air and maintenance of positive pressure in the process rooms relative to adjacent processing areas. HEPA filtration operation is monitored for performance; air quality is monitored monthly by Quality Assurance. Operators may not bring materials into process areas where HEPA filtration is not functioning to specification. Operators sign-off on the lot batch ticket for temperature and humidity and record the temperature and humidity on the batch ticket. Quality Assurance is responsible for review of completed batch tickets.

Rooms and equipment used in manufacturing are approved for production only after cleaning, sanitization and quality inspection. Prior to qualification of the process room for production, as specified in the appropriate SOP, the blending room is sprayed from ceiling to floor with 145-160°F water. All large equipment having any product contact surfaces are thoroughly scrubbed / foamed with a neutral detergent cleaner, rinsed with cold water, sanitized with an acid/iodine based sanitizer at 50ppm and re-rinsed with cold water. The floor is sanitized with acid/iodine sanitizer at NLT 50 ppm.

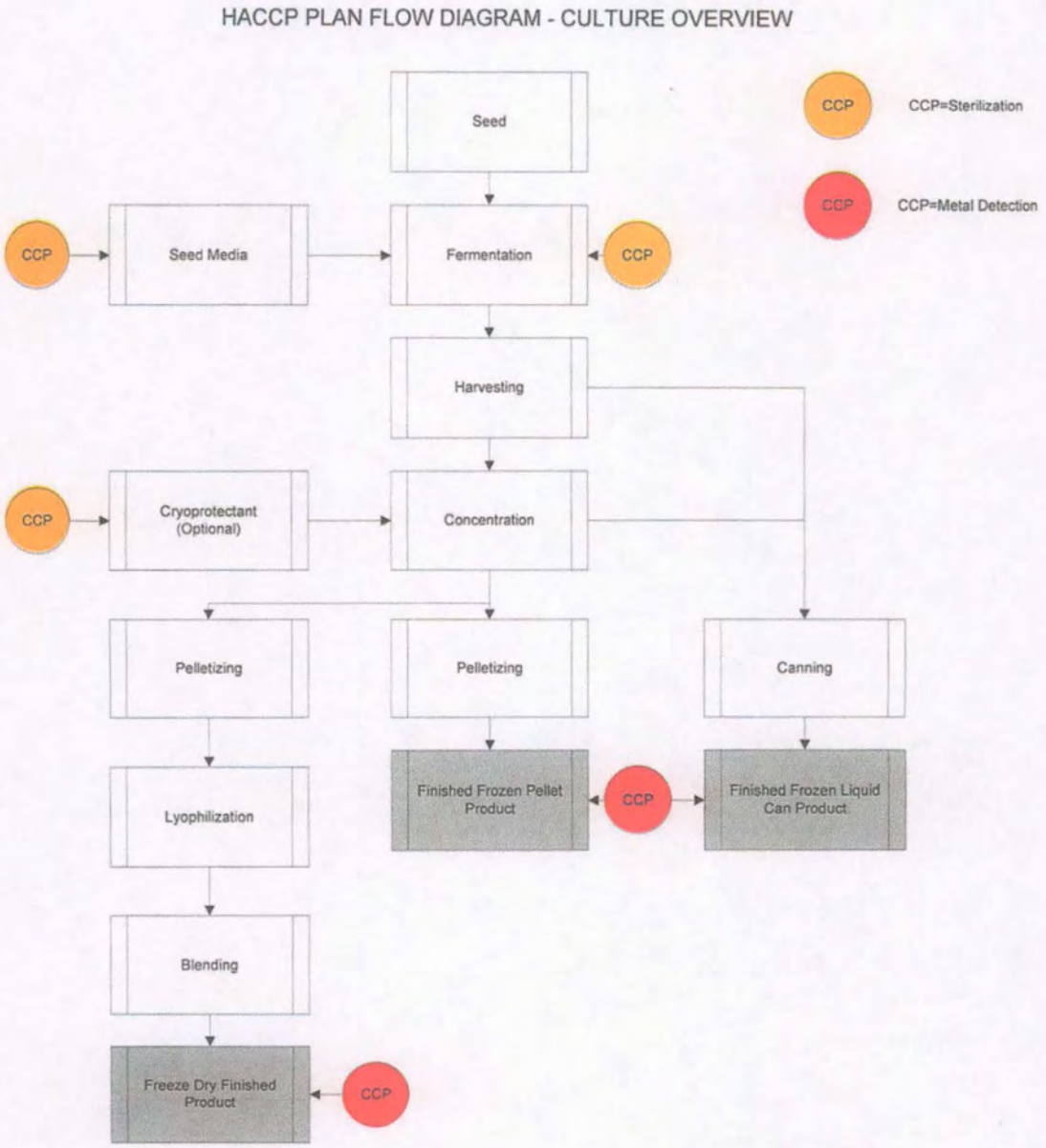
Process rooms and equipment are tested by Quality Assurance following cleaning and sanitation for microbial contamination and test results are entered on the batch tickets with Quality Assurance sign-

off. ATP and Microbiological swabs are taken after cleaning and sanitation. Room and equipment surfaces must be negative by test in order to qualify for use in production.

Batch records are maintained as per Standard Operating Procedures and are provided to Quality Assurance for each lot produced. Quality Assurance is responsible for batch ticket review

Specifications are listed in Table 2. A schematic overview of the manufacturing process is Figure 2 below.

Figure 2 Manufacturing Flow Diagram



Danisco USA Inc. Madison

September 2011

C. Specifications for food–grade material

Table 2 Product Specifications

Parameter	Specification	Method
Description	White to cream-colored free-flowing powder	
Particle size		
Color	Beige	
Odor	Characteristic	
Taste	Characteristic	
Viable cell Count	≥ 7 x10^{11} CFU/g	ISO 7889/IDF 117
Proximates		
Carbohydrate	90.17 g/100 g	
Protein	3.95 g/100 g	
Moisture		
Fats	0.55 g/100 g	
Fiber	0.3 g/100 g	
Sodium	0.11 g/100 g	
Heavy metals		
Lead	< 1ppm	EU regulation 629/2008
Cadmium	< 3pppm	EU regulation 629/2008
Mercury	< 3 ppm	EU regulation 629/2008
Microbiological purity		
Non-Lactic Cell Count	< 5,000/g	SMEDP, 17th Ed
Enterococci (CFU/g)	< 100/g	CMMEF, 4th Ed
Coliform (MPN)	Negative by test in 10 g	AOAC 966.24
Escherichia coli (MPN)	Negative by test in 0.3 g	AOAC 966.24
Staphylococcus (coagulase +)	Negative by test in 40 g	AOAC 975.55
Salmonella	Negative in 40 g	AOAC 2004.03
Listeria	Negative in 25 g	AOAC 999.06
Molds and Yeast	< 100 CFU/g	USP

Batch Analysis

Certificates of analysis of 4 non-consecutive batches of finished product are included in Appendix C. These indicate that the manufacturing process consistently meets product specifications and is not contaminated with heavy metals.

Stability Data

The stability of *Lactobacillus plantarum* Lp-115 was determined at 4°C, 25°C, and 30°C over a 24-month period by monitoring viable cell counts at regular intervals (Figure 3). The product is stable under these conditions. The results of a typical lot are presented in Figure 3.

Figure 3 Stability of Lp-115

Lot number:
1102322450

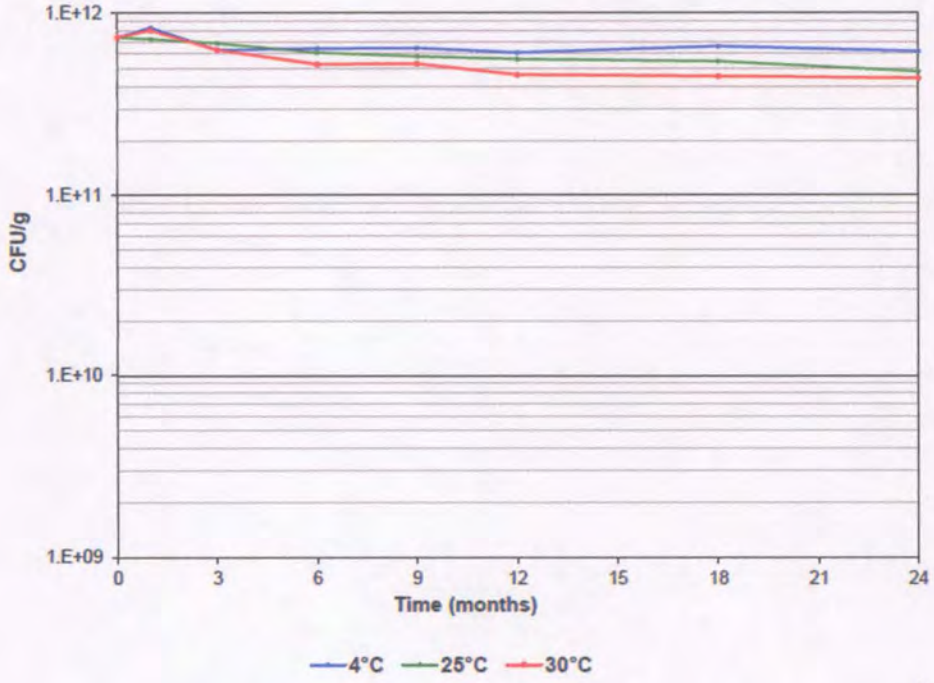
Excipient:
None

T zero Aw:
0.055

Format:
Culture concentrate

Packaging:
Foil sachet

Study start:
August 2014



GMO Status

DuPont Nutrition & Health certifies that *L. plantarum* Lp-115 is conventional (non-GMO). Any culture strain used in the manufacture of these products or any culture strain contained as part of this product has itself not been genetically modified according to Directive 2001/18/EC neither subject to the labeling requirement of (EC) 1830/2003 nor to the authorization procedure of Regulation (EC) 1829/2003 (Appendix D).

Allergens

The *L. plantarum* Lp-115 tested negative for wheat, other cereals containing gluten, crustacean shellfish, eggs, fish, peanuts, soybeans, milk (including lactose), nuts, celery, mustard sesame seed, sulfur dioxide and sulfites, lupin, and molluscs (Appendix D).

Part 3 – Dietary Exposure

A. Current Dietary Exposure of *L. plantarum* Lp-115

Lactobacillus plantarum Lp-115 is intended to be used in yogurt, and other dairy products, soy products, beverages, chewing gum, confectionary snacks and other foods and in supplement form including sachets, tablets and capsules. Uses are limited to foods that can sustain living *L. plantarum* Lp-115 during shelf life. It is intended to be added to conventional foods at initial levels as high as $5x10^{11}$ cfu/250g serving (i.e. $2x10^{9}$ cfu/g) to ensure at least 1×10^{10} CFU/250g serving throughout the shelf life of the product and in dietary supplements to ensure at least 5×10^{10} CFU/serving. The function of *Lactobacillus plantarum* Lp-115 is to serve as a probiotic microorganism to be consumed by the general population.

B. Intended Human Food Uses (Estimated Daily Intake)

DuPont Nutrition & Health has proposed the use of the *L. plantarum* Lp-115 in yogurt and other dairy products, soy products, beverages, chewing gum, confectionary snacks and other foods and also in supplement format including sachets, tablets and capsules. It is intended to be added to conventional foods at levels sufficient to ensure at least 1×10^{10} CFU/serving throughout the shelf of the product and in dietary supplements to ensure at least 5×10^{10} CFU/serving. The function of *L. plantarum* Lp-115 is to serve as a probiotic microorganism.

L. plantarum Lp-115 will not proliferate in the foods and beverages to which it is added but instead will decline over the shelf-life of the food. Considering the average individual consumes only about 20 servings/d of all food combined (Millen et al., 2006), a conservative estimate of the total estimated daily intake at 1×10^{9} CFU/serving times the 10 servings/d would estimate 1×10^{10} CFU/person/d. At the maximum daily intake in the dietary supplement form of 5×10^{10} CFU/day, the total estimated consumption would be 6×10^{10} CFU/person/d. This is a conservative estimate. It is unlikely that a consumer would consume 10 servings of foods containing Lp-115 and the number of CFU will decline over the shelf-life of the food. It is likely maximum ingestion is thus less than 6×10^{10} CFU/d and well within levels that have been shown to be safe. Supplements will be labeled recommending a single daily dose.

Part 4 – Self-limiting levels of use

There are no self-limiting intake levels but the use is restricted to foods that can sustain living *L. plantarum* for the shelf life of the food.

Part 5 – Experience based on common use in food before 1958

This GRAS conclusion is based on scientific procedures.

Part 6 – Narrative

A. Review of Safety Information

1. History of Consumption of L. plantarum

Lp-115 has been commercially available for at least 20 years. 78,000 kg have been marketed over the last 5 years as a dietary supplement under various trade names. This is equivalent to 5 billion servings.

2. Regulatory History of Lp-115 and related lactobacilli

In 2011, Purac submitted a GRAS notification (GRN 378) to the US. Food and Drug Administration (FDA) for a food ferment solution as a food ingredient (PURAC, 2011). The subject of the notice was cultured dairy sources, sugars, wheat, malt, and fruit- and vegetable-based sources fermented by *Lactobacillus plantarum* or other probiotic bacteria including (*Streptococcus thermophilus, Bacillus coagulans, Lactobacillus acidophilus, Lactobacillus paracasei* subsp. *paracasei, Lactobacillus sakei, Lactobacillus delbrueckii* ssp. *bulgaricus*, and/or *Propionibacterium freudenreichii* subsp. *shermanii*) or mixtures of these microorganisms for use as antimicrobial agents in a variety of food categories typically at levels of 0.1 to 4.5%, including meat and poultry, but excepting infant formula and infant foods. The use of *L. plantarum* and the other microorganisms was primarily as a processing aid. The Lp-115 was removed during processing so the final amount of Lp-115 in the culture dairy products was negligible. The FDA reviewed the GRAS notification GRN 378 and responded that it had no questions (CFSAN, 2012b).

House Wellness Food Corp submitted a GRAS notice (GRN 324) to the FDA on heat-killed *L. plantarum* for use as an ingredient in baked goods and baking mixes, beverages and beverage bases, breakfast cereals, dairy product analogs, fats and oils, frozen dairy desserts, grain products and pastas, milk and milk products, plant protein products, processed fruits and fruit juices, processed vegetables and vegetable juices, soft candy, and soups and soup mixes, excluding meat and poultry, at a maximum level of 150 milligrams per serving (House Wellness Foods Corp, 2014). In 2010 the FDA ceased to evaluate the notice at the request of the notifier (CFSAN, 2014b).

Various related *Lactobacillus* sp. (*Lactobacillus rhamnosus* GG, *Lactobacillus reuteri* DSM 17938, and *Lactobacillus rhamnosus* HN001) were reviewed and received letters of no objection from the FDA for example.

The FDA responded that it had no questions to a GRAS notice (GRN 231) submitted by Mead Johnson & Company for the use of *Lactobacillus rhamnosus* GG (LGG) at 10^8 CFU/g in hypoallergenic infant formula (CFSAN, 2008a). The infant formula is intended for use in term infants from date of birth at levels of 10^8 – 10^{10} CFU/day. In their submission, Mead Johnson concluded that LGG is nonpathogenic, non-toxigenic, and not known to produce exotoxin and thus, there is no evidence of a safety hazard and no adverse impact (Mead Johnson, 2007).

BioGaia AB (BioGaia) submitted a GRAS notification (GRN 254) for the use of *Lactobacillus reuteri* DSM 17938 for use as an ingredient in processed cheeses, yogurt, ice cream, fruit juices, fruit drinks, processed vegetables, processed vegetable drinks, beverage bases, energy bars, energy drinks, and chewing gum at a level up to 10^9 CFU per serving, and in a drinking straw at a level of 10^9 CFU per straw (BioGaia AB, 2008). BioGaia concluded that based on an extensive record of safety in preclinical and clinical safety trials in *L. reuteri* DSM 17938 as well as its parent strain *L. reuteri* ATC 55730, there is no evidence of a safety hazard based on these studies. BioGaia considered that *L. reuteri* DSM 17938 was nonpathogenic and had no demonstrable other risk factors. The FDA responded that it had no questions (CFSAN, 2008b).

The FDA responded that it had no questions to a GRAS notice (GRN 281) submitted by Fonterra Co-operative Group for use of *Lactobacillus rhamnosus* HN001 for use in powdered term infant formula that is intended for consumption from the time of birth (not intended for immunocompromised infants), as well as in milk-based powdered follow-on formula, at a level of 10^8 CFU/g of the formula powder (CFSAN, 2009a). Fonterra cited published literature supporting the nonpathogenic and nontoxigenic nature of *L. rhamnosus* strain HN001. Fonterra noted a safe history of use of *L. rhamnosus* strain HN001 in New Zealand for at least twenty years (Fonterra Co-operative Group, 2009b).

The FDA responded that it had no questions to a GRAS notice (GRN 288) submitted by Fonterra Co-operative Group for use of *L. rhamnosus* HN001 for use an ingredient in various foods, including certain beverages and beverage bases (excluding soft drinks); cheeses; milk drinks; milk products; meal replacements; energy bars; ready-to-eat cereals; fruit juices, nectars, ades, and drinks; confections;

chewing gum, and hard candies at a level to provide up to 10^9 CFU per standard serving (CFSAN, 2009b). Fonterra cited published literature supporting the nonpathogenic and nontoxigenic nature of *L. rhamnosus* strain HN001. Fonterra noted a safe history of use of *L. rhamnosus* strain HN001 in New Zealand for at least twenty years (Fonterra Co-operative Group, 2009a).

The FDA reviewed and did not object to the New Dietary Ingredient Notifications (NDIN) for *Lactobacillus plantarum* ($2 - 8 \times 10^9$ CFU/day) and fructooligosaccharide by Kups International in 2003 (CFSAN, 2014a).

In 2005, the Scientific Committee recommended to the European Food Safety Authority (EFSA) a generic approach to assess the safety of microorganisms used in food or feed and the production of food/feed additives (EFSA, 2007). This system was intended to be similar to the Generally Recognized as Safe (GRAS) definition used in the U.S. but modified to account for the regulatory practices in Europe. The system is referred to as Qualified Presumption of Safety (QPS). The Scientific Committee recommended policies and practices for the routine assessment of microorganisms based on taxonomy, familiarity, pathogenicity, and end use. If a microorganism is approved as QPS, it would not require further regulatory review prior to introduction into the food supply. Lactic acid bacteria (including *Lactobacillus* species) were among the microorganisms recommended to be reviewed in this initial document.

Lactobacillus species were reviewed under the QPS system in 2007, 2008, 2009, 2010, 2011, and 2013 (EFSA, 2008, 2009, 2010, 2011, 2012b, 2013). *L. plantarum* was among the taxonomic units included in the initial QPS review of lactobacilli. In the initial review, the Scientific Committee concluded that the weight of evidence available for these species was sufficient and provided as least the same degree of confidence as a case-by-case assessment (EFSA, 2007). The Scientific Committee reviewed the available evidence regarding the involvement of lactobacilli in human disease. Reviewing and summarizing the occasional reports of *Lactobacillus* bacteremia, the committee concluded lactobacillemia occurred primarily in immunocompromised or those suffering from severe underlying illness and that the *Lactobacillus* species described herein can be considered non-pathogenic to humans. They emphasized the long history of safe use in the food chain and reported no safety concerns.

Each subsequent QPS review (EFSA, 2007; EFSA, 2008; EFSA, 2009; EFSA, 2010; EFSA, 2011; EFSA, 2013) evaluated the totality of the scientific information each year and reaffirmed the QPS status of *L. plantarum*.

L. plantarum appears on the inventory of microorganisms with a documented history of use in human food that was compiled by the International Dairy Federation (IDF) in collaboration with the European Food and Feed Cultures Association (Morgensen et al., 2002) The inventory lists microbial strains used by the food industry that have a long history of use in food without reported adverse effects. In 2012, the IDF Bulletin 455 updated the inventory and once again included *L. plantarum* subsp *plantarum* as part of its inventory of microbial food cultures (MFC) safe for use in fermented food products (Bourdichon et al., 2012).

3. Safety of Lactic Acid Bacteria and Lactobacillus Species

Lactobacillus plantarum

L. plantarum has a long history of use in different types of fermented foods and has been used in other food products as an ingredient. It has been consumed as a component of fermented rice and fish dishes in Southeast Asia region at levels up to 10^{12} CFU/gm (Olympia et al., 1992; Orillo and Pederson, 1968; Tanasupawat et al., 1998) and worldwide in fermented vegetable dishes, pickles, sauerkraut, various types of cheeses, and fermented sausages at levels up to 10^{8} CFU/gm (Baruzzi et al., 2000; Garcia Fontan et al., 2007; Orillo et al., 1969; Plengvidhya et al., 2007; Rantsiou et al., 2006; Rebecchi et al., 1998; Tamminen et al., 2004). *L. plantarum* 299v has been consumed at levels up to 5×10^{10} CFU/gm as a constituent of a fermented oatmeal, fruit or yogurt drink, which is marketed under the name ProViva in parts of Europe (Molin, 2001a). *L. plantarum* is also a commensal bacterium commonly present as part of the normal, healthy intestinal flora of humans (Ahrne et al., 1998; Molin et al., 1993; Song et al., 2000).

Lactic acid bacteria have long been considered safe and suitable for human consumption. Very few instances of infection have been associated with these bacteria and several published studies have addressed their safety (Aguirre and Collins, 1993; Gasser, 1994; Gueimonde et al., 2004; Salminen et al., 1998). Lactobacilli have long been considered to be non-pathogenic despite a small number of opportunistic infections where immunocompromised hosts with underlying health issues experienced infection. A 69-year-old male (with untreated diabetes) was admitted to a hospital to undergo surgical resection of advanced laryngeal cancer and it was noted upon examination that a neck abscess was present with descending necrotizing mediastinitis (DNM). Because *L. plantarum* was the only bacterial strain isolated from the mediastinal abscess, it is believed that this strain was responsible for the infection. The patient was treated with antibiotics and released with full recovery from the infection (Nei et al., 2013).

L. plantarum is a versatile lactic acid bacterium that has been isolated from a range of environmental niches; for example, it has been shown to survive and colonize within the gastrointestinal tract of humans and other mammals (De Vries et al., 2006). *L. plantarum* may be considered a probiotic providing benefit to the host. *L. plantarum* strains isolated from fermented meat products have been demonstrated to produce bacteriocins that effectively prevent growth of pathogens, such as *Mycobacterium tuberculosis* (Todorov et al., 2014).

The safety and clinical benefit of various strains of *L. plantarum* has been reported in numerous animal studies and human clinical trials. A representative selection is described below.

In a 7-day, repeated oral dose, general toxicity study using 9-wk-old Wistar rats (n=6), the combined AB-LIFE *L. plantarum* strains (CECT 7527, 7528, and 7529) were compared 1.5 ml PBS controls (n=6). AB-LIFE (the combined AB-LIFE strains in a 1:1:1 ratio) was administered by gavage at 5×10^{10} CFU/kg bw daily for 2 consecutive days. Rats were weighed on days 0, 1, 3, 5, and 7. Total food and water

consumption was recorded and animal well-being was monitored every other day. On day 7 the animals were sacrificed and necropsies and histopathological examinations were conducted. Compared to the control group, no macroscopic adverse effects in organs or cavities, no clinical symptoms or alteration in the animal well-being, and no significant differences in weight, diet or water consumption were reported (Bosch et al., 2014).

Samples of mesenteric lymph node and liver were taken at sacrifice for culture to evaluate possible bacterial translocation. Tissue samples (5 mg) were homogenized and seeded in McConkey plates to assess growth of enterobacteria and MRS plates to assess the growth of lactic acid bacteria. Colonies were counted after 24 h and 48 h incubation at 37° C under micro-aerophilic conditions (5% CO_2). There were no differences between treated and control animals for either mesenteric ganglion or liver samples. These results suggest there was no bacterial translocation. The authors concluded that the strains "could be excellent candidates" as probiotics (Bosch et al., 2014).

A sub-chronic oral toxicity study was conducted to evaluate the potential repeated-dose toxicity of the blend of three AB-LIFE® *L. plantarum* strains CECT 7527, 7528 and 7529 in rats (Mukerji et al., 2016). Three groups of young adult male and female Crl:CD(SD) rats (10/sex/dose) were dosed by gavage for at least 90 days with AB-LIFE® Concentrate (*L. plantarum* CECT 7527, 7528 and 7529 in a 1:1:1 ratio), suspended in phosphate-buffered saline, at doses of 0, 300 or 1000 mg/kg bw/day (equivalent to 0, 5.55×10^{10} and 1.85×10^{11} CFU/kg bw/day). The study followed OECD, Section 4 (Part 408): Repeated Dose 90-Day Oral Toxicity Study in Rodents, Guideline for the Testing of Chemicals (1998) and U.S. FDA, Redbook 2000: IV.C.4.a: Subchronic Toxicity Studies with Rodents, Toxicological Principles for the Safety Assessment of Food Ingredients (2003).

Body weights, food consumption, and detailed clinical observations were evaluated at least weekly. Ophthalmological evaluations were performed prior to exposure and near the end of the exposure period. Clinical pathology parameters (hematology, coagulation, clinical chemistry, urinalysis), and anatomic pathology parameters (organ weights and gross and microscopic pathology) were evaluated at the end of the study. Additionally, fecal samples were collected at regular intervals for microbial and biochemical analyses and selected tissues (liver, mesenteric lymph nodes and whole blood) were collected at the time of sacrifice for microbial translocation evaluation.

There were no test substance-related effects on mortality, body weight, body weight gain, food consumption, food efficiency, clinical signs of toxicity, or ophthalmological evaluations. No test substance-related effects were reported on clinical pathology parameters (hematology, coagulation, clinical chemistry, and urinalysis), organ weights, or gross or microscopic pathology. One female at 1000 mg/kg/day died on test day 71 due to a dosing accident. This death was not test-substance related and did not impact the interpretation of the study data. Mean cell hemoglobin was higher in the 300 mg/kg bw/day group but was not considered treatment related because there was no dose-related pattern. Serum proteins and Ca were minimally higher (7% - 11% over controls) in the 1000 mg/kg bw/day female group and Ca was minimally elevated (4% over control) while urinary pH was lower. Based on the minimal magnitude, the lack of correlative differences in anatomic or clinical pathology parameters, the absence of corroborative differences in males, and the fact that all individual

values were within historical control ranges, these differences were considered spurious and non-adverse.

There was no evidence of systemic translocation of the test strains beyond the extra-intestinal sites (liver and mesenteric lymph nodes [MLN]). There was no evidence of systemic translocation in any animal at any dose, nor any evidence of contamination in any control animal. The low numbers of AB-LIFE® colonies cultured from the liver or MLN of treated animals, and the other bacterial colonies cultured from control and treated animals, were not considered to represent a safety concern. There were no test strains identified in control animals, and the results of the present study were consistent with previously published data (Gunji et al., 2006; Liong, 2008; Perdigon et al., 1995; Rodriguez et al., 2001). The absence of anaerobic bacteria in the blood indicated that the test strains had not spread beyond the extra-intestinal sites (live and MLN). The absence of high bacterial counts from extra-intestinal sites corresponded to the absence of any clinical, hematological or microscopic findings indicative of organ-specific bacterial infection or septicemia.

Administration of the test substance resulted in increased fecal *L. plantarum* CECT 7527, 7528 and 7529 strain counts at each of the monthly study intervals (week 4, 8, and 13), compared with the control group. The increased overall *L. plantarum* fecal counts occurred in a dose-dependent manner. Test strain counts were not detected in baseline samples, or in control samples throughout the dosing period, with the exception of a single control sample collected at week 8, which did not impact the interpretation of the study.

There were no adverse effects on any fecal chemical endpoint (bile acids, overall neutral sterols, short-chain fatty acids, branched chain fatty acids, or lactic acid) evaluated in males or females at either dose level. These results are described in more detail in Section D above.

Based on the available data and under the conditions of this study, the no-observed-adverse-effect level (NOAEL) for *L. plantarum* CECT 7527, 7528 and 7529 in male and female rats was 1000 mg/kg/day (equivalent to 1.85×10^{11} CFU/kg bw/day), the highest dose evaluated. The authors concluded that these strains are safe for human consumption (Mukerji et al., 2016).

L. plantarum CECT 7315 and CECT 7316 were administered to 50 healthy, institutionalized elderly subjects (mean age 70 y, age range 66 – 84 y, 26 male, 24 female in a randomized, double-blind pilot study. Subjects received either 5.1×10^8 CFU/day, 5.1×10^9 CFU/day or placebo daily for 12 weeks. The high probiotic group had increased T-suppressor (CD8+CD25+) and NK (CD56+ CD16+) cells, while low probiotic dose increased activated T-helper lymphocytes (CD4+CD25+), B lymphocytes (CD19+), and antigen presenting cells (HLA-DR+). Plasma TGFβ1 was increased in both probiotic groups and remained elevated for 12 wk after discontinuation of treatment. There was a non-significant trend towards an incidence of infections in the high probiotic group. There was a significant trend for mortality to be greater in the placebo group as compared to the probiotic groups. The authors concluded that although this study was underpowered to assess clinical outcomes, a positive effect of the probiotic supplementation on infection rates and survival were suggested by the data. There were no adverse

events reported during this trial and the authors concluded these properties "might result in better clinical outcomes in elderly subjects" (Mane et al., 2011).

The effects of CECT 7315/7316 in elderly, institutionalized volunteers in a randomized, double-blind, placebo-controlled human trial was reported by Bosch et al. (Bosch et al., 2012). Sixty subjects, age 65 – 85 y, were first immunized against the influenza virus and then 3 – 4 months later received either: group A (receiving 5 x10^9 CFU/day of *L. plantarum* CECT 7315/7316 in 20 g powdered skim milk), group B (receiving 5x 10^8 CFU/day of *L. plantarum* CECT 7315/7316 in 20 g powdered skim milk) and group C or placebo (20 g powered skim milk) daily for 3 months. Both probiotic treatment groups had increased the levels of influenza-specific IgA and IgG antibodies and there was a trend towards an increase in influenza-specific IgM antibodies. Additional safety parameters were not reported. The authors concluded the supplementation with *L. plantarum* was an efficient and safe method to improve the protective immune response in high risk groups.

L. plantarum has been isolated from the intestinal mucosa of adolescents and was shown to seemingly downregulate inflammatory cytokines IL-6 and IL-12 and to upregulate anti-inflammatory cytokine IL-10 in *in vitro* assays. This study demonstrated potential of *L. plantarum* as a probiotic candidate with potential of exhibiting positive immunomodulation on host health (Citar et al., 2015).

A single-blind, placebo-controlled, parallel-group clinical trial monitoring the symptoms of Japanese Cedar Pollinosis (JCP), compared those who ingested fruit juice fermented with *L. plantarum* YIT0132 to those who ingested unfermented citrus juice daily for 8 wk. The subjects (N=42, mean age 38.2 ± 10.2 y) all had symptoms of MCP on entry. The alleviation of allergenic symptoms in those who ingested the *L. plantarum* fermented juice is believed to be caused by the ability of *L. plantarum* to induce anti-inflammatory IL-10 cytokines. There were no drop outs or side effects reported and no differences in frequency of drug use or hospital visits between the groups. The authors conclude that continuous consumption "may help in the relief of allergy symptoms" (Harima-Mizusawa et al., 2014).

A randomized, double-blind, placebo-controlled clinical trial to monitor the effects of *L. plantarum* CJLP133 on atopic dermatitis in otherwise healthy but presenting with atopic dermatitis children (N=118, age 12 mo -13 y) was completed (Han et al., 2012). A dose of 2.5x10^{10} CFU/day was administered for 12 weeks. A significant improvement in symptoms was reported, as was a decrease in INF-γ, IL-4 and eosinophils in the *L. plantarum* group compared to the placebo. This clinical study also demonstrated the safety of *L. plantarum* in children ages 12 months to 13 years at a dose of 2.5x10^{10} CFU.

A clinical trial was conducted to determine if administration of *L. plantarum* strain 299v was beneficial in relief of abdominal pain in 81 adult patients (mean age 48 y) with Irritable Bowel Syndrome (IBS) over an 8-week period. While there was no association of alleviation of abdominal pain with the probiotic, the safety of *L. plantarum* at a dose of 5 x 10^9CFU/day was well tolerated. One adverse event of a rash was reported (Stevenson et al., 2014).

Soongisepp et al (Songisepp et al., 2012) evaluated the symptomatic efficacy of *L. plantarum* strain Tensia (DSM 21380) contained in semi-hard Edam-type cheese in an animal model and after

consumption of the probiotic cheese in three, double-blind randomized placebo-controlled human intervention studies with different age groups (60 subjects in total). In the animal model, NIH mice (10/group) received approximately 4 x 10^9 *L. plantarum* Tensia in cheese or control cheese for 30 d. There were no differences in physical appearance, behavior, mortality, food or water consumption, or on histologic evaluation of organs. There were no differences reported in counts of lactobacillus in cultures of heart blood, liver or spleen. In the clinical trials, the subjects consumed a daily dose of 50 or100 g/d or 1.5 x 10^9 CFU/serving *L. plantarum* Tensia cheese or control cheese for 3 wk. No harmful effects were reported on body mass index, inflammatory markers, or serum lipidograms. No negative effects on gastrointestinal welfare were reported, but the consumption of 100g/d for 3 weeks caused hard stools from the second week of the trial. The authors concluded that consumption of the cheese with or without the probiotic "produced no harmful effects" (Songisepp et al., 2012).

In a double blind, placebo-controlled, parallel-designed study, subjects with irritable bowel disease were randomized to receive either one capsule of 1 x 10^{10} CFU *L. plantarum* 299v (DSM 9843) or placebo daily for 4 wk. After 4 wk, pain severity, pain frequency, and bloating were lower in the treatment group than with placebo. No significant side effects were reported and the only adverse effect reported was transient vertigo. No changes in blood parameters were reported throughout the study. The treatment provided "effective symptom relief" (Ducrotte et al., 2012).

In a double-blind, randomized, placebo-controlled study to evaluate the effectiveness of two different probiotic blends, either mixed *L. plantarum* LP01 (LMG P-21021) and *Bifidobacterium breve* BR03 (DSM 16604) or *Bifidobacterium animalis* subspecies *lactis* BS01 (LMG P-21384) were administered to otherwise healthy volunteers presenting with evacuation disorders and intestinal discomfort. The *L. plantarum* group (n=110) received mixed *L. plantarum* LP01 and *B. breve* BR03 (2.5 x 10^9 CFU/d of each strain) daily for 30 d. This group reported a significant improvement in the number of weekly bowel movements and in the main troubles associated with evacuations, particularly consistency of feces and ease of expulsion. Decreased discomfort such as abdominal bloating and anal itching, burning, or pain were reported and the treatment provides "a useful tool for the management of such conditions" (Del Piano et al., 2010).

In a randomized, double-blind placebo-controlled crossover trial, *L. plantarum* MF1298 at 1 x 10^{10} CFU/d or placebo was administered to 16 patients with irritable bowel syndrome (IBS) daily for two 3-week periods with a 4-week washout period between arms. Compared to placebo, *L. plantarum* treatment resulted in less relief of symptoms and lower IBS score. This trial shows for the first time an unfavorable effect on symptoms in subjects with IBS after intake of a potential probiotic. One serious (cervicobrachialgia) and three minor adverse events were reported. The authors conclude that not all strains with in vitro demonstrated probiotic properties actually confer a health benefit and may actually be associated with unfavorable effects (Ligaarden et al., 2010).

In an open-label trial that included 39 patients with active ulcerative colitis (UC), subjects received Profermin® (a mixture of oats, water and a small amount of barley malt fermented with *L. plantarum* 299v) twice daily for 24 wk (Krag et al., 2012). The *L. plantarum* dose started at 1.25 x 10^{10} CFU/d and gradually increased over the course of the study to 4.5 x 10^{10} CFU/d as tolerated. The colitis activity

index was reduced and remission was achieved in 10 of 32 treated subjects. No major adverse events were observed and there were no drop-outs due to adverse events. The treatment was safe and well tolerated. In a separate randomized, controlled trial, the clinical efficacy of Profermin® was compared to a high-calorie, high-protein, fiber-free control (Krag et al., 2013). Seventy-four patients with mild to moderate UC received the treatments for 8 wk. Compared to the control group, the Profermin® group had a greater decrease in colitis activity, a greater incidence of achieving remission and a lower likelihood of dropping out. The authors concluded that the treatment was safe, well tolerated, palatable and effective.

Mangell et al. (2012) evaluated the effect of *L. plantarum* 299V on intestinal load of potentially pathogenic bacteria, bacterial translocation, and cell proliferation in elective colon surgery. In a randomized, double-blind, placebo-controlled clinical trial 75 patients (age 64 – 80 y) were administered either placebo or 1×10^9 CFU *L. plantarum* 10 days prior and 5 days post elective colon surgery. *L. plantarum* was established in the intestines, but no inhibitory effect on enteric bacteria, bacterial translocation, or postoperative complications was reported and no adverse effects were recorded.

Oudhuis et al. (2011) compared selective decontamination of the digestive tract (SDD) using polymyxin E, gentamicin, amphotericin B, enteral solution (same antibiotics), intravenous cefotaxime (first 4 days) to treatment with 5×10^9 CFU *L. plantarum* 299/299v plus fiber (LAB) on infection prevention and mortality in 250 intensive care patients. There were no statistical differences between the two groups in infection rate, mortality, gram-positive cocci and *Pseudomonas aerogenosa* in surveillance cultures. The LAB group had more *Enterobacteriaceae*. There was no difference in antibiotic resistance. The trial was terminated prematurely over a non-inferiority of LAB and after a study reported increased mortality in critically ill pancreatitis patients receiving probiotics (Besselink et al., 2008).

In a randomized, placebo-controlled trial, patients undergoing treatment for infections received either *L. plantarum* 299v (1×10^{10} CFU/d) or a placebo drink until a week after termination of antibiotic treatment (Lonnermark et al., 2010). The risk of developing loose or watery stools, and the development of nausea was lower in the probiotic-treated group. There were no differences in the incidence of diarrhea or in the number of toxin producing *C. difficile* in the feces. The authors concluded "*L. plantarum* could have a preventive effect on milder gastrointestinal symptoms".

Klarin et al. (2008) evaluated the effect of *L. plantarum* 299v (8×10^8 CFU/day contained in oatmeal gruel) on the risk of developing *Clostridium difficile*-associated disease (CDAD) in a randomized, placebo-controlled trial in 22 intensive care unit (ICU) patients (mean age 65 y). Colonization with *C. difficile* was reduced in the probiotic treatment group but there were no differences in bowel function or gut permeability. The treatment was well tolerated and there were no treatment-related adverse events.

In a separate randomized, placebo-controlled trial, 17 critically ill patients were administered *L. plantarum* 299v (1×10^9 CFU/day contained in oatmeal gruel) or placebo throughout their stay in the ICU (Klarin et al., 2005). The treatment duration ranged from 4 – 37 days. Biopsies of the rectal mucosa revealed four patients in the control group were colonized with *L. plantarum* at admission but thereafter

all their biopsies were negative. None of the treated patients was colonized at admission but three patients had *L. plantarum* adhered on the mucosa from the second or third biopsy and in the following samples. This demonstrates that *L. plantarum* has the ability to survive passage through the upper gastrointestinal tract and adhere to the rectal mucosa in critically ill, antibiotic-treated patients. There were no adverse events reported and the treatment was well tolerated.

McNaught et al. (2002) examined the effect of *L. plantarum* 299v (ProViva) on bacterial translocation in 129 surgical patients predisposed to postoperative septic morbidity. Subjects received either *L. plantarum* (2.5×10^{10} CFU/d) or placebo for at least 1 week prior to elective major abdominal surgery and during the postoperative period (median duration 5 days). There was no difference between the two groups in bacterial translocation to mesenteric lymph nodes, gastric colonization, systemic inflammatory response, or septic morbidity. The most common postoperative problems were unpalatability, nausea, and paralytic ileus. The authors conclude that preoperative administration *L. plantarum* 299v "has no effect on the human gut mucosal barrier".

McNaught et al. (2005) examined the effect of the probiotic *L. plantarum* 299v on gut barrier function and the systemic inflammatory response in randomized, placebo-controlled, clinical trial on 103 critically ill patients. Subjects received either placebo or 1×10^{10} CFU/day *L. plantarum* combined with oatmeal (ProViva) for a median duration of 9 days. There were no changes in the intestinal microflora, intestinal permeability, endotoxin exposure, septic morbidity or mortality. Late changes attenuation of the systemic inflammatory response was associated with lower serum IL-6 levels in the treatment group compared to controls.

Table 3 Studies on the Safety and Clinical Benefit of Various Strains of L. plantarum

Study Design	Subjects/Species	Strain/Dose	Duration	Results	Reference
Randomized, double-blind, placebo-controlled trial	50 hypercholesterolemic but otherwise healthy subjects (age range 18 – 65 y	AB-LIFE® (CECT 7527, 7528, and 7529 in a 1:1:1 ratio 1.2×10^9 CFU/day	12 wk plus 4 wk post treatment followup	No adverse events were reported and there were no treatment-related changes in body weight, BMI, glucose, creatinine, or liver enzymes (alanine aminotransferase, aspartate aminotransferase, and γ-glutamyl transferase)	(Fuentes et al., 2013)
Randomized, double-blind, placebo-controlled trial, pilot study	50 healthy subjects (mean age 70 y, 26 M, 24 F)	*L. plantarum* CECT 7315 and 7316 0, 5.1×10^8 CFU/d, or 5.1×10^9 CFU/d	12 wk	Underpowered to assess clinical outcomes, authors conclude a positive effect of the probiotic supplementation on infection rates and survival were suggested by data	(Mane et al., 2011)
Randomized, double-blind, placebo-controlled trial Subjects first immunized against influenza	60 healthy subjects (65 – 85 y)	*L. plantarum* CECT 7315 and 7316 0, 5.1×10^8 CFU/d, or 5.1×10^9 CFU/d	12 wk	Treatment increased levels of influenza-specific IgA and IgG antibodies. The authors concluded the supplementation was an efficient and safe method to improve the protective immune response in high-risk groups.	(Bosch et al., 2012)
Single-blind, placebo-controlled, parallel-group trial	42 subjects diagnosed with Japanese Cedar Pollinosis (mean age 38 y)	Sterilized, *L. plantarum* YIT0132 fermented fruit juice	8 wk	The *L. plantarum*-fermented juice alleviated allergenic symptoms, no side effects reported, no differences in frequency of drug use or hospital visits	(Harima-Mizusawa et al., 2014)
Randomized, double-blind, placebo-controlled trial	118 children with atopic dermatitis (1 – 13 y)	L. plantarum CJLP133 2.5×10^{10} CFU/d	12 wk	The treated group had improved symptoms, decreased inflammatory cytokines. The authors concluded this treatment was safe and efficacious	(Han et al., 2012)
Randomized, double-blind, placebo-controlled trial	81 adult subjects with Irritable Bowel Syndrome (mean age 48 y)	*L. plantarum* 299V 5×10^9 CFU/d	8 wk	There was no association of treatment with alleviation of pain. The treatment was well tolerated with only one adverse event (rash) reported	(Stevenson et al., 2014)
Randomized, double-blind,	60 adult, healthy subjects	*L. plantarum* DSM 21380	3 wk	No harmful effects on BMI, inflammatory markers, or serum lipidograms. No negative effects on	(Songisepp et al., 2012)

placebo-controlled trial (3)		1 or 2 servings of cheese containing 1.5×10^9 CFU/serving		gastrointestinal welfare, hard stools were reported	
Randomized, double-blind, placebo-controlled trial	Adult subjects with Irritable Bowel Syndrome	*L. plantarum* 299V 1×10^{10} CFU/d	4 wk	Treated group had decreased pain severity, pain frequency, and bloating. No significant side effects were reported. No changes in blood parameters. Some subject reported transient vertigo.	(Ducrotte et al., 2012)
Randomized, double-blind, placebo-controlled trial	110 adult subjects with evacuation disorders and intestinal discomfort	*L. plantarum* LP01 and Bifidobacterium breve BR03 2.5×10^9 CFU of each strain	30 d	The *L. plantarum*-treated group reported significant improvement in the number of weekly bowel movements and issues associated with evacuations, bloating, and pain.	(Del Piano et al., 2010)
Randomized, double-blind, placebo-controlled trial crossover study	16 adult subjects with Irritable Bowel Syndrome	*L. plantarum* MF1298 1×10^{10} CFU/d	3 wk	The *L. plantarum*-treated group had less relief of symptoms and lower IBS score. One serious (cervicobrachialgia) and 3 minor adverse events were reported.	(Ligaarden et al., 2010)
Open-label trial	39 Patients with active ulcerative colitis	Profermin (oats, water and barley malt fermented with *L. plantarum* 299V $1.25 \times 10^{10} - 4.5 \times 10^{10}$ CFU/d	24 wk	The colitis activity index was reduced and remission was achieved in 10 or 32 treated subjects. No major adverse events were reported. The treatment was safe and well tolerated.	(Krag et al., 2012)
Randomized, controlled trial treatment comparison	74 patients with mild to moderate ulcerative colitis	Profermin (oats, water and barley malt fermented with *L. plantarum* 299V $1.25 \times 10^{10} - 4.5 \times 10^{10}$ CFU/d compared to Fresubin	8 wk	The Profermin-treated group had a greater decrease in colitis activity, a greater incidence of achieving remission, and a lower likelihood of dropping out. The authors concluded the treatment was safe, well tolerated and effective	(Krag et al., 2013)
Randomized, double-blind,	75 patients undergoing elective colon surgery Age (64 – 80 y)	*L. plantarum* 299V 1×10^9 CFU/d	10 d prior to surgery and 5 d post surgery	*L. plantarum* was established in the intestines, no inhibitory effect on enteric bacteria, no bacterial	(Mangell et al., 2012)

placebo-controlled trial				translocation, or postoperative complications, no adverse effects reported	
	254 intensive care patients	*L. plantarum* 299/299v 5×10^9 CFU or Selective decontamination with antibiotics	4 d	No statistical differences between selective decontamination treatment and *L. plantarum* treatment in infection rate, mortality, pathogenic bacteria but the trial was terminated prematurely over non-inferiority of *L. plantarum* and concerns about clinical reports of complications in pancreatitis patients.	(Oudhuis et al., 2011)
Randomized, double-blind, placebo-controlled trial	Patients undergoing antibiotic treatment for infections	*l. plantarum* 299v 1×10^{10} CFU/d	Until termination of antibiotic treatment	The *L. plantarum*-treated group had a lower risk of watery stools and the development of nausea. No differences in incidence of diarrhea or toxin producing *C. difficile*.	(Lonnermark et al., 2010)
Randomized, double-blind, placebo-controlled trial	44 intensive care unit patients (mean age 65 y)	*L. plantarum* 299v 8×10^8 CFU/d in an oatmeal gruel 6-100 gm bolus daily, the 2-50 gm bolus until discharge from ICU	Until discharge from ICU (mean 5.5 d)	*C. difficile* colonization was reduced but there were no differences in bowel function or gut permeability. The treatment was well tolerated and there were no treatment-related adverse events	(Klarin et al., 2008)
Randomized, double-blind, placebo-controlled trial	17 critically ill patients	*L. plantarum* 299v 1×10^9 CFU/d in an oatmeal gruel until discharge from ICU	Until discharge from ICU ranged from 4 – 37 d	*L. plantarum* survived passage through the upper gastrointestinal tract, adhered to the rectal mucosa, and there were no adverse events observed. The treatment was well tolerated.	(Klarin et al., 2005)
Randomized, double-blind, placebo-controlled trial	103 critically ill patients	*L. plantarum* 299v 1×10^9 CFU/d combined with oatmeal	9 d	No changes in intestinal microflora, intestinal permeability, endotoxin exposure, septic morbidity or mortality.	(McNaught et al., 2005)
Prospective randomized trial	129 surgical patients predisposed to postoperative septic morbidity	*L. plantarum* 299v 1×10^9 CFU/d combined with oatmeal	1 wk prior to elective abdominal surgery and during the postoperative	No difference between the two groups in bacterial translocation to mesenteric lymph nodes, gastric colonization, systemic inflammatory response, or septic morbidity. The most common	(McNaught et al., 2002)

			period (median duration 5 d)	postoperative problems were unpalatability, nausea, and paralytic ileus.	

Lactic acid bacteria and Lactobacilli

Lactic acid bacteria (LAB) and lactobacilli have a long history of safe use in foods (Bernardeau et al., 2008; Salminen et al., 1998). Lactobacilli are intrinsically resistant to some antibiotics. Because this antibiotic resistance is not transferable and LAB are sensitive to many antibiotics in common clinical use they present no particular safety concern. Lactobacillemia induced by food, particularly fermented dairy products, is extremely rare and only occurs in predisposed patients (Bernardeau et al., 2008). Lactobacilli are found wherever substances rich in carbohydrates are available (Bernardeau et al., 2008).

The Food and Agriculture Organization and World Health Organization expert consultation reported that "lactobacilli have a long history of use as probiotics without established risk to humans, and this remains the best proof of their safety....no pathogenic or virulence properties have been found for lactobacilli" (FAO/WHO, 2002). The safety of probiotic bacteria was recently reviewed (Sanders et al., 2010; Sanders et al., 2007). Taken as a whole, any probiotic strain, including members of the genera *Lactococcus, Lactobacillus*, and *Bifidobacterium* is considered safe, as long as the strain is devoid of any transferable antibiotic resistance genes.

Infections in humans by these genera are extremely rare. There have been 180 cases of lactobacillemia and 69 cases of infective endocarditis attributed to lactobacilli reported during the past 30 years. In most cases of endocarditis, dental surgery occurred in the days or weeks preceding the disease. These infections resulted from native sources of these genera and not from consumption of probiotics products. Only two cases of *Lactobacillus* infection were linked with probiotic consumption. Increasing consumption of probiotic lactobacilli has not led to an increase in such opportunistic infections in consumers. Thus, the risk of infection by these genera is in the "negligible" range, considering that exposure to them is universal and persistent, not only through probiotic products but also as common colonizers of the human body (the digestive tract and oral and vaginal cavities). This lack of pathogenicity extends across all age groups (including preterm infants and pregnant women). However, caution is recommended for immunocompromised and critically ill patients such as those suffering from acute pancreatitis, bone marrow transplant or recently operated patients and/or those given parenteral nutrition (Sanders et al., 2010; Sanders et al., 2007).

In a comprehensive, evidence-based review and meta-analysis of the literature regarding the safety of probiotics, 622 peer-reviewed research articles were evaluated (Hempel et al., 2011). Of these, 235 studies reported only nonspecific safety statements such as "well tolerated" but did not indicate specific adverse events or what kinds of events were monitored. The remaining 387 studies predominantly investigated *Lactobacillus*, alone or in combination with other genera, most often *Bifidobacterium*. These studies were pooled to evaluate the relative risks (RR) of use of probiotics, active or lyophilized, single ingredients or in combination, in all delivery vehicles when used to improve health. The following key relative risk results germane to the current report are listed along with 95% confidence intervals (CI), p value, and the number of randomized clinical trials (RCT) included in the pool.

- There was **no evidence of increased risk from interventions with probiotics** compared to control groups

a. based on the number of participants with adverse events
(RR 0.98, CI: 0.93 – 1.04, p=0.537, 121 RCT)
b. based on the number of adverse-event incidences
(RR 1.00, CI: 0.93 – 1.07, p=0.999, 208 RCT)
c. **"None of the case series, controlled clinical trials, or parallel and crossover RCT reported an infection caused by the administered probiotics"** though few reported that they monitored for this

- There **was no indication that participants using probiotic organisms experienced more:**
  a. **Gastrointestinal events**
  (RR 1.03, CI: 0.89 – 1.18, p=0.693, 126 RCT)
  b. **Infections**
  (RR 1.00, CI: 0.87 – 1.16, p=0.967, 65 RCT)
  c. **Or other adverse events**
  (RR 1.01, CI: 0.91 – 1.12, p=0.923, 131 RCT)

- Stratified by probiotic genus, there was **no indication that participants using *Lactobacillus* experienced an increased risk.**
(RR 0.98, CI: 0.87 – 1.11, p=0.785)

- **Stratified by age, there was no indication of increased risk of adverse events for children, adults, or elderly.**
Although case studies have reported serious adverse events in health compromised, not generally healthy participants, **subgroup analyses of RCT did not show an increased risk of adverse events** in either:
  a. **Medium health-compromised participants**
  (RR 1.03, CI: 0.94 – 1.13, p=0.491)
  b. **Critically ill patients**
  (RR 0.79, CI: 0.51 – 1.22, p=0.286)

- There was **no indication that consumption of probiotics lead to hospital admission or lengthened hospitalization**. Most of these studies were based on *Lactobacillus* interventions.
(RR 1.06, CI: 0.97 – 1.16, p=0.201, 66 RCT)

- There was **no indication that consumption of probiotics increased the risk of adverse events in individuals concomitantly taking:**
  a. **Antibiotics**
  (RR 1.07, CI: 0.94 – 1.23, p=0.271)
  b. **Corticosteroids**
  (RR 1.04, CI: 0.88 – 1.22, p=0.650)

The strength of these conclusions is somewhat mitigated by the inconsistency between the results of RCT and case studies, the lack of systematic reporting of adverse events, and poor documentation in the studies evaluated. The authors conclude the RCT-based evidence does not indicate an increased risk of adverse events. "The available evidence in RCTs does not indicate an increased risk; however, rare adverse events are difficult to assess and despite the substantial number of publications, the current literature is not well equipped to answer questions on the safety of probiotic interventions with confidence."

4. Animal Studies

The toxicity of Lp-115 was evaluated in an up and down acute toxicity study in rats (Mukerji, 2015). The study was conducted in compliance with U.S. FDA Good Laboratory Practice Standards under U.S. EPA Health Effects Test Guidelines OPPTS 870-1100 (2002), U.S. FDA Redbook 2000: IV.C.2: Acute Oral Toxicity Tests (1993) and OECD Guideline for the Testing of Chemicals Section 4 (Part 425) (2008). A single dose of Lp-115 (5000 mg/kg bw, 4.2×10^{12} CFU.kg body wt) was administered by gavage to fasted, 10 week-old, female Crl: CD(SD) rats, with dosage corresponding to a range of 8.62_x10^{11} to 9.05_x10^{11} cfu/animal. The rats were monitored for 14 days after dosing and then necropsied to detect grossly observable evidence of organ or tissue damage. There were no incidents of mortality, clinical abnormalities, or body weight loss and there were no gross lesions evident in organs or tissues. The acute oral LD_{50} is greater than 5000 mg Lp-115/kg bw, the limit dose and the only dose tested. The Lp-115 was not considered to be acutely toxic. In the absence of test substance related mortality, an LD50 was not calculated. The tested dose is equivalent to 3.05×10^{14} CFU Lp-115 for a 73 kg adult.

The following studies were not designed with safety endpoints in mind. In the absence of reported mortality or safety issues these studies provide some support that *L. plantarum* administered orally at levels of up to 10^{11} CFU/ animal is not overtly toxic.

Daniel et al (Daniel et al., 2006) studied the role of three lactobacilli on intestinal inflammation and bacterial translocation in mice. The probiotic strains tested included *L. plantarum* Lp-115, *L. salivarius* Ls-33, and *L. acidophilus* NCFM as well as a non-probiotic *L. paracasei* strain as control. The survival in the gastrointestinal tract of the lactobacilli was examined *in vitro* by testing resistance to pepsin, pancreatin, bile, and adherence to Caco-2 cells. Lp-115 demonstrated a higher resistance to pepsin and bile than the other probiotic strains and a slightly lower resistance to pancreatin. Healthy BALB/c mice were administered 1×10^{10} CFU of each bacterial strain (n=5/strain) *via* gavage for 4 consecutive days and fecal samples were collected and cultured to evaluate survival in the gastrointestinal tract. Lp-115 was recovered until day 13, the last day evaluated. Healthy BALB/c mice (n=5/strain) were administered 1×10^{10} CFU of each bacterial strain (n=5/strain) *via* gavage for 5 consecutive days without any potential adverse effect on mouse activity, weight, or colon inflammation. Colonic tissue myeloperoxidase (MPO) levels remained low and did not differ from the buffer control group. Cultures of mesenteric lymph nodes (MLN), spleen liver, and kidneys were negative indicating an absence of bacterial translocation or dissemination. Mice treated with trinitrobenzene sulfonic acid (TNBS) served as a model of induced

acute colitis. Treatment with Lp-115 did not have a significant preventive effect in the TNBS model and did not have any negative effects on activity score, weight loss, or colonic inflammation. Bacteria was recovered at low levels in the liver and kidney but not MLN or spleen in one mouse (of the five treated with Lp-115). The authors conclude that Lp-115 has an "acceptable safety profile" for use as a probiotic.

Foligne et al. (Foligne et al., 2007) evaluated 13 strains of lactobacilli (including Lp-115) for their ability to stimulate the release of interleukins from peripheral blood mononuclear cells (PBMC) and their prophylactic capacity in the TNBS-induced colitis model in BALB/c and C57BL6 mice (n=10/group). The PBMC were exposed to 1×10^9 CFU/mL bacteria and the mice were administered 1×10^8 CFU via gavage daily for 6 days. Lp-115 treatment induced a low IL-10/IL-12 release in PBMC and an intermediate level of protection in the TNBS colitis model. Lp-115 improved the colitis score based on weight loss, rectal bleeding, stool consistency, diarrhea, lethargy, or histological evaluation of epithelial lesions, numbers of goblet and crypt cells, inflammatory infiltrates, and colon wall thickness. The authors concluded strains displaying an in vitro capacity offer the best protection in the in vivo colitis model and provide a useful pre-selection of probiotics prior to testing in animals and humans.

Dichi et al. (2015) used a high fructose diet as a model for metabolic syndrome in male Wistar rats. Experimental groups (n=10/group) were administered either a standard diet (G1), high fructose diet (G2), high fructose diet plus fermented milk containing 10^9 CFU Lp-115/mL (G3), high fructose diet plus yacon root powder (G4), or high fructose diet plus Lp-115 and yacon root (G5) via gavage daily for 8 weeks. It was reported that in the Lp-115 group (G3) there were no effects on measures of oxidative stress, while the synbiotic G5 group had decreased lipid peroxidation, increased protein sulfhydryl levels, and increased nitric oxide levels, indicating lower oxidative stress. The Lp-115-treated group was protective against the effects of the high fructose diet. Compared to the standard diet control, the G2 group had decreased weight and HDL cholesterol levels, increased serum glucose and insulin levels, and insulin resistance. Compared to G2, the G3 group had increased weight, decreased insulin, and decreased insulin resistance. The synbiotic group (G5) had only decreased insulin resistance compared to G2. No safety parameters were reported. The authors concluded that *L. plantarum* Lp-115 reduces insulin resistance in Wistar rats fed a high fructose diet, which is consistent with results obtained with other strains of *L. plantarum*.

Paroschi et al. (2015) compared the impact of various nutritional interventions on the production of colonic tissue MPO in a TNBS-induced model of colitis in male Wistar rats. The rats were divided into 5 groups (n=10/group); control (G1), sulfasalazine (G2), sulfasalazine plus $10^8 - 10^9$ CFU/g Lp-115 (G3), sulfasalazine plus n-3 fatty acids (G4), or sulfasalazine plus $10^8 - 10^9$ CFU/g Lp-115 plus n-3 fatty acids (G5) via gavage, daily for 14 days prior to and 7 days after challenge with TNBS. At the conclusion of treatment, the animals were sacrificed, colons were excised, and MPO activity was evaluated. The Lp-115 treated group (G3) had lower levels of MPO compared to control. No safety parameters were reported. The authors conclude that treatment with *L. plantarum* in combination with sulfasalazine provide beneficial effects in this animal model of colitis.

5. Human Studies

There are no published clinical studies designed to specifically evaluate the safety of Lp-115, however Lp-115 has been used in clinical studies. One study evaluated safety parameters on a mixture of probiotic ingredients that included Lp-115 (Zhang et al., 2013).

In a randomized, double-blind, placebo-controlled pilot study on 83 healthy volunteers aged 18-62 years [mean/median, males and/or females], subjects consumed 1 x 10^{10} CFU (twice per d) of either Lp-115 or one of six different probiotic strains or placebo for 3 weeks prior to challenge (Paineau et al., 2008). The subjects were challenged with oral cholera vaccine at day 7 through day 14. Saliva and serum samples were analyzed for specific IgA, IgG, and IgM at day 21 and day 28. Compared to the placebo group, the Lp-115-treated group (5M, 4F) had increased serum IgG response at day 21 ($p<0.09$) and increased IgM response at day 28. No safety data was reported. The authors conclude that Lp-115 may "act as an adjuvant to the humoral response following oral vaccination".

In a parallel arm comparison trial, 24 postmenopausal subjects (mean age 67 y) ingested either 80 mL/d non-fermented milk (n=12) or 80 mL/d milk fermented with 1.25 10^{7} CFU/g Lp-115 (n=12) for 90 d (Barreto et al., 2013). The group receiving fermented milk had lower glucose and homocysteine levels than the non-fermented milk group. Compared to baseline, both groups had decreased total cholesterol, γ-glutamyl transpeptidase, and LDL-cholesterol levels (trend only for the fermented group) and both groups had reduction in IL-6. There were no differences in weight, BMI, waist circumference, systolic or diastolic blood pressure, triglycerides, HOMA-IR, C reactive protein, or TNF. Randomization was not described; no safety data was reported. The authors concluded that *L. plantarum* showed favorable results in "relation to cardiovascular risk factors in postmenopausal women with metabolic syndrome".

In a case controlled study of 67 adult liver transplant patients, enteral treatment of fiber plus a mixture of 6 different probiotic bacteria that included 5 x 10^{9} CFU Lp-115 (19M, 15F) was compared to retrospectively selected patients (17M, 16F) that had received fiber treatment without probiotic bacteria (Zhang et al., 2013). The mean age of the probiotic group was 57 ± 10 years. The treatment duration was 16 ± 3 days. The incidence of bacterial infections and the duration of antibiotic therapy were lower in the probiotic treated group. Wound infection was the most frequent site of infection and enterococci the most frequently isolated bacteria. None of the probiotic bacteria were members of the *Enterococcus* genus. The authors reported that the treatment was well tolerated. There were no treatment-related adverse events. Adverse events included diarrhea (n=2 probiotic, n=1 control) and abdominal cramps (n=3 probiotic, n=6 control). All side effects disappeared after temporary reduction of the enteral nutrition. The authors conclude that treatment with the Lp-115-containing mixture of probiotics could "lower the incidence of bacterial infections ... shorten the duration of antibiotic therapy... and does not cause resistant strains or serious side effects".

In a time course study on 61 healthy adult subjects (22M, 39F) aged 19 – 65 years, subjects consumed daily doses of fermented milk containing 2 x 10^{11} CFU Lp-115 for 0, 15, 30, 45, 60 and 90 days (Costa et al., 2014). Analysis of Lp-115 in fecal samples using quantitative PCR was performed at 0, 15 and 45 days post-consumption. Compared to the baseline group, Lp-115 was elevated at the conclusion of treatment. Lp-115 levels returned to control levels after discontinuation of treatment. No safety data was reported. The authors conclude that daily intake of probiotics is necessary to maintain levels in the gastrointestinal tract.

B. Inconsistent Information

DuPont Nutrition & Health (formerly Danisco) and the convened expert panel has reviewed the available data and information and are not aware of any data and information that are, or may appear to be, inconsistent with this conclusion of GRAS status.

C. Expert Panel Evaluation

DuPont Nutrition & Health has concluded that *Lactobacillus plantarum* Lp-115 is GRAS for use in conventional foods and dietary supplements on the basis of scientific procedures. This GRAS conclusion is based on the totality of evidence generally available in the public domain pertaining to the safety of Lp-115, as discussed herein, and on consensus among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of infant formula ingredients and food ingredients. The Expert Panel consisted of the following qualified scientific experts: Dr. Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine) and Dr. Michael W. Pariza (University of Wisconsin). Dr. Michael Falk (LSRO Solutions LLC) served a Technical Advisor to the Expert Panel. The Expert Panel convened by DuPont Nutrition & Health independently and critically evaluated all data and information presented herein, and concluded that Lp-115 is GRAS for use in conventional foods and dietary supplements based on scientific procedures. As part of their evaluation, the Expert Panel relied on a decision tree for determining the safety of microbial cultures to be consumed by humans and animals (Pariza et al., 2015). A summary of data and information reviewed by the Expert Panel, and evaluation of such data as it pertains to the proposed GRAS uses of *L. plantarum* Lp-115 is presented in Appendix E.

D. Common Knowledge Elements of GRAS Conclusion

The first common knowledge element for a conclusion of GRAS status is that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing published, peer-reviewed scientific journals for the safety assessment. The animal studies and human clinical studies on which this GRAS conclusion has been based have been published in the scientific literature.

The second common knowledge element required for a GRAS determination is that consensus among qualified scientists about the safety of the substance with its intended use must be demonstrated. The panel agrees there is adequate data in the scientific literature to conclude that *L. plantarum* Lp-115 is a common component of food sources for man and animals, and is nutritionally efficacious without any evidence of adverse effects.

Finally, *L. plantarum* Lp-115 is consumed as a dietary supplement in the United States and internationally, *L. plantarum* is QPS in Europe, and *L. plantarum* is in common use as in food preparation in fermented foods.

E. Final Conclusion

Based on scientific procedures, the above data, and the information presented herein, DuPont Nutrition & Health has concluded the intended uses of *L. plantarum* Lp-115 is GRAS when consumed in conventional foods at 1×10^{10} CFU/serving and does not present a significant or unreasonable risk of illness or injury under conditions of use in dietary supplements at levels up to 5×10^{10} CFU/serving. General recognition of DuPont Nutrition & Health's GRAS determination is supported by the unanimous consensus rendered by an independent Expert Panel, qualified by experience and scientific training to evaluate the proposed uses of *L. plantarum* Lp-115.

This declaration is made in accordance with FDA's standard for food ingredient safety, i.e., reasonable certainty of no harm under the intended conditions of use.

The Panel offers the following conclusion:

> The Expert Panel has concluded that *Lactobacillus plantarum* Lp-115 --which is produced in accordance with Good Manufacturing Practices requirements and which meet the purity specifications as set forth in Section III of this safety evaluation, is considered to be Generally Recognized As Safe when consumed in foods at levels sufficient to ensure 1×10^{10} organisms per serving and at levels up to 5×10^{10} organisms per serving as a dietary supplement for the proposed uses as defined in 21 CFR 170.3(o)(20).

Part 7 – List of supporting data and information in GRAS notice

All data and information used in accordance with the above document are generally available.

Aguirre, M., Collins, M.D., 1993. Lactic acid bacteria and human clinical infection. J. Appl. Bacteriol 75, 95-107.

Ahrne, S., Nobaek, S., Jeppsson, B., Adlerberth, I., Wold, A.E., Molin, G., 1998. The normal Lactobacillus flora of healthy human rectal and oral mucosa. J. Appl. Microbiol 85, 88-94.

Aires, J., Doucet-Populaire, F., Butel, M.J., 2007. Tetracycline resistance mediated by tet(W), tet(M), and tet(O) genes of Bifidobacterium isolates from humans. Appl. Environ. Microbiol 73, 2751-2754.

Axelsson, L., 2004. Lactic acid bacteria: Classification and physiology, in: S. Salminen, W.A. von (Eds.), Lactic acid bacteria: Microbiology and functional aspects. Marcel Dekker, New York, pp. 1-73.

Barreto, F.M., Colado Simao, A.N., Morimoto, H.K., Batisti Lozovoy, M.A., Dichi, I., da Silva, H., 2013. Beneficial effects of Lactobacillus plantarum on glycemia and homocysteine levels in postmenopausal women with metabolic syndrome. Nutrition.

Baruzzi, F., Morea, M., Matarante, A., Cocconcelli, P.S., 2000. Changes in the Lactobacillus community during Ricotta forte cheese natural fermentation. J. Appl. Microbiol 89, 807-814.

Bernardeau, M., Vernoux, J.P., Henri-Dubernet, S., Gueguen, M., 2008. Safety assessment of dairy microorganisms: the Lactobacillus genus. Int J Food Microbiol 126, 278-285.

Besselink, M.G., van Santvoort, H.C., Buskens, E., Boermeester, M.A., van, G.H., Timmerman, H.M., Nieuwenhuijs, V.B., Bollen, T.L., van, R.B., Witteman, B.J., Rosman, C., Ploeg, R.J., Brink, M.A., Schaapherder, A.F., Dejong, C.H., Wahab, P.J., van Laarhoven, C.J., van der Harst, E., van Eijck, C.H., Cuesta, M.A., Akkermans, L.M., Gooszen, H.G., 2008. Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. Lancet 371, 651-659.

BioGaia AB, 2008. Generally Recognized as Safe (GRAS) Determination of Lactobacillus reuteri Strain DSM 17938. GRN 254, FDA.

Bosch, M., Fuentes, M.C., Audivert, S., Bonachera, M.A., Peiro, S., Cune, J., 2014. Lactobacillus plantarum CECT 7527, 7528 and 7529: probiotic candidates to reduce cholesterol levels. J. Sci. Food Agric 94, 803-809.

Bosch, M., Mendez, M., Perez, M., Farran, A., Fuentes, M.C., Cune, J., 2012. Lactobacillus plantarum

CECT7315 and CECT7316 stimulate immunoglobulin production after influenza vaccination in elderly. Nutr. Hosp 27, 504-509.

Bourdichon, F., Boyaval, P., Casaregola, S., Dupont, J., Farrokh, C., Frisvad, J.C., Hammes, W.P., Huys, G., Jany, J.L., Laulund, S., 2012. The 2012 Inventory of Microbial Species with technological beneficial role in fermented food products. Bulletin of the International Dairy Federation 455, 22-61.

CFSAN, 2008a. Agency Response Letter GRAS Notice No. GRN 000231, FDA.

CFSAN, 2008b. Agency Response Letter GRAS Notice No. GRN 000254, FDA.

CFSAN, 2009a. Agency Response Letter GRAS Notice No. GRN 000281, FDA.

CFSAN, 2009b. Agency Response Letter GRAS Notice No. GRN 000288, FDA.

CFSAN, 2014a. 75-day premarket notification of new dietary ingredients Lactobacillus plantarum and Fructooligosaccharide (Gastroplan and GastroplanCF), FDA.

CFSAN, 2014b. Agency response letter to GRAS Notifice No. GRN 000324, FDA.

Citar, M., Hacin, B., Tompa, G., Stempelj, M., Rogelj, I., Dolinsek, J., Narat, M., Matijasic, B.B., 2015. Human intestinal mucosa-associated Lactobacillus and Bifidobacterium strains with probiotic properties modulate IL-10, IL-6 and IL-12 gene expression in THP-1 cells. Benef. Microbes 6, 325-336.

Collado, M.C., Meriluoto, J., Salminen, S., 2007. Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. Letters in applied microbiology 45, 454-460.

Collado, M.C., Meriluoto, J., Salminen, S., 2008. Adhesion and aggregation properties of probiotic and pathogen strains. European Food Research and Technology 226, 1065-1073.

Colomer-Lluch, M., Jofre, J., Muniesa, M., 2011. Antibiotic resistance genes in the bacteriophage DNA fraction of environmental samples. PLoS. One 6, e17549.

Costa, G.N., Marcelino-Guimaraes, F.C., Vilas-Boas, G.T., Matsuo, T., Miglioranza, L.H., 2014. Potential fate of ingested Lactobacillus plantarum and its occurrence in human feces. Appl. Environ. Microbiol 80, 1013-1019.

Daniel, C., Poiret, S., Goudercourt, D., Dennin, V., Leyer, G., Pot, B., 2006. Selecting lactic acid bacteria for their safety and functionality by use of a mouse colitis model. Appl. Environ. Microbiol 72, 5799-5805.

Danisco, 2014. Technical Memorandum: Lactobacillus plantarum Lp-115.

De Vries, M.C., Vaughan, E.E., Kleerebezem, M., de Vos, W.M., 2006. Lactobacillus plantarum—survival, functional and potential probiotic properties in the human intestinal tract. International Dairy Journal

16, 1018-1028.

Del Piano, M., Carmagnola, S., Anderloni, A., Andorno, S., Ballare, M., Balzarini, M., Montino, F., Orsello, M., Pagliarulo, M., Sartori, M., Tari, R., Sforza, F., Capurso, L., 2010. The use of probiotics in healthy volunteers with evacuation disorders and hard stools: a double-blind, randomized, placebo-controlled study. J Clin. Gastroenterol 44 Suppl 1, S30-S34.

Dichi, I., Mari, N.L., Bregano, J.W., Simao, A.N.C., Lozovoy, M.A.B., Bonifacio, K.L., Alfierei, D.F., Miglioranza, L.H.S., 2015. Lactobacillus plantarum reduces insulin resistance and yacon or symbiotic reduces oxidative stress in rats with metabolic syndrome. J Nutrition Energy Balance 1, 1-10.

Douillard, F.P., De Vos, W.M., 2014. Functional genomics of lactic acid bacteria: from food to health. Microb. Cell Fact 13 Suppl 1, S8.

Ducrotte, P., Sawant, P., Jayanthi, V., 2012. Clinical trial: Lactobacillus plantarum 299v (DSM 9843) improves symptoms of irritable bowel syndrome. World J Gastroenterol 18, 4012-4018.

EFSA, 2007. Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. Opinion of the Scientific Committee (Question No EFSA-Q-2005-293). The EFSA J 587, 1-16.

EFSA, 2008. Scientific Opinion The maintenance of the list of QPS microorganisms intentionally added to food or feed. Scientific Opinion of the Panel on Biological Hazards (Question No EFSA-Q-2008-006), EFSA J

Adopted on 10 December 2008, pp. 1-48.

EFSA, 2009. Scientific Opinion on the maintenance of the list of QPS microorganisms intentionally added to food or feed (2009 update), EFSA J, 12 ed, p. 1431.

EFSA, 2010. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2010 update). EFSA on Biological Hazards (BIOHAZ). EFSA J 8, 1944.

EFSA, 2011. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food or feed (2011 update). Scientific Opinion of the Panel on Biological Hazards (Question No EFSA-Q-2011-00070), EFSA J

Adopted on 7 December 2011, p. 2497.

EFSA, 2012a. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP); Guidance on the

assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA J 10, 2740.

EFSA, 2012b. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. Scientific Opinion of the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). EFSA J 10, 2740.

EFSA, 2013. Scientific Opinion on the maintenance of the list of QPS biological agents intentionally added to food and feed (2013 update) (Question No EFSA-Q-2013-00019). EFSA Journal 11, 3449.

Egervarn, M., 2009. Antibiotic resistance in Lactobacillus reuteri and Lactobacillus plantarum, Faculty of Natural Resources and Agricultural Sciences Department of Microbiology. University of Uppsala.

Ewaschuk, J.B., Naylor, J.M., Zello, G.A., 2005. D-lactate in human and ruminant metabolism. J. Nutr 135, 1619-1625.

Foligne, B., Nutten, S., Grangette, C., Dennin, V., Goudercourt, D., Poiret, S., Dewulf, J., Brassart, D., Mercenier, A., Pot, B., 2007. Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. World J. Gastroenterol 13, 236-243.

Fonterra Co-operative Group, 2009a. Generally Recognized as Safe (GRAS) Determination for the Use of Lactobacillus rhamnosus Strain "HN001 (DR20™) in Conventional Foods GRN 000288, FDA.

Fonterra Co-operative Group, 2009b. Generally Recognized as Safe (GRAS) Determination for the Use of Lactobacillus rhamnosus Strain "HN001 (DR20™) in Infant Formula. GRN 000281, FDA.

Fuentes, M.C., Lajo, T., Carrion, J.M., Cune, J., 2013. Cholesterol-lowering efficacy of Lactobacillus plantarum CECT 7527, 7528 and 7529 in hypercholesterolaemic adults. Br. J. Nutr 109, 1866-1872.

Garcia Fontan, M.C., Lorenzo, J.M., Parada, A., Franco, I., Carballo, J., 2007. Microbiological characteristics of "androlla", a Spanish traditional pork sausage. Food Microbiol 24, 52-58.

Gasser, F., 1994. Safety of lactic acid bacteria and their occurrence in human clinical infections. Bulletin de l'Institut Pasteur 92, 45-67.

Gueimonde, M., Ouwehand, A.C., Salminen, S., 2004. Safety of probiotics. Food & Nutrition Research 48, 42-48.

Gunji, H., Scarth, S., Carlson, G.L., Warhurst, G., Little, R.A., Hopkins, S.J., 2006. Variability of bacterial translocation in the absence of intestinal mucosal damage following injury and the influence of interleukin-6. Pathophysiology 13, 39-49.

Han, Y., Kim, B., Ban, J., Lee, J., Kim, B.J., Choi, B.S., Hwang, S., Ahn, K., Kim, J., 2012. A randomized trial of Lactobacillus plantarum CJLP133 for the treatment of atopic dermatitis. Pediatric Allergy and Immunology 23, 667-673.

Harima-Mizusawa, N., Tohru, I.I.N.O., Onodera-Masuoka, N., Moriko, K.N., Kiyoshima-Shibata, J.,

Atsushi, G.O.M.I., Shibahara-Sone, H., Mitsuyoshi, K.A.N.O., SHIDA, K., Sakai, M., 2014. Beneficial Effects of Citrus Juice Fermented with Lactobacillus plantarum YIT 0132 on Japanese Cedar Pollinosis. Bioscience of microbiota, food and health 33, 147.

Hempel, S., Newbery, S., Ruelaz, A., Wang, Z., Miles, J.N.V., Suttorp, M.J., Johnsen, B., Shanman, R., Slusser, W., Fu, N., Smith, A., Roth, E., Polak, J., Motala, A., Perry, T., Shekelle, P.G., 2011. Safety of Probiotics to Reduce Risk and Prevent or Treat Disease. Evidence Report/Technology Assessment No. 200. (Prepared by the Southern California Evidence-based Practice Center under Contract No. 290-2007-10062-I.), Rockville, MD.

Holzapfel, W.H., Haberer, P., Geisen, R., Bjorkroth, J., Schillinger, U., 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. Am. J. Clin. Nutr 73, 365S-373S.

House Wellness Foods Corp, 2014. GRAS exemption claim for a heat-killed Lactobacillus plantarum (HK-LP) ingredient, FDA.

Hudson, M., Pocknee, R., Mowat, N.A., 1990. D-lactic acidosis in short bowel syndrome--an examination of possible mechanisms. Q. J. Med 74, 157-163.

Klarin, B., Johansson, M.L., Molin, G., Larsson, A., Jeppsson, B., 2005. Adhesion of the probiotic bacterium Lactobacillus plantarum 299v onto the gut mucosa in critically ill patients: a randomised open trial. Crit Care 9, R285-R293.

Klarin, B., Wullt, M., Palmquist, I., Molin, G., Larsson, A., Jeppsson, B., 2008. Lactobacillus plantarum 299v reduces colonisation of Clostridium difficile in critically ill patients treated with antibiotics. Acta Anaesthesiol. Scand 52, 1096-1102.

Krag, A., Israelsen, H., von Ryberg, B., Andersen, K.K., Bendtsen, F., 2012. Safety and efficacy of Profermin(R) to induce remission in ulcerative colitis. World J Gastroenterol 18, 1773-1780.

Krag, A., Munkholm, P., Israelsen, H., von Ryberg, B., Andersen, K.K., Bendtsen, F., 2013. Profermin is efficacious in patients with active ulcerative colitis--a randomized controlled trial. Inflamm. Bowel. Dis 19, 2584-2592.

Ligaarden, S.C., Axelsson, L., Naterstad, K., Lydersen, S., Farup, P.G., 2010. A candidate probiotic with unfavourable effects in subjects with irritable bowel syndrome: a randomised controlled trial. BMC. Gastroenterol 10, 16.

Liong, M.T., 2008. Safety of probiotics: translocation and infection. Nutr Rev 66, 192-202.

Lonnermark, E., Friman, V., Lappas, G., Sandberg, T., Berggren, A., Adlerberth, I., 2010. Intake of Lactobacillus plantarum reduces certain gastrointestinal symptoms during treatment with antibiotics. J Clin. Gastroenterol 44, 106-112.

Lu, F.C., 1988. Acceptable daily intake: inception, evolution, and application. Regul Toxicol Pharmacol 8, 45-60.

Mane, J., Pedrosa, E., Loren, V., Gassull, M.A., Espadaler, J., Cune, J., Audivert, S., Bonachera, M.A., Cabre, E., 2011. A mixture of Lactobacillus plantarum CECT 7315 and CECT 7316 enhances systemic immunity in elderly subjects. A dose-response, double-blind, placebo-controlled, randomized pilot trial. Nutr. Hosp 26, 228-235.

Mangell, P., Thorlacius, H., Syk, I., Ahrne, S., Molin, G., Olsson, C., Jeppsson, B., 2012. Lactobacillus plantarum 299v does not reduce enteric bacteria or bacterial translocation in patients undergoing colon resection. Dig. Dis. Sci 57, 1915-1924.

Marshall, B.M., Ochieng, D.J., Levy, S.B., 2009. Commensals: underappreciated reservoir of antibiotic resistance. Microbe 4, 231-238.

Mathur, S., Singh, R., 2005. Antibiotic resistance in food lactic acid bacteria--a review. Int. J. Food Microbiol 105, 281-295.

McNaught, C.E., Woodcock, N.P., Anderson, A.D., MacFie, J., 2005. A prospective randomised trial of probiotics in critically ill patients. Clin. Nutr 24, 211-219.

McNaught, C.E., Woodcock, N.P., MacFie, J., Mitchell, C.J., 2002. A prospective randomised study of the probiotic Lactobacillus plantarum 299V on indices of gut barrier function in elective surgical patients. Gut 51, 827-831.

Mead Johnson, 2007. GRAS Exemption Claim and Exemption Notification for the addition of Lactobacillus Casei, Subspecies Rhamnosus GG, (LGG) to the exempt infant formula, Nutramigen LIPIL. GRN 000231, FDA.

Molin, G., 2001a. Probiotics in foods not containing milk or milk constituents, with special reference to Lactobacillus plantarum 299v. Am. J. Clin. Nutr 73, 380S-385S.

Molin, G., 2001b. Probiotics in foods not containing milk or milk constituents, with special reference to Lactobacillus plantarum 299v. The American journal of clinical nutrition 73, 380s-385s.

Molin, G., 2008. The role of Lactobacillus plantarum in foods and in human health, in: E.R. Farnworth (Ed.), Handbook of fermented functional foods. CRC Press: Boca Raton, FL, pp. 353-393.

Molin, G., Jeppsson, B., Johansson, M.L., Ahrne, S., Nobaek, S., Stahl, M., Bengmark, S., 1993. Numerical taxonomy of Lactobacillus spp. associated with healthy and diseased mucosa of the human intestines. J. Appl. Bacteriol 74, 314-323.

Morgensen, G., Salminen, S., O'Brien, J., Holzapfel, A., Shortt, C., Fonden, R., Miller, G.D., Donohue, D., Playne, M., Crittenden, R., Salvadori, B., Zink, R., 2002. Inventory of microorganisms with a documented

history of use in food, Health Benefits and Safety Evaluation of Certain Food Components., 377 ed.

Mukerji, P., 2015 (unpublished). Lactobacillus plantarum Lp-115: Acute oral toxicity study in rats - up-and-down procedure. E.I. du Pont de Nemours and Compayn, DuPont Haskell Global Centers, pp. 1-31. DuPont Report 21058-834.

Mukerji, P., Roper, J.M., Stahl, B., Smith, A.B., Burns, F., Caverly Rae, J., Yeung, N., Lyra, A., Svard, L., Saarinen, M.T., Alhoniemi, E., Ibarra, A., Oewehand, A.C., 2016. Safety evaluation of AB-LIFE (Lactobacillus plantarum CECT 7527, 7528, and 7520): Antibiotic resistance and 90-day repeated-dose study in rats (publication pending). Food Chem Tox.

Nawaz, M., Wang, J., Zhou, A., Ma, C., Wu, X., Moore, J.E., Millar, B.C., Xu, J., 2011. Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Curr. Microbiol 62, 1081-1089.

Nei, T., Inai, S., Mikami, I., Sato, A., Okamoto, J., Yokoshima, K., Nakamizo, M., Haraguchi, S., Sonobe, K., Saito, R., 2013. Descending necrotizing mediastinitis associated with Lactobacillus plantarum. BMC. Infect. Dis 13, 398.

Olympia, M., Ono, H., Shinmyo, A., Takano, M., 1992. Lactic acid bacteria in fermented fishery product, burong bangus and its amyloytic enzyme. Journal of fermentation and bioengineering 73, 193-197.

Orillo, C.A., Pederson, C.S., 1968. Lactic acid bacterial fermentation of burong dalag. Appl. Microbiol 16, 1669-1671.

Orillo, C.A., Sison, E.C., Luis, M., Pederson, C.S., 1969. Fermentation of Philippine vegetable blends. Applied microbiology 17, 10-13.

Oudhuis, G.J., Bergmans, D.C., Dormans, T., Zwaveling, J.H., Kessels, A., Prins, M.H., Stobberingh, E.E., Verbon, A., 2011. Probiotics versus antibiotic decontamination of the digestive tract: infection and mortality. Intensive Care Med 37, 110-117.

Ouoba, L.I., Lei, V., Jensen, L.B., 2008. Resistance of potential probiotic lactic acid bacteria and bifidobacteria of African and European origin to antimicrobials: determination and transferability of the resistance genes to other bacteria. Int. J. Food Microbiol 121, 217-224.

Paineau, D., Carcano, D., Leyer, G., Darquy, S., Alyanakian, M.A., Simoneau, G., Bergmann, J.F., Brassart, D., Bornet, F., Ouwehand, A.C., 2008. Effects of seven potential probiotic strains on specific immune responses in healthy adults: a double-blind, randomized, controlled trial. FEMS Immunol. Med. Microbiol 53, 107-113.

Paroschi, T.P., Breganó, J.W., Simão, A.N.C., Dichi, I., Miglioranza, L.H.S., 2015. Effects of Sulfasalazine, Lactobacillus Plantarum (Lp-115) and Fish Oil in Experimental Colitis. SM J Food Nutri Disord 1, 1005.

Perdigon, G., Alvarez, S., Rachid, M., Aguero, G., Gobbato, N., 1995. Immune system stimulation by probiotics. J Dairy Sci 78, 1597-1606.

Plengvidhya, V., Breidt, F., Jr., Lu, Z., Fleming, H.P., 2007. DNA fingerprinting of lactic acid bacteria in sauerkraut fermentations. Appl. Environ. Microbiol 73, 7697-7702.

PURAC, 2011. GRAS notification claim for food ferment solutions: GRN000378. FDA.

Rantsiou, K., Drosinos, E.H., Gialitaki, M., Metaxopoulos, I., Comi, G., Cocolin, L., 2006. Use of molecular tools to characterize Lactobacillus spp. isolated from Greek traditional fermented sausages. Int. J. Food Microbiol 112, 215-222.

Rebecchi, A., Crivori, S., Sarra, P.G., Cocconcelli, P.S., 1998. Physiological and molecular techniques for the study of bacterial community development in sausage fermentation. J. Appl. Microbiol 84, 1043-1049.

Renwick, A.G., 1990. Acceptable daily intake and the regulation of intense sweeteners. Food Addit Contam 7, 463-475.

Rodriguez, A.V., Baigori, M.D., Alvarez, S., Castro, G.R., Oliver, G., 2001. Phosphatidylinositol-specific phospholipase C activity in Lactobacillus rhamnosus with capacity to translocate. FEMS Microbiol Lett 204, 33-38.

Rulis, A.M., Levitt, J.A., 2009. FDA'S food ingredient approval process: Safety assurance based on scientific assessment. Regul. Toxicol. Pharmacol 53, 20-31.

Salminen, S., von, W.A., Morelli, L., Marteau, P., Brassart, D., De Vos, W.M., Fonden, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S.E., Mattila-Sandholm, T., 1998. Demonstration of safety of probiotics -- a review. Int J Food Microbiol 44, 93-106.

Sanders, M.E., Akkermans, L.M., Haller, D., Hammerman, C., Heimbach, J., Hormannsperger, G., Huys, G., Levy, D.D., Lutgendorff, F., Mack, D., Phothirath, P., Solano-Aguilar, G., Vaughan, E., 2010. Safety assessment of probiotics for human use. Gut Microbes 1, 164-185.

Sanders, M.E., Gibson, G., Gill, H.S., Guarner, F., 2007. Probiotics: Their Potential to Impact Human Health, pp. 1-20.

Schmidt, E.G., Claesson, M.H., Jensen, S.S., Ravn, P., Kristensen, N.N., 2010. Antigen-presenting cells exposed to Lactobacillus acidophilus NCFM, Bifidobacterium bifidum BI-98, and BI-504 reduce regulatory T cell activity. Inflamm. Bowel. Dis 16, 390-400.

Song, Y., Kato, N., Liu, C., Matsumiya, Y., Kato, H., Watanabe, K., 2000. Rapid identification of 11 human intestinal Lactobacillus species by multiplex PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA. FEMS Microbiol. Lett 187,

167-173.

Songisepp, E., Hutt, P., Ratsep, M., Shkut, E., Koljalg, S., Truusalu, K., Stsepetova, J., Smidt, I., Kolk, H., Zagura, M., Mikelsaar, M., 2012. Safety of a probiotic cheese containing Lactobacillus plantarum Tensia according to a variety of health indices in different age groups. J Dairy Sci 95, 5495-5509.

Stevenson, C., Blaauw, R., Fredericks, E., Visser, J., Roux, S., 2014. Randomized clinical trial: effect of Lactobacillus plantarum 299 v on symptoms of irritable bowel syndrome. Nutrition 30, 1151-1157.

Tamminen, M., Joutsjoki, T., Sjoblom, M., Joutsen, M., Palva, A., Ryhanen, E.L., Joutsjoki, V., 2004. Screening of lactic acid bacteria from fermented vegetables by carbohydrate profiling and PCRGÇôELISA. Letters in applied microbiology 39, 439-444.

Tanasupawat, S., Okada, S., Komagata, K., 1998. Lactic acid bacteria found in fermented fish in Thailand. J. Gen. Appl. Microbiol 44, 193-200.

Todorov, S.D., Franco, B.D.G.M., Wiid, I.J., 2014. In vitro study of beneficial properties and safety of lactic acid bacteria isolated from Portuguese fermented meat products. Beneficial microbes 5, 351-366.

Turroni, S., Vitali, B., Bendazzoli, C., Candela, M., Gotti, R., Federici, F., Pirovano, F., Brigidi, P., 2007. Oxalate consumption by lactobacilli: evaluation of oxalyl-CoA decarboxylase and formyl-CoA transferase activity in Lactobacillus acidophilus. J. Appl. Microbiol 103, 1600-1609.

Vankerckhoven, V., Huys, G., Vancanneyt, M., Vael, C., Klare, I., Romond, M.-B., Entenza, J.M., Moreillon, P., Wind, R.D., Knol, J., 2008. Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. Trends in Food Science & Technology 19, 102-114.

Wang, H.H., Manuzon, M., Lehman, M., Wan, K., Luo, H., Wittum, T.E., Yousef, A., Bakaletz, L.O., 2006. Food commensal microbes as a potentially important avenue in transmitting antibiotic resistance genes. FEMS Microbiol. Lett 254, 226-231.

Zhang, Y., Chen, J., Wu, J., Chalson, H., Merigan, L., Mitchell, A., 2013. Probiotic use in preventing postoperative infection in liver transplant patients. Hepatobiliary surgery and nutrition 2, 142.

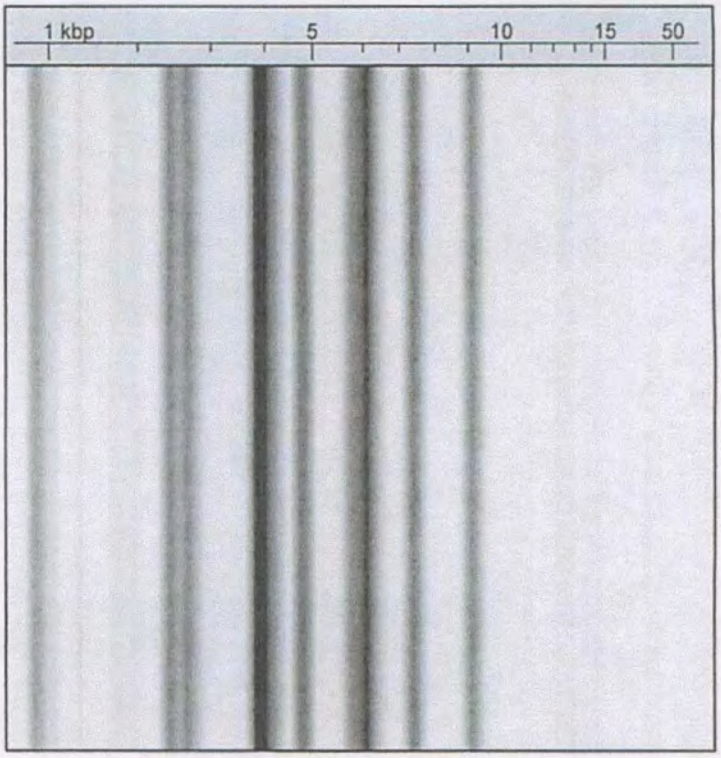
Zhang, Y., Zhang, L., Du, M., Yi, H., Guo, C., Tuo, Y., Han, X., Li, J., Zhang, L., Yang, L., 2011. Antimicrobial activity against Shigella sonnei and probiotic properties of wild lactobacilli from fermented food. Microbiol. Res 167, 27-31.

Appendix A

RiboPrinter® Microbial Characterization System
Sample 475-163-S-8 Report

DuPont Qualicon

Created	2/26/2014 10:27:06 AM
Batch Extraction Status	Passed
Process	KHA
Enzyme	EcoRI
Isolated From	STDUT
Label	LP115
Sample Comment	1102235215
Batch	475-163
Operator	JLW



	Type	Number	Similarity	Label	RiboPrint™ Pattern
1	Custom ID	Madison_EcoRI-6	0.98	Lactobacillus plantarum	
2	RiboGroup	ECORI 475-57-S-8	0.97		

Identification
PASS / FAIL
Signature: (b) (6)
Date: 3-4-14

Appendix B

Danisco USA, Inc.
3329 Agriculture Drive
Madison, WI 53716
800-255-6837 Tel
608-395-2603 Fax

FOOD GRADE STATEMENT

Date: 30 September 2015

Customer: LSRO Solutions, LLC

Product: *Lactobacillus plantarum* (Lp-115)

The above listed Danisco freeze dried microbial culture is produced from food grade ingredients, in compliance with the U.S. FDA's current Good Manufacturing Practices guidelines (21 CFR 110), in an FDA registered and inspected facility.

This information is given in respect of DuPont's policy of openness and transparency with its customers.

Sincerely,

(b) (6)

Don Scheffel
Quality & Food Safety Manager
DuPont - Nutrition & Health

Appendix C

DANISCO
First you add knowledge...

Certificate of Analysis

Date: 07 Oct 2014
Our ref. no.: 0
Your ref.

Material:	M85543A	LP-115 400B - 20 KG	
Batch No.:	1102331048	Best before date:	24 Jun 2016
		Production date:	25 Jun 2014

Test	Result	Specification	Unit	Reference
Viable Cell Count	6.88E+11	4.00E+11	/g	ISO 7889/IDF 117
Enterococcus	< 100	< 100	/g	CMMEF, 4TH EDITION
Non Lactics	< 5000	< 5000	/g	ISO 13559
Coliforms	< 10.0	< 10.0	/g	AOAC
E. coli, neg. by test (<0.3/g)	Negative	Negative		AOAC
Staph. aureus, neg. by test (<10/g)	Negative	Negative		AOAC
Salmonella, negative in 40 g	Negative	Negative		AOAC
Listeria, negative in 25 g	Negative	Negative		AOAC

Comments

Exceeds 400 billion CFU/gm of freeze dried Lb. plantarum.

The above product has been analyzed by Danisco and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

Best if used before the date listed above when stored at or below 4°C.

AOAC references above reflect the current edition of AOAC.

Culture identity is confirmed to Genus/species level (or sub-species level where applicable) based on DNA

DANISCO
First you add knowledge...

Certificate of Analysis

Date: 07 Oct 2014
Our ref. no.: 0
Your ref.

Material:	M85543A	LP-115 400B - 20 KG	
Batch No.:	1102331048	Best before date:	24 Jun 2016
		Production date:	25 Jun 2014

Fingerprinting Analysis generated by Automated Ribotyping.

This certificate is generated automatically

(b) (6)

Phil Ihrke

Quality Control Department

DANISCO
First you add knowledge...

Certificate of Analysis

Date: 07 Oct 2014
Our ref. no.: 0
Your ref.

Material:	M85543A	LP-115 400B - 20 KG	
Batch No.:	1102350591	Best before date:	20 Jul 2016
		Production date:	21 Jul 2014

Test	Result	Specification	Unit	Reference
Viable Cell Count	7.50E+11	4.00E+11	/g	ISO 7889/IDF 117
Enterococcus	< 100	< 100	/g	CMMEF, 4TH EDITION
Non Lactics	< 5000	< 5000	/g	ISO 13559
Coliforms	< 10.0	< 10.0	/g	AOAC
E. coli, neg. by test (<0.3/g)	Negative	Negative		AOAC
Staph. aureus, neg. by test (<10/g)	Negative	Negative		AOAC
Salmonella, negative in 40 g	Negative	Negative		AOAC
Listeria, negative in 25 g	Negative	Negative		AOAC

Comments

Exceeds 400 billion CFU/gm of freeze dried Lb. plantarum.

The above product has been analyzed by Danisco and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

Best if used before the date listed above when stored at or below 4°C.

AOAC references above reflect the current edition of AOAC.

Culture identity is confirmed to Genus/species level (or sub-species level where applicable) based on DNA

Danisco US - Madison Plant
Maintenance Dept.
3322 Agriculture Dr.
MADISON WI 53716

DANISCO
First you add knowledge...

Certificate of Analysis

Date: 07 Oct 2014
Our ref. no.: 0
Your ref.

Material:	M85543A	LP-115 400B - 20 KG	
Batch No.:	1102350591	Best before date:	20 Jul 2016
		Production date:	21 Jul 2014

Fingerprinting Analysis generated by Automated Ribotyping.

This certificate is generated automatically

(b) (6)

Phil Ihrke

Quality Control Department

DANISCO
First you add knowledge...

Certificate of Analysis

Date: 07 Oct 2014
Our ref. no.: 0
Your ref.

Material:	M85543A	LP-115 400B - 20 KG	
Batch No.:	1102378520	Best before date:	04 Sep 2016
		Production date:	05 Sep 2014

Test	Result	Specification	Unit	Reference
Viable Cell Count	6.65E+11	4.00E+11	/g	ISO 7889/IDF 117
Enterococcus	< 100	< 100	/g	CMMEF, 4TH EDITION
Non Lactics	< 5000	< 5000	/g	ISO 13559
Coliforms	< 10.0	< 10.0	/g	AOAC
E. coli, neg. by test (<0.3/g)	Negative	Negative		AOAC
Staph. aureus, neg. by test (<10/g)	Negative	Negative		AOAC
Salmonella, negative in 40 g	Negative	Negative		AOAC
Listeria, negative in 25 g	Negative	Negative		AOAC

Comments

Exceeds 400 billion CFU/gm of freeze dried Lb. plantarum.

The above product has been analyzed by Danisco and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

Best if used before the date listed above when stored at or below 4°C.

AOAC references above reflect the current edition of AOAC.

Culture identity is confirmed to Genus/species level (or sub-species level where applicable) based on DNA

Danisco US - Madison Plant
Maintenance Dept.
3322 Agriculture Dr.
MADISON WI 53716

DANISCO
First you add knowledge...

Certificate of Analysis

Date: 07 Oct 2014
Our ref. no.: 0
Your ref.

Material:	M85543A	LP-115 400B - 20 KG	
Batch No.:	1102378520	Best before date:	04 Sep 2016
		Production date:	05 Sep 2014

Fingerprinting Analysis generated by Automated Ribotyping.

This certificate is generated automatically

(b) (6)

Phil Ihrke

Quality Control Department

DANISCO
First you add knowledge...

Certificate of Analysis

Date: 13 Mar 2015
Our ref. no.: 0
Your ref.

Material:	M85538B	LP-115 400B - 1KG	
Batch No.:	1102458986	Best before date:	18 Jan 2017
		Production date:	19 Jan 2015

Test	Result	Specification	Unit	Reference
Viable Cell Count	6.80E+11	4.00E+11	/g	ISO 7889/IDF 117
Enterococcus	< 100	< 100	/g	CMMEF, 4TH EDITION
Non Lactics	< 5000	< 5000	/g	ISO 13559
Coliforms	< 10.0	< 10.0	/g	AOAC
E. coli, neg. by test (<0.3/g)	Negative	Negative		AOAC
Staph. aureus, neg. by test (<10/g)	Negative	Negative		AOAC
Salmonella, negative in 40 g	Negative	Negative		AOAC
Listeria, negative in 25 g	Negative	Negative		AOAC

Comments

Exceeds 400 billion CFU/gm of freeze dried Lb. plantarum.

The above product has been analyzed by Danisco and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

Best if used before the date listed above when stored at or below 4°C.

AOAC references above reflect the current edition of AOAC.

Culture identity is confirmed to Genus/species level (or sub-species level where applicable) based on DNA

DANISCO
First you add knowledge...

Certificate of Analysis

Date: 13 Mar 2015
Our ref. no.: 0
Your ref.

Material:	M85538B	LP-115 400B - 1KG	
Batch No.:	1102458986	Best before date:	18 Jan 2017
		Production date:	19 Jan 2015

Fingerprinting Analysis generated by Automated Ribotyping.

This certificate is generated automatically

(b) (6)

Phil Ihrke

Quality Control Department

CULTURES DIVISION
cultures@danisco.com
www.danisco.com

Appendix D

DANISCO
First you add knowledge...

Valid from: May 29, 2013

PRODUCT DESCRIPTION - PD 204425-6.0EN

Material no. M85538B

Lp-115 400B - 1 KG

Description

Freeze-dried probiotic powder. White to cream-color in appearance.

Directions for use

See Danisco Probiotic Usage & Handling Guide

Composition

Lactobacillus plantarum (Lp-115)

Microbiological specifications

Cell count	> 4.00E+11 / g
Non-Lactic Count	< 5000 / g
Enterococci	< 100 / g
Coliforms	< 10 / g
E. coli	neg. by test (< 0.3 / g)
Staphylococcus (coag. pos.)	neg. by test < 10 / g
Salmonella	neg. (40 g enrichment)
Listeria	neg. (25 g enrichment)

Storage

Shelf life is 24 months when stored in the original, sealed package at or below 4°C. Frozen storage will extend shelf life.

Packaging

High barrier foil laminate bags

Quantity

1 kg

Purity and legal status

Local regulations should always be consulted concerning the status of this product, as legislation regarding its intended use may vary from country to country.

Safety and handling

MSDS is available on request.

Kosher status

Circle K certification

Halal status

IFANCA certification

Allergens

Below table indicates the presence of the following allergens and products thereof:

Yes	No	Allergens	Description of components
	X	wheat	
	X	other cereals containing gluten	
	X	crustacean shellfish	
	X	eggs	
	X	fish	
	X	peanuts	
	X	soybeans	
	X	milk (including lactose)	
	X	nuts	
	X	celery	
	X	mustard	
	X	sesame seeds	
	X	sulphur dioxide and sulphites (> 10 mg/kg)	
	X	lupin	
	X	molluscs	

Local regulation has always to be consulted as allergen labelling requirements may vary from country to country.

CULTURES DIVISION
cultures@danisco.com
www.danisco.com

Valid from: May 29, 2013

DANISCO
First you add knowledge...

PRODUCT DESCRIPTION - PD 204425-6.0EN

Material no. M85538B

Lp-115 400B - 1 KG

Additional information

Country of Origin: USA

GMO status

Lp-115 400B - 1 KG does not consist of, nor contains, nor is produced from genetically modified organisms according to the definitions of Regulation (EC) 1829/2003 and Regulation (EC) 1830/2003 of the European Parliament and of the Council of 22 September 2003.

Appendix E

Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Status of the Proposed Conventional Food and Dietary Supplement Uses of *Lactobacillus plantarum* Lp-115

Introduction

Danisco USA convened a panel of independent scientists (the "Expert Panel"), qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, to conduct a critical and comprehensive evaluation of the available pertinent data and information on *Lactobacillus plantarum* Lp-115 and to conclude whether the proposed uses in food production would be Generally Recognized as Safe (GRAS) based on scientific procedures. The Expert Panel consisted of the below-signed qualified scientific experts: Michael W. Pariza, Ph.D. (University of Wisconsin) (Chair) and Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), with Michael C. Falk, Ph.D. (LSRO Solutions LLC) serving as technical advisor to the Expert Panel.

The Expert Panel, independently and collectively, critically evaluated a comprehensive package of scientific information and data compiled from the literature. The information was presented in a dossier provided by LSRO Solutions LLC ("Comprehensive GRAS Assessment of *Lactobacillus plantarum* Lp-115: Food Usage Conditions for General Recognition of Safety"; December 22, 2016). To the best of our knowledge, this determination is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of this ingredient in food. The Expert Panel evaluated other information deemed appropriate or necessary.

Summary and Basis for GRAS

The Expert Panel based its conclusions on the following information.

Lactobacillus plantarum Lp-115 is intended to be added to: ready-to-eat breakfast cereals; bars (e.g. breakfast, energy, nutrition); milk, milk drinks (e.g. flavored milks), milk products (e.g. butter), fermented milks (e.g. Kefir, sour cream, buttermilk), yogurt, cheese (incl. cheese food, cheese spreads) and ice cream; soy drinks and soy products; bottled water and teas; dry beverages including sports nutrition beverages; fruit juices, fruit nectars, fruit "ades", fruit drinks, jams and jellies; chewing gum; medical foods; nut and peanut spreads; margarines; snack foods (e.g. cookies, crackers, chips, granola); meal replacements; sauces, condiments; confections (e.g. bars, candy, coatings, drops, cookie filling); excluding infant formula. It is intended to be added to conventional foods at concentrations consistent with cGMP needed to provide at least 1×10^{10} CFU/serving throughout the shelf of the product. It will also be marketed as a dietary supplement in the form of sachets, tablets and as capsules at $1 \times 10^{9} - 5 \times 10^{10}$ CFU/serving. *Lactobacillus plantarum* Lp-115 is intended to serve as a probiotic microorganism. It will not proliferate in the foods and beverages to which it is added but instead will decline over the shelf-life of the food.

The *L. plantarum* Lp-115 was isolated from plants, is well characterized, and has been deposited in various strain depositories (e.g. Danisco Global Culture Collection and the American Type Culture Collection).

Analysis of *L. plantarum* Lp-115 confirmed the absence of transferable antibiotic resistance elements, the absence of virulence factor, infectivity elements, and toxins, the uniqueness of the strain, and the identity of the strains to the *L. plantarum* species.

The *L. plantarum* Lp-115 strain is susceptible to various common antibiotics, produces both D- and L-lactic acid, does not show unusual adherence capability, adverse metabolic activity or infectivity, has demonstrated survivability in the gastrointestinal tract, ability to bind to pathogenic bacteria and prevent the adherence of biogenic bacteria to intestinal mucus, and provides probiotic benefits to the host.

L. plantarum Lp-115 is produced using standard, well-documented fermentation techniques under current GMP manufacturing conditions using approved food grade materials. The strain is produced reproducibly and meets standard food grade specifications.

The safety of *L. plantarum* Lp-115 was evaluated in an acute toxicity study in five female Crl: CD(SD) rats. No adverse effects were reported during the 14 day observation period after an oral dose of 5000 mg/kg bw (4.2×10^{12} CFU/kg bw). There were no incidents of mortality, clinical abnormalities, or body weight loss and there were no gross lesions evident in organs or tissues. The acute oral LD_{50} is greater than 5000 mg/kg, the limit dose (OECD, Section 4 (Part 425): Acute Oral Toxicity: Up-and-Down Procedure, Guideline for the Testing of Chemicals (2008)).

L. plantarum Lp-115 was orally administered to healthy, female BALB/c mice , mice challenged with trinitrobenzene sulfonic acid (n=5/treatment group), and male, Wistar rats (n=10/group) in daily doses ranging from 1×10^8 CFU to 1×10^{10} CFU/animal for periods up to 8 weeks. Although these studies were not designed to evaluate the toxicity of Lp-115, the treatments were well-tolerated, no adverse events were reported, and the treatments reportedly ameliorated the symptoms of acute colitis, oxidative stress, and hyperglycemia.

In four studies, 168 healthy, male and female adult subjects and 67 adult male and female liver transplant patients consumed *Lactobacillus plantarum* Lp-115 at daily doses up to 2×10^{11} CFU, either as part of a mixture of probiotic strains or in milk fermented with Lp-115 for treatment periods up to 90 days. Although these studies were not designed to evaluate the safety of Lp-115, the authors reported that the treatments were well-tolerated, there were no treatment-related adverse events, and any side effects disappeared after temporary reduction of the dose. The safety and clinical benefit of various strains of *L. plantarum* has been reported in clinical trials at doses up to 5×10^{10} CFU for periods up to 12 weeks in healthy subjects without adverse effects.

The safety of *L. plantarum* Lp-115 was further evaluated using the decision tree procedure of Pariza *et al.* (2015). Based on the outcome of the decision tree for determining the safety of microbial cultures for consumption by humans and animals (Table 1), including strain characterization and genome sequencing, screening for undesirable attributes and metabolites, and experimental evidence of safety by appropriately designed safety evaluation studies, it was concluded that *L. plantarum* strain Lp-115 is not pathogenic and not toxigenic and is safe for use as probiotics in the foods and beverages identified above.

The history of safe use of *L. plantarum* in foods and as a dietary supplement has been demonstrated by the ubiquitous presence of *Lactobacillus plantarum* Lp-115 as a minor component in the bowel microflora, their ability to inhibit the growth of pathogenic microorganisms, the long history of safe use in fermented and non-fermented foods, the studies evaluating the efficacy and safety of various closely related strains of *L. plantarum* in human clinical trials, and the European Food Safety Authority (EFSA) classification of *L. plantarum* as an organism having a Qualified Presumption of Safety (QPS) and thus being "freed from the need for further safety assessment." Thus, *L. plantarum* strains are safe for use in foods. *L. plantarum* appears on the inventory of microorganisms with a documented history of use in food as compiled by the International Dairy Federation.

Common Knowledge Elements of GRAS Determinations

The first common knowledge element for a GRAS determination is that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing published, peer-reviewed scientific journals for the safety assessment. The animal studies and human clinical studies on which this GRAS determination has been based have been published in the peer-reviewed scientific literature.

The second common knowledge element required for a GRAS determination is consensus among qualified scientists that the safety of the proposed uses of the substance has been demonstrated. The Expert Panel agrees there is adequate data in the scientific literature to conclude that *L. plantarum* Lp-115 is a common component of food sources for man and animals and that the weight of the available evidence demonstrates that the proposed uses are safe without any evidence of adverse effects.

Finally, *L. plantarum* Lp-115 is available as a dietary supplement in the United States and internationally, *L. plantarum* is QPS in Europe, and *L. plantarum* is in common use in food preparation in the United States.

Conclusion

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

Based on our critical evaluation of the information on the safety of Lactobacillus plantarum Lp-115 summarized above, we unanimously conclude that DuPont's blend of *Lactobacillus plantarum* Lp-115, manufactured consistent with cGMP and meeting food grade specifications, is Generally Recognized As Safe (GRAS) based on scientific procedures for addition to various foods including, but not limited to, ready-to-eat breakfast cereals, bars, cheese, mild drinks and milk products, bottled water, teas, fruit juices, fruit nectars, fruit "ades", fruit drinks, chewing gum, and confections, at levels sufficient to ensure 1 X 10^{10} organisms per serving throughout the shelf life of the product. We also conclude that Lactobacillus plantarum Lp-115, manufactured consistent with cGMP and meeting food grade specifications, is safe and does not present a significant or unreasonable risk of illness or injury under the conditions of use in dietary supplements, at levels up to 5 x 10^{10} CFU per serving.

It is our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusion.

Michael W. Pariza, Ph.D., Chair
Emeritus Director Food Research Institute
Professor Emeritus Department of Food Sciences
University of Wisconsin

(b) (6)

Signature

11 Jan 2017

Date

Joseph F. Borzelleca, Ph.D.
Professor Emeritus
Pharmacology and Toxicology
School of Medicine
Virginia Commonwealth University

(b) (6)

Signature

01 January 2017

Date

Michael C. Falk, Ph.D.
LSRO Solutions LLC
Advisor to the Expert Panel

(b) (6)

Signature

12 Jany 2017

Date

Table 1: Decision Tree Analysis for Determining the Safety of Microbial Cultures for Consumption

1. Has the **strain**[i] been characterized for the purpose of assigning an unambiguous genus and species name using currently accepted methodology? [ii] (If YES, go to 2. If NO, the strain must be characterized and unambiguously identified before proceeding).	YES
2. Has the **strain** genome been sequenced? (If YES, go to 3. If NO, the genome must be sequenced before proceeding to 3.)[iii]	YES
3. Is the **strain** genome free of genetic elements[iv] encoding virulence factors[v] and/or toxins [v] associated with pathogenicity? [vi] (If YES, go to 4. If NO, go to 15.)	YES
4. Is the **strain** genome free of functional and transferable antibiotic resistance gene DNA? [vii] (If YES, go to 5. If NO, go to 15.)	YES
5. Does the **strain** produce antimicrobial substances? [viii] (If NO, go to 6. If YES, go to 15.)	NO
6. Has the **strain** been genetically modified using rDNA techniques? (If YES, go to 7. If NO, go to 8.)	NO
7. Do the expressed product(s) that are encoded by the introduced DNA have a history of safe use in food[ix]? (If YES, go to 8. If NO, the expressed product(s) must be shown to be safe before proceeding to 8.)[x]	YES
8. 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[xi] and characterizing[xii] component (not simply an 'incidental isolate')? (If YES, go to 9. If NO, go to 13.)[xiii]	NO
9. Has the **species**, to which the strain belongs, undergone a comprehensive peer-reviewed safety evaluation and been affirmed to be safe for food use by an authoritative group of qualified scientific experts?[xiv] (If YES, go to 10. If NO, go to 13.)	
10. Do scientific findings published since completion of the comprehensive peer-reviewed safety evaluation cited in question 9a continue to support the conclusion that the **species,** to which the strain belongs, is safe for use in food? (If YES, go to 11. If NO, go to 13.)	
11. Will the intended use of the **strain** expand exposure to the **species** beyond the group(s) that typically consume the species in "traditional" food(s) in which it is typically found (for example, will a strain that was isolated from a fermented food typically consumed by healthy adults be used in food intended for an 'at risk' group)? (If NO, go to 12. If YES, go to 13.)	

12. Will the intended use of the **strain** expand intake of the **species** (for example, increasing the number of foods beyond the traditional foods in which the species typically found, or using the strain as a probiotic rather than as a fermented food starter culture, which may significantly increase the single dose and/or chronic exposure)? (If NO, go to 14. If YES, go to 13.)	
13. Does the **strain** induce undesirable physiological effects in appropriately designed safety evaluation studies? [xv] If yes, go to 15. If no, go to 14.)	NO
14. The strain is deemed to be safe for use in the manufacture of food, probiotics, and dietary supplements for human consumption.	YES
15. The strain is NOT APPROPRIATE for human or animal consumption.[xvi]	

[i] A strain is a "population of organisms that descends from a single organism or pure culture isolate." P. 392, Prescott, Harley and Klein, 1996, Microbiology, Wiley. We recognize that the genotype and/or phenotype of a strain may change slightly when carried in culture, but such changes are irrelevant to safety considerations because there is no known mechanism or precedent for isolated strains in culture to begin spontaneously expressing pathogenic traits, unless that potential was already present in the genome at the time of isolation.

[ii] Whole Genome Sequencing provides distinct advantages for identification and characterization of microorganisms. In-depth analysis, including functional and comparative genomic studies, is afforded by sequencing the whole genome. This technology can provide a wealth of information that can be used for identification and characterization, including evidence of genetic evolution for adaptation of a species to a nutrient-rich environment, such as dairy products or the gastrointestinal tract (Pfeiler, EA, Klaenhammer, TR. 2007. The genomics of lactic acid bacteria. TRENDS in Microbiol, 15(12); 546-553). Less comprehensive molecular analysis, such as RAPD, FISH, and MLST, may also provide adequate information for identification, but the characterization ability is often times limited within a bacterial species (Gosiewski, T, Chmielarczyk, A, Strus M, Brzychczy-Wloch M, Heczko PB. 2012. The application of genetics methods to differentiation of three *Lactobacillus* species of human origin. Ann Microbiol 62:1437-1445).

[iii] The genomic sequence provides the tools to mine the genome for a number of functions, uncovering information spanning from safety to host-cell interactions (Callanan, M. 2005. Mining the Probiotic Genome: Advanced Strategies, Enhanced Benefits, Perceived Obstacles. Current Pharmaceutical Design, 11: 25-36). From a regulatory perspective, the ability to show percentage/regions of similarity and differentiation between a new strain of interest in comparison with a *type strain,* or an accepted strain with history of safe use, is beneficial (U.S. FDA; July 2011. Draft Guidance for Industry: Dietary Supplements: New Dietary Ingredient Notifications and Related Issues). The genome sequence is analogous to a chemical specification for a food ingredient, that is, it defines precisely what is being evaluated and permits a genetic assessment of pathogenic and toxigenic potential. Isolates from a type-strain culture collection, or a strain collection held by a commercial culture manufacturer, may be considered to have the same safety characteristics as, and to be *substantially equivalent* to, the original source pure culture, so in

these cases the requirement for genome sequencing may be satisfied by sequencing the genome of the original source pure culture.

[iv] The term "genetic elements" refers to gene sequences encoded in the chromosome or extra-chomosmal DNA.

[v] Known genetic element sequences for virulence factors and protein toxins are searchable, e.g. the MvirDb database of microbial virulence factors (http://mvirdb.llnl.gov) [ref Nucl. Acids Res.(2007) 35 (suppl 1): D391-D394.doi: 10.1093/nar/gkl791].

[vi] In considering the issue of "pathogenicity" and the potential to produce an infection, it is important to distinguish between *true pathogens* (i.e., microbes that possess virulence factors and are therefore capable of crossing or evading non-compromised host barriers) versus *opportunistic pathogens* (i.e., microbes that do not possess the required virulence factors to produce an infection in a non-compromised host). Typically this can be accomplished via genome analysis for known virulence factors coupled with a comprehensive search of the peer-reviewed scientific literature for infectious potential.

[vii] A functional antibiotic resistance gene results in an antibiotic resistance phenotype.

[viii] In this context, the term 'antimicrobial substances' refers to antibiotics that are used in medical or veterinary applications, for example substances that are positive in the JECFA test (FAO. 1981. *FAO Food and Nutrition Paper: 25th Session of the Joint FAO/WHO Expert Committee on Food Additives*, Appendix A, pp. 317–318, FAO/WHO, Geneva, Switzerland.)

[ix] The use of the terms "food" and "feed" includes supplements, which are in most jurisdictions considered to be a subset of the general categories.

[x] Demonstration of the safety of the expressed product may be accomplished by testing, e.g. toxicological testing as required by various regulatory bodies such as the US FDA Redbook 2000 (http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm2006826.htm) or by establishing a substantial equivalence of the test article to a substance with a safe history of use in food, or, in the case of animal feed additives, establishing a substantial equivalence of the test article to a substance with a history of safe use in target animal feeds.

[xi] Food fermentations, e.g. Cheddar cheese or yogurt, commonly result in "substantial" microbial food culture populations of 10^6-10^8 colony forming units per gram of the food. Significance should be judged relative to the fermented food, i.e. numbers of different organisms in a microbial population may change during the course of the life of the fermented food, e.g. Lactobacilli counts in Cheddar cheese are routinely low in the initial stages of cheese maturation, but begin to increase in numbers while the Lactococci, responsible for initial acid production, count decreases as the cheese ripens and pH decrease. [Spatial and temporal distribution of non-starter lactic acid bacteria in Cheddar cheese. N.A. Fitzsimons, T.M. Cogan, S. Condon, T. Beresford. Journal of Applied Microbiology 90(4): 600–608, 2001; Kosikowski, F. V., and V. V. Mistry. Cheese and Fermented Milk Foods. 1997. 3rd Ed. F. V. Kosikowski, L. L. C. Westport, CT.]

[xii] A species is a "characterizing" component of a food if it has a measurable impact on flavor, texture, stability or preservation properties that are characteristic of the food, e.g. typical color and flavor of "blue" cheeses derived

from *Penicillium roqueforti*; or surface texture, flavor and odor of Limburger cheese resulting from *Brevibacterium linens* growth on the surface. The color and flavor of "blue" cheese and the aroma, flavor and texture of Limburger cheese are characteristic of the food and the microbial cultures that are responsible for these traits are characterizing components.

[xiii] A strain that was isolated from a type-strain or a commercial culture, with a history of safe use in food fermentations, is deemed to have satisfied this requirement and may proceed to 9a.

[xiv] For example, the Qualified Presumption of Safety list (http://www.efsa.europa.eu/en/topics/topic/qps.htm) prepared and periodically updated by the European Food Safety Authority is the output from a systematic safety review of the included microorganisms by qualified experts.

[xv] Experimental evidence of safety is required. Such evidence may include, but is not necessarily limited to, studies in appropriate animal models, and clinical trials in humans.

[xvi] In some cases, the strain may be shown to be appropriate by test and re-application of the decision tree, e.g., where an undesirable genetic element has been removed from a strain's genome.
AB-LIFE® has been marketed as a food supplement in various European countries since 2012.