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Notice to US Food and Drug Administration of the Conclusion that the Intended Use of *Lactobacillus plantarum* ECGC 13110402 (LPLDL®) is Generally Recognized as Safe

Submitted by the Notifier:

ProBiotix Health Ltd.
Cavalier Suite The Barracks
Wakefield Road
WF8 4HH Pontefract
United Kingdom

Prepared by the Agent of the Notifier:

AIBMR Life Sciences, Inc
2800 E. Madison, Suite 202
Seattle WA 98112

February 21, 2019

February 21, 2019

#8477

Susan Carlson, PhD
Division Director
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
Department of Health and Human Services
5001 Campus Drive
College Park, MD 20740

Dear Dr. Carlson:

In accordance with regulation 21 CFR Part 170 Subpart E (Generally Recognized as Safe (GRAS) Notice), on behalf of ProBiotix Health Ltd. (the notifier), the undersigned, AIBMR Life Sciences submits, for FDA review, the enclosed notice that *Lactobacillus plantarum* ECGC 13110402 (LP_{LDL}®) is GRAS for use in foods.

Should you have any questions or concerns regarding this notice, please contact me at 253-286-2888 or amy@aibmr.com.

Sincerely,

[redacted]

Amy Clewell, ND, DABT (agent of the notifier)
Vice President, Scientific and Regulatory Affairs
AIBMR Life Sciences, Inc. ("AIBMR")

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Figures and Tables

Part 1: Signed Statements and Certification

1.1 Submission of GRAS Notice

ProBiotix Health Ltd. (the notifier) is submitting a new GRAS notice in accordance with 21 CFR Part 170, Subpart E, regarding the conclusion that *Lactobacillus plantarum* ECGC 13110402 (LP_{LDL}®) is Generally Recognized as Safe (GRAS) for its intended use, consistent with section 201(s) of the Federal Food, Drug and Cosmetic Act.

1.2 Name and Address of the Notifier and Agent of the Notifier

Notifier

ProBiotix Health Ltd.
Cavalier Suite The Barracks
Wakefield Road
WF8 4HH Pontefract
United Kingdom
lgosalbez@optibiotix.com

Agent of the Notifier

Amy Clewell, ND, DABT
Vice President, Scientific and Regulatory Affairs
AIBMR Life Sciences, Inc.
2800 E. Madison
Seattle, WA 98112
Tel: (253) 286-2888
amy@aibmr.com

1.3 Name of the Substance

The name of the substance is *Lactobacillus plantarum* ECGC 13110402 (LP_{LDL}®), known commercially and hereafter referred to as *L. plantarum* LP_{LDL}®.

1.4 Intended Conditions of Use

L. plantarum LP_{LDL}® is intended to be used as an ingredient added to foods where standards of identity do not preclude such use. It is not intended to be added to infant formula, or any products that would require additional regulatory review by USDA. The intended addition level to foods is up to 1×10^{10} CFU per serving.

1.5 Statutory Basis for GRAS Conclusion

The conclusion of GRAS status of *L. plantarum* $LP_{LDL}^{®}$ for its intended conditions of use, stated in Part 1.4 of this notice, has been made based on scientific procedures.

1.6 Not Subject to Premarket approval

We have concluded that *L. plantarum* $LP_{LDL}^{®}$ is GRAS for its intended conditions of use, stated in Part 1.4 of this notice, and, therefore, such use of *L. plantarum* $LP_{LDL}^{®}$ is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act.

1.7 Data and Information Availability Statement

The data and information that serve as the basis for this GRAS conclusion will be available for review and copying during customary business hours at the office ProBiotix Health Ltd., Cavalier Suite The Barracks, Wakefield Road, WF8 4HH Pontefract, United Kingdom, or will be sent to FDA upon request.

1.8 Exemption from Disclosure under the Freedom of Information Act

None of the data and information in Parts 2 through 7 of this GRAS notice are considered exempt from disclosure under the Freedom of Information Act (FOIA) as trade secret or commercial or financial information that is privileged or confidential.

1.9 Certification of Completion

We herby certify that, to the best of our knowledge, this GRAS notice 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 *L. plantarum* $LP_{LDL}^{®}$.

[redacted]

February 22, 2019

Stephen O'Hara (Notifier)
Chief Executive Officer
ProBiotix Health Ltd.
spohara@optibiotix.com

Date

Part 2: Identity, Manufacture, Specifications, and Physical or Technical Effect

2.1 Identification

LP_{LDL}® is a strain of the genus *Lactobacillus*, species *plantarum* initially isolated before 1993 from fruit juice (specifically tomato) fermentation and is not genetically modified. LP_{LDL}® is deposited in accordance with the terms of the Budapest Treaty of 1977 at the European Culture General Collection (Salisbury, United Kingdom) with accession number ECGC 13110402. Genomic markers align LP_{LDL}® with other *L. plantarum* strains present in vegetable material, clearly differentiating it from other natural sources such as animal or human microbiota or meat products.[1]

The taxonomic lineage of the strain is:

Kingdom: Bacteria

Phylum: Firmicutes

Class: Lactobacillales

Family: Lactobacilleae

Genus: Lactobacillus

Species: Lactobacillus plantarum

Strain: LP_{LDL}®

The *Lactobacillus* genus contains over 220 species, and is the major genus of the lactic acid bacteria (LAB) grouping, which produce lactic acid as the major end-product of hexose sugar fermentation.[2, 3] LAB are generally gram-positive, nonsporulating, facultative anaerobic or microaerophilic, cocci or rod shaped bacteria which occur naturally in and are utilized in fermented dairy and non-dairy products such as fermented vegetables, meats, and beverages.[2-5] In nature, they are ubiquitous in plant matter and are found in the gastrointestinal, vaginal, and urogenital tracts of humans and other animals.[2, 3] They are found wherever substances rich in carbohydrates are available, and are generally considered to be non-toxic and non-pathogenic.[5-7]

The genus' of LAB are diverse, but commonly include *Lactobacillus, Enterococcus, and Lactococcus,* amongst many others.[4] Some *Lactobacillus* species are exclusively found naturally in specific habitats (e.g., *L. helveticus* and *L. delbrueckii* ssp. *bulgaricus* in dairy products, *L. johnsonii* and *L. gasseri* in vertebrate gastrointestinal tracts) whereas other species, such as *L. plantarum* and *L. casei*, may be found in a variety of different environments.[8] In healthy humans, *Lactobacilli* are normally present at a population density of approximately 10^3–10^7 CFU/g in the oral cavity, 10^3–10^7 CFU/g in the ileum, 10^4–10^8 CFU/g the colon, and are the dominant microorganism in the vagina.[5]

L. plantarum is one of the best characterized species within the *Lactobacillus* genus,[3] which owes its name to its recurrent occurrence, in high numbers, in spontaneously-fermented plant matter. It has been reported to be a dominant naturally occurring species in fruits and vegetables such as grapes, cabbage and lettuce, and is commonly found in human fecal microbiota.[4] It is used in fermented foods in the human diet as well as in the production of fermented animal feed (silage).[5] It is a rod-shaped, gram-positive, facultative heterofermentative (meaning it can ferment hexoses to lactic acid, and can also degrade pentoses and gluconate, producing acetic acid, ethanol and formic acid), facultative anaerobe bacterium with a relatively large genome (> 3 Mb circular chromosome).[4, 6] It is a metabolically diverse species with an strong capacity to adapt to different conditions.[4]

2.1.1 Phenotypic and Biochemical Identification

Similarly to other *L. plantarum* strains, LP_{LDL}® forms round, opaque cream, raised colonies on solid culture media such as de Man, Rogosa and Sharpe (MRS) medium.[9] It is a facultative anaerobe (aerotolerant) organism with optimal growth conditions at pH 6 and a temperature of 37°C.

L. plantarum LP_{LDL}®'s capability to use and ferment different sugars as carbon sources was assessed following the API 50 CH system (Biomeréux, France), as established by the Bergey's Manual of Systematic Bacteriology for *Lactobacilli*.[10] Results are shown in Table 1 below. The results show that its profile is characteristic of *L. plantarum* strains (e.g. strain 266v, GRN 685).

Table 1. Sugar fermentation capacity (API 50 CH) of *L. plantarum* LP_{LDL}®

Substrate	Growth	Substrate	Growth	Substrate	Growth
Control	-	Inositol	-	D-Melezitose	+
Glycerol	-	D-Manitol	+	D-Raffinose	+
Erytrol	-	D-Sorbitol	+	Starch	-
D-Arabinose	-	Methil-αD-Manopyranoside	-	Glycogen	-
L-Arabinose	+	Methil-αD-Glucopyranoside	-	Xylitol	-
D-Ribose	+	N-Acetylglucosamine	+	Gentiobiose	+
D-Xylose	-	Amygdaline	+	D-Turanose	+
L-Xylose	-	Arbutine	+	D-Lyxose	-
D-Adonidol	-	Esculin	+	D-Tagatose	-
Metil-βD-Xylopyranoside	-	Salicin	+	D-Fucose	-
D-Galactose	+	D-Celobiose	+	L-Fucose	-
D-Glucose	+	D-Maltose	+	D-Arabitol	-
D-Fructose	+	D-Lactose	+	L-Arabitol	-
D-Mannose	+	D-Melibiose	+	Potassium Gluconate	+

L-Sorbose	-	D-Saccharose	+	Potassium 2-Ketogluconate	-
L-Rhamnose	-	D-Trehalose	+	Potassium 5-Ketogluconate	-
Dulcitol	-	Inulin	-		

2.1.2 Genetic Identification

Following FAO/WHO guidelines for probiotic characterization and identification,[8] a large fragment of the *L. plantarum* LP_{LDL}® 16S rRNA-encoding gene was amplified and sequenced. For this, a stationary culture of the strain was subjected to DNA extraction using the GenElute Bacterial Genomic DNA Kit (Sigma Aldrich, USA). The DNA preparation was subsequently used as template for a PCR using targeting the V1–V3 region of the 16S rRNA gene, universally considered the most appropriate for species-level identification.[10-12] The resulting amplicon of approximately 600 base pairs was sequenced and then analyzed using the CLC Genomics Workbench software v. 10.1.1. (CLC bio, Denmark). The sequence was subjected to nucleotide BLAST against the database "Nucleotide collection (nt/nt)", setting the search against the "Type Strains" database.

The alignment (Figure 1) of the nucleotide sequence of *L. plantarum* LP_{LDL}® showed 100% identity (i.e. 609 nucleotides out of 609 were identical) with the 16S rRNA gene of the *L. plantarum* type strain JCM1149 (GenBank accession no. NR_117813.1). The strain LP_{LDL}® is therefore identified as a member of the species *L. plantarum*.

(b) (4)

Additionally, the entire genome of *L. plantarum* LP_{LDL}® was sequenced with high-throughput pyrosequencing technology, a depiction of which is shown in Figure 2 below.

(b) (4)

2.2 Manufacturing of LP_{LDL}®

2.2.1 Manufacturing Overview

For each batch of *L. plantarum* LP_{LDL}® produced, the master and working seed are tested to assure that the strain is free of microbiological contaminants as shown in Table 2. An outline of the major steps of the manufacturing process is depicted in Figure 3 and the steps are further described in the text below.

Table 2. Tests for microbiological purity for *L. plantarum* LP$_{LDL}$® seed culture

Parameter	Specification	Method*
Morphological control	Circular colony, medium-large, irregular margin, bright green colored with halo light beige; some colonies of gray-green hue (polymorphic strain)	Manufacturer's internal method
Enterococci	Absent / mL	Manufacturer's internal method
Enterobacteriaceae	Absent / mL	ISO 21528-2 (SOP CQ-105)

ISO= International Organization for Standardization. *Detailed methods available upon request.

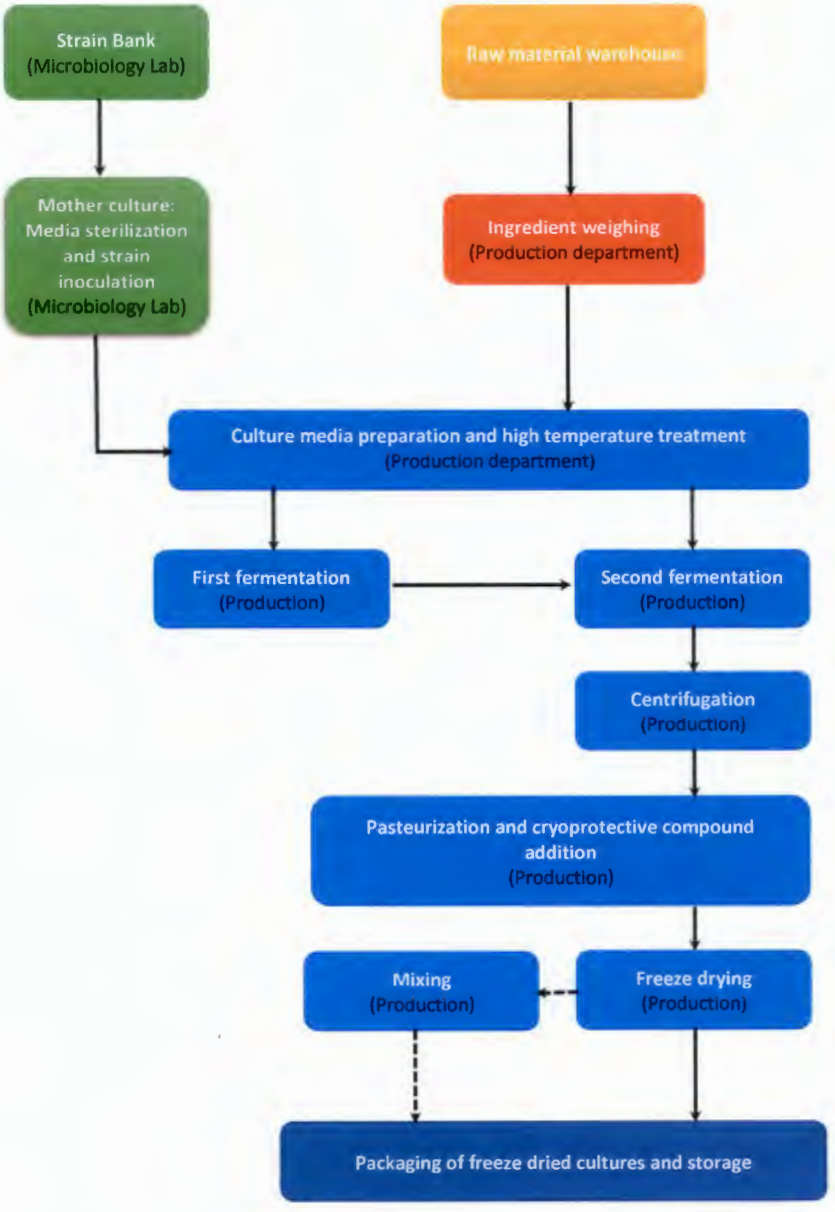

Figure 3. Manufacturing Flowchart for *L. plantarum* LP$_{LDL}$®

2.2.1.1 Strain Revival

A solution of MRS-X broth medium is sterilized at 121 °C for 15 minutes, then lowered to 34–40°C. The growth medium is composed of purified water and the following pharmaceutical and or food-grade substances: inorganic salts, sugars, amino acids, protein sources such as yeast extract and peptones. The medium is inoculated with 1–4% (v/v) *L. plantarum* $LP_{LDL}^{®}$ culture. Incubation proceeds until the pH reaches 5.0 ± 0.5, maximum 24 hours.

2.2.1.2 Preparation of Inoculum

The growth medium is composed of purified water and the following pharmaceutical and/or food-grade substances: inorganic salts, sugars, amino acids and protein sources such as yeast extract and peptones. A suitable vessel containing the medium is sterilized by raising the temperature to 121°C to obtain F0 value over 15, and then the temperature is lowered to 34–40°C. The medium is inoculated at 1–4% (v/v). *L. plantarum* $LP_{LDL}^{®}$ culture is prepared as per the previous step. Incubation proceeds until the pH reaches 5.0 ± 0.5, for a maximum 24 hours.

2.2.1.3 Production Pre-Fermentation

This step increases the fermentation volume approximately 50-fold. The growth medium is composed of purified water and the following pharmaceutical and/or food-grade substances: inorganic salts, sugars, amino acids and protein sources such as yeast extract and peptones, polysorbate and silicon emulsion.

The medium is sterilized by raising the temperature to 121°C for 7 minutes, in a tank, previously sterilized at 135°C for 15 minutes. The temperature is then reduced to 34–40 °C in preparation for inoculation. *L. plantarum* $LP_{LDL}^{®}$ seed culture is prepared as described above and is inoculated at 1–4% (v/v). The growth medium is maintained at this temperature under stirring until the pH decreases to a value of 5.0–6.0.

The pre-fermentation is regarded as having reached stationary phase when the pH shows no further tendency to decrease. The tank is then cooled at a temperature lower than 10°C under slow stirring and is maintained as such until the next fermentation step (production fermentation).

2.2.1.4 Production Fermentation

This step increases the fermentation volume 50-fold again. The growth medium is composed of purified water and the following pharmaceutical and or food-grade substances: inorganic salts, sugars, amino acids and protein sources such as yeast extract and peptones, polysorbate and silicon emulsion.

The medium is sterilized by a 14-second exposure at 142 °C, collected in a sterilized tank (at 105 °C for 90 minutes) and the temperature is reduced to 34–40 °C. *L. plantarum* $LP_{LDL}^{®}$ seed culture is prepared as in the previous step and is inoculated at 2–4% (v/v). As fermentation proceeds the pH is maintained at 5–6 by the use of

food-grade ammonium hydroxide. The medium is agitated as little as possible, but sufficiently to maintain the pH of the solution within the specified range.

The fermentation is stopped after approximately 8 hours based on measurement of ammonium hydroxide consumption from the culture medium by the organisms. The tank is then cooled at a temperature lower than 10 °C under slow stirring and maintained as such until the following step, in which the solution is centrifuged in order to increase the concentration of *L. plantarum* LP_{LDL}® approximately 20-fold.

2.2.1.5 Freeze-Drying

Harvested *L. plantarum* LP_{LDL}® is further processed to produce a freeze-dried product. A cryoprotectant is added to the centrifuged solution to suitably prepare the cells for the freeze-drying step.

The cryoprotectant solution is composed of purified water and the following pharmaceutical and/or food-grade substances: organic salts, sugars, carbohydrates. The solution, obtained by mixing centrifuged fermented medium containing the live culture and cryoprotectant, is loaded into a freeze-dryer and dried under vacuum. Once dried, the pellets are milled and packed into heat-sealed poly/foil bags. Before packing, the product is passed in an in-line magnet to detect any metal particles.

2.2.2 Good Manufacturing Practice

All production steps are performed under an approved Hazard Analysis and Critical Control Points (HACCP) plan and are consistent with current Good Manufacturing Practice (cGMP).

2.2.3 Raw Materials

Raw materials used in the production of ProBiotix's *L. plantarum* LP_{LDL}® are of appropriate food grade and/or suitable for use in this process.

2.3 Specifications

The specifications for the food-grade product *L. plantarum* LP_{LDL}®, along with the specification methods, are listed in Table 3 below. Note that currently ProBiotix is producing two main product concentrations — a $\geq$ 200 billion CFU/g product and a $\geq$ 400 billion CFU/g product, as is reflected in the specifications and in the batch analyses shown below. In the future, additional product concentrations may be developed. Ultimately, any product concentration may be diluted accordingly to meet the specified intended use CFU concentrations in final food products.

Table 3. *L. plantarum* LP_{LDL}® Specifications

Tested Parameters	Specification	Method
L. plantarum LP_{LDL}® cell count (CFU/g)	≥ 200 billion or ≥ 400 billion	SOP CQ-102 (ISO 27205-IDF 149)
Particle size	> 40 mesh	Ph.Eur. (sieves method)
AW	≤ 0.15	SOP CQ-146 (Ph. Eur.)
Appearance	Fine and smooth freeze-dried granulated powder	SOP CQ-194 (visual method)
Heavy Metals		
Arsenic (ppm)	< 0.2	External lab (ICP-MS method)
Cadmium (ppm)	< 0.2	External lab (ICP-MS method)
Mercury (ppm)	< 0.2	External lab (ICP-MS method)
Lead (ppm)	< 0.2	External lab (ICP-MS method)
Microbiological Tests		
Coliforms (CFU/10 g)	< 10	SOP CQ-097 (ISO 4031-4832)
Enterobacteriaceae (CFU/10 g)	< 100	SOP CQ-105 (ISO 21528-2)
Enterococci (CFU/10 g)	< 100	SOP CQ-103 (IDF 149)
Non-lactic acid bacteria (CFU/g)	≤ 5000	SOP CQ-090 (ISO 13559)
Total yeast and moulds (CFU/g)	< 100	SOP CQ-105 (ISO 6611)
Escherichia coli (per g)	Absent	SOP CQ-104 (AFNOR BRD 07/01-07/93)
Salmonella (per 25 g)	Absent	SOP CQ-184 (ISO 6785/IDF 93)
Staphylococcus aureus (per g)	Absent	SOP CQ 122 (ISO 6888-1)
Listeria monocytogenes (per 25 g)	Absent	SOP CQ -158 (ISO 11290-1/A1 – AFNOR BRD 07/04-09/98)

AFNOR BRD, Association Française de Normalisation; AW, water activity; ICP-MS, Inductively Coupled Plasma Mass Spectrometry; IDF, International Dairy Federation; ISO, International Organization for Standardization; Ph. Eur, European Pharmacopoeia.

2.3.1 Batch Analysis

Production conformity and consistency of ProBiotix's *L. plantarum* LP_{LDL}® are tested in production lots. Batch analyses of three non-consecutive lots are shown in Table 4 below and are reasonably consistent and met all of the product specifications.

Table 4. *L. plantarum* LP_{LDL}® Batch Analyses

Tested Parameters	Specification	Lot No./Date of Manufacture		
		[redacted]	[redacted]	[redacted]
Cell count (CFU/g)	≥ 200 billion / ≥ 400 billion	2.01×10^{11}	4.5×10^{11}	4.3×10^{11}
Particle size	> 40 mesh	Conforms	Conforms	Conforms
AW	≤ 0.15	Conforms	Conforms	Conforms
Appearance	Fine and smooth freeze-dried granulated powder	Conforms	Conforms	Conforms
Heavy Metals				
Arsenic (ppm)	< 0.2	Conforms	Conforms	Conforms
Cadmium (ppm)	< 0.2	Conforms	Conforms	Conforms
Mercury (ppm)	< 0.2	Conforms	Conforms	Conforms
Lead (ppm)	< 0.2	Conforms	Conforms	Conforms
Microbiological Tests				
Coliforms (CFU/10 g)	< 10	Conforms	Conforms	Conforms
Enterobacteriaceae (CFU/10 g)	< 100	Conforms	Conforms	Conforms
Enterococci (CFU/10 g)	< 100	Conforms	Conforms	Conforms
Non-lactic acid bacteria (CFU/g)	≤ 5000	Conforms	Conforms	Conforms
Total yeast and moulds (CFU/g)	< 100	Conforms	Conforms	Conforms
Escherichia coli (per g)	Absent	Absent	Absent	Absent
Salmonella (per 25 g)	Absent	Absent	Absent	Absent
Staphylococcus aureus (per g)	Absent	Absent	Absent	Absent
Listeria monocytogenes (per 25 g)	Absent	Absent	Absent	Absent

AW= water activity; [a] = specification was ≥ 200 billion CFU/g; [b] = specification was ≥ 400 billion CFU/g

2.3.2 Shelf–Life Stability

L. plantarum LP_{LDL}® has been subjected to a stability study in order to verify its ability to maintain bacterial counts. Tests were run at a temperature of 5 ± 3 °C in a dry atmosphere (corresponding to ICH 'refrigerated' conditions) and at 25 ± 2 °C with a relative humidity of 60 ± 5% (corresponding to ICH Zone II, 'Mediterranean-subtropical zone' conditions).

Results from this real-time, long-term (18 months) assessment of stability of LP_{LDL}® are shown in Table 5 and Figure 4. The results suggest that the strain is reasonably stable in a variety of environmental and storage conditions.

Table 5. Stability data of *L. plantarum* LP_{LDL}® at 5 °C and 25 °C from 0–18 months

Time (months)	Count (CFU/g)	
	5°C	25°C
0	8.00×10^{11}	8.00×10^{11}
1	6.70×10^{11}	4.30×10^{11}
3	6.47×10^{11}	3.20×10^{11}
6	5.63×10^{11}	3.25×10^{11}
12	4.86×10^{11}	2.27×10^{11}
18	3.83×10^{11}	1.45×10^{11}

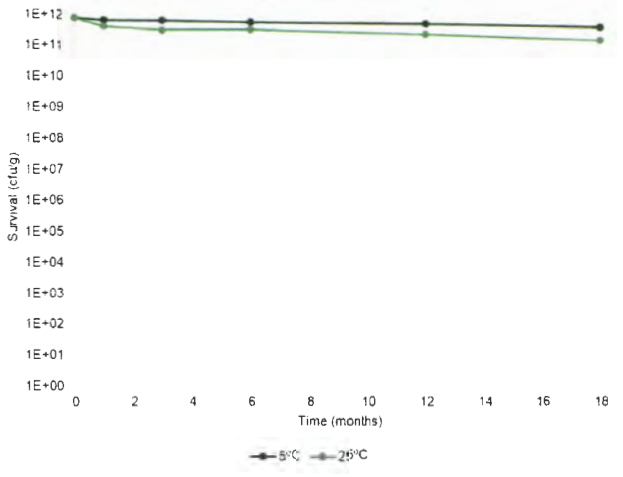

Figure 4. Stability data of *L. plantarum* LP_{LDL}® at 5 °C and 25 °C from 0–18 months

2.4 Antibiotic Resistance

Resistance to therapeutic antibiotics by microbial pathogens is currently considered one of the greatest challenges in medicine and public health, as some infectious diseases may become virtually untreatable if they become non-respondent to current therapies. Antibiotic resistance may be classified into two types;

- intrinsic/natural (when resistance is inherent to a bacterial species, and is a trait generally shared by all members of that species), or
- extrinsic/acquired (when a strain of a typically susceptible species is resistant to a given antimicrobial drug).

Extrinsic/acquired resistance can occur either from the gain of exogenous DNA or mutation of indigenous genes.[14, 15] While intrinsic resistance likely presents a very low risk of dissemination, extrinsic/acquired resistance, especially when the relevant genes are associated with mobile genetic elements such as plasmids and transposons, can be transferred to pathogens or other commensal bacteria.[16] It is generally recommended that resistance to antibiotics be assessed in all probiotic strains prior to marketing.[17 14, 18-20]

Antibiotic resistance is a complex phenomenon, in which microbial genetics and environmental stimuli both play important roles. Assessing resistance both phenotypically and genotypically is generally recommended. As detailed below, antibiotic susceptibility of *L. plantarum* LP_{LDL}® was evaluated using both approaches.

Phenotypic evaluation of antibiotic resistance involves testing the capacity of a microorganism to survive in a medium containing different concentrations of

antibiotics. Whereas most microorganisms can survive at low concentrations of many antibiotics, resistance is defined as the capacity to grow at antibiotic concentrations similar to those reached in the human body during therapeutic intervention.

Following EFSA recommendations and guidelines, ProBiotix Health Ltd. assessed the phenotypic susceptibility of *L. plantarum* LP_{LDL}® to the antibiotics detailed in the guidelines for *L. plantarum* strains, namely ampicillin, gentamycin, kanamycin, erythromycin, clindamycin, tetracycline and chloramphenicol.[14] Two additional antibiotics, streptomycin and vancomycin, were also tested as they are recommended for *Lactobacilli* although not specifically for *L. plantarum*. The EFSA guidelines define a bacterial strain as sensitive or susceptible to an antibiotic when it is inhibited at a concentration of a specific antimicrobial equal or lower than the established cut-off value for that particular compound.

The assays on *L. plantarum* LP_{LDL}® were performed following the Clinical and Standards Institute's (CLSI; www.clsi.org) protocol to test broth dilution on antimicrobial susceptibility (methods M07-A8 and M100-S22), using the culture medium recommended by EFSA (mixture of IST medium 90% and 10% MRS medium; Thermo Fischer Scientific, Waltham, MA, USA). Bacterial cultures were incubated anaerobically at 37 °C for 48 hours. The minimum inhibitory concentration (MIC) was established as the minimum concentration in which the antibiotic exerted a clear inhibition (lack of bacterial growth). Results are shown in Table 6 and indicate that *L. plantarum* LP_{LDL}® is phenotypically sensitive to all antibiotics included in EFSA's guidelines for the *L. plantarum* species.

(b) (4)

Genotypic evaluation of antibiotic resistance is a procedure in which the whole bacterial genome (chromosome and peripheral genetic elements, if any) is screened for putative genes of antibiotic resistance, as described in genetic databases. It is therefore a complementary procedure to the phenotypic assessment, in which the main objective is to discard the potential of transferring putative genes of antibiotic resistance to other microbes.

The genome of *L. plantarum* $LP_{LDL}^{®}$ was screened for antibiotic resistance genes in-house using the Comprehensive Antibiotic Resistance Database (CARD). Briefly, predicted genes were aligned to the databases using the usearch_local alignment algorithm of the USEARCH software,[21] which is a fast BLAST[22] alternative with similar sensitivity and specificity to BLAST. Alignments with sequence identity >85% and query coverage >80% or target coverage >80% were kept.

Genes encoded within the genome of *L. plantarum* $LP_{LDL}^{®}$ did not match the CARD database using the parameters stated above. Based on these findings *L. plantarum* $LP_{LDL}^{®}$ is unlikely to contain any known antibiotic resistance genes.

2.5 Genomic Analysis for Virulence and Pathogenicity

Genomic analysis of *L. plantarum* $LP_{LDL}^{®}$ was screened using the Virulence Factors of Pathogenic Bacteria (VFDB) database. Briefly, predicted genes were aligned to the databases using USEARCH software.[21] Again, alignments with sequence identity >85% and query coverage >80% or target coverage >80% were kept.

As expected, genes encoded in the genome of *L. plantarum* $LP_{LDL}^{®}$ did not match the VFDB database within these parameters. Based on these findings the *L. plantarum* $LP_{LDL}^{®}$ is unlikely to have known genes coding for virulence or pathogenicity factors.

2.6 Resistance to Gastric Acidity and Bile Salts

The extremely acidic environment of the stomach (pH of approximately 2.0–3.0) kills the majority of potentially pathogenic bacteria, preventing infection. Non-pathogenic bacteria, such as *Lactobacilli*, show different degrees of tolerance to extreme pH conditions as well as to hepatic bile salts, which are detergent-like compounds.

To determine *L. plantarum* $LP_{LDL}^{®}$'s ability to survive in low pH, the strain was grown for 17 h and cells were harvested, washed in phosphate buffered saline (PBS) and re-suspended in PBS adjusted at pH 2.0, 2.5, 3.0, and 7.0 as a control. Cell suspensions were incubated at 37°C for 3 h. Viability of cells at time 0 h and 3 h was evaluated by plate counts on MRS agar (37°C, 48 h, incubated anaerobically). Percentage of survival was calculated as referred to 0 h and compared to pH 7.0 values (control). Results, as shown in Table 7, showed fairly low *in vitro* resistance

to acidic pH values after 3 hours of exposure. These results are in line with those described for other *Lactobacilli* when exposed to similar conditions.[23, 24]

Table 7. *L. plantarum* LP_{LDL}® counts obtained after 3 h incubation at different pH values

pH	CFU/mL ± SD	survival (%) ± SD*
7.0 (control)	$1.37 \times 10^7 \pm 9.94 \times 10^4$	101.00 ± 0.05
3.0	$1.67 \times 10^2 \pm 7.07$	0.0015 ± 0.002
2.5	<10	-
2.0	<10	-

CFU=colony forming units, SD=standard deviation
* Results are the average of two assays ± SD

To determine *L. plantarum* LP_{LDL}®'s capacity to resist bile salt concentrations typical to the upper small intestine, cultures were harvested following 17 h anaerobic incubation, washed in PBS and re-suspended in PBS containing 0.3%, 0.5%, 1% and 2% (w/v) bile salts (Oxgall, Thermo Fischer Scientific, Waltham, MA, USA). Cell suspensions were incubated at 37 °C for 3 hours. Cell viability was tested at 0 h and 3 h by plate counts on MRS agar (37 °C, 48 h, incubated anaerobically). Percentage of resistance was calculated referred to 0 h and compared with 0% bile salt incubation. The results, as shown in Table 8 and Figure 5, suggest a strong resistance of the strain to bile salt compounds in vitro.

Table 8. *L. plantarum* LP_{LDL}® counts obtained after 3 h incubation at different bile salt concentrations

Assay	CFU/mL ± SD*	survival (%) ± SD
0% Oxgall	$1.37 \times 10^7 \pm 9.90 \times 10^4$	101.00 ± 0.05
0.3% Oxgall	$1.43 \times 10^7 \pm 8.13 \times 10^4$	104.93 ± 5.50
0.5% Oxgall	$1.43 \times 10^7 \pm 1.10 \times 10^5$	104.07 ± 1.60
1% Oxgall	$1.32 \times 10^7 \pm 7.07 \times 10^4$	97.09 ± 6.20
2% Oxgall	$1.32 \times 10^7 \pm 5.66 \times 10^4$	97.52 ± 9.75

CFU=colony forming units, SD=standard deviation
*Results are the average of two assays ± SD

L. plantarum LP_{LDL}® also shows the capacity to grow in the presence of bile salts in vitro. Cultures of the strain were harvested following 17 h anaerobic incubation, washed in PBS and re-suspended in MRS broth containing 0.3%, 0.5%, 1% and 2% (w/v) bile salt (Oxgall, Thermo Fischer, Waltham, MA, USA). Cell suspensions were incubated anaerobically at 37 °C for 17 h in a microplate reader (Multiskan) and growth monitored by OD_{595} measurements throughout the assay. At 19 h, growth was evaluated by plate counts on MRS agar (37 °C, 48 h, incubated anaerobically). Results are shown in Table 9 and Figure 6.

Table 9. *L. plantarum* LP_{LDL}® counts expressed in CFU/mL, obtained after 19 h incubation at different bile salt concentrations

Assay	CFU/mL ± SD
0% Oxgall	$7.33 \times 10^9 \pm 1.41 \times 10^8$
0.3% Oxgall	$7.75 \times 10^9 \pm 1.31 \times 10^8$
0.5% Oxgall	$1.64 \times 10^9 \pm 6.41 \times 10^7$
1% Oxgall	$6.28 \times 10^8 \pm 3.43 \times 10^6$
2% Oxgall	$1.55 \times 10^8 \pm 7.07 \times 10^4$

CFU=colony forming units, SD=standard deviation
*Results are the average of two assays ± SD

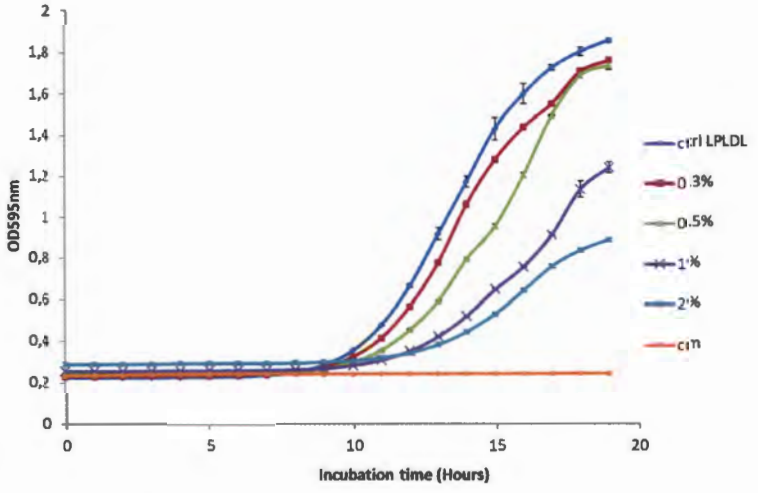

Figure 6. Growth curve (as OD_{595} values) obtained for *L. plantarum* LP_{LDL}® in the presence of different bile salt concentrations. Results are the average of two assays ± SD.

The high tolerance of *L. plantarum* LP_{LDL}® to bile salts, as demonstrated above, may be due to a high bile salt hydrolase (BSH) enzymatic activity (which catalyzes the hydrolysis of conjugated bile salts) expressed by this strain. The resulting bile acids are less efficient in emulsifying biological membranes rendering them less aggressive towards microorganisms. High BSH activity is naturally found in many microbes that inhabit the human gastrointestinal tract and in microorganisms for human use, such as *Lactobacillus*,[25-37] *Bifidobacterium*,[36, 38-41] *Enterococcus*,[36, 42] *Clostridium*,[32, 36] and *Bacteroides*,[36, 43, 44] each of which expresses different isoenzyme forms varying in activity, specificity towards conjugated bile acid substrates, and yielding different catabolytes.[45] One of the criteria used in the selection of the *L. plantarum* LP_{LDL}® strain was its high BSH activity, as described in the human clinical study published by Costabile et al. (2017).[46]

It has been suggested that certain secondary bile acids may have undesirable health effects,[36, 47-52] although other studies contradict these findings or even suggest their implication in normal, physiological processes.[36, 53] In order to generate these secondary bile acids from the conjugated bile salts released by the liver, a series of biochemical steps are necessary, of which BSH activity is only one.[53] Bacterial genera most commonly used as probiotics, such as *Bifidobacteria* and *Lactobacilli*, are generally not capable of performing the further chemical modifications required to generate secondary bile acids, according to the literature.[36, 53-56] The main enzyme related to the generation of potentially harmful compounds, 7-α-dehydroxylase, has not been reported in any LAB, and appears to be exclusive to a limited group of microorganisms such as *E. coli*, and members of the genera *Bacteroides*, *Enterobacter, Eubacterium* and *Clostridium* in the human intestinal microbiome.[36, 57, 58] Thus the majority of the breakdown products of BSH activity by a probiotic will be precipitated and excreted in feces, not being subject to any further chemical modification,[36] or even accumulated inside bacterial cells responsible for the BSH activity.[59, 60]

2.7 Biogenic Amine Formation

Some species and/or strains of LAB are able to produce biogenic amines (organic, basic, nitrogenous compounds formed mainly by the decarboxylation of amino acids), likely for use as metabolic energy and/or to increase acid resistance.[7] These amines are present in a wide range of foods (e.g. fermented food products), and although they are involved in many natural physiological processes, consuming large quantities of these amines can have undesirable consequences in some individuals. For example, if they are not properly detoxified in the body, they can cause release of adrenaline/noradrenaline, cause gastric acid secretion, increased cardiac output, heart rate, and blood pressure, migraines, and increased blood sugar.[7] Biogenic amine formation in fermented foods has been reviewed by EFSA (2011)[61] and Spano (2010).[7] Histamine and tyramine are considered the most concerning with regard to food safety.[61]

Generally, detection of strains possessing amino acid decarboxylase deaminase activity is important to help mitigate the accumulation of these amines in food products. As some strains of *L. plantarum* have been reported to produce biogenic amines,[61-64] the capacity of *L. plantarum* LP_{LDL}® to produce them was evaluated.

L. plantarum LP_{LDL}® was grown in MRS broth supplemented with 0.1% of the corresponding amino acid precursor (histidine, tyrosine, lysine and ornithine) and 0.005% of the cofactor pyridoxal-5′-phosphate, or in MRS broth not supplemented with those precursors, as a control. The culture supernatant was harvested after 48 hours. *L. plantarum* LP_{LDL}® culture supernatants and a standard amine solution were derivatized with benzoyl chloride.

Concentrations of biogenic amines in the 24 hour fermentation supernatants and standards were quantified by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with a photodiode array (PDA) detector set at 254 nm. Sample quantification was carried out using calibration curve standards for histamine (histamine dihydrochloride, Sigma Aldrich, USA), tyramine (tyramine hydrochloride, Sigma Aldrich, USA), cadaverine (cadaverine dihydrochloride, Sigma Aldrich, USA) and putrescine (putrescine dihydrochloride, Sigma Aldrich, USA). Peak identification was achieved via retention time comparison with the standards. MRS and MRS supplemented with the amino acid precursors were analyzed as controls. The chromatograms obtained are shown as Figure 7 and 8 below; and in conclusion, *L. plantarum* LP_{LDL}® did not show biogenic amine production.

(b) (4)

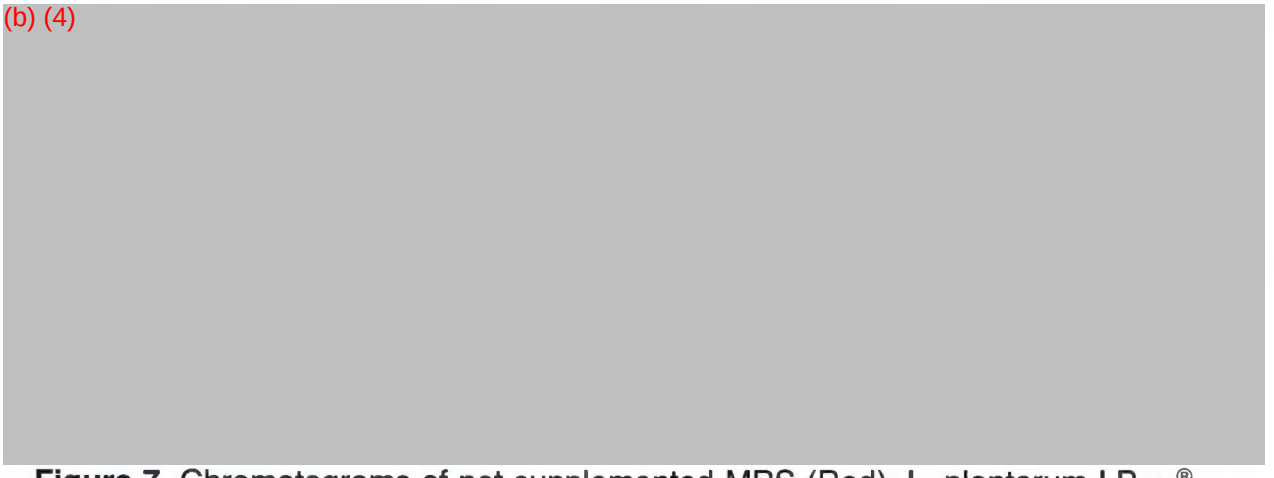

Figure 7. Chromatograms of not supplemented MRS (Red), L. plantarum LP_{LDL}® culture supernatant in MRS broth with no amino acid precursors (Blue) and standards (Black, 1 Putrescine, 2 Cadaverine, 3 Histamine and 4 Tyramine).

(b) (4)

Figure 8. Chromatograms of MRS broth supplemented with amino acid precursors (red), *L. plantarum* LP_{LDL}® culture supernatant in MRS broth supplemented with amino acid precursors (blue) and standards (black, 1 Putrescine, 2 Cadaverine, 3 Histamine and 4 Tyramine).

2.8 Production of D-Lactate

L. plantarum produces lactic acid (lactate) from the fermentation of carbohydrates. Lactate exists in two forms, a dextrorotary enantiomer (D-lactate) and a levorotary enantiomer (L-lactate). In humans, over 99% of lactate found in the blood is L-lactate. Testing D-lactate production by food microorganisms has been historically recommended likely because until relatively recently, it was believed that humans had a poor capacity of metabolizing D-lactate.[18] LAB (including *L. plantarum* species), as well as several other members of the intestinal microflora produce a mixture of L- and D-lactate.[65] More recent studies have shown that much of the human gut microbiota produces D-lactate with no evidence of D-lactic acidosis, and in fact, humans are able to metabolize this isoform.[66-72] D-lactate accumulation may only occur in cases of impaired D-lactate metabolism and/or in subjects with a disturbed gastrointestinal function following bowel resection or Short Bowel Syndrome.[68, 72-75]

L. plantarum LP_{LDL}® D- and L-lactate isomer production quantification was performed using an assay kit by Megazyme (Wicklow, Ireland). The results of this experiment showed that 57% of the lactate was in the D-form, which is very similar to that reported for other *L. plantarum* strains (e.g. Lp-115, GRN 722).

In order to assess the potential buildup of lactate (L- and D-forms) as well as other organic acids in conditions analogous to the human intestine, a series of in vitro fecal batch cultures were carried out, on fecal samples obtained from three healthy

adults, after addition of galactooligosaccharides (1% wt/vol) ± *L. plantarum* $LP_{LDL}^{®}$ using appropriate fermentation vessels and medium.

Batch cultures were run for a period of 24 h, and samples were obtained from each vessel at 0, 8 and 24 h for bacteriology using fluorescent *in situ* hybridization (FISH) and organic acids analysis using gas chromatography (GC). Organic acids analysis was generally carried out using the gas chromatography (GC) method described by Richardson *et al.* (1989).[76]

Organic acid data obtained throughout fecal fermentation in the presence and absence of *L. plantarum* $LP_{LDL}^{®}$ suggest that the addition of *L. plantarum* $LP_{LDL}^{®}$ in the fecal cultures had no impact on lactate, acetate, propionate, or butyrate profiles in the mixture. The behavior of the microbial community in this respect was very similar both in the absence and in the presence of *L. plantarum* $LP_{LDL}^{®}$.

2.9 Physical or Technical Effect

L. plantarum $LP_{LDL}^{®}$ is not intended to produce any physical or other technical effects that are relevant to the safety of the ingredient.

Part 3: Dietary Exposure

L. plantarum LPLDL®, manufactured in accordance with current GMP, is intended to be used as an ingredient added to foods where standards of identity do not preclude such use. It is not intended to be added to infant formula, or any products that would require additional regulatory review by USDA. The intended addition level to foods is up to 1 x 10^{10} CFU per serving (which is similar to levels of lactic acid bacteria found in traditionally fermented food products).[77]

The addition of *L. plantarum* LPLDL® to some foods may be substitutive with regard to previous GRAS *L. plantarum* strain intended uses (GRN 685 and GRN 722), or with regard to traditional uses of *L. plantarum*. However, uses in other foods may be considered more novel and additive with regard to exposure. A publication from the USDA's Center for Nutrition Policy and Promotion (October 2000) states that males aged 51 and older consume the largest number of servings of food per day, at 18.2 servings/day.[78] Comparatively, women aged 19–24 consumed the least, at 12.5 servings/day. This data came from detailed 14-day food diaries from 5,752 adults in the 1992–1994 time period. Using a most conservative estimation of consumption, if 100% of food servings contained *L. plantarum* LPLDL®, males aged 51 and older would consume approximately 1.82 x 10^{11} CFU/day (using a standard 70 kg body weight, this is equivalent to 2.6 x 10^{9} CFU/kg bw/day). This estimation is considered extremely conservative, as realistically, most foods will not contain *L. plantarum* LPLDL® due to the standards of identity of many foods, the fact that it will not be added to foods requiring additional USDA regulatory review, market share limitations, and the fact that the ingredient will likely be "invisible" to many consumers, who may realize they are consuming a fermented food but likely will not be aware of the specific strain that they are consuming, reducing the likelihood that only food products containing this strain will be chosen and consumed. If a more realistic (but still highly conservative) estimate that 50% of food servings will contain *L. plantarum* LPLDL®, males aged 51 and older would consume approximately 9.1 x 10^{10} CFU/day (using a standard 70 kg body weight, this is equivalent to 1.3 x 10^{9} CFU/kg bw/day).

Part 4: Self-limiting Levels of Use

There are no known inherent self-limiting levels of *L. plantarum* strain LP$_{LDL}$® use in foods.

Part 5: Experience Based on Common Use in Food Prior to 1958

The GRAS conclusion for *L. plantarum* strain LP$_{LDL}$® is based on scientific procedures, and thus, experience based on common use in food prior to 1958 is not considered pivotal information. Nevertheless, the historical use of foods fermented with LAB, *Lactobacilli* and specifically *L. plantarum*, is extensively discussed in Section 6.

Part 6: Narrative

6.1 History of Consumption

Lactic acid bacteria (or "milk-souring organisms" as they were called around the turn of the 20th century) have long been considered safe and suitable for human consumption.[4, 18, 20] The fermentation process has been used to produce a wide variety of foods from the earliest record of human food preservation, and *Lactobacilli* bacteria were among the first organisms to be used for this purpose.[5]

L. plantarum specifically has a known long history of safe human use as well. For example it has traditionally been used in the fermentation of cheese, kefir, sauerkraut, meats, vegetables, and beverages.[2, 5, 79] Around the world it is a part of various traditional fermented ethnic foods such as gundruk (Nepal), kishk (Egypt), koumiss (Kazakhstan), and Zincica (Czech Republic).[5] In agriculture, *L. plantarum* has long been used to preserve grass or maize in the form of silage.[5] In more recent times, *L. plantarum* has become commercially available as a probiotic dietary supplement, manufactured and sold by numerous companies.[2, 79] Overall it is one of the more prominent *Lactobacillus* species associated with this long history of human consumption.[79]

With regard to levels of intake from fermented foods, it has been estimated that fermented cheeses and milk contain at least 10^7 CFU of *Lactobacilli* per gram, but may be as high as 10^9 CFU/g and approaching 10^{10} CFU/g.[5, 77] Yogurt products containing at least 10^8 CFU/g at the time of manufacture in the United States may use the "live and active" seal from the National Yogurt Association.[77] Note that consumption of a 100 g serving of a fermented food containing $10^{8\text{-}10}$ CFU/g is equivalent to consuming $10^{10\text{-}12}$ CFU/serving. FDA's Reference Amount Customarily Consumed for yogurt is currently 170 g per serving.

L. plantarum is listed in the International Dairy Federation (originally a collaboration with the European Food and Feed Culture's Association)'s inventory of microbial species with technological beneficial roles in fermented food products, emphasizing again its long history of use.[80] *L. plantarum* is additionally a commensal bacterial species, found in the healthy human gastrointestinal, vaginal, and urogenital tracts.[2, 79] As stated previously, there are successful FDA GRAS and new dietary ingredient notifications for several unique L. *plantarum* strains, for intended uses in certain foods as well as in dietary supplements.

6.2 Past Sales of *L. plantarum* LP_{LDL}® and Reported Adverse Events

According to ProBiotix, *L. plantarum* LP_{LDL}® has been sold in different functional food products in Germany since July 2017, in New Zealand since May 2018, and in Spain, England, Greece, Cyprus, and Bulgaria since October 2018. No adverse events have been reported from consumers to date.

A search of the FDA Adverse Events Reporting System (FAERS Public Dashboard reveals only 1 case of an adverse event (no death) related to *L. plantarum* from 2010 (no further information is available about the report). FDA's Center for Food Safety and Applied Nutrition Adverse Event Reporting System did not uncover any specific mention of *L. plantarum* products. Databases were accessed on February 1, 2019.

6.3 Regulatory Opinions

6.3.1 Europe

The European Food Safety Authority (EFSA) has developed the Qualified Presumption of Safety (QPS) system for the assessment of microorganisms based on their taxonomic group, familiarity, pathogenicity, and end use. If a microorganism species is approved as QPS, new strains do not require further EFSA regulatory review prior to introduction into the food supply, other than satisfying any qualifications specified in the status. *L. plantarum,* along with numerous other members of the genus *Lactobacillus*, was included in the initial QPS review. The Scientific Committee concluded that the weight of evidence available was sufficient regarding safety for this species. Each subsequent QPS review evaluated the totality of current scientific evidence and reaffirmed the QPS status of *L. plantarum*. While there are occasional reports of lactobacillemia in the literature, EFSA determined that such rare cases primarily occurred in immunocompromised individuals, or those suffering from underlying illnesses, and the bacteria are not considered pathogenic to humans.

The only EFSA qualification for *L. plantarum* is the generic qualification for all QPS bacterial taxonomic units, which is that individual strains should not harbor any acquired antimicrobial resistance genes to clinically relevant antimicrobials.[81, 82]

6.3.2 United States

6.3.2.1 FDA GRAS

There have been a number of FDA GRAS notices related to members of the *Lactobacillus* genus, and three notices specifically related to use of *L. plantarum*, as described below.

- GRN 378: PURAC received FDA's no objection letter in 2012 for its cultured dairy sources, sugars, wheat, malt, and fruit- and vegetable-based sources fermented by various bacterial species including *L. plantarum*. The bacteria are removed during processing, so final bacterial levels are negligible.
- GRN 685: Probi AB received FDA's no objection letter in 2017 for its *L. plantarum* strain 299v. The intended use is as an ingredient in conventional foods at an addition level of up to 1 x 10^{11} CFU/serving to ensure that an actual intended use level of 1 x 10^{10} CFU/serving will be maintained throughout the shelf-life. The food categories include but are not limited to: wet chilled and ambient products such as fruit drinks, yogurts, milk; and plant-based products; dry chilled products; dry and shelf-stable products such as cereals, candy, bars, cookies, gums and confectionary. Exposure to the strain was estimated as 1 x 10^{10} CFU/day by assuming consumption of one serving of food containing the strain daily.
- GRN 722: Dupont received FDA's no objection letter in 2018 for its *L. plantarum* strain Lp-115 for use as an ingredient in conventional foods, including yogurt and other dairy products, soy products, beverages, chewing gum and confectionary snacks at an addition level of up to 5 x 10^{11} CFU/serving to ensure that an actual intended use level of 1 x 10^{10} CFU/serving will be maintained throughout the shelf-life. Exposure to the strain was estimated as 1 x 10^{11} CFU/day based on an estimated consumption of 10 servings per day, with a more realistic exposure estimated as 6 x 10^{10} CFU per day based the fact that individuals are unlikely to consume 10 servings per day as well as the fact that CFU will decline over the shelf-life of the product.

6.3.2.2 FDA New Dietary Ingredient Notifications

There have been a number of FDA New Dietary Ingredient (NDI) notifications related to members of the *Lactobacillus* genus, and two notifications that are specifically related to use of *L. plantarum* as a dietary supplement, as described below.

- RPT171: Kups International received FDA's acknowledgement with no comments letter in 2003 for its *L. plantarum* (ATCC 202195) product for use as a dietary ingredient, at an exposure of up to 8 x 10^{10} (80 billion) CFU per

serving, and a recommended use of one to two servings per day (i.e. up to 1.6 x 10^{11} CFU/day).

- RPT900: CJ Cheil Jedang received FDA's acknowledgement with no comments letter in 2016 for its *L. plantarum* CJLP133™ product for use as a dietary ingredient, at an exposure of up to 0.5 x 10^{10} CFU/serving, and a recommended use of up to two servings per day (i.e. up to 1 x 10^{10} CFU/day).

6.4 Safety Information

Literature on *L. plantarum* was summarized and evaluated by FDA for GRAS notices GRN 685 and GRN 722, which were submitted on December 20, 2016 and August 2, 2017, respectively. As we agreed with the publicly available safety data and conclusions summarized within those notices, they are only briefly mentioned in the current GRAS instead of being discussed in detail. New safety data published after August 2, 2017 were obtained through a comprehensive search of the scientific literature published through February 1st, 2019. Of note, EFSA also reviewed new published data on *Lactobacillus* species published through March 2018 (including 565 new papers).[82] While six articles were found by EFSA to raise possible safety concerns, none involved *L. plantarum*, and ultimately the findings did not result in the revoking of QPS status for any *Lactobacillus* species previously listed.

6.4.1 Toxicological Studies

As was described in GRN 722, Mukerji et al., (2016) conducted a 90-day repeated dose oral toxicity study on AB-LIFE (a product containing equal parts of three *L. plantarum* strains; CECT 7527, 7528, and 7529) in rats, at doses of 300 and 1000 mg/kg bw/day.[83] The NOAEL of the study was the highest dose tested, equivalent to 1.85 x 10^{11} CFU/kg bw/day.

Not described in previous GRAS notices, in 2018, Fareez et al. published an acute and 90-day repeated dose oral toxicity study in rats on microencapsulated *L. plantarum* LAB12 (a strain isolated from tempeh).[84] In the acute study, six male animals were given a single 1 x 10^{11} CFU/kg bw dose of the test article via gavage, and were observed for body weight, mortality, and detailed clinical observations weekly. On day 15, the rats were euthanized, and blood was collected for biochemical and hematological analyses. Vital organs were weighed and underwent macroscopic examination. No toxicologically relevant findings related to treatment were observed.

In the sub-chronic 90-day study, 6 rats/sex/group were fed with basal control diet, or control diet containing the test article, and doses were aimed at 1 x 10^8, 1 x 10^9 and 1 x 10^{10} CFU/kg bw/day.[84] Rats were observed for clinical signs twice daily, and body weights were measured once per week and on the day of necropsy. Food and water intake were recorded once weekly. Rats were euthanized on day 91, after urine was collected for urinalysis. Blood was collected for biochemical and

hematological analyses, and the heart, kidneys, lungs, liver, spleen, and gonads were weighed and utilized for gross and histopathological examinations. There was no mortality and no treatment related findings occurred in terms of clinical signs, body weight, water or food consumption, gross organ or histopathology. Liver weights were significantly decreased in the female mid- and high-dose groups and increased in the male high-dose group compared to controls, however the values were within the range of previously published control data, and no related histopathological findings were noted. While ALT and ALP were increased in male low dose and high dose rats, respectively, there was no specific indication of dose-response, no findings in females, and levels were within normal historical limits. With regard to hematology, the only finding was elevated platelet counts in the low- and high-dose males and low and mid-dose females compared to controls, however levels all fell within reference ranges. There were no differences in urinalysis results other than a significant rise in urobilinogen in high-dose males, however there were no other changes suggestive of effects on renal function within the urinalysis or clinical chemistry parameters, and there were no histopathological findings in the kidneys, thus the finding was considered incidental. The NOAEL for microencapsulated *L. plantarum* LAB12 was determined to be the highest dose tested at 2.5 x 10^{10} CFU/kg bw/day for both genders.

Pradhan et al.(2018) studied *L. plantarum* MTCC 5690 in a mouse colitis model, and found no pathogenicity or toxicity of the strain, even under the conditions of impaired intestinal permeability.[85] This same strain was studied in various toxicological experiments published in 2019.[86] A 14-day repeated dose oral toxicity study in Swiss albino mice given 4 x 10^{10} CFU/g bw/day by gavage resulted in no mortality and no toxicologically relevant findings compared to controls in clinical signs, weight gain, feed/water intake, organ weights, or in hematological, clinical chemistry, or histopathological examinations. No bacterial growth occurred when blood and tissue samples were plated.

The authors also performed 28-day and 90-day studies on *L. plantarum* MTCC 5690, although in both experiments, only a single treatment dose group was included. In both studies, two groups of eight male Swiss albino mice were administered 4 x 10^{7} CFU/g bw/day or sterile PBS as the control for 28- or 90-days, depending on the study, by gavage. Results of the 28-day study revealed no clinical signs, but slightly lower body weight gain in the treated group with no differences in food or water intake. There were no relevant variations in hematological or clinical chemistry parameters, or in macroscopic analysis of the organs. A statistically significant increase in liver weights was observed in the treated group compared to controls, although no differences were noted in relative body weights, suggesting the finding may have been due to overall animal weights. Results of the 90-day study also showed no clinical signs or differences in body weight or feed intake, although body weight gain was higher in the MTCC 5690 mice in this study. No hematological findings occurred, however treated mice had decreased total leucocyte count, SGOT and SGPT levels, which fell within historical control levels.

No differences in macroscopic examination or absolute/relative organ weights were noted. In both sub-chronic studies, no viable bacteria were detected in visceral swab or blood cultures, or from plated samples of organs. As the studies only contained a single dose group, a NOAEL could not be established, although no toxicological findings occurred in the study at the dose of 4 x 10^7 CFU/g bw/day (4 x 10^4 CFU/kg bw/day).

Numerous other animal studies have been published using *L. plantarum* strains as the test articles, and while they were not specifically designed as toxicological studies, they support the overall safety of this species by showing a lack of signs of adverse events under various conditions, doses, and periods of time.

6.4.2 Human Studies

The ProBiotix *L. plantarum* LP_{LDL}® strain was formally investigated in a randomized, double-blinded placebo-controlled clinical trial involving 49 healthy, normal to mildly hypercholesterolemic adults (ages 30 to 65 years), and included evaluation of various safety and tolerance outcomes.[46] The study was conducted by the University of Reading Department of Food and Nutritional Sciences. The subjects (34 females and 15 males) in the active group ingested 2 x 10^9 CFU encapsulated *L. plantarum* LP_{LDL}® twice daily (before breakfast and dinner) for 12 weeks, with a four-week washout period at the end of the study (clinical trials.gov ID: NCT03263104). The placebo control group received capsules containing maltodextrin and sucrose. The strain was well tolerated with no impact on gastrointestinal function (number of bowel movements or stool consistency) or other effects as recorded in a daily gastrointestinal symptom diary. No serious side effects were observed during the study. The active group showed no significant findings related to body weight, body mass index or waist circumference compared to the control group, and there was no impact on blood pressure or cholesterol markers that would be considered negative or of concern. There were also no differences between groups with regard to immune markers or fecal metagenomics (the microbiota composition in the feces did not significantly change over the 12 weeks) and blood and urine metabonomic profile analyses did not reveal significant changes related to intake of *L. plantarum* LP_{LDL}®.

GRN 685 describes 37 prospective human studies (nearly all double-blinded and placebo-controlled) on *L. plantarum* 299v, consisting of 1,502 individual subjects, with dosing up to 2 x 10^{11} CFU/day for as long as 90 days. No adverse effects were reported in any of the studies in healthy adults or children. In the 20 studies in which the strain was administered to compromised adults, it was generally well tolerated without adverse effects at levels up to 2 x 10^{11} for up to 56 days.

GRN 722 also summarizes what was considered a representative selection of clinical studies on *L. plantarum* found in the literature (~19 studies). Doses in the studies were up to 2.5 x 10^{10} CFU/day in both adults and children, with a duration

of up to 12 weeks. Overall the *L. plantarum* test articles were well tolerated with no significant side effects attributed to intake.

Clinical trials have been published on various *L. plantarum* strains since the literature reviews of the two recent GRAS notices' literature searches. A summary of these published studies (August 2, 2017 through February 1st, 2019) is provided in Table 10 below. Overall, the results of these studies support a conclusion that intake of *L. plantarum* strains do not result in adverse effects.

Table 10. Summary of Recent Human Clinical Trials

	Dose & Description	Length	# of Subjects and Study Type	Condition	Comments (Results)
Lim, 2018[87]	Synbiotic treatment (1 x 10^{10} CFU/day of *L. plantarum* LP01 and *Bifidobacterium lactis* BB12 plus inulin-oligofructose)	12 weeks	N=85 (Adults) Randomized, double-blind, placebo-controlled trial	Constipation	Adverse events were not mentioned or discussed; the test article appeared to be well tolerated.
Abbasi et al., 2017[88]	Treatment with two probiotics, including 1 x 10^9 CFU/day of *L. plantarum* CETC7879, in addition to standard medical treatment for the infection.	10 days	N=209 (Adults) Randomized, double-blind, placebo-controlled trial	*Helicobacter pylori* infection	There were no significant differences in adverse events reported by subjects in the active versus placebo groups.
Nielsen et al., 2018 Date[89]	75 g/day of pasteurized or unpasteurized lacto-fermented sauerkraut dietary consumption (fermentation was dominated by natural *L. plantarum*)	6 weeks	N=34 Randomized, double-blind, placebo-controlled trial	Irritable bowel syndrome	Irritable bowel syndrome symptoms improved, side effects that led to drop-outs related to treatment were similar in number in both the pasteurized and unpasteurized groups.
Panigrahi et al., 2017[90]	Synbiotic treatment (~ 1 x 10^9 CFU/day) *L. plantarum* ATCC-202195 plus fructooligosaccharide)	7 days	N=4,556 newborn infants Randomized, double-blind, placebo-controlled trial	Prevention of sepsis	The preparation was well tolerated.
Toshimitsu et al., 2018[91]	Heat-killed *L. plantarum* OLL2712 cells (1 x 10^{10} CFU/day)	12 weeks	N=34 Open study	Insulin resistance	No adverse events were recorded in study diaries.

6.5 Opportunistic Infections

Infections caused by species of the commensal *Lactobacillus* genus have been described in the literature, but occur at a very low rate (estimated as one case per 10 million people, which has been considered "unequivocally negligible") and are rarely fatal.[1, 20, 92] Underlying disease and/or immunosuppression are commonly noted in such cases, and they are usually caused by the translocation of microbiota after medical/surgical procedures.[20, 92, 93] Most reported cases have been of

bacteriemia or endocarditis, and have been secondary to *L. rhamnosus* GG or *L. casei*.[5, 93] Adawi et al. (2002) tested the potential implication of *L. plantarum* in endocarditis by inoculating a rat model of the disease with 10^8 CFU of *L. plantarum* DSM 9843 with a catheter directly into the lumen of the left cardiac ventricle. After 96 hours of the intervention, none of the animals died and there was no *L. plantarum* in the treated hearts.[94] Therefore, as a member of that species, *L. plantarum* LP_{LDL}® has a very low likelihood to behave as an infectious or pathogenic agent. There is overall little evidence that ingested bacteria pose a risk of infection greater than that associated with endogenous commensal strains.[5, 95]

6.6 Allergenicity

L. plantarum LP_{LDL}® does not contain or have added, and is manufactured in a facility free of, all eight major allergens (milk, egg, fish, Crustacean shellfish, tree nuts, wheat, peanuts, and soybeans) identified, and required to be disclosed in labeling, in the Food Allergen Labeling and Consumer Protection Act (FALCPA). Additionally, *L. plantarum* LP_{LDL}® does not contain gluten, celery, mustard, sesame seeds, sulfur dioxide and sulfites or lupin, or any products of the aforementioned.

6.7 Data and Information that are Inconsistent with the GRAS Conclusion

We have reviewed the available data and information and are not aware of any data and information that are, or may appear to be, inconsistent with our conclusion of GRAS status.

6.8 Information that Privileged or Confidential

There are no data or information in this report that are considered trade secret or commercial or financial information that is privileged or confidential.

6.9 Basis for the GRAS Conclusion

ProBiotix's *L. plantarum* LP_{LDL}® has been the subject of a thorough safety assessment as described above. The totality of evidence supporting safety is comprised of data and information that establish the safety of *L. plantarum* LP_{LDL}® under the conditions of its intended use and data and information that is corroborative of safety. The general availability and general acceptance, throughout the scientific community of qualified experts, of the data and information that establish the safety of *L. plantarum* LP_{LDL}® under its intended conditions of use establish the general recognition of this data and information. Together, the establishment of safety based on scientific procedures and its general recognition

form the basis for ProBiotix's conclusion of GRAS status of *L. plantarum* LP_{LDL}® for its intended use.

6.9.1 Data and Information that Establish Safety

The scientific data, information, and methods forming the basis of this conclusion are:

- The establishment of LP_{LDL}®'s identity via whole genome sequencing including the 16S rRNA gene, demonstrating unequivocally that it is a strain of the *L. plantarum* species, with established phenotypic characteristics;
- *L. plantarum* LP_{LDL}® lacks resistance to clinically relevant antibiotics, as is demonstrated both phenotypically and genotypically, and also lacks known genes related to virulence and pathogenicity;
- The methods of manufacture, specifications, and batch analyses, demonstrating the safe production and the high quality control standards for *L. plantarum* LP_{LDL}®;
- The intended use as an ingredient in foods at an addition level of up to 1 x 10^{10} CFU per serving, which is in line with addition levels for other GRAS microbial ingredients as well as with levels of *Lactobacilli* found naturally in various fermented foods, with an estimated exposure of 9.1 x 10^{10} CFU/day (1.3 x 10^{9} CFU/kg bw/day) by conservatively assuming consumption in 50% of all food servings daily;
- The documented long history of safe human (and livestock) consumption of *L. plantarum* as a common bacterial species in fermented foods;
- *L. plantarum*'s history of EFSA QPS status, suggesting no further regulatory review prior to introduction of new strains into the European food supply, other than the qualification that it may not harbor acquired antimicrobial resistance genes;
- Agreement in the literature that it is highly unlikely that a microorganism maintained in pure culture, with a history of safe use, would become unsafe as a result of mutation, production changes, or delivery format changes;[96-98]
- US FDA GRAS notices GRN 685 and 722 for two different *L. plantarum* strains received no objection letters related to safety conclusions for their specified intended uses with exposure estimates ranging from 1 x 10^{10-11} CFU/day, and two NDI notifications for *L. plantarum* strains for use in dietary supplements at exposures up to 1.6 x 10^{11} CFU/day were also filed without comment;
- Published sub-chronic repeated dose oral toxicity studies in rodents on several *L. plantarum* strains, with NOAELs of the highest levels tested, up to 1.85 x 10^{11} CFU/kg bw/day;
- Published clinical trials demonstrating no adverse effects of consumption of various *L. plantarum* strains at levels of up to 2 x 10^{11} CFU/day for up to 90 days.

In the 90-day toxicological study by Mukerji et al. (2016) on three *L. plantarum* strains, the NOAEL was the highest level tested at 1.85 x 10^{11} CFU/kg bw/day in male and female rats. Based on the extremely conservative exposure estimate for *L. plantarum* LP_{LDL}® of 1.3 x 10^{9} CFU/kg bw/day, the NOAEL allows for an adequate margin of safety (NOAEL/Exposure; 1.85 x 10^{11} CFU/kg / 1.3 x 10^{9} CFU/kg) of 142. The NOAEL of the ninety-day study by Fareez et al. (2018) was also the highest level tested in the study (2.5 x 10^{10} CFU/kg bw/day), which adds additional support to safety, even though the highest level tested was lower than that in the Mukerji et al. study.

6.9.2 Data and Information that is Corroborative of Safety

Data and information that are corroborative of safety include additional phenotypic characterization of *L. plantarum* LP_{LDL}® (the strain is reasonably sensitive to gastric acidity but resistant to bile salts, does not produce significant biogenic amines, and produces levels of D- and L-lactate that are similar to other *L. plantarum* strains). The original isolation of the strain from fruit juice fermentation also suggests a history of safe consumption. The published human study on ProBiotix's *L. plantarum* LP_{LDL}® at a consumption level of 4 x 10^{9} CFU/day showed that the strain was well tolerated with no impact on gastrointestinal function or any noted side effects, which, even though doses were lower than the current intended uses, adds to the totality of safety evidence.

6.9.3 General Recognition

The scientific data, information, and methods herein reported, that provide the basis of this GRAS conclusion by scientific procedures are published and available in the public domain. Part 7 of this GRAS notice contains the citations for the published studies. These publicly available data and information fulfill the requirement of the GRAS standard for general availability of the scientific data, information, and methods relied on to establish the safety of *L. plantarum* LP_{LDL}® for its intended conditions of use. The peer-review of the published studies and lack of Letters to the Editor or other dissenting opinions provide ample evidence of general recognition among qualified experts that there is reasonable certainty that consumption of *L. plantarum* LP_{LDL}® for its intended use is not harmful. The general availability and acceptance of these scientific data, information, and methods satisfy the criterion of the GRAS standard that general recognition of safety requires common knowledge throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food that there is reasonable certainty that the substance is not harmful under the conditions of its intended use.

Part 7: Supporting Data and Information

Literature searches for the safety assessment described in Part 6 of this GRAS notice were conducted through February 1, 2019.

7.1 Data and Information that are *not* Generally Available

All of the data and information described in this GRAS notice, other than the tests characterizing the *L. plantarum* LP_{LDL}® strain described in Part 2, are generally available.

7.2 References that *are* Generally Available

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