JHeimbach LLC

September 15, 2022

Susan J. Carlson, Ph.D., Director
Office of Food Additive Safety (HFS-200),
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Dr., College Park, MD 20740

Dear Dr. Carlson:

Pursuant to 21 CFR Part 170, Subpart E, Advanced Enzyme Technologies, Ltd., through me as its agent, hereby provides notice of a claim that the addition of *Lactiplantibacillus plantarum* 022AE to conventional foods is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because Nestle Nutrition has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

A virus-free CD is enclosed containing Form 366 and the GRAS monograph in a zip directory produced through COSM.

If you have any questions regarding this notification, please feel free to contact me at 202-320-3063 or jh@jheimbach.com.

Sincerely,

[redacted]

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

Encl.

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Generally Recognized as Safe (GRAS) Determination for ProbioSEB LP 022AE (*Lactiplantibacillus plantarum* 022AE)

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L.I.C. Service Road, Louiswadi,
Thane (W) – 400 064, INDIA

Edited by: JHeimbach LLC

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List of Abbreviations

%	Percentage
µg	Microgram
µm	Micrometer
ACLAME	A Classification of Mobile Genetic Elements
ADI	Acceptable Daily Intake
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSL-1	Biosafety level 1
bw	Body weight
°C	Degrees Celsius
CARD	Comprehensive Antibiotic Resistance Database
CFR	Code of Federal Regulations
CFU	Colony Forming Units
cGMP	Current Good Manufacturing Practice
CLSI	Clinical and Laboratory Standards Institute
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
d	Day
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
FASTER	Food Allergy Safety, Treatment, Education, and Research
FDA	U.S. Food and Drug Administration
FSSAI	Food Safety and Standards Authority of India
g	Gram
GI	Gastrointestinal
GRAS	Generally Recognized as Safe
GRN	GRAS Notice
h	Hour
HACCP	Hazard Analysis and Critical Control Points
kg	Kilogram
LD_{50}	Median Lethal Dose
mg	Milligram
MIC	Minimum Inhibitory Concentration
mL/ml	Milliliter
n	Number

NAv	Not Available
NCBI	National Center for Biotechnology Information
NCMR	National Center for Microbial Resources
NLT	Not Less Than
NMT	Not More Than
NOAEL	No Observed Adverse Effect Level
NR	Not Required
OECD	Organization for Economic Co-operation and Development
P. aeruginosa	*Pseudomonas aeruginosa*
QPS	Qualified Presumption of Safety
R	Resistant
RH	Relative Humidity
RNA	Ribonucleic Acid
S	Susceptible
S9	Metabolic Activation System or Metabolic Activator
USDA	United States Department of Agriculture
USP	United States Pharmacopeia
VFDB	Virulence Factor Database
EP	European Pharmacopeia
JP	Japanese Pharmacopeia
IP	Indian Pharmacopeia
AOAC	Association of Official Analytical Collaboration
BLASTX	Basic Local Alignment Search Tool- searches protein database using a translated nucleotide query
BLASTN	Basic Local Alignment Search Tool-searching translated nucleotide database using a protein query
ATCC	American Type Culture Collection
FOIA	Freedom of Information Act
FSIS	Food Safety and Inspection Service
rRNA	Ribose RNA/ Ribose ribonucleic acid
tRNA	Transfer RNA/ transfer ribonucleic acid
BLAST	Basic Local Alignment Search Tool
PGAP	Prokaryotic Genome Annotation Pipeline
CIA	Critically important antimicrobials
HIA	Highly important antimicrobials
GABA	Gamma-Aminobutyric acid
EPA	Environmental Protection Agency
COG	Clusters of Orthologous Groups of proteins
ISO	International Organization for Standardization

LDPE	Low Density Polyethylene
HDPE	High-Density Polyethylene
LAB	Lactic Acid Bacillus
FAO	The Food and Agriculture Organization
WHO	World Health Organisation
IDF	The International Dairy Federation
EFFCA	European Food and Feed Cultures Association
USA	United States of America
FSANZ	Food Standards Australia New Zealand
GLP	Good Laboratory Practice
TOS	Total Organic Solids
PCEs	Polychromatic erythrocytes
CHO	Chinese hamster ovary
DSS	dextran sodium sulfate
MTCC	The Microbial Type Culture Collection and Gene Bank
ICU	Intensive Care Unit
AIDS	Acquired Immune Deficiency Syndrome
HIV	Human Immunodeficiency Virus
WBC	White Blood Cells
RBC	Red Blood Cells
IBS-D	Irritable Bowel Syndrome with Diarrhea

Part 1. 21 CFR 170.225: Generally Regarded as Safe (GRAS) Notice-Exemption Claim

1.1 Exemption Claim for *Lactiplantibacillus plantarum* 022AE

Advanced Enzymes Technologies Ltd. submits this GRAS notice in accordance with 21 CFR part 170, subpart E. Advanced Enzyme Technologies, Ltd. has concluded that *Lactiplantibacillus plantarum* 022AE is GRAS by scientific procedures in accordance with both 21 CFR 170.30 (a) and (b) and is thereby exempt from pre-market approval requirements of the Food, Drug and Cosmetic Act.

1.2 Name and Address of Notifier

APPLICANT

Name:	Advanced Enzyme Technologies Ltd.
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PERSON RESPONSIBLE FOR THE DOSSIER

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Country:	India
Tel. no:	+91 22 25830284
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AGENT WHO IS AUTHORIZED TO ACT ON BEHALF OF THE NOTIFIER

Name:	James T. Heimbach, Ph.D., F.A.C.N. JHeimbach LLC
Address:	923 Water Street #66
Postal code and City:	Port Royal, Virginia 22535
Country:	USA
Tel. no:	+1 804-742-5543
E-mail:	jh@jheimbach.com

1.3 Name of Notified Microorganism

'*Lactiplantibacillus plantarum* strain 022AE'. '022AE' is the designation of the proprietary *Lactiplantibacillus plantarum* strain of Advanced Enzyme Technologies Ltd. The strain is deposited at the National Centre for Microbial Resources (NCMR), India, under accession number MCC0537. *Lactiplantibacillus plantarum* is the new name for the bacterium previously identified as *Lactobacillus plantarum*.

The product *Lactiplantibacillus plantarum* 022AE is a microbial culture. Commercial preparations are known as ProbioSEB LP 022AE, BioSEB LP, ProFood LP, LP 22AE, SEBPlantarum, SEB LP.

In this GRAS notice, the *Lactiplantibacillus plantarum* strain 022AE is referred to by names such as *'L. plantarum* 022AE'*; 'Lactiplantibacillus plantarum* 022AE*'*, '*Lactiplantibacillus plantarum* 022AE (MCC 0537)',and '*Lactobacillus plantarum* 022AE'.

1.4 Intended Conditions of Use

L. plantarum 022AE is intended to be used in the following food categories:

Yogurt, other milk based products, dairy analogs, soy products, fruit drinks, frostings, frozen dairy desserts and mixes, fruit and water ices, drinking water, sports drinks, chewing gum, extracts, and flavorings, condiments, herbs, seeds, spices, seasonings, blends, nuts and nut products, plant-protein products, cereals, processed fruits, processed vegetables and vegetable juices, snack foods, and toppings at a maximum level of approximately 1.0×10^9 to 10×10^9 colony forming units (CFU)/serving.

Based upon the estimated number of servings of food consumed per day, i.e. 18.2, in the US and the highest intended addition level of *L. plantarum* 022AE per serving of 10 billion CFU, the estimated daily intake (EDI) of the strain is 1.82×10^{11} CFU/day. This EDI would be reached only if all target foods indeed contained *L. plantarum* at the maximum addition level.

The intended use of *L. plantarum* 022AE is similar to those stated for other *L. plantarum* strains i.e., GRN 685, 722, 847 and 953 to which FDA has no objection. It therefore provides an alternate source of the microbial culture added to these foods but would not result in any change in exposure to the species.

L. plantarum 022AE is not intended for use in foods that are targeted toward infants, such as infant formulas or foods formulated for infants, nor in meat and poultry products that come under USDA jurisdiction.

1.5 Statutory Basis for GRAS Status

Advanced Enzyme Technologies, Ltd., has determined that the intended use of *L. plantarum* 022AE is GRAS through scientific procedures in accordance with 21 CFR §170.30(a) and (b).

1.6 Premarket Exempt Status

Since Advanced Enzyme Technologies Ltd. has determined that the intended use of *L. plantarum* 022AE is GRAS, the use of the notified substance is exempt from pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act.

1.7 Data Availability

Advanced Enzyme Technologies Ltd. agrees to make the data and information that are the basis for the determination of GRAS status available to FDA upon request. Such data and information may be sent by Advanced Enzyme Technologies Ltd. to FDA either in electronic format or on paper or reviewed during customary business hours at the home office of JHeimbach LLC, located at 923 Water Street, Port Royal VA 22535.

1.8 FOIA Statement

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

1.9 Certification

To the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information as well as favorable information known to the notifier and pertinent to the evaluation of the safety and GRAS status of the intended use of *L. plantarum* 022AE.

1.10 FSIS Statement

Not applicable.

1.11 Signature of Responsible party/ Agent

James T. Heimbach, Ph.D., F.A.C.N.
President, JHeimbach LLC
Agent to Advanced Enzyme Technologies Ltd.

Part 2. 21 CFR 170.230: Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity/Identification

The substance that is covered in this GRAS notification is a preparation of *L. plantarum* 022AE isolated from fermented dairy milk. The diluents used in the manufacturing of *L. plantarum* 022AE are approved as either food additives or GRAS substances.

2.1.1 *SCIENTIFIC NAME, TAXONOMY AND OTHER NAMES*

Name of the food ingredient: *L. plantarum* 022AE

Synonyms: *Lactobacillus plantarum* strain 022AE/ *L. plantarum* 022AE/ *Lactiplantibacillus plantarum* 022AE

Taxonomy:

Kingdom: Bacteria

Phylum: Firmicutes

Class: Bacilli

Order: Lactobacillales

Family: Lactobacillaceae

Genus: *Lactiplantibacillus*

Species: *plantarum*

Strain: 022AE

2.1.2 *DESCRIPTION/SOURCE INFORMATION AND GENOTYPIC, PHENOTYPIC CHARACTERIZATION OF THE ORGANISM*

L. plantarum 022AE is a nonpathogenic, non-toxicogenic, non-spore-forming bacterium that was isolated from fermented milk. *L. plantarum* 022AE preparation is off-white to brown colored powder having a total viable count not less than 400 billion CFU/g. *L. plantarum* 022AE is deposited at the National Centre for Microbial Resources (NCMR), India, with deposit number MCC 0537.

2.1.2.1 Genotypic Characterization

Genotypic characterization of *L. plantarum* 022AE was carried out following 16S rRNA and *phe*S gene analysis and whole genome sequencing of the microorganism. The *L. plantarum* 022AE genome is sequenced and analyzed for safety. Whole-genome information was deposited in the NCBI/GenBank database under the accession number CP031127. The parameters described below were assessed to establish the safety of *L. plantarum* 022AE.

a) 16S rDNA

L. plantarum 022AE was identified following the *phe*S gene and 16S rRNA genes as phylogenetic markers. As 16S rRNA gene sequence alone does not allow the correct identification of closely related species, the housekeeping *phe*S gene, which encodes the phenylalanyl-tRNA synthase alpha subunit, was selected as an alternative phylogenetic marker. The bacterial culture 022AE was identified as *L. plantarum* by BLAST analysis of the 16S rRNA and *pheS* sequences.

b) Whole Genomic Sequencing

Hybrid assembly of the sequenced genome was generated from Ilumina and Nanopore reads using MaSuRCA Hybrid Assembler (Aleksey et al., 2013). *L. plantarum* HAC01strain was used as a reference genome. The final genome assembly was 3,234,271 bp in size with 44.55% GC content. Gene prediction was done for the assembled genome using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). The whole-genome data was deposited in the NCBI/GenBank database with accession number CP031127.

The assembled genome of *L. plantarum* 022AE was compared with other bacterial genomes present in the Ref Seq genome database using NCBI-BLASTN (Altschul et al., 1990) considering *L. plantarum* (taxid:1590) as references database for NCBI-BLASTN. The BLASTN results indicated 100% sequence homology of the *de-novo* assembled genome with the genome of the reference strain *L. plantarum* HAC01.

c) Determination of mol G+C%

The genomic DNA G+C content, defined as the proportion of guanines and cytosines within the overall number of nucleotides in the genome is one of the features in taxonomic descriptions of micro-organisms (Meier-Kolthoff et al., 2014). The mol % G+C for *L. plantarum* 022AE genome assembly of 3,234,271 bp in size equals 44.55%. Similar results were also reported by Florez A. B and Mayo B, 2018; Goel A et al., 2020 for the strains of *L. plantarum* LL441, DKP1 and DHCU 17.

d) Safety assessment concerning antibiotic resistance genes

A homology search between the assembled genome of *L. plantarum* strain 022AE and antibiotic resistance genes/proteins was performed using the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017). In this case, BLASTX was used with the criteria (similarity >30%, coverage >70% and e-value < 1e-02) for the identification of significant hits. Critically important antimicrobials (CIAs) or highly important antimicrobials (HIAs), as per WHO, 2016 and EFSA, 2012, were screened in the data, which was analyzed post homology alignment of the assembled genome of strain 022AE and CARD.

Antibiotic resistance genes identified on the genome were: tetracycline resistance MFS efflux pump (1), chloramphenicol acetyltransferase (DUT87_14410) (1) and daunorubicin resistance protein (1). These genes are inherent to the species and hence referred to as intrinsic resistance (Zang et al., 2012, https://www.uniprot.org/uniprot/A0A1E3KSF9).

The presence/absence of mobile elements in the flanking regions of these genes was analyzed within the assembled genome using ISfinder web-based software (Siguier et al., 2006) and ACLAME database (Leplae et al., 2009), and through the homology search against the assembled genome. None of the genes coding for or contributing to the resistance to antimicrobials had mobile elements in their flanking regions; therefore, the presence of these genes associated with antimicrobial resistance does not pose any safety concerns.

To support the genotypic analysis on antimicrobial resistance, a phenotypic analysis on *L plantarum* 022AE was carried out to assess its antibiotic sensitivity/resistance against seven antibiotics (EFSA 2012) viz., ampicillin, gentamicin, kanamycin, erythromycin, clindamycin, tetracycline, and chloramphenicol. The minimum inhibitory concentration (MIC) values obtained for *L. plantarum* 022AE were below or equal to the breakpoint values as described by EFSA (2012) for the tested antibiotics. *L. plantarum* 022AE was sensitive to all the tested antibiotics including chloramphenicol, despite the presence of chloramphenicol acetyltransferase, which indicates that the gene responsible for the chloramphenicol resistance is non-functional. Similar results were also reported by Zang et al., 2012 for the strain *L. plantarum* JDM1.

e) Analyses of risk associated with virulence factor genes

Virulence factor genes/proteins were downloaded from the Virulence Factor Database (VFDB) (Chen et al., 2004). A homology search between the assembled genome of *L. plantarum* strain 022AE and virulence factor proteins was performed using BLASTX (criteria: similarity >30%, coverage >70% and e-value < 1e-02) to identify significant hits. A total of 369 proteins showed resemblance with virulence factor proteins by the COG database. These proteins belonged to the following functions: inorganic ion transport and metabolism (65), defense mechanisms (60), cell wall/membrane/envelope biogenesis (44), signal transduction mechanisms (34), lipid transport and metabolism; secondary metabolites biosynthesis, transport and catabolism; general function prediction only (25), posttranslational modification, protein turnover, chaperones (22), cell motility; intracellular trafficking, secretion, and vesicular transport (20), inorganic ion transport and metabolism; coenzyme transport and metabolism (19), signal transduction mechanisms; transcription (24), amino acid transport and metabolism (13), carbohydrate transport and metabolism; cell wall/membrane/envelope biogenesis (13), cell cycle control, cell division, chromosome partitioning (7), lipid transport and metabolism (6), cell wall/membrane/envelope biogenesis; carbohydrate transport and metabolism (4), lipid transport and metabolism; secondary metabolites biosynthesis, transport and catabolism (2), amino acid transport and metabolism; signal transduction mechanisms (1), cell wall/membrane/envelope biogenesis; intracellular trafficking, secretion, and vesicular transport (1), cell wall/membrane/envelope biogenesis; translation, ribosomal structure and biogenesis (1), energy production and conversion (1), nucleotide transport and metabolism (1), posttranslational modification, protein turnover, chaperones; intracellular trafficking, secretion, and vesicular transport (1), replication, recombination and repair (1) and general function prediction only (4).

Though multiple putative virulent factor genes were identified, they cannot be considered

harmful, as a majority of them are related to transport mechanisms. No invasion or toxin proteins, which are offensive virulence factors, were identified. Also, few of the genes identified were related to the extracellular structures which could be correlated to the adhesion property, a trait required for adhesion of the microorganisms. *Lactobacilli,* in general, lack tissue-destructive functions and genuine virulence factors, and the overall result of their interactions with the host are generally beneficial for the host (Lebeer et al., 2008).

To further confirm the non-virulence nature of the strain 022AE, *in vitro* cytotoxicity testing against Vero cells was carried out (EFSA, 2018). The fluorescence values observed for the samples from *L. plantarum* 022AE were less than 20% compared with the fluorescence of the positive control, indicating that the strain does not have any cytotoxic effect. (Refer to section 2.1.4.)

f) Identification of genes involved in biogenic amine production

Protein sequences of the biogenic amine producing genes (amino acid decarboxylase) were downloaded from Uniprot database. BLASTX between the assembled genome and biogenic amine producing proteins resulted in the identification of one glutamate decarboxylase (DUT87_06365). The presence of glutamage decarboxylase was also reported for *L. plantarum* JDM1 strain by Zhang et al., 2012. Evanovich et al., 2019 in their study on comparative genome analysis of *L. plantarum,* observed that the gene encoding glutamate decarboxylase is common to all the studied *L. plantarum* strains. Glutamate decarboxylase (l-glutamate-1-carboxylase, GAD; EC 4.1.1.15) is a pyridoxal-5'-phosphate-dependent enzyme that catalyzes the irreversible α-decarboxylation of l-glutamic acid to γ-aminobutyric acid (GABA) and carbon dioxide. GABA is a non-protein amino acid with several well-characterized physiological functions (Li and Cao, 2010). Toxicology trials on GABA indicated that it does not produce any toxic effects in terrestrial animals including humans, rats, and mice (EPA Publication Number: EPA 730-F-98-019). *L. plantarum* 022AE, therefore, does not comprise any genes of concern that may result in biogenic amines.

g) Identification of mobile elements in the assembled genome

Mobile elements are DNA sequences that can move around the genome by changing their copy number or simply by changing their location, often affecting the activity of nearby genes. In the present study, mobile elements were predicted from the assembled genome by using web-based software, ISfinder. In addition, all the nucleotide sequences, including plasmids, viruses, and prophages were downloaded from the ACLAME database (version 0.4). A homology search (BLASTN) was performed between the downloaded nucleotide sequences (1,25,190) from the above-mentioned database and the assembled genome. A total of 119 regions in the assembled genome had a significant hit (coverage >=50% and e-value <=1e-05) against the nucleotide sequences (i.e. mobile elements) downloaded from the ACLAME database. A total of 25 insertion sites (11 repeat sequences of ISP2, 1 repeat sequence of IS153, and 1 ISLmo8) were identified in the assembled genome. ISP2 encodes for transposase-encoding region, IS153 transposases encode bacterial insertion sequences belonging to the IS3 family. None of the regions of concern,

such as antibiotic resistance genes, virulence factor genes, and genes leading to biogenic amine production were observed in the vicinity of the predicted mobile elements in the assembled genome, thus ensuring the stability of the genome and constant safe use of the strain.

h) Identification of genes involved in putatively adverse metabolite

Gene mining was performed to identify genes responsible for enzymes involved in the formation of putatively adverse metabolites or side effects as described by Zang et al., 2012. These genes encode the enzymes beta-glucosidase, arylsulfatase, beta-glucuronidase, nitroreductase, FMN-dependent NADH-azoreductase, D-lactate dehydrogenase, conjugated bile salt hydrolase and bile salt 7-hydroxylase (BHX). The assembled genome of *L. plantarum* 022AE contained 9 beta-glucosidase (DUT87_03505, DUT87_03510, DUT87_04565, DUT87_06775, DUT87_05085, DUT87_06790, DUT87_06795, DUT87_09030, DUT87_12850), 4 nitroreductase (DUT87_02785, DUT87_04385, DUT87_07390 and DUT87_07440), 2 azoreductase (DUT87_07495 and DUT87_10960), 2 D-lactate dehydrogenase (DUT87_10630 and DUT87_15500), and 4 conjugated bile salt hydrolase (DUT87_02720, DUT87_06185, DUT87_06835 and DUT87_07460) genes. The assembled genome did not contain genes related to arylsulfatase and beta-glucuronidase. *L. plantarum* is a flexible and adaptive strain present in a variety of environmental niches, resulting in the presence of a relatively large number of metabolic enzymes. However, there is no evidence for any adverse reactions reported for the metabolites resulting from the activities of enzymes encoded by the aforementioned genes in *L. plantarum* (Zang et al., 2012)

i) Identification of CRISPR associated regions in assembled genome

The assembled genome of *L. plantarum* 022AE was screened for the presence of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) sequences using CRISPRCas Finder (Couvin et al., 2018). CRISPRs are direct repeats found in the DNA of many bacteria (~40% of sequenced bacterial genomes) and range between 23-47 bp in length. Each of these repeats is separated by spacers of similar lengths unique in each of the genomes. These spacers indicate the non-coding region of genomic sequences between the genes. Four CRISPRs were identified along with 1 Cas gene in the assembled genome. The presence of the CRISPR system indicates an advantage in promoting genome stability by acting as a barrier to the entry of foreign DNA elements.

Conclusion

The *de novo* assembled genome of *L. plantarum* 022AE, generated without gaps, is a single scaffold of 3,234,271 bp. The *de novo* assembled genome of *L. plantarum* 022AE showed 100% sequence homology with the reference strain *L. plantarum* strain HAC01. The assembled genome was annotated against several databases such as Uniprot, CARD, VFDB, KAAS, and COG. The genome analysis for antimicrobial resistance genes, virulence factors, biogenic amines, and prophage sequences showed no safety concern.

The genes conferring resistance to tetracycline, chloramphenicol, and daunorubicin were identified on the genome; however, these genes are inherent to the species and considered intrinsic and non-transferable as no mobile elements were found in their vicinity. During phenotypic analysis of antibiotic sensitivity/resistance for *L. plantarum* 022AE, sensitivity to all tested antibiotics, along with chloramphenicol, was observed, suggesting that the chloramphenicol acetyltransferase gene is non-functional.

Multiple putative virulence-factor genes, identified through the VFDB, are not harmful as the majority of them are related to transport mechanisms and extracellular structures. *L. plantarum* 022AE does not contain any biogenic amine producing genes of concern. The presence of CRISPR sequences in the assembled genome indicates an advantage in promoting genome stability by acting as a barrier to the entry of foreign DNA elements.

In conclusion, *L. plantarum* 022AE does not contain any sequences/genes in the genome that are risk associated, thus confirming the safety of the strain through the genome-based approach.

2.1.2.2 Phenotypic and Biochemical characterization

L. plantarum 022AE is a Gram positive, rod shaped non-motile bacterium. Cells appear as single rods, in pairs, and/or in long chains. The cell size ranges from 0.9 µm–1.2 µm in width and 1 µm–2 µm in length. Colonies of *L. plantarum* 022AE are white, raised, and smooth.

Biochemical studies were carried out on *L. plantarum* 022AE according to the tests cited in *Bergey's Manual of Systematics of Archaea and Bacteria* (Hammes and Hertel, 2015). *L. plantarum* 022AE was observed to be positive for methyl red, oxidase, casein hydrolysis, bile degradation tests, nitrate reductase enzymes, and L (+) lactic acid production. The strain showed a negative result for indol, catalase, Voges-Proskauer, citrate, urease, gelatinase, starch, haemolysis, and lecithinase. In the triple-sugar iron test, the strain showed no gas or hydrogen sulfide production with the butt and slant both turning yellow (acidic).

L. plantarum 022AE was able to ferment D-glucose, sucrose, lactose, maltose, dextrin, mannitol, xylose, rhamnose, D-fructose, D-galactose, D-mannose, D-trehalose, and L-arabinose.

The results of biochemical tests of *L. plantarum* 022AE strain were comparable to the reference strain of *L. plantarum* ATCC 8014 as presented in Table 1. These analyses further confirm the identity of the strain *L. plantarum* 022AE.

Table 1. Results of Morphological and Biochemical Tests

Test	Results	
	***L. plantarum* 022AE**	***L. plantarum* ATCC 8014**
Colony Characteristics	Colonies are white, raised, and smooth	Colonies are white, raised, and smooth
Gram Staining	Gram Positive	Gram Positive
Cell Morphology	Non-motile, rod-shaped cells	Non-motile, rod-shaped cells
Size	Cell size 0.9 µm–1.2 µm in width and 1 µm–2 µm in length	Cell size 0.9 µm–1.2 µm in width and 1 µm–2 µm in length
Arrangement	Single cells, in pairs, or in long chains	Single cells, in pairs, or in long chains
Catalase Test	Negative	Negative
Oxidase Test	Positive	Positive
Nitrate Reduction Test	Positive	Variable
Endospore stain	Absent	Absent
Indole Test	Negative	Negative
Methyl Red Test	Positive	Positive
Voges-Proskauer Test	Negative	Negative
Citrate Utilization Test	Negative	Negative
Urease Test	Negative	Negative
Triple Sugar Iron (H_2S) Test	No production of hydrogen sulfide, no gas production, yellow slant and butt	No production of hydrogen sulfide, no gas production, yellow slant and butt
Gelatin hydrolysis Test	Negative	Negative
Casein hydrolysis Test	Positive	Positive
Starch hydrolysis Test	Negative	Negative
Hemolysis test	Negative	Negative
L(+) Lactic acid	Positive	Positive
Lecithinase production	Negative	Negative
Bile degradation	Positive	Positive
Sugar Fermentation Tests		
D-Glucose	Acid produced, no gas produced	Acid produced, no gas produced
Sucrose	Acid produced, no gas produced	Acid produced, no gas produced
Lactose	Acid produced, no gas produced	Acid produced, no gas produced
Maltose	Acid produced, no gas produced	Acid produced, no gas produced
Starch	Negative	Negative
Dextrin	Acid produced, no gas produced	Acid produced, no gas produced
Glycerol	Negative	Negative
Mannitol	Acid produced, no gas produced	Acid produced, no gas produced
Xylose	Acid produced, no gas produced	Negative
Rhamnose	Acid produced, no gas produced	Acid produced, no gas produced
D-Fructose	Acid produced, no gas produced	Negative
D-Galactose	Acid produced, no gas produced	Acid produced, no gas produced
D-Mannose	Acid produced, no gas produced	Acid produced, no gas produced
L-Arabinose	Acid produced, no gas produced	Negative
Inulin	Negative	Negative
D-Sorbitol	Negative	Negative
D-Trehalose	Acid produced, no gas produced	Acid produced, no gas produced

2.1.3 *ANTIBIOTIC RESISTANCE (SUSCEPTIBILITY)*

Three batches of the *L. plantarum* 022AE strain were assessed for antibiotic susceptibility based on the minimum inhibitory concentration (MIC) of different antibiotics for *L. plantarum* 022AE using broth dilution assay. *Streptococcus pneumoniae* ATCC 49619 was used as a quality control strain as recommended by CLSI 2012 and CLSI 2016. Results were interpreted as the strains being "Sensitive (S) / Resistant (R)" by comparing the individual MIC values with the breakpoint MIC values of each antibiotic following EFSA (2012) and CLSI (2012b) guidelines.

Seven antibiotics (clindamycin, chloramphenicol, ampicillin, gentamicin, tetracycline, kanamycin, and erythromycin) were tested for *L. plantarum* 022AE using broth dilution assay and six antibiotics (clindamycin, chloramphenicol, ampicillin, tetracycline, vancomycin, and erythromycin) for control *S. pneumoniae* ATCC 49619. The results are provided in Table 2.

Table 2. Antibiotic Susceptibility of *L. plantarum* 022AE

Antibiotic	*Streptococcus pneumoniae* ATCC 49619			*L. plantarum* 022AE		
	MIC range[1] (µg/mL)	MIC (µg/mL)	Interpretation	MIC break-point[4] (µg/mL)	MIC (µg/mL)	Interpretation
Clindamycin	0.03-0.12	0.06	S[3]	2	0.015	S
Chloramphenicol	2-8	4	S	8	1	S
Ampicillin	0.06-0.25	0.25	S	2	0.03	S
Gentamicin	NAv[2]	NAv	NAv	16	16	S
Tetracycline	0.06-0.5	0.25	S	32	0.03	S
Kanamycin	NAv	NAv	NAv	64	32	S
Vancomycin	0.12-0.5	0.125	S	NR[5]	NR	NR
Erythromycin	0.03-0.12	0.125	S	1	1	S

1. Source: CLSI, 2012a
2. NAv = Not available in CLSI (2012b)
3. S = Susceptible
4. Source: EFSA, 2012
5. NR = Not required (EFSA, 2012a)

L. plantarum strain 022AE was sensitive to all tested antibiotics. Minimum inhibitory concentration (µg/ml) of all the antibiotics viz. clindamycin, chloramphenicol, ampicillin, gentamicin, tetracycline, kanamycin and erythromycin against *L. plantarum* strain 022AE, was within the recommended breakpoints (EFSA 2012).

2.1.4 *VIRULENCE ACTIVITY*

Members of the genus *Bacillus*, other than *Bacillus cereus* group species, were reported to produce enterotoxins and emetic toxins. From et al. (2005) screened 333 *Bacillus* strains; eight strains belonging to *B. subtilis*, *B. mojavensis*, *B. pumilus*, and *B. fusiformis* were found to produce cytotoxic and emetic toxins. The production of the *B. cereus*-like diarrheal enterotoxins by some strains of other *Bacillus* species was described in the SCAN opinion (EC, 2000). In the absence of animal models shown to be able to distinguish hazardous from non-hazardous strains,

a test for cytotoxicity using Vero cells was performed to demonstrate that *L. plantarum* 022AE is free from toxigenic potential (EFSA 2014).

The test is based on the principle that the DNA intercalating agent propidium iodide will stain DNA of cells having leaky cell membranes, thereby enhancing the resulting intracellular fluorescent signal. Positive control contained Triton x 100 treated cells with leaky cell membranes (100% fluorescence). The DNA of intact cells would not show any uptake of propidium iodide, resulting in basal level, negligible fluorescence. The study showed that the sample of *L. plantarum* 022AE did not elicit cytotoxicity on Vero cells (Table 3).

Table 3. Test for Detection of Cytotoxicity Using Vero cells

Test Article	Fluorescence Units in Live Cells	% Fluorescence with respect to positive control
Background	0.59	0.46
Positive control	129.29	100.00
Negative control	4.81	3.72
L. plantarum 022AE – 10 µl	21.30	16.48
L. plantarum 022AE – 50 µl	15.55	12.03
L. plantarum 022AE – 100 µl	14.65	11.33

The fluorescence values for *L. plantarum* 022AE samples were less than 20% compared with the positive control, indicating that the samples did not have any cytotoxic effects.

2.1.5 *ANTIMICROBIAL ACTIVITY*

L. plantarum 022AE was evaluated for its antimicrobial activity following CLSI (2012a) guidelines (EFSA 2012) against five reference microorganisms: *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Enterococcus faecalis* ATCC 29212.

Additionally, three microorganisms (*Escherichia coli* ATCC 8739, *Staphylococcus epidermis* ATCC 12228, and *Staphylococcus aureus* ATCC 6538), as recommended by the United States Pharmacopoeia (USP, 2008), were included in the study. *L. plantarum* 022AE showed an absence of antimicrobial activity against all these reference micro-organisms.

2.1.6 *ACID AND BILE SALT TOLERANCE*

L. plantarum 022AE was assessed for its viability in acidic pH and bile under *in vitro* conditions. The aqueous suspension of *L. plantarum* was exposed to different acidic pH (pH 1.5, 2.5, 3.0, 5.0 and 7.0) and bile concentrations (0.01, 0.10, 0.20, 0.30, 0.50, 0.70 and 1.00%) for up to 5 h. Samples were analysed for viable cell count in triplicates and results were expressed as log_{10} CFU/mL.

Table 4. *In vitro* stability of *L. plantarum* 022AE under different acidic pH (1.5 – 7.0). The viable cell count is expressed in log_{10} CFU/mL ± SD.

Different pH and viable count of *L. plantarum* (log_{10} CFU/mL)					
Time (h)	**pH 1.5**	**pH 2.5**	**pH 3.0**	**pH 5.0**	**pH 7.0**
0	9.30 ± 0.02	9.30 ± 0.02	9.30 ± 0.02	9.30 ± 0.02	9.30 ± 0.02
1	6.67 ± 0.10	9.10 ± 0.03	9.30 ± 0.02	9.30 ± 0.03	9.30 ± 0.03
2	6.67 ± 0.03	8.89 ± 0.12	9.30 ± 0.05	9.30 ± 0.01	9.30 ± 0.01
3	6.67 ± 0.05	8.86 ± 0.08	9.27 ± 0.02	9.30 ± 0.02	9.30 ± 0.04
4	6.67 ± 0.04	8.70 ± 0.01	9.17 ± 0.01	9.30 ± 0.03	9.30 ± 0.04
5	6.45 ± 0.03	8.70± 0.03	9.15 ± 0.03	9.30 ± 0.05	9.30 ± 0.02

Table 5.

Table 5. *In vitro* stability of *L. plantarum* 022AE under different bile concentrations (0.01 – 1.00%). The viable cell count is expressed in log_{10} CFU/mL ± SD.

Bile concentration (%) and viable count of *L. plantarum* (log_{10} CFU/mL)						
Time (h)	**0.01**	**0.10**	**0.30**	**0.50**	**0.70**	**1.00**
0	9.30 ± 0.00	9.30 ± 0.00	9.30 ± 0.00	9.30 ± 0.00	9.30 ± 0.00	9.30 ± 0.00
1	9.30 ± 0.01	9.30 ± 0.15	9.28 ± 0.03	9.17 ± 0.07	9.17 ± 0.08	9.14 ± 0.01
2	9.29 ± 0.03	9.30 ± 0.02	9.28 ± 0.10	9.15 ± 0.02	9.17 ± 0.03	9.12 ± 0.03
3	9.29 ± 0.03	9.28 ± 0.07	9.27 ± 0.06	9.02 ± 0.05	8.98 ± 0.02	8.96 ± 0.06
4	9.28 ± 0.04	9.24 ± 0.04	9.22 ± 0.02	8.99 ± 0.19	8.94 ± 0.03	8.63 ± 0.02
5	9.26 ± 0.03	9.22 ± 0.04	9.16 ± 0.04	8.96 ± 0.10	8.94 ± 0.08	8.51 ± 0.05

The results demonstrate that *L. plantarum* 022AE was stable under different acidic conditions and bile concentrations under *in vitro* experimental conditions.

2.1.7 *ENTEROTOXINS*

L. plantarum 022AE was checked for enterotoxin production by Duopath® Cereus Enterotoxins test kit, Merck and D-cultural technique. Positive controls were *Bacillus cereus* ATCC 14579 and *Bacillus cereus* ATCC 11778. It was observed that *L. plantarum* 022AE does not produce enterotoxins while the toxin was detected in positive control strains.

L. plantarum 022AE was concluded to be negative for non-hemolytic enterotoxins and emetic toxin.

2.1.8 D- LACTIC ACID PRODUCTION

All lactic acid bacteria (LAB) produce lactic acid through carbohydrate fermentation; the lactic acid can be produced in two different isomeric forms-- the dextrorotatory enantiomer (D-lactate) and the levorotatory enantiomer (L-lactate). It is well documented that almost all lactic acid bacteria produce a mixture of D- and L- lactic acid (Holzapfel WH, 2001; GRN 685; GRN 847 and GRN 953). FAO/WHO recommends investigation of D- lactate production by food microorganisms considering the earlier belief that the D- lactate cannot be metabolized by humans (FAO/WHO, 2002; Ewaschuk JB et al., 2005). Many recent and older reports demonstrate that D- lactic acid can be metabolized in adult humans as well as infants with normal gastrointestinal function (Uribarri J et al., 1998; de Vrese M et al., 1990; Connolly et al., 2005; Vitetta et al., 2017 and Lukasik et al., 2018).

Very old and initial studies like those of Stolley and Droese, 1971, reported the concern regarding supplementation of D- lactate producing microbes in infant formula acidified with D- and L-lactic acid. But subsequent studies like Connolly and Lonnerdal, 2004, and Connolly et al., 2005, found no evidence related to D-lactate acidosis due to supplementation of different LAB species studied during these studies. According to all the studies mentioned above, the safe use of LAB species producing a racemic mixture of D- and L- lactate will not have any toxic effects considering the normal individuals with functional small intestine (GRN 722, GRN 847 and GRN 685).

L. plantarum 022AE has also been analyzed for its D- and L- lactic acid production and it was found that almost 45.5% lactate is D-lactate and remaining 54.5 % is L- lactate. These values are similar to those of other *L. plantarum* strains like *L. plantarum* LP_{LDL} (GRN 847), and *L. plantarum* Lp-115 (GRN 722).

Conclusion

L. plantarum 022AE strain has been thoroughly analyzed for risk-associated factors following genome-based analyses and phenotypic/biochemical studies. Various studies/analyses carried out on this strain showed no safety concern and it has been concluded that the strain is safe for human consumption.

2.2 Manufacturing Process

2.2.1 *OVERVIEW*

Fermentation is a well-known process that has been used for the cultivation of microorganisms for decades. *L. plantarum* 022AE is produced in the form of vegetative cells by batch type submerged fermentation. Fermentation is carried out in accordance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis and Critical Control Points (HACCP). The typical fermentation batch size ranges from 10,000 to 20,000 L. The frequency of production is planned as per market demand. The manufacturing facility is ISO 9001:2015 , FSSC 22000, and GMP certified.

As shown below, the key steps for production of *L. plantarum* 022AE are fermentation, recovery, formulation, and packaging. The process is illustrated in Figure 1.

2.2.2 *FERMENTATION*

2.2.2.1 Raw materials

Materials used in the fermentation process (including inoculum development, seed preparation, and main fermentation) are all food-grade substances approved for this use. None of the ingredients used is based on milk, soy, or any of the top nine allergens (FASTER, 2021).

- Potable water
- A carbon source
- A nitrogen source
- Salts
- Vitamins (as a part of complex fermentation materials)
- pH adjustment agents
- Foam control agent (at ≤ 0.1%)

2.2.2.2 Inoculum (Seed) Development

A suspension of a pure culture of *L. plantarum* 022AE is aseptically transferred to an inoculum flask containing fermentation medium.

The culture is grown in the flask under optimum conditions to obtain a sufficient amount of biomass, which can subsequently be used as inoculum for the seed fermentation.

2.2.2.3 Seed Fermentation

The inoculum is aseptically transferred to the seed fermenter containing seed fermentation medium with due checking of purity of culture. When a sufficient amount of biomass has developed (typically up to 6-14 hours), the content of the seed fermenter is used for inoculation of the main fermenter.

2.2.2.4 Main Fermentation

During the main fermentation, the growth (cell-mass) of *L. plantarum* 022AE takes place in the form of vegetative cells.

The main fermentation is operated in a batch mode. Media ingredients are mixed properly and steam sterilized, followed by cooling to the desired fermentation temperature. Seed culture (inoculum) developed in the seed fermenter is aseptically transferred to the main fermenter containing pre-sterilized fermentation medium. The fermentation process is continued for a predetermined amount of time or until laboratory test data indicate the desired achievement of biomass or until the rate of biomass production starts decreasing below a predetermined production rate. Throughout the process of fermentation, typical parameters like temperature, pH, and culture purity are monitored and controlled to achieve the desired viable cell count.

2.2.3 *RECOVERY*

The purpose of the recovery process is to separate the suspended *L. plantarum* 022AE vegetative cells from the fermentation medium, concentrate, and dry the obtained biomass.

The steps of recovery include:

- Primary separation of vegetative cells (biomass) from the soluble media components by centrifugation
- Washing of concentrated vegetative cells (biomass)
- Lyophilization (Freeze Drying)

2.2.3.1 Primary Separation

The fermentation broth is passed through a high-speed centrifuge to separate the vegetative cells (biomass) from the production medium. The cell biomass is collected as a thick slurry and subjected to further processing. Temperature and pH are controlled during this step.

2.2.3.2 Washing

Sterilized and demineralized water is added to the collected biomass slurry. Slurry is again passed through high-speed centrifuge and the washed biomass is collected. Temperature and pH are controlled during this step.

2.2.3.3 Lyophilization (Freeze Drying)

The concentrated viable cells slurry is lyophilized (freeze dried) in presence of food grade non-allergenic stabilizer(s) to obtain the unformulated concentrate.

2.2.4 *FORMULATION AND PACKAGING*

L. plantarum 022AE is sold as a powder preparation of different viable cell counts, depending on the final intended application.

For the manufacturing of the dry vegetative cell preparation, the spray-dried unformulated concentrate (not less than 400 billion CFU/g) is further formulated with approved food grade formulating agents such as maltodextrin and adjusted to a desired viable cell count.

The *L. plantarum* 022AE preparation is tested by the Quality Control Department for all quality related aspects and released by the Quality Assurance Department. The final product is packed

in suitable food grade packaging material before storage. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for final preparations.

Manufacturing flow chart for *L. plantarum* 022AE

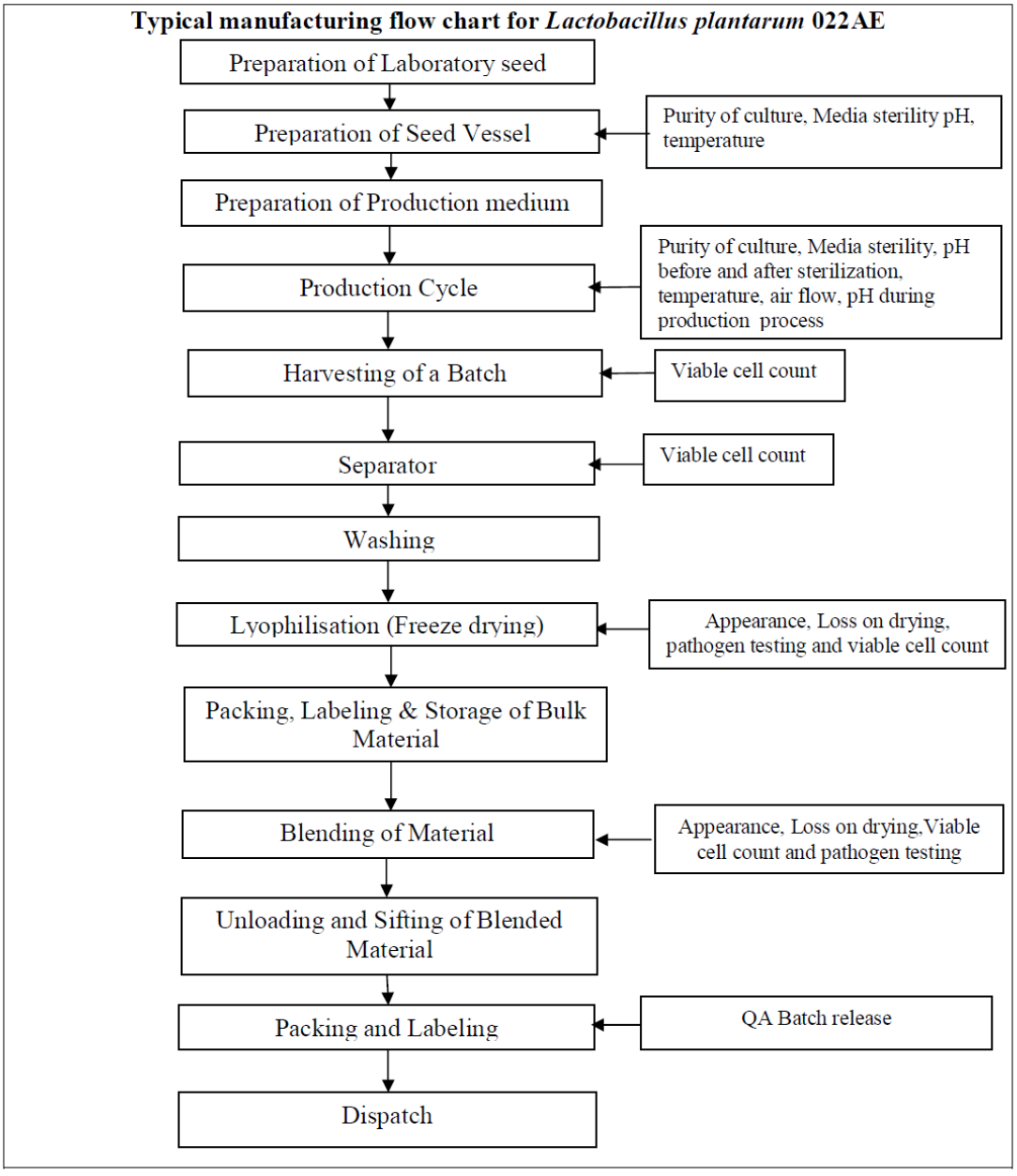

Figure 1. Manufacturing Process for L. plantarum 022AE

2.3 Product Specifications and Compositional Variability

2.3.1 *PRODUCT SPECIFICATIONS*

Specifications for *L. plantarum* 022AE preparation have been established by Advanced Enzyme Technologies Ltd. and are summarized in Table 6. All methods have been validated for this purpose.

Table 6. Product Specifications for *L. plantarum* 022AE

Product specification	Advanced Enzyme Technologies Ltd.	
	Limits	**Reference Method**
Total viable count/Assay (viable cell count/g)	Not less than 400 billion viable cell counts/g	Internal method
Appearance/ Description	Off-white to brown colored powder	Visual, Olfactory
Arsenic	Not more than 0.25 ppm	AOAC 2015.01, 21st Edition
Cadmium	Not more than 0.25 ppm	AOAC 2015.01, 21st Edition
Lead	Not more than 0.1 ppm	AOAC 2015.01, 21st Edition
Mercury	Not more than 0.1 ppm	AOAC 2015.01, 21st Edition
Total yeast & mold count	Not more than 100 CFU/g	Harmonized method (USP, EP and JP) and IP
Total coliforms	Not more than 10 CFU/10g	FDA Bacteriological Analytical Manual
E. coli	Absent in 10 g	Harmonized method (USP, EP and JP) and IP
Salmonella spp.	Absent in 10 g	Harmonized method (USP, EP and JP) and IP
Staphylococci spp.	Absent in 1 g	Harmonized method (USP, EP and JP) and IP
Listeria monocytogenes	Absent in 25 g	Internal method
Non-lactic-acid bacteria	Not more than 5000 CFU/g	ISO 13559:2002 (IDF153)
Enterobacteriaceae	Not more than 100 CFU/ 10g	Harmonized method (USP, EP and JP) and IP
Enterococci	Not more than 100 CFU/ 10g	Internal method

2.3.2 *COMPLIANCE WITH SPECIFICATIONS*

Three non-consecutive batches of *L. plantarum* 022AE were analyzed and the results compared with food-grade specifications as presented in Table 7. All tested batches were in compliance with the specifications, demonstrating that the production process is in control.

Table 7. Analysis of Compositional Variability of *L. plantarum* 022AE

Parameter	Specification	Batch		
		122052	122055	012117
L. plantarum 022AE viable cell count	Not less than 400 billion viable cell count/g	425 billion viable cell count/g	453 billion viable cell count/g	460 billion viable cell count/g
Description	Off white to brown colored powder	Yellowish colored powder	Yellowish colored powder	Yellowish colored powder
Heavy Metal Analysis				
Arsenic	Not more than 0.25 ppm	Complies	Complies	Complies
Cadmium	Not more than 0.25 ppm	Complies	Complies	Complies
Lead	Not more than 0.1 ppm	Complies	Complies	Complies
Mercury	Not more than 0.1 ppm	Complies	Complies	Complies
Microbial Analysis				
Total yeast & mold count	Not more than 100 CFU/g	Less than 10 CFU/g	Less than 10 CFU/g	Less than 10 CFU/g
Total coliforms	Not more than 10 CFU/10g	Less than 10 CFU/g	Less than 10 CFU/g	Less than 10 CFU/g
E. coli	Absent in 10g	Complies	Complies	Complies
Salmonella spp.	Absent in 10g	Complies	Complies	Complies
Staphylococci spp.	Absent in 1g	Complies	Complies	Complies
Listeria monocytogenes	Absent in 25g	Complies	Complies	Complies
Non-lactic-acid bacteria	Not more than 5000 CFU/g	Complies	Complies	Complies
Enterobacteriaceae	Not more than 100 CFU/ 10g	Complies	Complies	Complies
Enterococci	Not more than 100 CFU/ 10g	Complies	Complies	Complies

2.4 Shelf-Life Stability

L. plantarum 022AE has been assessed for its stability at different temperatures such as 5°±3°C and 25°C±2°C and 60% RH±5% for 24 months to confirm its ability to retain its viability throughout its shelf life.

The product is found to be stable for 12 months at 25°±2°C and 60% ±5% RH and for 18 months at 5°±3°C from the date of manufacture in double LDPE bags (tightly packed for air resistance) and kept in HDPE drum.

Based on the above observation, the proposed shelf life of *L. plantarum* 022AE is 18 months when stored at 5°±3°C in market packing [e.g. double polybag bag in HDPE drum (powder)].

Part 3: 21 CFR 170.235: Intended Use and Dietary Exposure

L. plantarum 022AE is intended to be added to conventional foods at a level not exceeding $10x10^9$ CFU/serving consistent with cGMP. Intended food applications include but are not limited to the following:

> Yogurt, other milk based products, dairy analogs, soy products, fruit drinks, frostings, frozen dairy desserts and mixes, fruit and water ices, drinking water, sports drinks, chewing gum, extracts, and flavorings, condiments, herbs, seeds, spices, seasonings, blends, nuts and nut products, plant protein products, cereals, processed fruits, processed vegetables and vegetable juices, snack foods and toppings.

The intended addition of *L. plantarum* 022AE and the food categories to which it will be added are similar to those stated for other *L. plantarum* strains (i.e., GRN 685, 722, 847 and 953) to which FDA had no objection. *L. plantarum* 022AE is intended to be added as a food ingredient in multiple food categories between 1.0×10^9 to 10×10^9 CFU/serving.

The No Observed Adverse Effect Level (NOAEL) of *L. plantarum* 022AE in Wistar rats, following oral administration for 28 days, was reported to be 1000 mg/kg bw/day. This dose corresponds to 425 billion CFU/kg bw/day as the strength of *L. plantarum* 022AE provided for the toxicity study was 425×10^9 CFU/g. The acceptable daily intake (ADI) concluded from the NOAEL dose of the 28-day toxicity study of *L. plantarum* 022AE, which provides a 100x safety factor, is 2.97×10^{11}cfu/person/day[1].

According to USDA Nutrition Insights, a publication of the USDA Center for Nutrition Policy and Promotion, October 2000, males aged 51 or older consume the greatest number of servings of food per day, about 18.2 servings. Based upon this number of servings and the highest intended addition level of *L. plantarum* 022AE per serving, the maximum estimated daily intake (EDI) is 1.82×10^{11} CFU per day,[2] which is less than the ADI derived from the NOAEL from the 28-day chronic oral toxicity study.

The EDI assumes that all foods to which the strain may be added contain it at the maximum intended level and that an individual consumes over 18 servings per day of these specific foods, indicating that the EDI is likely a considerable overestimate.

[1] ADI = NOAEL x 70/100 , where the body weight of a healthy individual is considered to be 70 kg and a safety factor of 100 is employed.

[2] Maximum Estimated Daily Intake of 182 x 10^9 CFUs per day of *L. plantarum* is calculated as follows: 10 x 10^9 CFUs/serving (highest possible additional level of *L. plantarum*) x 18.2 servings/day (greatest estimated serving of food).

Part 4: 21 CFR 170.240: Self-Limiting Levels of Use

There are no self-limiting levels of use of *L. plantarum* from *L. plantarum* 022AE in food applications.

Part 5: 21 CFR 170.245: Experience Based on Common Use in Food before 1958

The statutory basis for our conclusion of GRAS status in the notice is scientific procedures rather than common use in food prior to 1958.

Part 6: 21 CFR 170.250: Narrative

6.1. History of Consumption of Lactobacillus species and *L. plantarum*

There is a long history of consumption of *Lactobacillus* species in human food. It is also a natural inhabitant of the human and animal oral cavity, gastrointestinal tract, vagina, etc. (Corsetti and Valmorri, 2011). More than 250 species of *Lactobacillus* are known to man (Zhen et al., 2020).

A Food and Agriculture Organization and World Health Organization expert consultation (FAO/WHO 2002) stated that, "*lactobacilli* have a long history of use as probiotics without established risk to humans, and this remains the best proof of their safety" (Naidu et al., 1999; Saxelin et al., 1996) and concluded that, "no pathogenic or virulence properties have been found for *lactobacilli, bifidobacteria,* or *lactococci*" (Aguirre and Collins, 1993). Among the lactic acid bacteria, genus *Lactobacillus* include large number of GRAS species (Salvetti et al., 2012). Species belonging to the genus *Lactobacillus* are some of the most important bacteria in food microbiology and human nutrition due to their role in production of food and feed and preservation, as well as the probiotic qualities of select strains (Felis and Dellaglio, 2007). Bacteria of the genera *Lactobacillus* and *Bifidobacterium* are classified among the main probiotics considered safe for food and feed use, and they are produced in industrial scales (Siezen et al., 2011).

Lactobacillus species like *Lactobacillus acidophilus, L. casei, L. paracasei, L. rhamnosus, L. delbrueckii subsp. bulgaricus, L. brevis, L. johnsonii, L. plantarum,* and *L. fermentum are* indigenous inhabitants of the human intestinal tract (Fijan, 2014). Consequently, strains of *L. plantarum* are gaining in importance as potential probiotics among the LABs considering their ancient important uses in food preservation and fermentation (GRAS 685, 722 and 847).

L. plantarum has a long history of use in different types of fermented foods. It has traditionally been used in the fermentation of cheese, kefir, sauerkraut, meats, vegetables, and beverages. The occurrence of *L. plantarum* is frequently associated with lactic-acid fermented foods of plant origin, e.g. brined olives, sauerkraut, salted gherkins, sourdough, Nigerian *ogi* [made with maize or sorghum], Ethiopian *kocho* [made with starch from *Ensete ventricosum*], and Ethiopian sourdough made from *tef* [*Eragrostis tef*]. *L. plantarum* occurs in high numbers in most lactic-acid fermented foods like Nigerian ogi (Johansson 1995), Ethiopian kocho (Gashe 1987), Ethiopian sourdough *(*Gashe 1987) and grape juice and wine (Vaquero et al., 2004). This implies that *L. plantarum* can be naturally present in the human diet and potentially present in the human GI tract as well.

In Sweden, a nasogastric feeding formula containing oatmeal is supplemented with *L. plantarum* 299v. It is used as active ingredient in the food product (Molin, 2001). In agriculture, *L. plantarum* has been used to preserve grass or maize in the form of silage (Kacho, 2019). Furthermore, a food originated *L. plantarum* was isolated and studied for its evolutionary-

conserved characteristic to tolerate and metabolize high concentrations of bile acid (Prete et al. 2020).

The International Dairy Federation (IDF) and the European Food and Feed Cultures Association (EFFCA) have prepared an inventory of microbial strains used by the food industry that have a long history of use in food without adverse effects since 2002 (Morgensen et al., 2002). *L. plantarum* is among the organisms listed since then up to the most recent bulletin published in 2018 (Bourdichon et al., 2018).

Several commercial preparations containing *L. plantarum*, as described in GRN 685, 722, 847, 953 are being used commercially as food ingredients.

6.2 Regulatory History of *L. plantarum*

L. plantarum strains have long been known to be safely consumed by the general human population. *L. plantarum*-containing products are commercially available in the USA, European, and Asian markets. In the USA, strains of *L. plantarum* are commercially available in products like Florastor, Culturelle, and iFlora.

The European Food Safety Authority granted *L. plantarum* 'Qualified Presumption of Safety' (QPS) status in 2008 (EFSA, 2007) and has renewed its status annually since then. Further, *L. plantarum* does not appear on the list of pathogens in Annex III of Directive 2000/54/EC, as it is globally regarded as a safe microorganism.

The American Type Culture Collection (ATCC, 2020) has classified different strains of *L. plantarum* as Bio-safety Level 1, indicating that it is a well-characterized agent which does not cause disease in healthy humans.

The Food Safety and Standards Authority of India (FSSAI), includes *L. plantarum* in the list of permitted components in food (FSSAI, 2016).

Health Canada has approved *L. plantarum* as food containing probiotic microorganism under acceptable non-strain specific claims for the probiotics (Health Canada, 2009) and as Natural Health Products under schedule 1 (Health Canada, 2021).

Food Standards Australia New Zealand (FSANZ) identified no safety concerns associated with *L. plantarum* and considered as traditional use in foods in Australia and New Zealand (FSANZ, 2012).

The Japanese Ministry of Health and Welfare classified *L. plantarum* under the "Foods for Specific Health Use" (FOSHU) system has approved the use of *L. plantarum* products for modification of gastrointestinal conditions (MHLW, Japan 2021).

L. plantarum strains *L. plantarum* strain 299v (ProbiAB Sweden, GRN 685), *L. plantarum* Lp-115 (Danisco USA, GRN 722) and *L. plantarum* ECGC 13110402 (LP_{LDL}) (AIBMR Life Sciences USA, GRN 847) have received a "FDA has no question" letter from US FDA for their intended uses in food products like wet, chilled and ambient products (fruit drinks, yogurts,

cheese, milk and plant-based products), dry-chilled products, ready-to-eat breakfast cereals, milk and milk products (flavored milks, butter, Kefir, sour cream, buttermilk), nutrition beverages(fruit juices, fruit nectars, fruit drinks, jams and jellies); snack foods (cookies, crackers, chips, granola), meal replacements; sauces, condiments; confections (bars, candy, coatings, drops, cookie filling).

6.3 Safety of *L. plantarum* 022AE —Oral Toxicity and Genotoxicity Studies

The safety of *L. plantarum* 022AE and other strains has been evaluated in animal research, including acute, subacute, sub-chronic, and chronic studies of oral toxicity and genetic toxicity assays.

6.3.1. IN VITRO *AND ANIMAL STUDIES OF* L. PLANTARUM *022AE*

L. plantarum 022AE, the notified strain, has been investigated for its safety in a series of toxicity studies complying with OECD Guidelines and conducted in accordance with the principles of Good Laboratory Practice (GLP) as published by the OECD (ENV/MC/CHEM(98)17).

Acute oral toxicity test (OECD 423, 2001): Using the step-wise treatment method, 2 groups of n=3 female Wistar rats aged 8-9 weeks and weighing 165-178 g were dosed via gavage with 2000 mg/kg bw and observed for 14 days. No indications of toxicity were reported, and so two similar groups of n=3 female Wistar rats were gavaged with 2000 mg/kg bw of *L. plantarum* 022AE. Based on the results, the estimated LD_{50} for *L. plantarum* 022AE was greater than 2000 mg /kg bw.

Repeated-dose 28-day oral toxicity test (OECD 407, 2018): Groups of 5 male and 5 female Wistar rats, 6-7 weeks old and weighing 244-300 g (males, mean = 270.27 g) and 169-211 g (females, mean = 195.55 g) were assigned to receive daily oral gavage of doses of 0, 250, 500, and 1000 mg *L. plantarum* 022AE preparation/kg bw for 28 days and were sacrificed on day 29 to evaluate its toxicity. A concurrent control group of 5 male and 5 female rats was also maintained and received a vehicle, i.e. analytical grade water at 5 mL/kg bw for 28 days. Additionally, for assessment of reversibility, persistence or delayed occurrence of toxicity, two recovery groups of 5 rats per sex were maintained and were further observed for a period of 14 days following the 28-days treatment and administered with a vehicle at 5 mL/kg and the test item at the high dose level, i.e. 1000 mg/kg bw for 28 days

Rats were examined daily for signs of toxicity, morbidity, and mortality. They were subjected to detailed clinical examinations at day 0 and weekly thereafter during the treatment and recovery periods. At week 4, all animals were assessed for sensory reactivity, grip strength, and motor activity. Feed consumption and body weight were recorded weekly. Blood and urine samples were taken at the end of dosing and after recovery. All animals were subjected to necropsy and weights of adrenals, testes, ovaries, kidneys, liver, brain, thymus, heart, spleen, epididymides, uterus with cervix, prostate and seminal vesicles with coagulating glands were recorded. Histological evaluations were performed on all tissues from control and high-dose rats.

There was no incidence of mortality in rats of *L. plantarum* 022AE treated groups at and up to the dose of 1000 mg/kg during the treatment and recovery period. *L. plantarum* 022AE did not induce any remarkable or treatment related clinical abnormalities in rats treated at and up to the dose of 1000 mg/kg. Also, no mortality or abnormal clinical signs were observed in the animals of the vehicle control group.

The functional observations (neurological examinations) conducted in the fourth week of the study did not reveal treatment related incidence of neurological abnormalities at and up to the dose of 1000 mg/kg of *L. plantarum* 022AE.

Body weight and weight gain was not affected in male and female rats treated with *L. plantarum* 022AE at and up to the dose of 1000 mg/kg body weight and were found to be comparable to that of the control rats throughout the treatment period and during the recovery period. The group mean daily food consumption for groups treated with *L. plantarum* 022AE at and up to the dose of 1000 mg/kg bw was found to be comparable to those of the rats of control group.

The hematological parameters like hemoglobin, packed cell volume, total RBC count, total and differential WBC counts, RBC indices, platelet count, reticulocyte count, activated partial thromboplastin time, prothrombin time and morphology of blood cells of male and female rats treated with *L. plantarum* 022AE at and up to the dose level of 1000 mg/kg body weight were found to be comparable to those of the vehicle control animals at termination of the treatment and at the end of the recovery period.

L. plantarum 022AE at and up to the dose level of 1000 mg/kg body weight, did not alter the plasma levels of total protein, albumin, globulin (calculated), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), glucose, urea nitrogen, urea (calculated), creatinine, total cholesterol, total bile acid, triglycerides, total bilirubin, sodium, potassium, calcium, gamma glutamyl trans-peptidase, albumin/globulin (A/G) ratio (calculated) and phosphorus in male and female rats. Treatment of rats with test item, at and up to the dose of 1000 mg/kg body weight, did not induce any alterations in urine parameters including microscopic appearance of the centrifuged deposits.

The values of absolute and relative weights of adrenals, testes, ovaries, kidneys, liver, brain, thymus, heart, spleen, epididymis, uterus with cervix, prostate and seminal vesicles with coagulating glands of rats treated with *L. plantarum* 022AE at and up to the dose level of 1000 mg/kg body weight were found to be comparable with those of the control rats at the end of treatment period and also at the end of the recovery period.

L. plantarum 022AE at and up to the dose of 1000 mg/kg body weight did not induce any remarkable and treatment related gross pathological alterations in the tissues / organs of rats as evident at detailed necropsy examination carried out at termination. Microscopic evaluation of all tissues / organs of all the male and female rats from control and high dose groups in this study did not reveal incidence of any treatment related histopathological findings.

The no observed adverse effect level (NOAEL) of *L. plantarum* 022AE in the Wistar rat, following oral administration for 28 days, was the highest dose tested, 1000 mg/kg bw/day providing 4.25 $x10^{11}$ viable cell count/kg bw/day.

Bacterial reverse mutation test—Ames assay (OECD 471, 1997): The test was conducted using *Salmonella typhimurium* tester strains TA1535, TA97a, TA98, TA100, and TA102 in the presence and absence of S9 metabolic activation. The test was conducted in triplicate at concentrations of 50, 150, 500 and 1500 µg/plate. No significant increase in the number of histidine revertant colonies was reported, and it is concluded that, under the conditions of this study, *L. plantarum* 022AE is non-mutagenic.

***In vitro* mammalian chromosomal aberration test in human lymphocytes (OECD 473, 2016):** Cultures of human peripheral blood lymphocytes were exposed to *L. plantarum* 022AE at concentrations of 0, 1250, 2500, and 5000 µg/mL in the presence and absence of metabolic activation for 3 or 24 hours. No significant concentration related increase was reported in the incidence of structural chromosome aberrations at any tested concentration, and it was concluded that *L. plantarum* 022AE is non-clastogenic in the presence and absence of microsomal enzymes.

***In vivo* micronucleus test in mice (OECD 474, 2016):** A group of 5 male mice was administered *L. plantarum* 022AE formulated in analytical grade water by oral gavage at a dose of 2000 mg/kg bw. The animals were treated for two consecutive days at an interval of about 24 hours. A concurrent vehicle control group of 5 male mice received 10 mL/kg bw of analytical grade water, while another concurrent positive control group of 5 male mice received cyclophosphamide monohydrate at the dose of 15 mg/kg bw. All the animals were observed for signs of toxicity following the treatment and were sacrificed at about 45 hours after the last treatment. During the study period, animals were monitored for mortality, clinical signs, and body weight. Blood from each mouse was collected, fixed, and subsequently stained with Anti-Mouse CD71 and Anti-Rodent CD61 antibodies. About 20,000 polychromatic erythrocytes (PCEs, i.e. immature erythrocytes) per animal were examined using flow cytometer to detect the incidence of micronucleated PCEs (MN-PCEs). In addition, the proportion of immature erythrocytes was assessed for each animal as a measure of potential toxicity.

L. plantarum 022AE at 2000 mg/kg bw dose did not induce an increase ($p > 0.05$) in the frequency of micronucleated immature erythrocytes (% PCE) in the peripheral blood of mice when compared with the vehicle control group. No incidence of abnormal clinical signs indicative of systemic toxicity was noted in treated mice at the dose level of 2000 mg/kg bw. Based on the results obtained, it was concluded that *L. plantarum* 022AE is non-clastogenic under the conditions tested.

6.3.2. ANIMAL STUDIES OF OTHER STRAINS OF L. PLANTARUM

L. plantarum PS128^{TM} was assessed for its safety through genotoxicity and systemic toxicity studies (Liao et al., 2019) A 28-day repeated dose oral toxicity study (0, 400, 2400 mg/kg bw) of *L. plantarum* PS128^{TM} was conducted in ICR mice. There were no abnormal clinical signs,

histopathological lesions, and/or mortality detected in the treatment groups. The NOAEL of PS128^{TM} was set to be greater than 2.4 g/kg bw for both male and female ICR mice. Further, *L. plantarum* PS128^{TM} was assessed for its genotoxicity by bacterial reverse mutation test in five strains of *Salmonella typhimurium*, chromosomal aberrations test in CHO epithelial cells, and micronucleus assay in mice and found non-mutagenic and non-clastogenic and concluded safe for human consumption.

The safety of *L. plantarum* MTCC 5690 and *L. fermentum* MTCC 5689 strains in a dextran sodium sulfate (DSS)-induced colitis mouse model was reported by Pradhan et al., 2019. Both MTCC 5690 and MTCC 5689 strain did not induce any detrimental effects on the colitic mice, as was reflected by normal colon and cecum length, blood biochemistry, hematology, and absence of inflammation.

Acute and sub-chronic (90-day repeated dose) oral toxicity tests of microencapsulated *L. plantarum* LAB12 were conducted in rats (Fareez et al., 2018). In the acute study, 6 male animals were given a single $1x10^{11}$ CFU/kg bw dose. No treatment-related effects were observed after 14 days. In the sub-chronic toxicity study, rodents were randomly divided into 4 groups (6 rats/sex/group) and treated with 0, 8, 9 and 10 log CFU/kg bw of microencapsulated LAB12 in pellet form. There were no observed adverse effects on growth, feed consumption, cellular blood components, and vital organs of the treated animals. The NOAEL for microencapsulated LAB12 was 2.5×10^{10} CFU/kg bw for both genders. These results indicated that LAB12 is likely non-pathogenic and non-toxic.

Safety assessment of AB-LIFE(®) (*L. plantarum* CECT 7527, 7528, and 7529) was reported by Mukerji et al. (2016). AB-LIFE(®) was evaluated for potential subchronic oral toxicity in rats with dosages of 300 and 1000 mg/kg bw/day (equivalent to 5.55×10^{10} and 1.85×10^{11} CFU/kg bw/day). There were no effects on clinical pathology parameters (hematology, coagulation, clinical chemistry, and urinalysis), organ weights, or macroscopic and microscopic pathology attributable to administration of AB-LIFE®. The NOAEL for AB-LIFE(®) in male and female rats was 1000 mg/kg bw/day (1.85×10^{11} CFU of AB-LIFE(®)/kg bw/day). Also, whole genome sequencing was performed on each of the three strains. Antibiotic resistance was evaluated by genomic mining for resistance genes, and assessment for transferability. No risk of transfer potential was identified for any antibiotic resistance genes in *L. plantarum* CECT 7527, 7528, or 7529.

L. plantarum 299v along with *L. paracasei* DSM 13434, and *L. gasseri* 5B3 along with two *Bifidobacteriurn* strains were evaluated in Sprague-Dawley rats for 7 days in dextran-sulfate sodium-induced colitis and treatment continued for another 7 days (Osman et al., 2004). Colitis severity was assessed daily; after sacrifice, samples were taken of ileum and colon, arterial and portal blood, mesenteric lymph nodes, and liver for bacterial evaluation. The severity of the colitis was significantly lower in all probiotic groups as compared to controls. There was no mortality. No adverse effects from the probiotic administration were reported.

Ikhsani et al. (2020) reported administration of *L. plantarum* Mut-7 at a dose of 10^{11} CFU/mL to Sprague Dawley rats for 21 days. No deleterious effect on the performance of rats (feed intake, body weight, hematological concentration, physiological and stress markers, and gut morphology) were observed. Additionally, consumption of high doses of *L. plantarum* Mut-7 for 21 days did not cause bacterial translocation in the organs or blood of the rats.

6.4 Safety of *L. plantarum* —Human Studies

Several researchers have carried out studies on human subjects, including children and adults, with different *L. plantarum* strains and evaluated the safety aspects. These studies are summarized in Table 8.

Table 8. Human Studies of *L. plantarum*

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Adults					
Ducrotte et al., (2012)	Double blind, placebo-controlled, parallel-designed study to assess the symptomatic efficacy of *Lactobacillus plantarum* 299v (*L. plantarum* 299v, DSM 9843) for the relief of abdominal symptoms in a large subset of irritable bowel syndrome	214 subjects	*L. plantarum* 299v (DSM 9843) $10x10^9$ CFU/ capsule/day	4 weeks	No significant side-effect was reported in any group during the 4 week of treatment. The only adverse event reported was a transient vertigo onset by one of the patients who received *L. plantarum* 299v (DSM 9843)
Hirose et al.(2013)	Randomized, double-blind, placebo-controlled, parallel study. The study was for lowering the incidence of upper respiratory tract infection in healthy subjects with high levels of psychological stress	78 healthy subjects	50 mg of LP20 (heat killed *L. plantarum* L-137	12 weeks	All adverse events were mild and were judged to be unrelated to the dietary intervention. Among the safety assessments performed, albumin and diastolic blood pressure were significantly higher and lactate dehydrogenase was significantly lower in the HK L-137 group than in the control group, but the changes were within the ranges of the corresponding reference values.
Fuentesa et al. (2016)	Double-blind, placebo-controlled, randomized trial to assess the effects of a combination of three *L. plantarum* strains on low-density lipoprotein cholesterol (LDL-C) and other lipid parameters in hypercholesterolemic adults	60 patients	*L. plantarum*-containing probiotic (LpPRO) or placebo (PLBO) single capsule daily for 12 weeks Dose: 1.28×10^9 CFU/capsule	6 to 12 weeks	There were no adverse events related to the study products.

Table 8. Human Studies of *L. plantarum*

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Mujagic et al. (2017)	Randomized double-blind placebo-controlled cross-over trial to investigate the effects of three *L. plantarum* strains on *in-vivo* small intestinal barrier function and gut mucosal gene transcription in human subjects	10 healthy volunteers	*L. plantarum* WCFS1 (2.6×10^{10} CFU per dose), *L. plantarum* CIP48 (2.4×10^{10} CFU/ dose), *L. plantarum* TIFN101 (5.9×10^{10} CFU/ dose) or placebo.	7 days	None of the participants reported discomfort or possible side effects during the test and follow-up period, nor any significant differences in the outcomes of the self-report questionnaires.
de Vos et al. (2017)	Randomized placebo-controlled cross-over trial in healthy human subjects to determine whether *L. plantarum* can enhance immune response	10 subjects	*L. plantarum* WCFS1, 2.6×10^{9} CFU; CIP104448, 2.4×10^{9} CFU; and TIFN101, 5.6×10^{9} CFU	7 days	No side effects or complications were reported.
Oh et al. (2021)	Randomized, double-blind, placebo-controlled clinical trial. Evaluation of *L. plantarum* HAC01's effects on metabolic parameters of prediabetic human subjects.	40 subjects	$4x10^{9}$ CFU/ capsule of *L. plantarum* HAC01.	8 weeks	No serious adverse effects were observed, suggesting that *L. plantarum* HAC01 has potential as an effective lifestyle intervention to forestall or prevent the onset of T2D

Table 8. Human Studies of *L. plantarum*

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Liu et al. (2021)	Randomized, double-blind, placebo-controlled, pilot clinical trial to study and investigate the IBS-D-alleviating effects of a probiotic strain, *L. plantarum* CCFM8610, with multiple health-promoting effects.	75 patients	*L. plantarum* CCFM8610 Dose: $1x10^{10}$ CFU per day	12 weeks	It was observed that treatment with *L. plantarum* CCFM8610 did not have any side effects as compared with other treatments of IBS-D.
Litton et al. (2021)	Randomized, placebo-controlled, restoration of gut microflora in critical illness trial to determine whether early and sustained *L. plantarum* 299v therapy administered to adult ICU patients increased days alive and at home	218 patients	$2×10^{10}$ CFU of *L. plantarum* 299v per capsule	60 days	Early and sustained untargeted administration of probiotic therapy with *L. plantarum* 299v to adult patients admitted to the ICU is safe.
Infants & Children					
Rundles et al. (2000)	Prospective, randomized, double-blind, placebo-controlled study to determine whether oral administration of the probiotic *L. plantarum* 299v could improve nutrient status and promote growth in children congenitally exposed to HIV	15 immunocom-promised children with HIV	*L. plantarum* 299v Dose of $2x10^{10}$ CFU/day	1 month	No patient experienced bloating or other symptoms of intolerance, and none had to be withdrawn. The data suggest that *L. plantarum* 299v may be given safely to the immune-compromised host.

Table 8. Human Studies of *L. plantarum*

Reference	Study Design and Objective	Subjects	Strain and Dosage	Duration	Safety-Related Results
Guandalini et al. (2010)	Randomized, double-blind, placebo controlled, crossover trial to study the improvement in the symptoms in children with irritable bowel syndrome after probiotic treatment.	77 patients with a new diagnosis of IBS.	*L. plantarum* along with another 7 probiotic strains, concentration was 4.50×10^{11} VSL#3, 1 sachet daily.	16 weeks	All dropped patients from phase I were because of inability / unwillingness to complete questionnaires and dislike of the preparation given. No adverse event was recorded by any patient throughout the study.
Yang et al., (2014)	Randomized, double-blind, placebo-controlled, parallel trial with a washout period to study efficacy of probiotic therapy on atopic dermatitis (AD) children**.**	100 children with mild to moderate AD	Mixture of *L. casei*, *L. rhamnosus*, *L. plantarum*, and *B.lactis* 1×10^{9} CFU of each bacterial strain twice a day	6 weeks	The drop-out rate was higher than anticipated, with 13 patients (26%) in the probiotics group and 16 (32%) in the placebo group eliminated from the study.
Hakansson et al. (2019)	A randomized, double-blind, placebo-controlled clinical trial to study the effect of *L. plantarum* on peripheral immune response in children with celiac disease autoimmunity (CDA)	89 children at genetic risk for celiac disease and on a gluten-containing diet	*L. plantarum* HEAL9 and *L. paracasei* 8700 $2x10^{10}$ CFU/day	6 months	Out of 89, only 11 (12%) children dropped out after the initial visit. The main reasons for study dropout were low compliance and symptoms such as diarrhea or vomiting. One child was also excluded due to insufficient blood sample volumes. Three children in the probiotic group and four children in the placebo group reported adverse events during the study (pain, flatulence, or diarrhea) and one child in each group had gastrointestinal symptoms.

Table 8. Human Studies of *L. plantarum*

					Safety-Related Results
Ahren et al., (2020)	A randomized, double-blind, placebo-controlled trial to evaluate the efficacy of *L. plantarum* HEAL9 and *L. paracasei* 8700:2	131 children	1×10^9 CFU/sachet *Lactobacillus plantarum* HEAL9 and *Lactobacillus paracasei* 8700	3 months	No safety concerns were reported.
Mensi et al., (2021)	A comprehensive clinical, neurophysiological, neuroradiological and genetic assessment to study the role of *L. plantarum* PS128 for reduced symptoms of autism	131 autistic children	*L. plantarum* PS128 $3x10^{10}$ CFU/day if weight was less than 30 kg, and $6x10^{10}$/day CFU for higher weight.	6 months	Side effects were reported in six patients (on *L. plantarum* PS128): increased irritability in three patients (2.3%) and transitory diarrhea in another three patients (2.3%). Out of 131 patients one third of patients had gastrointestinal symptoms already before treatment of *L. plantarum* PS128.

6.5 Decision Tree

Pariza et al. 2015, proposed a 'decision tree' process to determine the safety of microorganisms for human and animal consumption. The decision tree is a step-wise approach addressing various aspects of safety including identity, history of safe use, genomic and phenotypic safety evaluation. The decision tree process considers scenario as substantially increased exposure to a culture that has an established record of safety in a more limited application; a new strain without a history of safe use that was isolated from a food or feed; a new strain isolated from a non-food or non-feed source. It is modeled on previous decision trees that are used worldwide to evaluate the safety of microbial enzymes for use in human food or animal feed (Pariza and Cook, 2010; Pariza and Johnson, 2001; Pariza and Foster, 1983).

The safety of *L. plantarum* 022AE has been assessed using the decision tree for determining safety of microbial culture to be consumed by humans or animals (Pariza et al. 2015)

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

2. Has the strain genome been sequenced? **YES**

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

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

5. Does the strain produce antimicrobial substances? **NO**

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

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

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

Conclusion: The strain is "deemed to be safe for use in the manufacture of food, and dietary supplements for human consumption".

6.6 Safety Assessment and GRAS Determination

This section presents an assessment that demonstrates that the intended use of *L. plantarum* 022AE preparation is safe and is GRAS based on scientific procedures.

This safety assessment and GRAS determination entail two steps. In the first step, the safety of the intended use of *L. plantarum* 022AE is demonstrated. Safety is established by demonstrating a reasonable certainty that the exposure of consumers to *L. plantarum* 022AE under its intended conditions of use is not harmful. In the second step, the intended use of *L. plantarum* 022AE is determined to be GRAS by demonstrating that the safety of the microbial culture under its intended conditions of use is generally recognized among qualified scientific experts and is based on generally available and accepted information.

The regulatory framework for establishing whether the intended use of a substance (or microorganism) is GRAS, in accordance with Section 201(s) of the Federal Food Drug and Cosmetic Act, is set forth under 21 CFR §170.30. As per the 21 CFR §170.30 intended use of a substance or microorganism is GRAS by following two things, either when it is safe for use in human food through scientific procedures under §170.30(b) or 2) by experience based on the common use of a substance to be GRAS used in food prior to January 1, 1958, under §170.30(c). The present GRAS determination is based on scientific procedures under §170.30(b).

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

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

The criteria outlined above for a scientific-procedures GRAS determination are applied below in an analysis of whether the intended use of *L. plantarum* 022AE preparation is safe for the intended uses and is GRAS.

6.6.1 *EVIDENCE OF SAFETY*

The food ingredient *L. plantarum* 022AE has been studied in detail to establish its safety for human consumption. Studies included a polyphasic approach for strain identification; genome analyses to evaluate the concerns of antibiotic resistance, virulence factors, biogenic amines, various toxins; safety of production process; toxicological studies in animal models including acute oral toxicity and 28 days repeated dose oral toxicity; and a detailed review of literature on the safety of *L. plantarum* for human consumption.

Identification of a microorganism is of paramount importance in determining its safety. *L. plantarum* 022AE was analyzed for 16S rRNA and *phe*S gene sequence, mol G +C % and phenotypic and biochemical characteristics to establish its identity. Phenotypic and biochemical characteristics of *L. plantarum* 022AE were also compared and found similar to a reference strain *L. plantarum* ATCC 8014. These studies unambiguously confirm the identity of the strain 022AE as *L. plantarum.*

L. plantarum 022AE was found sensitive to all tested antibiotics. Genome analysis confirmed the presence of horizontally non-transferable and intrinsic antibiotic resistance genes, which are not flanked by mobile elements, suggesting no safety concern with these genes. Antibiotic resistance is a strain-dependent phenomenon, intrinsic and non-transferable antibiotic resistance is not a safety concern, antibiotic sensitivity/resistance of various *L. plantarum* strains are described in GRN 685, 953, 847, and 722.

A homology search between the assembled genome of *L. plantarum* 022AE and virulence factor genes/proteins was performed using BLASTX. The analyses showed no safety concern with respect to virulence factors/genes. Further, to confirm the non-virulence of the strain, *in vitro* cytotoxicity testing against Vero cells was carried out. *L. plantarum* 022AE did not show any cytotoxic effect.

A BLASTX analyses was performed between the assembled genome and biogenic amine producing proteins. *L. plantarum* 022AE does not contain any biogenic amine producing gene of concern. None of the regions of concern, i.e., antibiotic resistance genes, virulence factor genes, and biogenic amine producing genes, were observed in the vicinity of predicted mobile elements in the assembled genome, thus ensuring the stability of the genome and constant safe use of the strain. Four CRISPRs were identified from the assembled genome of *L. plantarum* strain 022AE. The presence of a CRISPR system indicates an advantage in promoting genome stability by acting as a barrier to entry of foreign DNA elements.

L. plantarum 022AE was checked for enterotoxins by Duopath® Cereus Enterotoxins test kit, Merck and D-cultural technique. It was observed that *L. plantarum* 022AE does not produce enterotoxins while the toxin was detected in positive control strains.

L. plantarum 022AE was analyzed for its D- and L- lactic acid production and the results were similar to those of other GRAS strains of *L. plantarum*.

No indications of toxicity were found in acute and repeated-dose studies of oral toxicity or in genotoxicity assays in strain 022AE or other strains of *L. plantarum*. No adverse effects were reported when the vegetative cells of various *L. plantarum* strains were administered to humans. All these generally available findings support the conclusion that the intended use of *L. plantarum* 022AE preparation is safe for human consumption.

Part 7: 21 CFR 170.255: Bibliography

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DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 07/31/2022
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No._____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd):* _____

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd):* _____

SECTION B INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Ankit Kishor Rathi	Director of Quality & Regulatory
Organization *(if applicable)*	
Advanced Enzyme Technologies, Ltd.	
Mailing Address *(number and street)*	
Magnetica LIC Service Road, Louiswadi	

City	State or Province	Zip Code/Postal Code	Country
Thane	Maharashtra	400604	India

Telephone Number	Fax Number	E-Mail Address
+91 22 2583 0284		ankit.rathi@advancedenzymes.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
James T. Heimbach	President
Organization *(if applicable)*	
JHeimbach LLC	
Mailing Address *(number and street)*	
923 Water Street #66	

City	State or Province	Zip Code/Postal Code	Country
Port Royal	Virginia	22535	United States of America

Telephone Number	Fax Number	E-Mail Address
8047425543		JH@JHEIMBACH.COM

SECTION C GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Lactiplantibacillus plantarum 022AE

2. Submission Format: *(Check appropriate box(es))*

☒ Electronic Submission Gateway

☐ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☐ Yes *(Proceed to Item 5)* ☒ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☐ a) GRAS Notice No. GRN _____

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☐ e) Other or Additional *(describe or enter information as above)* _____

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Yogurt, other milk based products, dairy analogs, soy products, fruit drinks, frostings, frozen dairy desserts and mixes, fruit and water ices, drinking water, sports drinks, chewing gum, extracts, and flavorings, condiments, herbs, seeds, spices, seasonings, blends, nuts and nut products, plant-protein products, cereals, processed fruits, processed vegetables and vegetable juices, snack foods, and toppings at a maximum level of approximately 1.0×109 to 10×10E9 colony forming units (CFU)/serving.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E PARTS 2 7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

SECTION F SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Advanced Enzyme Technologies, Ltd.
(name of notifier)

has concluded that the intended use*(s)* of Lactiplantibacillus plantarum 022AE
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Advanced Enzyme Technologies, Ltd. agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.
(name of notifier)

Office of JHeimbach LLC (Agent), 923 Water Street, Port Royal VA 22535
(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
	James T. Heimbach, President, JHeimbach LLC	09/15/2022

SECTION G LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Form3667.pdf	Administrative
	L.plantarumGRASDossier.pdf	Administrative

From: James Heimbach <jheimbach@va.metrocast.net>
Sent: Tuesday, August 15, 2023 10:11 AM
To: Deng, Kaiping <Kaiping.Deng@fda.hhs.gov>
Subject: RE: [EXTERNAL] RE: blends or seasoning blends? -GRN 1108- L. plantarum

Dear Kaiping—

We confirm that the intended uses include only the food categories listed in Part 3 of GRN 001108 as revised in our amendment dated July 18, 2023. We further confirm that the ingredient is not intended for use in infant formula, foods formulated for infants, foods where standards of identity preclude its use, products under the jurisdiction of USDA, or alcoholic beverages.

Best regards,
Jim

James T. Heimbach, Ph.D., F.A.C.N.
JHeimbach LLC
923 Water Street #66
Port Royal VA 22535
USA
Tel: (+1) 804-742-5543
Cell: (+1) 202-320-3063
Email: jh@jheimbach.com

From: Deng, Kaiping <Kaiping.Deng@fda.hhs.gov>
Sent: Wednesday, August 9, 2023 10:16 AM
To: James Heimbach <jheimbach@va.metrocast.net>
Subject: RE: [EXTERNAL] RE: blends or seasoning blends? -GRN 1108- L. plantarum

Morning Jim -

Would you please help clarify one more question below? Thank you!

Kaiping

In Part 3 of GRN 001108 (page 26), the notifier states that the intended food uses of *Lactobacillus plantarum* MCC 0537 include but *are not limited to* the following food categories:

"Yogurt, other milk based products, dairy analogs, soy products, fruit drinks, frostings, frozen dairy desserts and mixes, fruit and water ices, drinking water, sports drinks, chewing gum, extracts, and flavorings, condiments, herbs, seeds, spices,

seasonings, blends, nuts and nut products, plant protein products, cereals, processed fruits, processed vegetables and vegetable juices, snack foods and toppings."

In the amendment dated July 18, 2023, the notifier removed drinking water (including bottled water, flavored water, and enhanced water) and sports drinks from the intended uses of the ingredient.

We request that the notifier further clarify the intended uses of *L. plantarum* MCC 0537:

a) Please confirm that the intended uses include ONLY the food categories listed in Part 3 of GRN 001108 except those that were removed in the amendment dated July 18, 2023. If you intend to use your ingredient in any additional food categories, please clearly specify these food categories.
b) If the intended uses include all conventional foods, please clearly state it, and confirm that the ingredient is not intended for use in infant formula, foods formulated for infants, foods where standards of identity preclude its use, products under the jurisdiction of USDA, and alcoholic beverages.

From: James Heimbach
To: Deng, Kaiping
Subject: [EXTERNAL] RE: blends or seasoning blends? -GRN 1108- L. plantarum
Date: Tuesday, August 8, 2023 1:28:35 PM
Attachments: image001.png

Dear Kaiping—

What my client intends is what I would write simply as "seasonings," which I would take as included both single- and multi-ingredient seasonings. I don't think it's necessary to call mixed seasonings "seasoning blends."

Hope this clarifies things.

Regards,
Jim

From: Deng, Kaiping <Kaiping.Deng@fda.hhs.gov>
Sent: Tuesday, August 8, 2023 10:20 AM
To: James Heimbach <jheimbach@va.metrocast.net>
Subject: blends or seasoning blends? -GRN 1108- L. plantarum

Hi Jim,

Could you please clarify whether it is "seasoning, blends" or "seasoning blends" in the food categories for intended uses? Please see the highlighted below. The food list can be found at Part 1.4 and Part 3 in the notice. If it is "blends", could you please let me know what blends they are?

"Yogurt, other milk based products, dairy analogs, soy products, fruit drinks, frostings, frozen dairy desserts and mixes, fruit and water ices, drinking water, sports drinks, chewing gum, extracts, and flavorings, condiments, herbs, seeds, spices, seasonings, blends, nuts and nut products, plant-protein products, cereals, processed fruits, processed vegetables and vegetable juices, snack foods, and toppings at a maximum level of approximately 1.0×109 to 10×109 colony forming units (CFU)/serving."

Thank you so much! I look forward to hearing from you.
Kaiping

Kaiping Deng, Ph.D.
Regulatory Review Scientist/Staff Fellow
Regulatory Review Branch
Division of Food Ingredients

Office of Food Additive Safety
FDA/CFSAN
Tel: 708-924-0622
kaiping.deng@fda.hhs.gov

From: James Heimbach
To: Deng, Kaiping
Cc: "Jim Heimbach"
Subject: RE: [EXTERNAL] RE: GRAS notice for L. plantarum
Date: Tuesday, July 18, 2023 7:46:36 PM

Sorry for the omission. Yes, sports drinks will also be removed from the intended use.

From: Deng, Kaiping <Kaiping.Deng@fda.hhs.gov>
Sent: Tuesday, July 18, 2023 5:34 PM
To: James Heimbach <jheimbach@va.metrocast.net>
Cc: 'Jim Heimbach' <jh@jheimbach.com>
Subject: RE: [EXTERNAL] RE: GRAS notice for L. plantarum

Dear Jim,

"Sport drinks" is included in the list of intended use for the notice of GRN 1108. Would you please confirm whether sport drinks will also be removed from the intended use? Thank you so much!

Kaiping

From: James Heimbach <jheimbach@va.metrocast.net>
Sent: Tuesday, July 18, 2023 2:23 PM
To: Deng, Kaiping <Kaiping.Deng@fda.hhs.gov>
Cc: 'Jim Heimbach' <jh@jheimbach.com>
Subject: RE: [EXTERNAL] RE: GRAS notice for L. plantarum

Dear Deng—

GRN 001108: Response to the query dated July 6, 2023

You indicated that your intended uses did not include foods in which standards of identity preclude the use of the ingredient. We noted that drinking water is one such category in which standards of identity exist. Please clarify the intended use of drinking water by indicating if this is bottled water, flavored and enhanced waters, etc.

Response:

We intend to remove drinking water from intended uses entirely, including bottled

water, flavored water, and enhanced water.

You provided revised specifications for heavy metals in the May 5, 2023 amendment. The limit of quantification and the results of the batch analyses were the same for arsenic, cadmium, and lead. However, the proposed specification for lead was 0.1 mg/kg, while the proposed specifications for cadmium and arsenic was 0.25 mg/kg each. In keeping with FDA's Closer to Zero initiative for heavy metals, please consider lowering the specifications for cadmium and arsenic to 0.1 mg/kg.

Response:
Revised specifications for heavy metals for *L. plantarum* MCC 0537 are provided below:

Heavy Metal	Limit of Quantification LOQ (mg/kg)	Heavy metal concentration in *L. plantarum* MCC 0537 batches			Specification (mg/kg)
		Batch No. 122052	Batch No. 122055	Batch No. 012117	
Arsenic	0.1	<0.1	<0.1	<0.1	Not more than 0.1 ppm
Cadmium	0.1	<0.1	<0.1	<0.1	Not more than 0.1 ppm
Lead	0.1	<0.1	<0.1	<0.1	Not more than 0.1 ppm
Mercury	0.025	<0.025	<0.025	<0.025	Not more than 0.1 ppm

We trust that these responses are satisfactory.

Regards,
Jim

From: James Heimbach
To: Deng, Kaiping
Cc: "Jim Heimbach"
Subject: RE: [EXTERNAL] RE: GRAS notice for L. plantarum
Date: Tuesday, July 18, 2023 2:23:36 PM
Attachments: image001.png
image002.png

Dear Deng—

GRN 001108: Response to the query dated July 6, 2023

You indicated that your intended uses did not include foods in which standards of identity preclude the use of the ingredient. We noted that drinking water is one such category in which standards of identity exist. Please clarify the intended use of drinking water by indicating if this is bottled water, flavored and enhanced waters, etc.

Response:

We intend to remove drinking water from intended uses entirely, including bottled water, flavored water, and enhanced water.

You provided revised specifications for heavy metals in the May 5, 2023 amendment. The limit of quantification and the results of the batch analyses were the same for arsenic, cadmium, and lead. However, the proposed specification for lead was 0.1 mg/kg, while the proposed specifications for cadmium and arsenic was 0.25 mg/kg each. In keeping with FDA's Closer to Zero initiative for heavy metals, please consider lowering the specifications for cadmium and arsenic to 0.1 mg/kg.

Response:

Heavy Metal	Limit of Quantification LOQ (mg/kg)	Heavy metal concentration in *L. plantarum* MCC 0537 batches			Specification (mg/kg)
		Batch No. 122052	Batch No. 122055	Batch No. 012117	
Arsenic	0.1	<0.1	<0.1	<0.1	Not more than 0.1 ppm
Cadmium	0.1	<0.1	<0.1	<0.1	Not more than 0.1 ppm
Lead	0.1	<0.1	<0.1	<0.1	Not more than 0.1 ppm
Mercury	0.025	<0.025	<0.025	<0.025	Not more than 0.1 ppm

Revised specifications for heavy metals for *L. plantarum* MCC 0537 are provided below:

We trust that these responses are satisfactory.
Regards,
Jim

From: James Heimbach <jheimbach@va.metrocast.net>
Sent: Saturday, May 6, 2023 11:24 AM
To: Deng, Kaiping <Kaiping.Deng@fda.hhs.gov>
Subject: RE: [EXTERNAL] RE: GRAS notice for L. plantarum

GRN 001108: Query Response

1. **You stated that the *Lactiplantibacillus plantarum* strain is deposited at the National Centre for Microbial Resources (NCMR), India, with deposit number MCC 0537.**
   a) **We recommend that a depository name should be used for a GRAS notice, to ensure the strain is available and can be verified.**
   b) **Please provide a reference which the taxonomical analysis was followed.**

   Response:
   a) **We recommend that a depository name should be used for a GRAS notice, to ensure the strain is available and can be verified.**
   We have no objection to using the depository name for our GRAS notice.
   b) **Please provide a reference which the taxonomical analysis was followed.**
   NCBI Taxonomy Browser: *Lactiplantibacillus plantarum*

2. **Please confirm that the proposed maximum use level for *L. plantarum* MCC 0537 of approximately 1.0 × 10^{10} colony forming unit (CFU/g) is the maximum addition level of the ingredient. If there is an overage amount added to food to compensate for the loss of viable cells over time, please specify the overage amount and revise the dietary exposure estimate to account for the overage.**

   Response:
   The proposed maximum use level for *L. plantarum* MCC 0537 of approximately 1.0 × 10^{10} colony forming unit (CFU/g) is the maximum addition level of the ingredient. There is no additional overage.

3. **We note that your intended uses of *L. plantarum* MCC 0537 include uses in foods for which standards of identity exist (e.g., drinking water). Please clarify as to whether**

the ingredient is not intended for use in foods where standards of identity preclude its use.

Response:
The ingredient is not intended for use in foods where standards of identity preclude its use.

4. **In Table 6 (page 23), the unit for total viable count is specified as "viable cell count/g". We note that the colony forming unit (CFU) is a measure of viable colonogenic cell numbers. For the record and consistency throughout the notice, please confirm that the unit for total viable count is CFU/g.**

Response:
The unit for total viable count is CFU/g.

5. **In Table 7 (page 24), the results for heavy metals from three non-consecutive batches are provided as "complies". Please provide the actual measured levels of heavy metals. If the levels are below the limit of detection (LOD) or limit of quantitation (LOQ) of the method, please provide the LOD or LOQ. We recommend that the specification limits for heavy metals should be reflective of the results of the batch analyses and be as low as possible.**

Response:
Revised specification for heavy metals for *L. plantarum* MCC 0537 is provided below:

Heavy Metal	Limit of Quantification LOQ (mg/kg)	Heavy metal concentration in L. plantarum MCC 0537 batches			Specification (mg/Kg)
		Batch No. 122052	Batch No. 122055	Batch No. 012117	
Arsenic	0.1	<0.1	<0.1	<0.1	Not more than 0.25 ppm
Cadmium	0.1	<0.1	<0.1	<0.1	Not more than 0.25 ppm
Lead	0.1	<0.1	<0.1	<0.1	Not more than 0.1 ppm
Mercury	0.025	<0.025	<0.025	<0.025	Not more than 0.1 ppm

6. **In part 2.1.2.1 (b), you stated that the strain has 100% sequence homology with the genome of the reference strain *L. plantarum* HAC01 based on BLASTN results. We note that the type-strain of *L. plantarum* is ATCC 14917 in the reference where *Lactobacillus* genus was taxonomically analyzed (Zheng *et al*, 2020, "A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of**

***Lactobacillaceae* and *Leuconostocaceae*", *Int J Syst Evol Microbiol* 70:2782–2858). Please describe why strain *L. plantarum* HAC01 was chosen for the genomic comparison.**

Response:

A whole genome sequence was carried out using the *de novo* assembly for the strain *L. plantarum* 022AE (*L. plantarum* MCC 0537) but the annotation of the genome was carried out based on the reference strain approach. In our experience, annotation is most accurate using a strain with maximum identity. Accordingly, the strain *L. plantarum* HAC01 was considered for annotation as well as for genome comparison.

7. **Please clarify if *L. plantarum* strain MCC 0537 is genetically modified.**

Response:

L. plantarum strain MCC 0537 is not genetically modified.

8. **You stated that a pure culture of *L. plantarum* MCC 0537 was used for inoculation. For the administrative record, please briefly specify how the purity of *L. plantarum* MCC 0537 inoculum is ensured.**

Response:

Purity of *L. plantarum* strain MCC 0537 is ensured throughout the process. Inoculum preparation is carried out by trained personnel in biosafety cabinets, installed in a class 100,000 cleanroom. Subsequent growth of the bacterium on a laboratory shaker is also carried out in a similar cleanroom environment. The purity of the initial inoculum is ensured by monitoring the inoculum's specific growth pattern, direct microscopic evaluation, gram staining, and reviewing the characteristics of viable growth on agar medium. Fermentation of *L. plantarum* strain MCC 0537 is carried out in a contained, sterile environment in FSSC 22000-certified facilities following cGMP and HAACP principles.

9. **Please clarify that the fermentation process is continuously monitored for contaminants.**

Response:

The fermentation process is conducted in an entirely closed and sterile environment. The production facilities are FSSC 22000-certified and have fully implemented HACCP plans that continuously monitor and control critical parameters and minimize the risk of contamination. At regular intervals, small aliquots are sampled aseptically from the main

fermentation tank to check for contamination through direct microscopic evaluation and the characteristics of viable growth on agar medium.

10. **Please conduct a literature search and discuss any opportunistic infection of the microorganism at species level. For example, we note that reference "Biesiada G. et al., 2019. Meningoencephalitis caused by *Lactobacillus plantarum* - case report, *Int J Neurosci.*, 129(7):715-718" is published.**

Response:

Lactobacilli are generally considered a non-pathogenic, non-acidogenic and highly beneficial family of bacteria (Chidre and Revanasiddapa 2017). Although cases of opportunistic infections caused by various lactobacillus species have been reported, very few are associated with *L. plantarum* specifically. These cases are summarized and discussed below.

Tena et al. in 2013 published a case study of a 57-year-old male patient with acute acalculous cholecystitis with peritonitis that may have been secondarily caused by *L. plantarum* on the basis of the presence of *L. plantarum* in his bile and peritonial fluid. The identification of the isolate strain was carried out using API 50 CHL system and 16s rDNA sequencing. The isolate was resistant to vancomycin, erythromycin and clindamycin and susceptible to penicillin, cefotaxime, imipenem, ciprofloxacin, and trimethoprim-sulfamethoxazole. The patient was treated with imipenem for 2 weeks. The patient recovered and did not have recurrence of the infection. (Tena et al., 2013)

Callaway et al. in 2013 proposed the use of reliable methods for identification etiopathogeny of infections due to lactobacilli. The authors used species-specific PCR, MALDI-TOF MS to analyze 87 carious dentin samples. The analyzed samples showed the presence of two *L. plantarum* species, among other isolated *Lactobacillus* species, in soft or hard carious dentin from 70 out of 87 first molars of children between 7 and 8 years old. (Callaway et al., 2013)

In the case study published in 2019 by Biesiada and group, confirmed meningoencephalitis was caused by *L. plantarum* in a 63-year-old male patient with newly diagnosed lung cancer. In the next two days, the patient developed hypercapnia, increased production of pulmonary secretions, and an irregular breathing pattern. Three days after initial symptoms, *Lactobacillus* spp. were isolated from both blood and CSF cultures using the automated microbial detection system BACT/ALERT 3D. The isolates were identified as *L. plantarum.* Gradually, after treatment and intravenous antimicrobial therapy, the authors observed an improvement in the patient's condition. Accordingly, *L. plantarum* may be indicative in the development of complexities during onset of meningoencephalitis (Biesiada et al., 2019).

References-

Biesiada G, Krycińska R., Czepiel J, Stażyk K., Kędzierska J & Garlicki A. 2019. Meningoencephalitis caused by *Lactobacillus plantarum* - case report. Int J Neurosci. 129(7):715-718.

Chidre P, Revanasiddappa Kelmani C. 2017. Probiotic potential of Lactobacilli with antagonistic activity against pathogenic strains: An in vitro validation for the production of inhibitory substances. Biomedical Journal 40, 270-283.

Callaway A, Kostrzewa M, Willershausen B, Schmidt F, Thiede B, Küpper H, Kneist S. 2013. Clin Lab. Identification of *Lactobacilli* from deep carious lesions by means of species-specific PCR and MALDI-TOF mass spectrometry. 59 (11-12):1373-9.

Tena D, Mariela N, Losa C, Fernández C, Medina M, Sáez-Nieto J. 2013. Acute acalculous cholecystitis complicated with peritonitis caused by *Lactobacillus plantarum* Diagnostic Microbiology and Infectious Disease. 76, 510–512

11. **In Part 6, you stated that "strains of *L. plantarum* are gaining in importance as potential probiotics among the LABs considering their ancient important uses in food preservation and fermentation (GRAS 685, 722 and 847)". In general, submissions should not include discussion of purported benefits or language implying dietary supplement uses or drug uses (e.g., "probiotic", dose, capsule, sachet, efficacy as an endpoint, health benefit, *etc.*). It should be noted that implications for such claim should not be included in a GRAS notice, which should focus on identity, intended use in conventional foods and safety.**

Response:

We accept your suggestion and will refrain from using the term "probiotic" in the GRAS notification.

12. **For supporting your safety conclusion, you listed three successful GRAS notices (#685, 722, 847) describing the uses of *L. plantarum* strains. As each GRAS notice stands on its own, for the administrative record, please provide a brief paragraph summarizing the information pertaining to safety of *L. plantarum* strains that are the subjects of these GRAS notices.**

Response:

The safety evaluation of Advanced Enzyme's *L. plantarum* MCC 0537 is completely based on its own safety information, which is provided in GRN 001108. This information includes WGS analysis, antibiotic resistance pattern, antimicrobial activity, virulence activity, enterotoxin production, systemic oral toxicity, and genotoxicity. It is not dependent on other successful GRAS notices (e.g., GRNs 685, 722, and 847). These were merely provided for comparison.

Please find below a brief paragraph summarizing the information pertaining to safety of *L. plantarum* strains that are the subjects of these GRAS notices.

GRN 685

GRN 685 concludes that *Lactobacillus plantarum* strain 299v has been adequately identified and characterized for both phenotypic and genotypic parameters, with no concerns existing regarding the safety of ingestion at levels up to 10^{11} cfu/day. Studies reported in the GRN confirmed that no genes encoding for antibiotic resistance, were identified in *L. plantarum* strain 299v, also it produces lactic acid and acetic acid at nonhazardous levels, hence posing a low risk of D-lactic acidosis. The animal studies conducted in rats, mice, and pigs with *L. plantarum* strain 299v showed no adverse effect with doses as high as 10^{10} cfu/day when administered for four weeks. Also, 37 studies with human adults and children, both healthy and diseased, showed no adverse effects. The safety of strain was further assessed by use of a Pariza et al. (2015) decision-tree and it was found to be safe for use in the production of food for human consumption.

GRN 722

Genotypic and phenotypic studies reported in GRN 722 showed that there are no safety concerns regarding consumption of *Lactobacillus plantarum* Lp-115 at doses up to $1x10^{10}$ cfu/day. The GRN concludes safety of *Lactobacillus plantarum* Lp-115 by proving the absence of transferable antibiotic resistance elements, the absence of virulence factor, infectivity elements, toxins, ability to produce to both D- and L- lactic acid, and absence of unusual adherence capability. The acute oral toxicity study showed no adverse effects at an acute oral dose of 5000 mg/kg bw. (LD_{50})(4.2×10^{12} CFU/kg bw). Human studies reported in the GRN confirmed no treatment-related adverse events at daily doses up to 2×10^{11} CFU of *L. plantarum* Lp-115 for the treatment period of 90 days in healthy subjects and liver-transplant patients. The safety of *L. plantarum* Lp-115 was further evaluated using the decision tree procedure of Pariza *et al.* (2015). It supports the safety of the intended use of *L. plantarum* Lp-115.

GRN 847

The safety of *L. plantarum* LPLDL® was established in GRN 847 for its intended usage as an ingredient in foods at a level of addition of up to 1×10^{10} CFU per serving. Genotypic and phenotypic analysis of *L. plantarum* LPLDL® showed lack of resistance to clinically relevant antibiotics and absence of known genes related to virulence and pathogenicity. The phenotypic characterization data on *L. plantarum* LPLDL® reported in the GRN proves its safety with the strain's sensitivity to gastric acidity, resistant to bile salts, inability to produce significant biogenic amines, and potential to produce D-and L-lactate at levels similar to other *L. plantarum* strains. The human study on *L. plantarum* LPLDL® involving 49 healthy, normal to mildly hypercholesterolemic adults at a consumption level of 2 x

10^9 CFU twice daily for 12 weeks reported no impact on gastrointestinal function or any other health related side effects.