November 25, 2024

Division of Food Ingredients
Office of Pre-Market Additive Safety
Office of Food Chemical Safety,
Dietary Supplements, and Innovation
Human Foods Program
U.S. Food and Drug Administration
5001 Campus Park Drive.
College Park, MD 20740

Dear Sir/Madam:

In accordance with regulation 21 CFR Part 170 Subpart E (Generally Recognized as Safe (GRAS) Notice), Sunway Co. LTD (the notifier), through me as its agent, hereby provides the enclosed notice of a claim that the addition of *Lacticaseibacillus paracasei* subsp. *paracasei* NTU 101 to conventional foods is GRAS under the conditions of its intended use in foods based on scientific information. Please note that this is a resubmission of the GRAS notification for *Lacticaseibacillus paracasei* subsp. *paracasei* NTU 101. Based on the recommendations provided in the response letter from the previous submission, the notifier has included a more detailed description of the intended use.

If you have any questions regarding this notification, don't hesitate to get in touch with me.

Sincerely,

Chun Chang Fang, PhD
ChemRite Professional Group
Tel: +1 818 730 3636
Email: chemrite@gmail.com

Generally Recognized as Safe (GRAS) Determination for the Intended Use of *Lacticaseibacillus paracasei* subsp. *paracasei* NTU 101

Submitted by the Notifier:

Sunway Biotech Co., Ltd.,
No.139, Xing'ai Rd., Neihu Dist.,
Taipei City 114, Taiwan (R.O.C.)

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Part 1. Signed statements and certification.

a. Submission of the GRAS Notice

Sunway Biotech Co., submits this notification of Generally Recognized as Safe (GRAS) notification in accordance with 21 CFR §170.225.

b. Name and Address of Notifier

Sunway Biotech Co., Ltd., No.139, Xing'ai Rd., Neihu Dist., Taipei City 114, Taiwan (R.O.C.)
TsungWei Shih, PhD
tw.shih@sunway.cc
+886-2-27929568 ext. 632

c. Names of Notified Microorganisms

The substance that has been determined as Generally Recognized as Safe (GRAS) in this notice is *Lacticaseibacillus paracasei* subsp. *paracasei* strain NTU 101 (*L. paracasei* NTU 101). *Lactobacillus* species have been re-classified, and some names have been changed (Zheng et al., 2020). The former name of *L. paracasei* NTU 101 is *Lactobacillus paracasei* subsp. *paracasei* strain NTU 101. This strain will be manufactured as a freeze-dried powder and marketed under the product name Vigiis-101 LAB.

d. Intended Conditions of Use

L. paracasei NTU 101 is manufactured in compliance with Good Manufacturing Practices as specified in Part 110 of the Title 21 Code of Federal Regulations (21 CFR).

This GRAS substance qualifies as a Nutrient Supplement as defined in 21 CFR §170.3. Furthermore, no specific medical or therapeutic claims have been made for this substance.

For the purpose of this GRAS notice, *L. paracasei* NTU 101 is intended to be used as an ingredient added to foods where standards of identity do not preclude such use. *L. paracasei* NTU 101 is intended for use in dairy products, soy products, juices, non-alcoholic and alcoholic beverages, cereals, confectionary snacks, ice cream, chocolate, chewing gum, and other foods. Additionally, it is intended for use in both fermented and unfermented foods, at a concentration of up to 1.0×10^{10} CFU/serving, to maintain 5.0×10^{9} CFU/serving throughout the product's shelf life. These addition levels do not exceed the acceptable daily intake (ADI) of 1.0×10^{11} CFU/60 kg BW/day.

e. Statutory Basis for GRAS Determination

L. paracasei NTU 101 has been determined to be GRAS through the use of scientific procedures, in accordance with 21 CFR §170.30 parts (a) and (b).

f. Exemption from Premarket Approval Requirements

L. paracasei NTU 101 is exempt from the requirements for premarket approval as detailed in the Federal Food, Drug, and Cosmetic Act based on the conclusion that the notified substance is GRAS under the conditions of intended use described above.

g. Data Availability

The data, information, and individual documents used to determine the GRAS status of *L. paracasei* NTU 101 are available to the FDA upon request.

Such documentation may be sent to the FDA in paper or electronic format by the designated contact person listed below:

Tsung Wei Shih
tw.shih@sunway.cc
+886-2-27929568 ext. 632

or viewed during standard business hours at Sunway Biotech Co's business address.

h. Freedom of Information Act

None of the information as listed in Parts 2 through 7 of this notice is exempt from disclosure under the Freedom of Information Act, 5 USC §552.

i. Certification and Signature

To the best of our knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of safety and GRAS status of the use of this substance.

TsungWei Shih, PhD
R&D division Manager
Sunway Biotech Co., Ltd

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

2. Identity

2.1. Name of the GRAS Organism

The notified organism is *Lacticaseibacillus paracasei* subsp. *paracasei* NTU 101 (*L. paracasei* NTU 101), which was previously named *Lactobacillus paracasei* subsp. *paracasei* NTU 101. The target strain was deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) under accession number 28047.

2.2. Sources of the GRAS Organism

L. paracasei NTU 101 was isolated from infant feces (Pan et al., 2002) and identified according to standard taxonomic guidelines.

2.2.1. The taxonomic lineage is:

Kingdom: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Lactobacillales
Family: Lactobacillaaceae
Genus: Lacticaseibacillus
Species: paracasei
Sub-species: paracasei
Strain: NTU 101

2.2.2. Descriptions of the GRAS Organisms

2.2.2.1. Morphologic and Phenotypic Identification

L. paracasei NTU 101 is an anaerobic, gram-positive, catalase-negative, and rod-shaped bacterium that does not form spores. A micrograph of the Gram staining of *L. paracasei* NTU 101 is shown in Figure 1. LAB-selective de Mann–Rogosa–Sharpe (MRS) agar plates revealed smooth, round, and off-white colonies (Figure 2). The fermentation profile of the strain was determined using the API 50 CHL Identification System (bioMérieux, France) and is shown in Table 1.

The morphology and fermentation profile of *L. paracasei* NTU 101 exhibited the characteristics of *Lactobacillus paracasei* subsp. *paracasei* (the former name of *Lacticaseibacillus paracasei* subsp. *paracasei*).

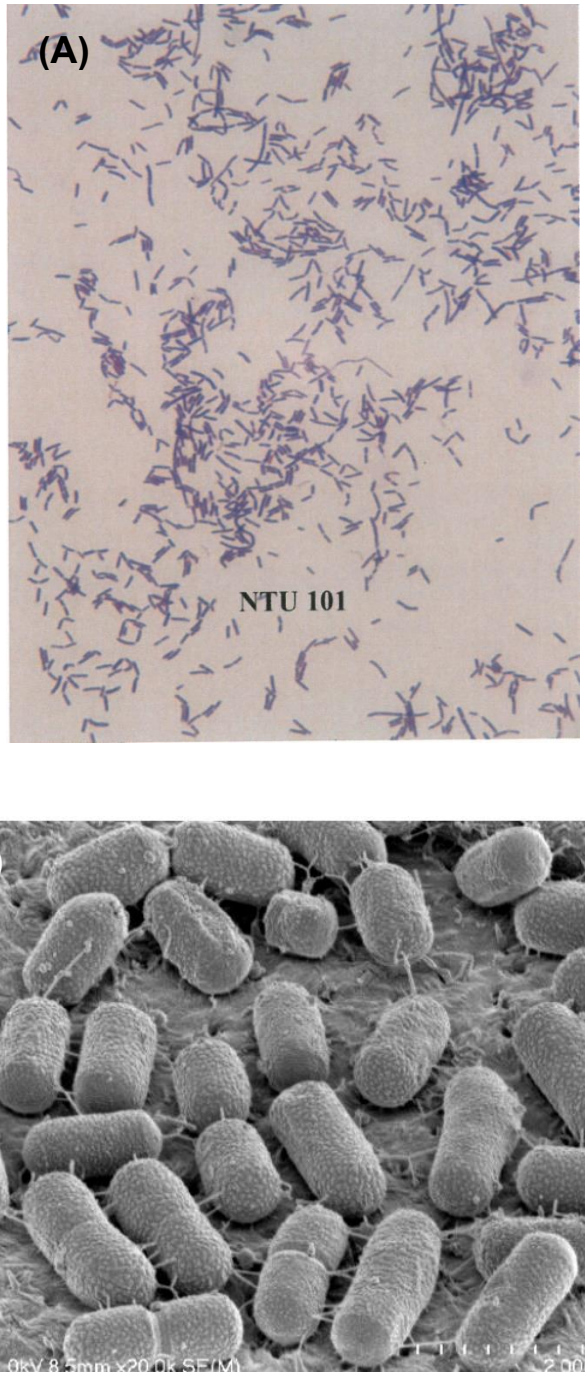

Figure 1. *L. paracasei* NTU 101 under (A) light microscope. (B) scanning electron microscope.

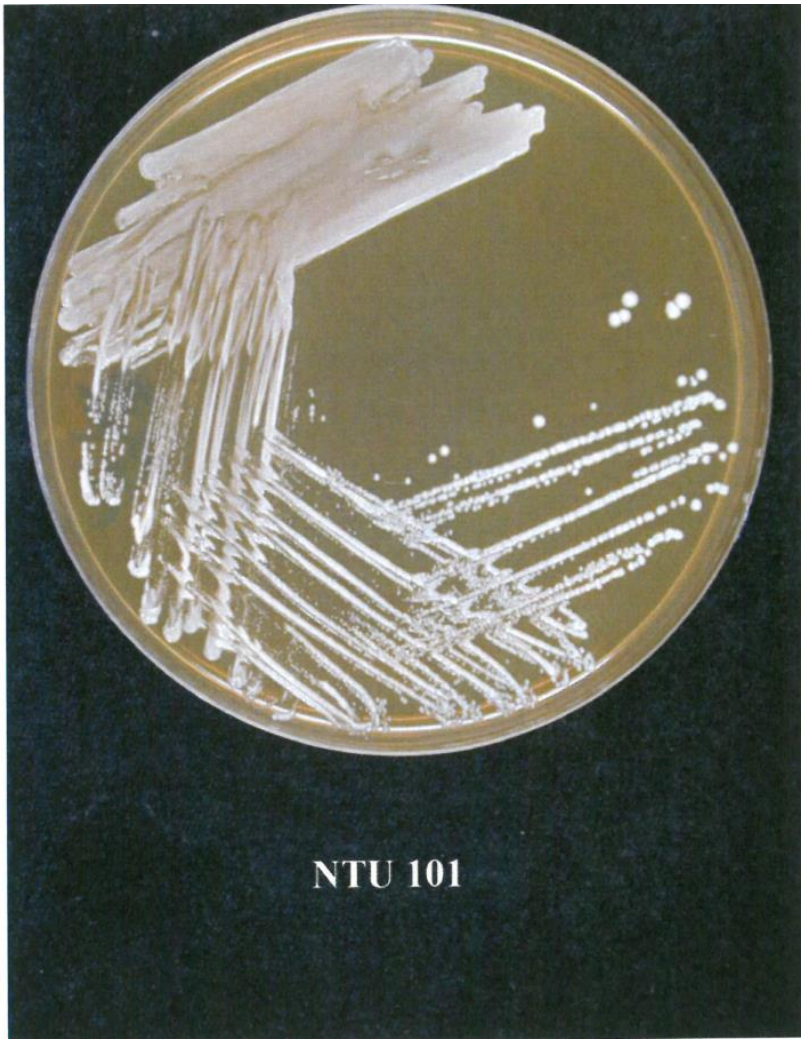

Figure 2. *L. paracasei* NTU 101 colonies grown under anaerobic conditions on MRS agar.

Table 1. API 50 CHL panel results of *L. paracasei* NTU 101. (Chen et al., 2024, supplementary material)

Strain: *Lacticseibacillus paracasei* subsp. *paracasei* NTU 101					
Phenotypic Characteristics: API 50CHL Identification Systems (bioMérieux)					
Tests	**Ingredients**	**Results**	**Tests**	**Ingredients**	**Results**
	Control	−	ESC	Esculin, ferric citrate	+
GLY	Glycerol	−	SAL	Salicin	+
ERY	Erythritol	−	CEL	D-Cellobiose	+
DARA	D-Arabinose	−	MAL	D-Maltose	+
LARA	L-Arabinose	−	LAC	D-Lactose (Bovine origin)	+
RIB	D-Ribose	+	MEL	D-Melibiose	−
DXYL	D-Xylose	−	SAC	D-Saccharose (Sucrose)	+
LXYL	L-Xylose	−	TRE	D-Trehalose	+
ADO	D-Adonitol	+	INU	Inulin	−
MDX	Methyl-βD-xylopyranoside	−	MLZ	D-Melezitose	+
GAL	D-Galactose	+	RAF	D-Raffinose	−
GLU	D-Glucose	+	AMD	Amidon (Starch)	−
FRU	D-Fructose	+	GLYG	Glycogen	−
MNE	D-Mannose	+	XLT	Xylitol	−
SBE	L-Sorbose	+	GEN	Gentiobiose	+
RHA	L-Rhamnose	−	TUR	D-Turanose	+
DUL	Dulcitol	−	LYX	D-Lyxose	−
INO	Inositol	−	TAG	D-Tagatose	+
MAN	D-Mannitol	+	DFUC	D-Fucose	−
SOR	D-Sorbitol	+	LFUC	L-Fucose	−
MDM	Methyl-αD-mannopyranoside	−	DARL	D-Arabitol	−
MDG	Methyl-αD-glucopyranoside	−	LARL	L-Arabitol	−
NAG	N-Acetyl-glucosamine	+	GNT	Potassium Gluconate	+
AMY	Amygdalin	+	2KG	Potassium 2-Ketogluconate	−
ARB	Arbutin	+	5KG	Potassium 5-Ketogluconate	−

+: Positive reaction; −: Negative reaction; /: Borderline

2.2.3. Genotypic Identification

L. paracasei NTU 101 was identified through 16S rRNA sequence analysis. The sequence similarity to *Lactobacillus paracasei* subsp. *paracasei* was 99.9% (Chen et al., 2024).

Zheng et al. (2020) proposed the re-classification of the genus *Lactobacillus* into 25 genera based on core genome phylogeny, (conserved) pairwise average amino acid identity, clade-specific signature genes, physiological criteria, and organism ecology. *Lactobacillus paracasei* subsp. *paracasei* was re-classified as *Lacticaseibacillus paracasei* subsp. *paracasei* (synonym: *Lactobacillus paracasei* subsp. *paracasei*) based on the Notification List of the International Committee on Systematics of Prokaryotes. Thus, *L. paracasei* NTU 101 was reassigned the scientific name *Lacticaseibacillus paracasei* subsp. *paracasei* strain NTU 101 based on the findings of Zheng et al. (2020).

A draft whole genome sequence of *L. paracasei* NTU 101 was obtained using whole genome shotgun sequencing and deposited in the National Center for Biotechnology Information (NCBI) database under GenBank accession number GCA_002901165.3 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_002901165.3/). The *L. paracasei* NTU 101 genome consists of a single circular chromosome of 3,010,957 bp and a plasmid of 50,630 bp (Chen et al., 2024). In terms of genomic similarity, the average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) value between *L. paracasei* NTU 101 and *Lacticaseibacillus paracasei* subsp. *paracasei* JCM 8130^{T} (type strain) were 98.7% and 88.2%, respectively (Chen et al., 2024). According to the standards for the taxonomy of prokaryotes published by IJSEM (Riesco and Trujillo, 2024), results with an ANI greater than 96% and dDDH above 70% can be considered the same species.

L. paracasei NTU 101 was confirmed as *Lacticaseibacillus paracasei* subsp. *paracasei* based on morphological, phenotypic, and genotypic characteristics (Chen et al., 2024).

2.2.4. Strain Level Identification

Strain typing tests were performed using multilocus sequence typing (MLST) and whole genome MLST through sequence analysis of genes. Sequence analysis revealed several nucleotide polymorphisms between *L. paracasei* NTU 101 and reference strains of *Lacticaseibacillus paracasei* subsp. *paracasei.* The molecular typing of *L. paracasei* NTU 101 at the strain level is supported by these data.

2.2.5. Summary

Sunway Biotech Co., commissioned Food Industry Research and Development Institute (FIRDI), Taiwan, to conduct the strain identification of *L. paracasei* NTU 101 in 2017. The morphological, phenotypic, and genotypic evaluation of *L. paracasei* NTU 101 demonstrated that this strain belongs to *Lacticaseibacillus paracase*i subsp. *paracasei*.

MLST analysis confirmed that the strain type of *L. paracasei* NTU 101 is different from the other reference strains.

2.3. Method of Manufacture

The production facility used to manufacture *L. paracasei* NTU 101 is located in Luzhu District, Kaohsiung Science Park, Taiwan (SYNBIO TECH INC.). *L. paracasei* NTU 101 is produced in full compliance with ISO 22000, National Sanitation Foundation Good Manufacturing Practices (NSF-GMP), and Food Safety System Certification 22000.

2.3.1. Maintenance of *L. paracasei* NTU 101

Synbio Tech maintains frozen reference stocks of *L. paracasei* NTU 101. Reference stocks are used to generate working stock under controlled conditions, maintaining effective acceptance criteria at the Synbio Tech Culture Collection and Data Bank. Both the reference and working stocks are verified to be *L. paracasei* NTU 101 through PCR. Stocks that fail any of the required tests are destroyed. Qualified stocks are stored at -80°C until fermentation. All steps for the preparation of stocks are documented in a specified database, allowing traceability of every seed preparation to each batch of raw material used.

2.3.2. Fermentation Process

A schematic of the manufacturing process for *L. paracasei* NTU 101 is shown in Figure 3.

All mixing tanks, heat exchangers, lines, fermenters, and centrifuges are cleaned using an automated clean-on-place system before fermentation. The systems are steamed before product contact.

Fermentation begins by withdrawing one of the working stocks and scaling it up via a series of fermentations until a commercial-sized batch is completed. The seed culture is used to inoculate the culture medium in the main fermenter and is cultured at a constant temperature until the bacteria reach the fermentation endpoint. The temperature and pH of the culture medium are used as quality controls.

The viability of lactic acid bacteria (LAB) is $\geq 3.0 \times 10^{11}$ CFU/g when fermentation is complete. Fermenters are normally cooled to 30–37°C, and

fermentation is stopped when the pH and base relative data indicate that fermentation has entered the stationary phase. The cooled fermentate is pumped through a centrifuge, and the bacteria are concentrated.

The concentrated bacterial slurry is mixed with good-grade cryoprotectants, frozen in liquid nitrogen, and freeze-dried. The freeze-dried pellets are subsequently lyophilized, resulting in substantially low water activity and ensuring culture stability.

The freeze-dried pellets are milled and sieved. Excipients are then added to the concentrate to standardize the blends and confer healing properties to dry powder. The standardized blend is packed into aluminum foil bags and stored at -18°C while awaiting approval from quality control personnel. The product is then released and made available for shipping.

Production Process Flow Chart of NTU 101

Process	Control point
Raw material[*1]	[*1]Time: 5 ~ 20 min
↓	
Sterilization[*2]	[*2]SIP Temperature: 105 ~ 121°C
↓	Time: 15 ~ 50 min
↓	UHT Temperature: 130 ~ 145°C
↓	Time: 3 ~ 30 sec
Cooling[*3]	[*3]Temperature: 30 ~ 37°C
↓	
Inoculation and Fermentation[*4]	[*4]Temperature: 30 ~ 37°C
↓	Time: 8 ~ 24 hr
End fermentation[*5]	[*5]Lactic acid bacteria: ≥ $1.0 \times 10^{8\text{-}10}$ CFU/g
↓	
Centrifugation	
↓	
Coating	
↓	
Freeze drying[*6]	[*6]Temperature: -50 ~ -45°C
↓	
Milling[*7]	[*7]Temperature: < 30°C
↓	Relative humidity: < 65%
Standardization[*8]	[*8]Temperature: < 30°C
↓	Relative humidity: < 65%
Sieving[*9]	[*9]Particle size: 40 mesh 99% pass
↓	
Magnetic inspection[*10]	[*10]Magnetic matters: ≥ 7000 GAUSS
↓	
Packaging[*11]	[*11]Appearance: white ~ yellowish-brown powder
↓	Water content: < 60%
↓	Lactic acid bacteria: ≥ $1.0 \times 10^{10\text{-}11}$ CFU/g
↓	Mold and Yeast: < 10 CFU/g
↓	Coliform: Negative
↓	*Staphylococcus aureus*: < 1.0×10^{2} CFU/g
↓	*Salmonella*: Negative
↓	*Listeria monocytogenes*: < 1.0×10^{2} CFU/g
↓	
Stored at -18°C or below for 2 years	

Figure 3. Production Process for *L. paracasei* NTU 101. SIP, sterilization-in-place. UHT, ultra-high-temperature processing.

2.3.3. Specifications

A quality control laboratory is maintained onsite. Quality control personnel are qualified through training and experience to test and release products based on the specifications (Table 2). These parameters are assessed using compendial methods. Data from three batches of NTU 101 demonstrate adequate control of the production process and compliance with product specifications.

Table 2. Product specifications for three batches of *L. paracasei* NTU 101 powder.

Parameter	**Specification**	**Lot No. /Month of manufacture**					**Method**
		220512 05/2022	**23360901 06/2023**	**23391501 09/2023**	**24310801 01/2024**	**24332501 03/2024**	
Appearance	White to light yellow powder	Passed	Passed	Passed	Passed	Passed	-
Lactic acid bacteria (CFU/g)	≧ 1.0×10^{11}	2.2×10^{11}	3.2×10^{11}	2.5×10^{11}	2.7×10^{11}	2.6×10^{11}	MOHW Food No. 1021950329 Announced: Methods of Test for Food Microorganisms-Test of Lactic Acid Bacteria. (MOHWM0013.01)
Water content (%)	< 6.0%	1.9%	1.6%	1.3%	2.3%	3.7%	FD-660 Infrared Moisture Analyzer, KETT Japan
Water activity (Aw)	< 0.20	0.058	0.042	0.057	0.052	0.077	HygroLab, Rotronic Switzerland
Total plate count	< 5.0×10^4 CFU/g	Passed	Passed	Passed	Passed	Passed	MOHW Food No. 1121900620 Announced: Methods of Test for Food Microorganisms-Test of Aerobic Plate Count. (MOHWM0014.02)
E. coli	Negative	Negative	Negative	Negative	Negative	Negative	Chromocult® Coliform Agar /AOAC 020902(Merck)、3M Petrifilm™ E. coli/ Coliform Count Plate

Mold and Yeast	< 10^2 CFU/g	Passed	Passed	Passed	Passed	Passed	MOHW Food No. 1021950329 Announced: Methods of Test for Food Microbiology- Test of Mold and Yeast Count. (MOHWM0008.01)
Coliform	< 10^2 CFU/g	Passed	Passed	Passed	Passed	Passed	Chromocult® Coliform Agar /AOAC 020902 (Merck)、3M Petrifilm™ E. coli/ Coliform Count Plate
Staphylococcus	Negative	Negative	Negative	Negative	Negative	Negative	CHROMagar™ Staph aureus (TPM)
Salmonella	Negative	Negative	Negative	Negative	Negative	Negative	Singlepath® Salmonella /AOAC 060401 (Merck)
Listeria	Negative	Negative	Negative	Negative	Negative	Negative	CHROMagar™ Listeria (TPM)

2.4. Stability

L. paracasei NTU 101 was packaged in a foil bag, frozen (-20°C), and refrigerated (5°C ± 3°C) to determine stability (viability of cells). These two storage conditions were evaluated over 24 months (Figure 4). The intended use of the microorganisms is limited to applications that can sufficiently support microbial cell viability throughout the shelf life of a product.

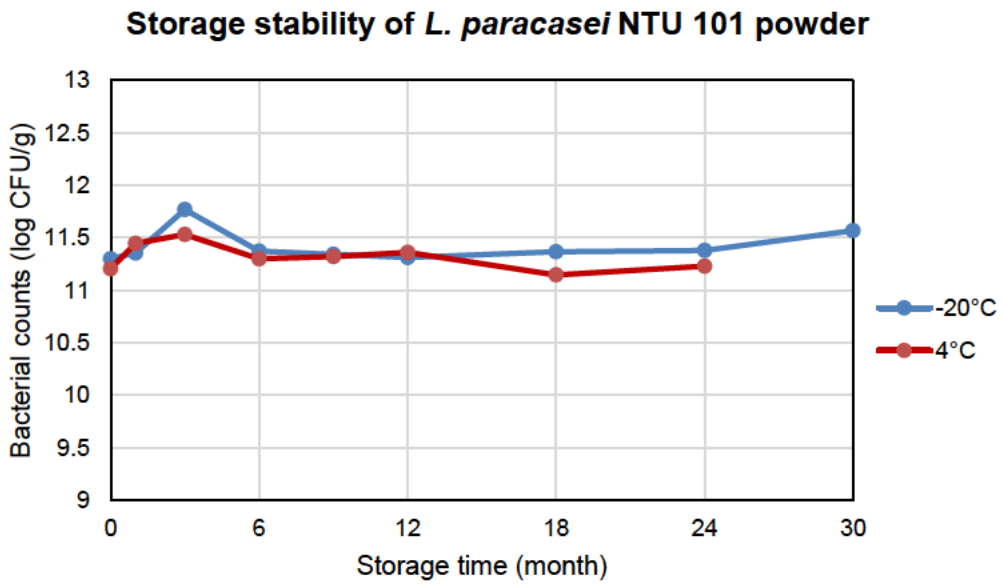

Figure 4. Storage stability on frozen and refrigeration conditions of *L. paracasei* NTU 101 powder over 24 months.

Part 3. Intended Use and Dietary Exposure

For the purpose of this GRAS notice, *L. paracasei* NTU 101 is intended to be used as an ingredient added to foods where standards of identity do not preclude such use. The intended applications of the substance include addition to the following food products as an ingredient: dairy products, soy products, juices, cereals, chocolate, alcoholic beverages, chewing gum, confectionary snacks, and other conventional foods. The amount of *L. paracasei* NTU 101 added to the aforementioned non-fermented food products generally ranges from 1.0×10^9 to 1.0×10^{10} CFU/serving. In fermented foods, such as yogurt or fermented soy milk, viable *L. paracasei* NTU 101 content ranges from 1×10^8 to 1×10^{10} CFU/serving, typically around 6.0×10^9 CFU/serving. The food categories to which *L. paracasei* NTU 101 will be added, as outlined in 21 CFR 170.3 (n), are listed in the provided Table 3, including but not limited to.

L. paracasei NTU 101 is not intended for use in product regulated by the FSIS/USDA.

Table 3. Proposed food uses of *L. paracasei* NTU 101.

Food Category
(1) Baked goods and baking mixes, including all ready-to-eat and ready-to-bake products, flours, and mixes requiring preparation before serving.
(2) Beverages, alcoholic, including malt beverages, wines, distilled liquors, and cocktail mix.
(3) Beverages and beverage bases, nonalcoholic, including only special or spiced teas, soft drinks, coffee substitutes, and fruit and vegetable flavored gelatin drinks.
(4) Breakfast cereals, including ready-to-eat and instant and regular hot cereals.
(5) Cheeses, including curd and whey cheeses, cream, natural, grating, processed, spread, dip, and miscellaneous cheeses.
(6) Chewing gum, including all forms.
(7) Coffee and tea, including regular, decaffeinated, and instant types.
(8) Condiments and relishes, including plain seasoning sauces and spreads, olives, pickles, and relishes, but not spices or herbs.
(9) Confections and frostings, including candy and flavored frostings, marshmallows, baking chocolate, and brown, lump, rock, maple, powdered, and raw sugars.
(10) Dairy product analogs, including nondairy milk, frozen or liquid creamers, coffee whiteners, toppings, and other nondairy products.
(20) Frozen dairy desserts and mixes, including ice cream, ice milks, sherbets, and other frozen dairy desserts and specialties.
(21) Fruit and water ices, including all frozen fruit and water ices.

(22) Gelatins, puddings, and fillings, including flavored gelatin desserts, puddings, custards, parfaits, pie fillings, and gelatin base salads.
(23) Grain products and pastas, including macaroni and noodle products, rice dishes, and frozen multicourse meals, without meat or vegetables.
(24) Gravies and sauces, including all meat sauces and gravies, and tomato, milk, buttery, and specialty sauces.
(25) Hard candy and cough drops, including all hard type candies.
(26) Herbs, seeds, spices, seasonings, blends, extracts, and flavorings, including all natural and artificial spices, blends, and flavors.
(27) Jams and jellies, home-prepared, including only home-prepared jams, jellies, fruit butters, preserves, and sweet spreads.
(28) Jams and jellies, commercial, including only commercially processed jams, jellies, fruit butters, preserves, and sweet spreads.
(29) Meat products, including all meats and meat containing dishes, salads, appetizers, frozen multicourse meat meals, and sandwich ingredients prepared by commercial processing or using commercially processed meats with home preparation.
(30) Milk, whole and skim, including only whole, lowfat, and skim fluid milks.
(31) Milk products, including flavored milks and milk drinks, dry milks, toppings, snack dips, spreads, weight control milk beverages, and other milk origin products.
(32) Nuts and nut products, including whole or shelled tree nuts, peanuts, coconut, and nut and peanut spreads.
(33) Plant protein products, including the National Academy of Sciences/National Research Council “reconstituted vegetable protein” category, and meat, poultry, and fish substitutes, analogs, and extender products made from plant proteins.
(35) Processed fruits and fruit juices, including all commercially processed fruits, citrus, berries, and mixtures; salads, juices and juice punches, concentrates, dilutions, “ades”, and drink substitutes made therefrom.
(36) Processed vegetables and vegetable juices, including all commercially processed vegetables, vegetable dishes, frozen multicourse vegetable meals, and vegetable juices and blends.
(37) Snack foods, including chips, pretzels, and other novelty snacks.
(38) Soft candy, including candy bars, chocolates, fudge, mints, and other chewy or nougat candies.

3.1. Estimated Dietary Intake (EDI) from The Intended Use in Conventional Foods

Three approaches were used to estimate the anticipated daily intake:

1) Following the estimation method described in GRN 1131 (FDA, 2023), based on the USDA Nutrition Insights publication by the USDA Center for Nutrition Policy and Promotion (Basioties et al., 2000), which reports the consumption levels of grains, fruits, vegetables, milk, meat, fats, oils, and sweets across various age and gender groups. Among these groups, men aged 51 and older had the highest total daily consumption at 18.2 servings/day. Assuming 50% of the daily intake includes *L. paracasei* NTU 101 at an addition level of 1.0×10^{10} CFU/serving, the estimated daily intake (EDI) would be 9.1×10^{10} CFU/day.
2) Referring to the method mentioned in GRN 1114 (FDA, 2023) and based on a publication on dietary consumption in healthy adults (Millen et al., 2006), the average total daily consumption is approximately 20 servings. Similarly, assuming 50% of the daily intake includes *L. paracasei* NTU 101 at an addition level of 1.0×10^{10} CFU/serving, the EDI would be 1.0×10^{11} CFU/day.
3) Following the calculation method described for *Lactobacillus casei* Shirota (FDA GRN 429, 2012), yogurt, the most common LAB-containing food, was used to estimate the EDI. According to data from *What We Eat in America*, NHANES 2017-2018 (USDA, 2020), the mean per capita yogurt consumption in the U.S. is 0.06 cup equivalents per day, or approximately 14.7 g/day (refer to methodology document, Bowman et al., 2020). The survey on Dietary Quality by Food Source and Demographics in the United States, 1977–2018, also reports that 4.0% of the population consumed yogurt daily during 2017 to 2018 (Lin et al., 2023, Figure 20). Based on these figures, the mean daily yogurt intake among consumers is estimated at 367.5 g/day (14.7/0.04). Given a reference serving size of yogurt (170 g), this corresponds to 2.16 servings of *L. paracasei* NTU 101, as defined by the FDA's Reference Amounts Customarily Consumed (FDA, 2018). Following FDA guidance (FDA, 2006) for the 90th percentile consumption, which is calculated by doubling the mean intake, the approximate serving size reaches 4.32 servings at the 90th percentile. With an addition level of 1.0×10^{10} CFU/serving, the maximum EDI of *L. paracasei* NTU 101 is approximately 4.3×10^{10} CFU.

3.2. Acceptable Daily Intake (ADI)

Based on findings from a 90-day oral toxicity study in animals, the no-observed-adverse-effect-level (NOAEL) for *L. paracasei* NTU 101 was established at 2000 mg/60 kg body weight (BW)/day (see part 6). Given that *L. paracasei* NTU 101 powder contains a viable cell count of 1.0×10^{11} CFU/g, an ADI of 1.0×10^{11} CFU/60 kg BW/day was derived using a 120-fold safety factor to account for interspecies scaling.

Considering the results from the three methods used to estimate the EDI, it is evident that, under the assumption of adding 10^9 CFU of *L. paracasei* NTU 101/serving, all values remain below the ADI for *L. paracasei* NTU 101. Thus, the likelihood of *L. paracasei* NTU 101 intake surpassing the ADI under typical dietary conditions is low, indicating that consuming foods containing *L. paracasei* NTU 101 at this addition level is safe.

Furthermore, similar to the case of *L. paracasei* subsp. *paracasei* F-19 GRAS (FDA GRN 840, 2019), *L. paracasei* NTU 101 will not proliferate in the foods and beverages to which it is added. Instead, its viability will decrease gradually throughout the shelf life of the food. Therefore, its likely maximum intake is less than 100 billion CFU/day, which is well within safe levels.

Part 4. Self-Limiting Levels of Use

In the fields of use described in this document, NTU 101 does not have any self-limiting levels of use.

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

Although *L. paracasei* are part of the lactic acid bacterial culture used to ferment many traditional foods, *L. paracasei* NTU 101 has not been used in human food products. However, the *L. paracasei* species has been used in several food and beverage products. Thus, this GRAS notification is not based on common use of the *L. paracasei* NTU 101 strain in food under 21 CFR §170.30 parts (a) and (c).

Part 6. Narrative

6.1. Safety Assessment Based on Phenotypic and Genotypic Analyses

The safety assessment of *L. paracasei* NTU 101 was published in a peer-reviewed journal in 2024 under the title "Comprehensive Safety Assessment of *Lacticaseibacillus paracasei* subsp. *paracasei* NTU 101 Through Integrated Genotypic and Phenotypic Analysis" (Chen et al., 2024). Part 6 will elaborate on the safety data of *L. paracasei* NTU 101 based on the published findings and additional supporting information.

6.1.1. Whole-genome Sequence, Annotation, and Search Against Gene Database

The complete genome sequence of *L. paracasei* NTU 101 stored in GenBank (accession no. GCA_002901165.3) and that obtained through Nanopore sequencing (Oxford Nanopore Technologies, UK) were used for gene prediction and annotation, which were performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Subsequent gene function and safety analyses were conducted using the *L. paracasei* NTU 101 complete genome sequence or annotated protein sequence files. The *L. paracasei* NTU 101 complete genome sequence was used to predict antibiotic resistance using the Comprehensive Antibiotic Resistance Database (CARD), Resfinder, and AMRfinderplus. Furthermore, this sequence was used to predict mobile genetic elements using the Mobile Element Finder. Protein sequences were used for Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation and virulence factor analysis using the Virulence Factor Database (VFDB).

The thresholds for screening the results of antimicrobial resistance, toxigenicity, and pathogenicity gene analyses were based on the standards outlined in the "EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain" (EFSA, 2021). According to these standards, query sequence hits with at least 80% identity (at the protein or nucleotide level, as provided in the database) and 70% of the length of the subject sequence should be reported. Potential risk genes identified through various databases were verified using the BLASTp algorithm to search the NCBI reference proteins database (RefSeq protein) and confirm the latest gene names and functions, as necessary.

6.2. Antibiotic Resistance

6.2.1. Phenotypic Analysis

An antibiogram of *L. paracasei* NTU 101 was constructed using the method outlined in ISO 10932:2010. Recorded minimal inhibitory concentrations (MICs) are displayed in Table 4 (Chen et al., 2024). All MIC values, except for that of

chloramphenicol, were below the cut-off value defined for *Lactobacillus paracasei* (EFSA, 2018).

Table 4. Antimicrobial minimum inhibitory concentrations (MIC) results of *L. paracasei* NTU 101 and BCRC 12248^T.

	Phenotypic analysis – MIC		
Antibiotic	***L. paracasei* cut-off* (mg/L)**	***L. paracasei* subsp. *paracasei* NTU 101**	***L. paracasei* subsp. *paracasei* BCRC 12248^T**
Ampicillin	4	2	1
Vancomycin	n. r.	-	-
Gentamicin	32	4	4
Kanamycin	64	64	64
Streptomycin	64	64	64
Erythromycin	1	0.5	0.25
Clindamycin	4	0.5	0.25
Tetracycline	4	4	2
Chloramphenicol	4	8	8

* EFSA, 2018

6.2.2. Genotypic Analysis

6.2.2.1. Search Against Antibiotic Resistance Gene Database

We used search engines built into the CARD, ResFinder, and AMRfinderPlus databases to identify antibiotic resistance genes. First, we used the Resistance Gene Identifier (RGI; version 6.0.3) to compare against the CARD (version 3.2.9) data. The results yielded one strict hit and no perfect hits. According to the RGI, "the strict algorithm detects previously unknown variants of known AMR genes." Therefore, the function of strict hits must be confirmed. RGI prediction indicated that this strict hit was *qacJ*, a gene related to resistance to the disinfectant benzalkonium chloride.

Further analysis confirmed that *qacJ* encodes the EmrE transporter, a member of the small multidrug resistance transporter family, which functions as a multidrug efflux pump. Previous studies have shown that the EmrE transporter in *E. coli* does not confer resistance to chloramphenicol (Yerushalmi et al., 1995). This gene is also found in other *Lacticaseibacillus* species, suggesting that it is likely intrinsic to *Lacticaseibacillus* rather than being an acquired gene. (Chen et al., 2024). In previous analyses, we found 284 loose hits. Because "the loose algorithm provides detection of new, emergent threats and more distant homologs of AMR genes," no potential risk genes were found after filtering with thresholds of 80% identity and 70% length

coverage of the subject sequence.

Additionally, Chen et al. (2024) demonstrated that ResFinder (version 4.5.0) and AMRfinderPlus (version 3.12.8) were used to detect acquired antimicrobial and disinfectant resistance genes, with neither method detecting any resistance genes. Furthermore, analysis with MobileElementFinder identified no mobile genetic elements harboring resistance genes. Accordingly, *L. paracasei* NTU 101 can be regarded as safe with regard to antibiotic resistance.

6.2.2.2. Chloramphenicol resistance-related genes

Antibiotic MIC tests indicated that *L. paracasei* NTU 101 is only resistant to chlormphenicol. In accordance with the EFSA (2012) guidelines, we investigated whether *L. paracasei* NTU 101 possesses any resistance genes. We further determined if these genes are endogenous to Lacticaseibacillus and whether they have the potential for gene transfer.

As indicated by the database analysis results in CARD, ResFinder, and AMRfinderPlus, no genes associated with chloramphenicol resistance were detected. Furthermore, we downloaded the chloramphenicol resistance genes from the GenBank database and performed a BLASTn search against the *L. paracasei* NTU 101 complete genome sequence and annotated gene protein sequence, respectively. No homologous gene fragments were identified for chloramphenicol resistance genes in the *L. paracasei* NTU 101 genome.

6.3. Virulence Factor

A BLAST search of the *L. paracasei* NTU 101 genome was performed against sequences from the VFDB (version 24-07-2024) to identify potential virulence genes. After screening, no potential virulence genes were detected in the full DNA and protein datasets, respectively. According to the VFDB definition, the full datasets cover all genes related to known and predicted virulence factors in the database. By lowering the identity threshold to 70%, the protein database analysis identified four potential virulence genes. A search for similar sequences in the NCBI RefSeq protein database showed that these genes are commonly found in *Lacticaseibacillus* species. (Chen et al., 2024). Thus, we concluded that they posed no toxicity risk, owing to their low potential for mobility and lack of pathogenic functions.

6.4. Hemolysis

6.4.1. Phenotypic Analysis

The hemolytic properties of *L. paracasei* NTU 101 were analyzed as described by Ficoseco et al. (2018), Casarotti et al. (2017), Buxton (2005), and Koh et al.

(2018). Briefly, *L. paracasei* NTU 101 was cultured on blood agar (Columbia Agar with 5% sheep blood) and compared with *Staphylococcus aureus* BCRC 12154 as the β-hemolysis positive strain to determine its hemolytic properties.

L. paracasei NTU 101 exhibited α-hemolysis when cultured under aerobic conditions. However, no hemolysis (also called gamma hemolysis) was observed when *L. paracasei* NTU 101 cells were cultured under anaerobic conditions. In addition, three other *L. paracasei* strains (*L. paracasei* subsp. *paracasei* BCRC 12248T, BCRC 14023, and BCRC 17002) and the *Lacticaseibacillus rhamnosus* BCRC 16000 strain also exhibited α-hemolysis (Chen et al., 2024).

α-Hemolysis (also known as partial hemolysis) is the reduction of the red blood cell hemoglobin to methemoglobin in the medium surrounding the colony. This causes green or brown discoloration of the medium (Buxton, 2005). According to Barnard and Stinson (1996), α-hemolysis is caused by the hydrogen peroxide produced by *Streptococcus*. Goldstein et al. (2015) noted that *Lactobacillus* (the former name of *Lacticaseibacillus*) usually exhibits α-hemolysis on blood agar. Moreover, the GRAS notice of *Lactobacillus casei* subsp. *paracasei* Lpc-37 (FDA GRN 736, 2018) revealed that Lpc-37 also exhibits α-hemolysis.

Based on this information, we can infer that α-hemolysis is a common phenomenon for LAB, and that *L. paracasei* strains commonly exhibit α-hemolysis.

6.4.2. Genotypic Analysis

6.4.2.1. α-Hemolysis

We searched for genes related to the production of hydrogen peroxide to perform α-hemolysis genotypic analysis. *Lactobacillus* can produce hydrogen peroxide through *nfr1* and *nfr2*, which exhibit hemolytic activity (Barnard and Stinson, 1996; Hertzberger et al., 2014). The results of the gene search and comparison showed no gene similar to *nfr1* (accession no. Q74HL7) and *nfr2* (accession no. Q74HL8) in the *L. paracasei* NTU 101 genome, indicating that there is no *nfr1*/*nfr2* system in the *L. paracasei* NTU 101 genome.

Zotta et al. (2017) discovered that LAB may produce hydrogen peroxide through two mechanisms in the presence of oxygen: carbohydrate metabolism or respiration. The pathway through which hydrogen peroxide is generated during carbohydrate metabolism is catalyzed by POX-ACK kinase activity. Furthermore, pyruvate may be converted to acetate through pyruvate oxidase-acetate kinase (POX-ACK) activities in the presence of oxygen. This leads to the production of CO_2 and hydrogen peroxide (POX activity), as well as ATP generation (ACK activity). The regulation of *pox* and *ack* genes depends on sugar and oxygen availability.

Proteomic analysis of *L. paracasei* NTU 101 demonstrated the expression of the

pyruvate oxidase (*pox*) and acetate kinase (*ack*) genes. Zotta et al. (2017) reported that the POX-ACK pathway is active when the glucose concentration limits growth (usually in the stationary phase). Similar results were found in a study by Ocaña et al. (1999), in which the highest levels of hydrogen peroxide were produced by two *Lactobacillus paracasei* strains (F2 and F28) during the stationary phase.

Another possible pathway for hydrogen peroxide generation in LAB is respiration. However, activation of the respiratory chain in LAB requires supplementation with hemin and menaquinone (Zotta et al., 2017). The minimal respiratory chain of LAB includes an electron donor (NADH dehydrogenase), a quinone shuttle (menaquinone), and a terminal oxidase (heme-binding cytochrome bd-I oxidase) (Lechardeur et al., 2011; Pedersen et al., 2012). Cytochrome bd-I oxidase subunit-coding genes (*cydABCD*) were identified in the *L. paracasei* NTU 101 genome; however, this strain lacks the NADH dehydrogenase gene and, therefore, is unable to perform respiratory reactions.

LAB accumulate reactive oxygen species (ROS), including hydrogen peroxide, in aerobic environments. This can affect their survival and lead to the production of harmful substances in fermented foods. To counteract the harmful effects of ROS, LAB have evolved antioxidant systems, such as peroxidase, superoxide dismutase, and catalase (Zotta et al., 2017). Peroxidase genes were identified in the *L. paracasei* NTU 101 genome, indicating their ability to remove hydrogen peroxide. Because the human intestinal tract is anaerobic, *L. paracasei* NTU 101 does not produce sufficient hydrogen peroxide to damage red blood cells, suggesting that the likelihood of *L. paracasei* NTU 101 producing large amounts of hydrogen peroxide in the intestines is low. LAB-fermented foods have a long history of consumption without reports of links to hemolysis. Therefore, the safety concerns related to the α-hemolysis properties of *L. paracasei* NTU 101 are likely minimal.

6.4.2.2. β-hemolysis

Annotation searches were conducted using the *L. paracasei* NTU 101 whole-genome protein sequence to evaluate hemolysin genes related to β-hemolysis. The VFDB search identified three hemolytic toxin-coding genes in the *L. paracasei* NTU 101 genome, which may belong to the hemolysin III family and the hemolysin family. The analysis results revealed that these proteins are highly conserved across *Lacticaseibacillus*, encoded by *YafA* and *TlyC* (Chen et al., 2024). A comparison of the BLASTp search results revealed that this protein is a highly conserved hemolysin III protein of *Lacticaseibacillus*, with the predicted membrane channel-forming protein *YqfA*, a conserved functional region of the hemolysin III family. Although species within the genus *Lacticaseibacillus* generally harbor hemolysin III

(Chokesajjawatee et al., 2020), there are no documented safety concerns associated with this gene. Furthermore, the presence of the *TlyC*-encoded hemolysin protein family in other *L. paracasei* strains and *Lacticaseibacillus* species was confirmed by a search of the NCBI RefSeq protein database (Chen et al., 2024). In addition, phenotypic analysis of the blood agar test showed that *L. paracasei* NTU 101 did not exhibit β-hemolysis. Therefore, it is suggested that *L. paracasei* NTU 101 does not pose a hemolytic risk.

6.4.2.3. Summary of Hemolysis Analysis

We elucidated the cause of α-hemolysis, where hydrogen peroxide can be produced by *L. paracasei* NTU 101, and speculated on the conditions required for its production. In addition, analysis of β-hemolysis confirmed the presence of conservative genes belonging to *Lacticaseibacillus*. However, no β-hemolysis was observed in the blood agar phenotypic test. Additionally, no safety concerns related to hemolysis have been reported in previous studies on *L. paracasei.* Therefore, it can be concluded that *L. paracasei* NTU 101 poses no significant risk for hemolysis.

6.5. Biogenic Amine

6.5.1. Phenotypic Analysis

Biogenic amines generally result from the decarboxylation of amino acids by decarboxylases. Common biogenic amines in fermented foods include histamine, tyramine, tryptamine, 2-phenylethylamine, cadaverine, agmatine, putrescine, spermidine, and spermine. Histamine has clear regulatory limits in various countries, typically ranging from 50 to 200 ppm (Mah et al., 2019). Some studies have suggested a limit of 30 ppm for 2-phenylethylamine, with variations depending on the type of food (Brink et al., 1990). The EFSA limits tyramine intake to 600 mg per person per meal, whereas the intake limit for monoamine oxidase inhibitors ranges from 6 to 50 mg.

We analyzed the biogenic amine content in the fermentation broth of *L. paracasei* NTU 101 in MRS medium, milk, and soymilk using HPLC. The results showed a considerable increase in biogenic amine content. Cadaverine was produced (4.18 ppm) during MRS fermentation whereas putrescine (4.54 ppm), cadaverine (2.85 ppm), 2-phenylethylamine (3.79 ppm), and spermine (22.47 ppm) were detected during milk fermentation. In contrast, histamine (2.44 ppm) and tyramine (12.73 ppm) were produced during soymilk fermentation.

6.5.2. Genotypic Analysis

6.5.2.1. Biogenic Amine-Related Genes Annotated by KEGG

The gene protein sequences of *L. paracasei* NTU 101 were analyzed using the KofamKOALA gene function annotation tool available on the KEGG website. The version used was kofam 1.3.0, with the database version 2024-05-01. The threshold was set at an E-value lower or equal to 0.01.

6.5.2.2. Biogenic Amine-Related Gene Analysis

Biogenic amine-related genes were identified and analyzed based on literature or KEGG pathway databases that list the enzymes or channel proteins involved in the metabolism of various biogenic amines. Genes that encode histidine decarboxylase and tyrosine decarboxylase, which are responsible for histamine and tyramine production, respectively, were not detected in the *L. paracasei* NTU 101 genome. Similarly, genes related to phenylalanine decarboxylase and spermine synthase, which are involved in the catalytic decarboxylation of 2-phenylethylamine, spermine, and tyramine from amino acids, were not detected in the *L. paracasei* NTU 101 genome, nor were any other potential synthetic pathway enzyme genes identified.

The *L. paracasei* NTU 101 genome contained the gene that encodes L-ornithine decarboxylase, which originates from *L. paracasei* and is widely distributed among *L. paracasei* strains. L-ornithine decarboxylase catalyzes the production of putrescine from l-ornithine. Moreover, the genes that encode the four subunits of the channel protein responsible for putrescine and *potABCD* transport were found to be intact. Additionally, a gene encoding a putative lysine decarboxylase, which catalyzes cadaverine production from lysine, was identified in the *L. paracasei* NTU 101 genome. However, conserved domain analysis indicated that it may not correspond to this enzyme. (Chen et al., 2024).

The synthetic enzymes associated with some biogenic amines were not identified (i.e., 2-phenylethylamine, tyramine, and spermine), possibly due to the presence of genes encoding related enzymes that could not be accurately annotated.

Fermented dairy products generally contain biogenic amines (EFSA, 2011), with average histamine, tyramine, putrescine, and cadaverine levels in the 0.3 - 65.1, 0.3 - 335, 0.7 - 449, and 1.9 - 628 ppm ranges, respectively. Biogenic amine levels are typically higher in acid curd cheese (51.3 - 628 ppm) than those in yogurt (0.5 - 3.2 ppm). The levels of various biogenic amines in fermented soy products are similar to those in fermented dairy products, with varying concentrations found in different types of foods (Mah et al., 2019). Although spermine levels in fermented dairy products are generally low (0.02 - 0.8 ppm), spermine is commonly found in other foods at concentrations higher than those in *L. paracasei* NTU 101 (Ali et al., 2011), such as lean beef (27.3 - 36.4 ppm). Spermine levels typically range from 1.3 to 242 ppm in fermented soy products (Mah et al., 2019). Considering the typical

consumption amounts, the amount of spermine produced by *L. paracasei* NTU 101 is not expected to be excessively high. The biogenic amine content of *L. paracasei* NTU 101 is relatively low compared to that of fermented or general foods, suggesting a low likelihood of harm.

The GRAS safety sections for *Lactobacillus casei* Shirota (FDA GRN 429, 2012) and *Lactobacillus rhamnosus* HN001 (FDA GRN 288, 2009) state the following:

"If the production of biogenic amines is indeed a safety concern, it is usually associated with spoilage or long fermentation processes in the production of fermented dairy products using Lactobacillus *species, rather than their use as probiotics (Bernardeau et al., 2008). Furthermore, Naidu et al. (1999) noted that probiotic* lactobacilli *reduce intestinal pH by producing lactic acid, which in turn inhibits the growth of many potential putrefactive bacteria that produce harmful biogenic amines."*

Based on these results, it can be inferred that *L. paracasei* NTU 101 produces biogenic amines at relatively low concentrations in fermented foods. Additionally, gene analysis did not reveal the complete set of genes encoding biogenic amine-producing enzymes. Therefore, it is unlikely that biogenic amines produced by *L. paracasei* NTU 101 in fermented foods pose a significant risk.

6.6. D-lactic Acid Production

The *L. paracasei* NTU 101 production of L- and D-lactate was assessed. The ratio between L- and D-lactic acids was determined, revealing that 96.3% of the lactate produced was the L-enantiomer. D-lactic acid is produced from pyruvate by D-lactate dehydrogenase. Gene annotation shows that a gene encoding D-2-hydroxyacid dehydrogenase is present in the *L. paracasei* NTU 101 genome, an enzyme with D-lactate dehydrogenase activity, suggesting that the D-lactic acid detected in the fermentation broth is generated by *L. paracasei* NTU 101, which is consistent with the phenotypic results (Chen et al., 2024). D-lactic acidosis, a rare form of lactic acidosis, is most commonly observed in patients with short bowel syndrome (Kowlgi et al., 2015). Although symptomatic patients typically exhibit elevated D-lactate levels, the risk of D-lactic acidosis in healthy individuals is considered low (Bang et al., 2021). Relative to other lactic acid bacteria, *L. paracasei* NTU 101 produces a comparatively low proportion of D-lactate. For instance, *Limosilactobacillus reuteri* NCIMB 3053 produces 54.5% D-lactate, while *Lactobacillus delbrueckii* ATCC 11842 produces 52.2% (Sulemankhil et al., 2012).

Considering strains such as *L. rhamnosus* GG (FDA GRN 1013, 2021) and

Bifidobacterium animalis ssp. *lactis* BB-12 (FDA GRN 856, 2019), which have a long history of consumption and are GRAS, a fermentation ratio of >95% L-lactic acid can be considered safe.

6.7. Bile Salt Hydrolase Activity

The putative bile salt hydrolase (BSH) gene was identified by searching the protein sequence of *L. paracasei* NTU 101. A BLASTp search against the NCBI RefSeq database indicated that this gene encodes a linear amide C-N hydrolase. This gene belongs to an enzyme family comprising several hydrolases that cleave carbon-nitrogen and peptide bonds in linear amides. Intestinal bacteria encode the BSH protein, which is involved in transformation reactions and catalyzes the hydrolysis of taurine or glycine at the C-24 position of conjugated bile salts (Zhang et al., 2009; Ridlon et al., 2006).

Lactobacillus acidophilus possesses BSH activity (Dashkevicz and Feighner, 1989; McAuliffe et al., 2005; Tanaka et al., 1999). Alignment of the potential BSH protein sequence of *L. paracasei* NTU 101 with that of *L. acidophilus* revealed a sequence similarity of only 24.6%. Zhang (2009) suggested that the linear amide C-N hydrolase of *Lactobacillus casei* Zhang exhibits BSH activity. Alignment of the linear amide C-N hydrolase protein sequences of *L. paracasei* NTU 101 with *L. casei* Zhang (accession no. ACC93573.1) showed 100% similarity, suggesting that this gene segment of *L. paracasei* NTU 101 possesses BSH functionality. Furthermore, Elkins et al. (2001) demonstrated that the BSH activity of LAB is related to bile salt transporters. However, there is limited research on *Lacticaseibacillus* in this regard. Furthermore, it is speculated that the BSH activity of *L. paracasei* NTU 101 may be influenced by the bile salt transporter function.

Begley et al. (2006) noted that excessive deconjugated bile salts may have undesirable effects on the human host, including impaired digestive function and disruption of normal intestinal conditions and/or gallstones. Bile acids are also considered potential carcinogens. BSH activity increases bacterial tolerance in the digestive tract and prolongs survival. Moreover, it is believed to reduce cholesterol levels. However, the phenotypic experimental results of *L. paracasei* NTU 101 indicated no BSH activity compared with the positive control group (Chen et al., 2024). Given the long history of consumption of *L. paracasei*, BSH gene does not pose a significant risk.

6.8. Mucin Degradation and Intestinal Mucosal Barrier

Mucin degradation is an undesirable trait of probiotics because of its potential to compromise the intestinal mucosal barrier, which is vital for host mucosal defense.

Damage or disruption to this mucin layer can have adverse effects (Zhou et al., 2001; Ruas-Madiedo et al., 2008).

6.8.1. Phenotypic Analysis

The ability of *L. paracasei* NTU 101 to hydrolyze mucin was assessed as described previously (Abe et al., 2010; Casarotti et al., 2017; Zhou et al., 2001), and its mucin hydrolysis capability was analyzed using porcine stomach mucin (PSM). *L. paracasei* NTU 101 did not grow on PSM-only plates, and no mucin hydrolysis was observed. In contrast, the positive control strain *Salmonella enterica* subsp. *enterica* BCRC 10747 grew normally on PSM-only plates (Chen et al., 2024).

6.8.2. Genotypic Analysis

Mucin-degrading microbes typically possess glycosyl hydrolases (GHs) that cleave specific glycan linkages (Glover et al., 2022). The core GH-ome for mucin degradation, as reported by Glover et al. (2022), includes GH33, 29, 95, and 20/35. Analysis of the *L. paracasei* NTU 101 whole-genome protein sequence annotations against the carbohydrate-active enzyme database (CAZy db) revealed that *L. paracasei* NTU 101 only possessed genes related to GH29 and 20/35 (Chen et al., 2024). Therefore, it lacks the complete capability to degrade mucin. This result is consistent with those of the mucin degradation phenotype experiment. To further evaluate the potential impact of *L. paracasei* NTU 101 on the intestinal barrier, the C2BBe1 intestinal epithelial cell line was utilized to establish a monolayer membrane model mimicking the intestinal barrier. No translocation of *L. paracasei* NTU 101 was observed in this model with intact barrier function (Chen et al., 2024).

In conclusion, *L. paracasei* NTU 101 does not degrade mucin nor compromise the integrity of the intestinal mucosal barrier.

6.9. Toxicological Study and Animal Study

The *L. paracasei* NTU 101 animal study was conducted by Super Laboratory Co., Ltd., New Taipei City, Taiwan, using the Health Food Safety Assessment methods of the Taiwan Food and Drug Administration, Ministry of Health and Welfare. Additionally, these studies were conducted in compliance with Good Laboratory Practice for Nonclinical Laboratory Studies (21 CFR Part 58) by the United States FDA (1987); OECD Principles on Good Laboratory Practice (ENV/MC/CHEM (98) 17, 1998); Good Laboratory Practice for Nonclinical Laboratory Studies by the Taiwan Food and Drug Administration, Ministry of Health and Welfare (2006); and General Requirements for the Competence of Testing and Calibration Laboratories (ISO/IEC 17025) by the International Organization for

Standardization (2005).

We conducted a series of toxicological studies of *L. paracasei* NTU 101, which have been published in peer-reviewed journals (Tseng et al., 2015; Chen et al., 2024). These studies included a 28-day oral toxicity assay, a repeat-dose 90-day oral toxicity study, a bacterial reverse mutation test, an *in vitro* mammalian cell chromosomal aberration test, and a rodent peripheral blood micronucleus test (refer to the Table 5). The animal and *in vitro* models used are also listed in Table 5. Based on these findings, the no-observed-adverse-effect level (NOAEL) of *L. paracasei* NTU 101 powder for the 90-day repeated-dose oral toxicity study in rats was determined to be 2000 mg/kg body weight (equivalent to 2×10^{11} CFU/kg body weight), which is 120 times the recommended daily human intake (1000 mg/60 kg body weight, equivalent to 1×10^{11} CFU/60 kg body weight).

In summary, *L. paracasei* NTU 101 demonstrated safety as a food product and showed no potential concerns.

Oral toxicity studies have also been conducted on other *L. paracasei* strains. In a 90-day oral toxicity study of *L. paracasei* GW080, Jia et al. (2011) reported a NOAEL of 5.0 g/kg body weight in rats, which corresponds to a human equivalent dose of 5×10^9 CFU/kg body weight. Lu et al. (2021) investigated a 28-day repeated oral dose of *Bifidobacterium lactis* BL-99, *L. paracasei* K56, and ET-22, finding that the NOAELs for *L. paracasei* K56 and ET-22 in rats were both 2.62×10^{11} CFU/kg body weight, which, when applying a 120-fold safety factor, translates to a human intake level of 1.31×10^{11} CFU for a 60 kg individual. Furthermore, Chen et al. (2024) reported that a 90-day oral toxicity study of *L. paracasei* ET-66 in rats demonstrated a NOAEL of 1.5 g/kg body weight, corresponding to a human intake level of 2.04×10^{11} CFU/60 kg body weight based on the provided data.

Collectively, these findings indicate the safety of *Lacticaseibacillus paracasei* strains for human consumption.

Table 5. Toxicological studies on *L. paracasei* NTU 101.

Study Type/Duration	**Animal Number (Strain)/Group**	**Dose Groups/ Concentration**	**NOAEL Conclusion/ Findings**
28-day oral toxicity assay	10 Wistar rats /sex/group	Pelleted diet and distilled water supplied to rats. **Test group** Control group: 0 mg/kg B.W. Low dose group:	There was no evidence of toxicity in the 28-day oral toxicity assay at 5000 mg/kg/day in rats.

		300 mg/kg B.W. Middle dose group: 1500 mg/kg B.W. High dose group: 5000 mg/kg B.W.	
Repeat dose 90-day oral toxicity study in rat oral gavage	10 Sprague-Dawley rats/sex/group	**Control group** RO water **Dose group** 500 mg/kg B.W. 1000 mg/kg B.W. 2000 mg/kg B.W.	NOAEL value: 2000 mg/kg B.W., equivalent to 1.0 × 1011 CFU/60 kg B.W. in 120-fold safety factor. No any abnormal clinical signs on hematology, clinical biochemistry, urine examination and histopathological examination.
Bacterial reverse mutation test (Ames test)	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, and TA1537	**Control** Positive control S9 mix (+): Benzo [a] pyrene (4.0 μg/plate) Positive control S9 mix (-): 4-Nitroquinoline-N-oxide (0.5 μg/plate)	No *Salmonella typhimurium* reverse mutation occurs when test article "NTU 101 powder" is treated or not treated with S9 mix.
In vitro mammalian cell chromosomal aberration test	Chinese hamster ovary cells (CHO-K1)	**Control** Negative control S9 mix (+) and S9 mix (-): Ham's F-12 medium with 10% FBS, 1% DMSO Positive control S9 mix (+): 25 g/mL cyclophosphamide monohydrate Positive control S9 mix (-): 2.5 g/mL mitomycin C **Test group**	The results indicate that NTU 101 powder had a negative effect under the condition in the *in vitro* mammalian chromosomal aberration test.

		S9 mix (+): 3 h S9 mix (-): 3 and 20 h	
Rodent peripheral blood micronucleus test	5 Male ICR mice/group	**Control** Negative control: RO water Positive control: cyclophosphamide, 50 mg/kg **Test group** Low dose group: 875 mg/kg B.W. Medium dose group: 1750 mg/kg B.W. High dose group: 3500 mg/kg B.W. Mouse blood from each group was sampled at 48 and 72 h.	There were no differences between the peripheral blood reticulocyte counts and micronucleated reticulocyte counts of test group and negative control group. The result indicates NTU 101 powder exhibited a negative result in the rodent peripheral blood micronucleus test.

6.10. Human Study

A human clinical trial investigating the effects of Vigiis 101-LAB (capsules made from NTU 101 powder) on gut microbiota, gastrointestinal motility, immune function, and antioxidant capacity in healthy individuals has been conducted (Table 6). Based on the findings of the study, administering Vigiis 101 not only provides health benefits by improving gut microflora, peristalsis, immunity, and antioxidative capacity but also does not induce any adverse effects.

Table 6. Human clinical study of *Lacticaseibacillus paracasei* subsp. *paracasei* NTU 101.

Reference	Study Design & Objective	Subjects	Strain & Daily Dose	Duration	Safety-Related Results
Chen et al., 2020	**Clinical trial I:** Randomized, double-blind clinical criteria for effects of Vigiis 101-LAB capsule I on gut	36 healthy adults aged 20 to 65 years. n = 18 at treatment group.	Capsule I: 5×10^9 CFU of *L. paracasei* NTU 101 with lactose, crystalline cellulose,	4 weeks capsule administration, orally once per day, one capsule each time. Fecal sampling at	During the trial period, the clinician carried out consultations once every 2 weeks. No subjects experienced abnormal

	flora.		and excipient were made into capsules.	weeks 0, 1, 2, 3, and 4.	reactions during the trial.
	Clinical trial II: Randomized, double-blind clinical criteria for effects of Vigiis 101-LAB capsule II on peristalsis, immunity, and anti-oxidative capacity.	54 healthy adults aged 20 to 65 years. n = 27 at treatment group.	Capsule II: 1×10^{10} CFU of *L. paracasei* NTU 101 with lactose, crystalline cellulose, and excipient were made into capsules.	4 weeks capsule administration, orally once per day, one capsule each time. Blood samples were drawn on the first day of the first week before taking the capsules, and then at weeks 4 and 6.	In the test of biochemical blood biomarker, there were no significant changes in the treatment group or placebo group. In this trial, there were no safety concerns with the administration of the Vigiis 101-LAB capsule II or the placebo.

6.11. Qualified Presumption of Safety (QPS) status

Refer to explanation of QPS of EFSA website,
"EFSA asked the Panel on Biological Hazards (BIOHAZ) to deliver a scientific Opinion on the maintenance of the list of qualified presumption of safety (QPS) biological agents. The QPS approach was developed by the EFSA Scientific Committee to provide a harmonized generic pre-evaluation to support safety risk assessments of biological agents intentionally introduced into the food and feed chain, in support of the concerned scientific Panels and Units in the frame of market authorizations." Since 2014, the updates of QPS list are carried out and published every 3 years. (https://zenodo.org/records/8124409).

In the introductory section of the first QPS document (EFSA, 2007), various microbial species are approved for use in food and feed production. Some species have a long history of safe use whereas others have limited information, which may pose risks to consumers. Therefore, a tool that assesses the risks posed by microbes used in food and feed production is required. To meet this demand, the EFSA proposed a system for the pre-market safety assessment of specific microbes, resulting in the "Qualified Presumption of Safety (QPS)." The primary criterion for confirming whether a microorganism belongs to a QPS list is its taxonomic classification, which determines its eligibility. If listed, a limited safety assessment is required for consumption; otherwise, a comprehensive safety assessment is necessary and only the assessed strain can be consumed.

L. paracasei has been listed on the QPS since its first edition and has not been removed in subsequent updates and safety reviews. It remains on the latest version (EFSA 2024), demonstrating the safety of this species. According to the present QPS specifications, *L. paracasei* can be used in food as long as the strain does not contain acquired antimicrobial resistance genes relevant to clinical antimicrobial drugs. Based on the MIC results for antibiotic resistance and analysis of resistance genes, *L. paracasei* NTU 101 poses no risk of acquiring antimicrobial resistance genes, making it safe for use in food.

6.12. GRAS Notification of *L. paracasei* Species

Several strains or mixed strains of *L. paracasei* (synonymous with *Lactobacillus paracasei*) and the closely related *L. casei* (synonymous with *Lactobacillus casei*) have received FDA approval (Table 7). Some strains have undergone multiple human clinical trials without causing any harm to humans, demonstrating the safety of these bacterial species. As a strain of *L. paracasei*, NTU 101 has shown safety in both animal studies and human clinical trials, with no reported adverse reactions.

Table 7. The list of *Lacticaseibacillus casei* and *Lacticaseibacillu paracasei* strains which have FDA's no questions letter to GRAS notice.

GRN no.	Substance	Date of closure	FDA's Letter	Note
1085	*Lactobacillus casei* strain KCTC 12398BP	Oct 27, 2023	FDA has no questions	
840	*Lactobacillus paracasei* strain F19	Aug 27, 2019	FDA has no questions	
736	*Lactobacillus casei* subsp. *paracasei* Lpc-37	Apr 11, 2018	FDA has no questions	Reclassified to *Lacticaseibacillus paracasei*
429	*Lactobacillus casei* strain Shirota	Dec 10, 2012	FDA has no questions	Reclassified to *Lacticaseibacillus paracasei*
378	Cultured [dairy sources, sugars, wheat, malt, and fruit- and vegetable-based sources] fermented by [*Streptococcus thermophilus*, *Bacillus coagulans*, *Lactobacillus*	Mar 26, 2012	FDA has no questions	

	acidophilus, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactobacillus bulgaricus* and *Proprionibacterium freudenreichii* subsp. *shermanii* or mixtures of these strains]			
240	Corn, cane, or beet sugar cultured with *Lactobacillus paracasei* subsp. *paracasei*, *Bacillus coagulans* LA-1, or *Propionibacterium freudenreichii* subsp. *shermanii*, or mixtures of these microorganisms	Oct 24, 2008	FDA has no questions	

6.13. Safey Assessment and GRAS Determination of *L. paracasei* NTU 101

Since its first publication in 2002, several studies have evaluated the basic characteristics and probiotic properties of *L. paracasei* NTU 101. In some of these studies, cell and animal experiments were conducted, which not only demonstrated the probiotic effects of *L. paracasei* NTU 101 but, more importantly, showed no adverse reactions in the test cells or animals.

In part 6, we have outlined safety assessment data for *L. paracasei* NTU 101. These are as follows:

(1) *L. paracasei* NTU 101 poses no risk of antibiotic resistance. This strain exhibits no risk of β-hemolysis and only demonstrates α-hemolysis, a common characteristic of *Lactobacillus* strains. Furthermore, this strain has no previous associated pathogenic reports.
(2) It harbors a few genes that encode biogenic amine-producing enzymes, resulting in the low-level production of biogenic amines in some fermented foods.
(3) *L. paracasei* NTU 101 lacks markers for pathogenic or toxigenic gene variants.

The primary lactate produced by this strain is L-lactic acid, with D-lactic acid constituting less than 5% of the total lactate content. Although *L. paracasei* NTU 101 possesses the BSH gene, no positive results were observed in the phenotypic experiments. Furthermore, *L. paracasei* NTU 101 is incapable of degrading mucin, indicating that it does not pose a risk to the integrity of the intestinal barrier.

(4) Animal studies have shown no genetic mutations or adverse reactions, even at higher doses, and no safety concerns were observed in human clinical trials, indicating that the consumption of *L. paracasei* NTU 101 is safe.

Based on the data analysis described in this section, it can be concluded that *L. paracasei* NTU 101 is GRAS.

6.14. Conclusions and General Recognition of the Safety of *L. paracasei* NTU 101

Based on the scientific procedures performed, Sunway Biotech concluded that these uses of *L. paracasei* NTU 101 are GRAS. The proposed use is safe within the terms of the Federal Food, Drug, and Cosmetic Act, which meets the standard of reasonable certainty of no harm. It is also GRAS according to the 21 CFR.

Sunway Biotech is unaware of any information that would be inconsistent with the finding that the proposed use of *L. paracasei* NTU 101 meets the appropriate specifications and that its use according to cGMP is GRAS. Finally, recent review of scientific literature has revealed no potential adverse events or health concerns.

Part 7. List of supporting data and information

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Form Approved: OMB No. 0910-0342; Expiration Date: 08/31/2025
(See last page for OMB Statement)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

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

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
001232	Nov 26, 2024
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
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1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
Chunchang Fang, PhD	

Organization *(if applicable)*

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SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

Lacticaseibacillus paracasei subsp. paracasei NTU 101

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.

For the purpose of this GRAS notice, L. paracasei NTU 101 is intended to be used as an ingredient added to foods where standards of identity do not preclude such use. L. paracasei NTU 101 is intended for use in dairy products, soy products, juices, non-alcoholic and alcoholic beverages, cereals, confectionary snacks, ice cream, chocolate, chewing gum, and other foods. Additionally, it is intended for use in both fermented and unfermented foods, at a concentration of up to 10 billion CFU/serving, to maintain 5 billion CFU/serving throughout the product's shelf life. These addition levels do not exceed the acceptable daily intake (ADI) of 100 billion CFU/60 kg BW/day.

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 SunWay Biotech Co., Ltd
(name of notifier)

has concluded that the intended use*(s)* of Lacticaseibacillus paracasei subsp. paracasei strain NTU 101
(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. SunWay Biotech Co., Ltd *(name of notifier)* 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.

No.139, Xing'ai Rd., Neihu Dist., Taipei City 114, Taiwan (R.O.C.)
(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)
TsungWei Shih 數位簽署者：TsungWei Shih 日期：2024.11.26 14:32:37 +08'00'	TsungWei Shih	11/25/2024

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
	Tsengetal_NTU101_28day_2015.pdf	Administrative
	Chenetal_NTU101_clinical_study_2020.pdf	Administrative
	Chenetal_NTU101_safety_assessment_2024.pdf	Administrative
	L.paracasei_NTU101_GRAS_notice_20241126_signed.pdf	Administrative