

Lactobacillus rhamnosus

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Expert Committee	Non-botanical Dietary Supplements

In accordance with the Rules and Procedures of the Council of Experts, the Non-botanical Dietary Supplements Expert Committee has revised the *Lactobacillus rhamnosus* monograph. The purpose for the revision is to make a correction to the *Acceptance criteria for Identification A. Microscopic Identification*.

Prior to this Revision Bulletin, a revision of *Lactobacillus rhamnosus* HN001 was published in *USP 2021 Issue 1* (to be official on May 1, 2021) to merge strain HN001 and new strain GG. In addition, the name of the monograph was changed to *Lactobacillus rhamnosus*.

The *Lactobacillus rhamnosus* Revision Bulletin supersedes the currently official monograph.

Should you have any questions, please contact Maria Monagas, Scientific Liaison (301-230-7422 or mjm@usp.org).

Change to read:

Lactobacillus rhamnosus ▲▲ (USP 1-May-2021)

DEFINITION

Change to read:

▲ *Lactobacillus rhamnosus* is a gram-positive, rod-shaped, heterofermentative non-spore-forming bacterium. Cells are nonmotile rods often with square ends, occurring singly or in chains. Arginine is not hydrolyzed. Suitable cryoprotectants may be added to the concentrated bacteria following fermentation, after which the product is frozen and then freeze-dried. The formulated product may be blended with suitable diluents and/or bulking agents. It contains NLT 100% of the labeled viable cell count of the appropriate strain of *Lactobacillus rhamnosus*.

- **Strain HN001:** Strain HN001 is a pure, specific strain of *Lactobacillus rhamnosus*. It is a non-D-lactate producer strain. ▲ It contains plasmids. ▲ (RB 1-May-2021)
- **Strain GG:** Strain GG is a pure, specific strain of *Lactobacillus rhamnosus*. It is a non-D-lactate producer strain. ▲ (USP 1-May-2021) ▲ It contains no plasmid. ▲ (RB 1-May-2021)

IDENTIFICATION

Change to read:

• **A. ▲ MICROSCOPIC IDENTIFICATION**

Analysis: Proceed as directed in the Assay and perform microscopic examination.

Acceptance criteria:

- **Strain HN001:** Presents as rods of varying length, occurring in short chains. ▲▲ (RB 1-May-2021)
- **Strain GG:** Presents as small uniform rods, occurring in chains. ▲▲ (RB 1-May-2021) ▲ (USP 1-May-2021)

Change to read:

• **▲ B.▲ (USP 1-MAY-2021) NUCLEIC ACID-BASED IDENTIFICATION**

[NOTE—In all cases for *Identification* ▲ B ▲ (USP 1-May-2021), "sterile water" refers to sterile, nuclease-free water acceptable for use in molecular biology.¹]

Buffer: Use a molecular biology-grade 10 mM tris-hydrochloride, 1 mM ethylenediaminetetraacetic acid (EDTA) sodium buffer.²

Sample solution: 100 mg/mL of the freeze-dried probiotic powder in *Buffer*

▲ **Primer sets:** See [Table 1](#).

Table 1. Primer Sets and Acceptance Criteria

Strain	Primer Set ^a	Forward Primer Sequence	Reverse Primer Sequence	Acceptance Criteria ^b
HN001	1	(5'–3') CACCTGCGATCAAAGCGAAAC	(5'–3') GCTCCACCGGCACATTA	Amplification product of 340 base pairs.
GG	1	(5'–3') GTGATCGGAACATCTTTGCCACTTGTGG	(5'–3') ACTTAATCAAACAAGGCAAGCTGGACGATG	Amplification product of 552 base pairs; it may result in different amplicons in other strains.

Strain	Primer Set ^a	Forward Primer Sequence	Reverse Primer Sequence	Acceptance Criteria ^b
	2	(5'–3') CTCCACATTGCGCCAAACA	(5'–3') ACAACCTTCACCACGACCAA	Amplification product of 388 base pairs with 1 single-nucleotide polymorphism (SNP); it may result in a similar amplicon in other strains. The SNP sequence is identified as (underlined in the following 21 base pair sequence) ACCAATGTAA <u>a</u> CATACCCATC and the location is 250 base pairs from the 3' end of the forward primer described in <i>Primer set 2</i> . The sequence of the amplicon should be determined by validated, standard sequencing technologies such as Sanger sequencing.
	3	(5'–3') GCCGGTTGTCAAGTACCATA	(5'–3') AGACAAATGCCTGGATGCCT	Amplification product of 293 base pairs with 2 SNPs; it may result in a similar amplicon in other strains. The first SNP is identified as (underlined in the following 21 base pair sequence) TCTTCCGAC <u>t</u> TTAGCAGGGA and the location is 71 base pairs from the 3' end of the forward primer described in <i>Primer set 3</i> . The second SNP is identified as (underlined in the following 21 base pair sequence) TCAGGCGCA <u>t</u> TATGAAGATGG and the location is 98 base pairs from the 3' end of the forward primer described in <i>Primer set 3</i> . The sequence of the amplicon should be determined by validated, standard sequencing technologies such as Sanger sequencing.
	4	(5'–3') TGCAACACATCAAGCGATTGG	(5'–3') TCATAACCAGCATACGCCGG	Amplification product of 490 base pairs. There should be no amplification product of 367 base pairs; it may result in a similar amplicon in other strains.

^a DNA primers are commercially available (custom manufacture) from Integrated DNA Technologies (www.idtdna.com/pages) and other commercial sources.

^b Acceptance criteria for each primer set must be met to prove strain identity.

Primer dilution: Primers should be diluted with *Buffer* to a stock concentration of 100 µM, and then further diluted with *Buffer* to 25 µM, and stored at –20°.▲ (USP 1-May-2021)

Polymerase chain reaction (PCR) sample preparation: 1 µL of the *Sample solution*, ▲12.5▲ (USP 1-May-2021) µL of mastermix polymerase, ▲3▲ (USP 1-May-2021) 1 µL of diluted forward primer (25 µM), 1 µL of diluted reverse primer (25 µM), and ▲9.5▲ (USP 1-May-2021) µL of sterile water

PCR negative control: Prepare as directed for the *PCR sample preparation*, replacing the 1 µL of *Sample solution* with 1 µL of sterile water.

▲**PCR positive control:** Prepare as directed for the *PCR sample preparations*, either replacing the *Sample solution* with 1 µL of the genomic DNA of the reference strain or replacing the strain specific primers with species specific universal primers for *Lactobacillus rhamnosus*.▲ (USP 1-May-2021)

PCR amplification: Perform PCR on the *PCR sample preparation*, and the *PCR negative control* ▲and *PCR positive control*▲ (USP 1-May-2021) using an appropriate thermal cycler.▲ Incubate at 95° for ▲10▲ (USP 1-May-2021) min (step 1), 95° for 30 s (step 2), 57.0° for 30 s (step 3), and at 72° for ▲1 min▲ (USP 1-May-2021) (step 4). Repeat steps 2–4 for 34 cycles, then incubate at 72° for 5 min, and hold at 4°.

Analysis: Analyze the products of the *PCR amplification* for the *PCR sample preparation* and *PCR negative control* using an automated on-chip electrophoresis system with a DNA kit.▲ Follow the manufacturer's instructions for analysis. Alternatively, analysis and visualization may be accomplished by using gel electrophoresis. Prepare or use a commercially available ▲2%▲ (USP 1-May-2021) (w/v) agarose gel in a 1X tris-acetic acid–EDTA buffer (40 mM tris-hydrochloride, 1% glacial acetic acid, and 1 mM EDTA). Stain the gel with 0.5 mg/mL of ethidium bromide in water and destain with deionized water. [CAUTION—Ethidium bromide is considered a toxic substance and a potential mutagen. Use appropriate personal protective equipment, including nitrile gloves when handling this reagent.] Use a DNA ladder standard (1 kilobase plus)▲ suitable for determining the size of linear double-stranded DNA fragments ▲▲ (USP 1-May-2021) amplified with the *Primer sets* described in [Table 1](#). The ladder standard should be used in the first and last lanes on the gel to allow for proper comparison of amplicons.

▲If using genomic DNA of the strains as positive control, analysis of *PCR positive control* must result in the amplification of specific base pairs as indicated in the acceptance criteria. If using species specific universal primers as positive control, analysis of *PCR positive control* must result in the amplification of specific base pairs as found in published literature.▲ (USP 1-May-2021) Analysis of the *PCR negative control* must result in the absence of any amplification products or the preparation of the *PCR sample preparation* and *PCR negative control* must be repeated, followed by *PCR amplification* and *Analysis*.

Acceptance criteria: ▲See [Table 1](#).▲ (USP 1-May-2021)

ASSAY

Change to read:

● **ENUMERATION**

▲**Analysis:** Proceed as directed in general chapter [Probiotic Tests \(64\)](#).▲ (USP 1-May-2021)

Acceptance criteria: NLT 100% of the labeled viable cell count, in cfu/g

CONTAMINANTS

[NOTE—The methods of microbial analysis included in this section as examples represent currently accepted methods commonly used in industry. Users may substitute other validated test methods for the methods in this section.]

Delete the following:

▲◆ **MICROBIAL ENUMERATION TESTS (2021):** The total combined molds and yeasts count does not exceed 10² cfu/g.▲ (USP 1-May-2021)

Delete the following:

▲◆ **NON-LACTIC ACID BACTERIA:** ISO international standard number 13559 (IDF 153), available from the International Organization for Standardization (www.iso.org). The total non-lactic acid bacterial count is less than 5 × 10³ cfu/g.▲ (USP 1-May-2021)

Delete the following:

▲◆ **ABSENCE OF SPECIFIED MICROORGANISMS (2022), [Test Procedures, Test for Absence of Escherichia coli](#) and [Test for Absence of Salmonella Species](#):** It meets the requirements of the test for absence of *Escherichia coli*. It meets the requirements of the test for absence of *Salmonella* species in 40 g.▲ (USP 1-May-2021)

Add the following:

▲● **PROBIOTIC TESTS** (64), *Contaminants*, *Contaminant Microorganisms*: The total combined molds and yeasts count does not exceed 10^2 cfu/g. The total non-lactic acid bacterial count is less than 5×10^3 cfu/g. ▲ (USP 1-May-2021)

Add the following:

▲● **PROBIOTIC TESTS** (64), *Contaminants*, *Specified Microorganisms*: It meets the requirements of the test for absence of *Escherichia coli* in 10 g. It meets the requirements of the test for absence of *Salmonella* species in 40 g. ▲ (USP 1-May-2021)

● **LISTERIA**: (See *Food Chemicals Codex*, Appendix XV.) It meets the requirements of the test for absence of *Listeria* in 25 g.

ADDITIONAL REQUIREMENTS

● **PACKAGING AND STORAGE**: Preserve in high barrier foil laminate bags and store at or below 4°.

Change to read:

● **LABELING**: This ingredient should be labeled with the genus, species, and strain names and with the formulated enumeration in cfu/g (or similar units). This monograph applies only to *Lactobacillus rhamnosus* ▲strains HN001 and GG, ▲ (USP 1-May-2021) and no other strains of *Lactobacillus rhamnosus* cultures.

¹ Suitable PCR-Certified Waters, RNase and DNase Free, are available from Teknova (www.teknova.com).

² Suitable buffers (e.g., TE Buffer 1X, Molecular Biology Grade) are available from Promega (www.promega.com).

³ AmpliTaq Gold 360 Master Mix from Applied Biosystems (www.thermofisher.com) or equivalent.

⁴ Suitable thermal cyclers are available from Eppendorf (www.eppendorf.com).

⁵ Suitable automated on-chip electrophoresis systems with DNA kits (e.g., Agilent 2100 Bioanalyzer with Agilent DNA 1000 Kit) are available from Agilent (www.genomics.agilent.com).

⁶ Suitable 1-kilobase-plus DNA ladders are available from Life Technologies (<https://www.thermofisher.com/us/en/home.html>).

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