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▲⟨64⟩ PROBIOTIC TESTS

INTRODUCTION

This chapter provides testing procedures for identification, enumeration, contamination, and other requirements for probiotics. Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host. Probiotics are typically identified at the strain level as their characteristics are considered strain-specific.

This chapter applies to probiotics produced in specialized fermenters under strict hygiene conditions for dietary supplements or pharmaceutical applications. Fermentation media are tailored to the specific requirements of the microbial species or strain and typically contain nutrients such as proteins, carbohydrates, vitamins, and minerals. The culture is allowed to multiply and grow under carefully defined conditions. After the microbial cells are grown, the cultured cells are harvested, often by centrifugation. Suitable protectants may be added to the concentrated probiotic microorganisms (biomass), and the biomass is freeze-dried or spray-dried to a powdered form. The dried biomass then undergoes formulation, which may involve blending one or more strains with suitable excipients. Formulated probiotic ingredients can be further processed into dosage forms, e.g., tablets, capsules, softgels, powders, or gels.

IDENTIFICATION

Probiotics as defined in this chapter can be identified at the genus and species level or at the strain level according to the monograph requirements. Since regulatory filings for commercial probiotics are commonly made at the strain level supported by information from safety assessment and human studies, identification at the strain level may be required to verify the strain declared in labeling of commercial products. In this chapter, strain-specific identification using polymerase chain reaction (PCR) with strain-specific primers is recommended for the identification of probiotics. Currently, a testing procedure for identification of *Lactobacillus* and *Bifidobacterium* strains by PCR with specific primers is listed in this chapter. Additional testing procedures for identification of probiotic strains including non-spore-forming bacteria, spore-forming bacteria, and yeasts or molds will be listed in this chapter once they become available.

• **IDENTIFICATION FOR NON-SPORE-FORMING BACTERIAL STRAINS**

Identification for *Lactobacillus* and *Bifidobacterium* strains by PCR with specific primers

Unless otherwise specified in the individual monograph, use the following general procedure. All reagents and solutions used in this test should be molecular biology grade.

Buffer: 10 mM tris-hydrochloride, 1 mM sodium ethylenediaminetetraacetic acid (EDTA) buffer[1]

PCR master mix: *Taq* DNA polymerase (62.5 U/mL), 2.5X *Taq* reaction buffer [125 mM potassium chloride (KCl), 75 mM tris-hydrochloride pH 8.3, and 4 mM magnesium (Mg^{+2})], and 500 µM of each deoxynucleotide (dNTP). Prepared *PCR master mix* solutions are commercially available.[2]

Sample: 100 mg/mL suspension of the probiotic powder in *Buffer*

Primer set: Use the primers for the strain specified in the individual monograph. Dilute the forward and reverse primers in *Buffer* to a stock concentration of 100 µM, and dilute further with *Buffer* to 25 µM. The diluted forward primer and diluted reverse primer 25-µM dilutions can be stored at –20°.[3]

PCR sample preparations: For each *Primer set*, prepare a solution containing 1 µL of the *Sample*, 10 µL of *PCR master mix*, 1 µL of the diluted forward primer (25 µM), 1 µL of the diluted reverse primer (25 µM), and 12 µL of *Sterile Purified Water*.

PCR negative control: Prepare as directed for the *PCR sample preparations*, replacing the *Sample* with 1 µL of *Sterile Purified Water*.

PCR positive control (if available)**:** Prepare as directed for the *PCR sample preparations*, replacing the *Sample* with 1 µL of the corresponding Reference Standard.

Analysis: Perform PCR amplification on each of the *PCR sample preparations*, *PCR positive control*, and the *PCR negative control* using an appropriate thermal cycler.[4] Unless otherwise specified in the individual monograph, incubate at 95° for 7 min (step 1) for direct heat-induced DNA extraction.

Thermal cycle: 95° for 30 s (step 2); annealing temperature specified in the individual monograph for 30 s (step 3); and at 72° for 30 s (step 4). Repeat steps 2–4 for between 30 and 34 cycles, incubate at 72° for 5 min, and hold at 4°. Perform electrophoretic separation of the PCR amplification products for each of the *PCR sample preparations*, *PCR positive control*, and for the *PCR negative control*. An automated on-chip electrophoresis system with a DNA kit can be used.

Prepare or use a commercially available 1% (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 de-stain with 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 KB plus)[5] suitable for determining the size of linear double-stranded DNA fragments between 100 and 12,000 base pairs. The ladder standard should be used in the first and last lanes on the gel to allow for proper comparison of amplicons. Analysis of the *PCR negative control* must result in the absence of any amplification products. If amplification occurs, repeat the *PCR sample preparations*, and the *PCR negative control*, followed by PCR amplification and analysis.

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[1] Suitable buffers (e.g., TE Buffer 1X, molecular biology grade) are available from Promega (www.promega.com).

[2] 5 PRIME MasterMix Polymerase from VWR Scientific (www.vwr.com), AmpliTaq Gold DNA Polymerases from ThermoFisher Scientific (www.thermofisher.com), or other chemical/microbiology suppliers.

[3] DNA primers are commercially available (custom manufacture) from Integrated DNA Technologies (www.idtdna.com) and other commercial sources.

[4] Suitable thermal cyclers are available from Eppendorf (www.eppendorf.com/OC-en/).

[5] Suitable 1 KB plus DNA ladders are available from ThermoFisher Scientific (www.thermofisher.com).

Acceptance criteria: Refer to the acceptance criteria in the individual monograph.

ENUMERATION

Currently, a testing procedure for enumeration of *Lactobacillus* and *Bifidobacterium* species is listed in this chapter. Additional testing procedures for enumeration of probiotic strains, including non-spore-forming bacteria, spore-forming bacteria, and yeasts and molds, will be listed in this chapter once they become available. Alternative microbiological procedures, including automated methods, may be used provided that their equivalence has been demonstrated.

• **ENUMERATION FOR NON-SPORE-FORMING BACTERIA STRAINS**

***Lactobacillus* and *Bifidobacterium* species**

Unless otherwise specified in the individual monograph, use the following general procedure.

***Lactobacilli* MRS agar** (medium for *Lactobacillus* and *Bifidobacterium* species)**:** Prepare according to *Table 1* or use a commercially available mixture.[6]

Table 1. *Lactobacilli* MRS Agar

Reagent	Quantity (g)
Proteose peptone #3[a]	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate[b]	0.05
Dipotassium phosphate	2.0
Agar	15.0

[a] A suitable peptone for microbiological analysis is available from BD Bacto™ (www.bd.com).
[b] A suitable manganese sulfate is available from Sigma-Aldrich (www.sigmaaldrich.com).

Suspend the *Lactobacilli MRS agar* in 1 L of *Purified Water* in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat to boiling with stirring on a hot plate. Allow to boil for 1 min to completely dissolve the medium, and then autoclave the solution at 121° for 15 min. Cool to 45° and use immediately. Boiled agar medium may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. The agar can be stored at a temperature between 2° and 8° in a sterile well-closed container unless it is intended for immediate use. Do not use the medium for a longer storage period than has been validated. Allow the agar to come to room temperature before use.

***Lactobacilli* MRS broth:** Prepare according to *Table 2* or use a suitable commercially available broth.[7]

Table 2. *Lactobacilli* MRS Broth

Reagent	Quantity (g)
Proteose peptone #3[a]	10.0
Beef extract	10.0
Yeast extract	5.0
Dextrose	20.0
Polysorbate 80	1.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulfate	0.1
Manganese sulfate[b]	0.05
Dipotassium phosphate	2.0

[a] A suitable peptone for microbiological analysis is available from BD Bacto™ (www.bd.com).

[6] Difco™ *Lactobacilli* MRS Agar, or equivalent. Suitable *Lactobacilli* MRS agars are available from VWR Scientific (www.vwr.com) or other chemical/microbiological suppliers.
[7] Difco™ *Lactobacilli* MRS Broth or equivalent. Suitable *Lactobacilli* MRS broths are available from VWR Scientific (www.vwr.com) or other chemical/microbiological suppliers.

[b] A suitable manganese sulfate is available from Sigma-Aldrich (www.sigmaaldrich.com).

Suspend *Lactobacilli MRS broth* in 1 L of *Purified Water* in an appropriately sized conical flask or beaker (sufficiently large to not boil over). Cover the flask or beaker with aluminum foil and heat to boiling with stirring on a hot plate. Allow to boil for 1 min to completely dissolve the broth ingredients, and then autoclave the solution at 121° for 15 min. Broth may also be aseptically transferred into individual media bottles in 100- or 200-mL aliquots before sterilizing, and then autoclaved and stored for later use. The broth can be stored at a temperature between 2° and 8° in a sterile well-closed container unless it is intended for immediate use. Do not use the medium for a longer storage period than has been validated. Allow broth to come to room temperature before use.

Peptone diluent: Prepare a solution of 0.1% peptone[8] in water (w/v) and adjust with a lactic acid solution to a pH of 7.0. Using an autoclave, steam sterilize the solution at 121° for NLT 15 min, and then allow to cool in the unopened autoclave. Dispense into sterile containers as needed for preparing samples.

Sample preparation: Unless otherwise specified in the individual monograph, transfer an amount equivalent to 11.0 g of probiotic powder into a sterile polyethylene bag, commercially known as a stomacher bag. Add 99 mL of previously sterilized (room temperature) *Lactobacilli MRS broth* to the bag. Blend in a mechanical blender until a uniform mixture is achieved or in a stomacher[9] at 230 rpm for 30 s. Hold the mixture at room temperature for 30 min to allow rehydration of the sample, and then blend for an additional 30 s at 230 rpm. This is the primary 10^{-1} dilution. Using sterilized, filtered pipet tips, make serial dilutions by aseptically transferring 1.0 mL of the primary 10^{-1} dilution to sterile media bottles, each containing 99.0 mL of *Peptone diluent* (10^{-3} dilution). Repeat this operation until the desired dilution series is obtained. Dilutions are expected to contain 25–250 cfu/mL. Shake the media bottles to mix completely before proceeding with the *Analysis*.

Analysis: Using three sterile, filtered 1-mL pipet tips, aseptically transfer 1.0 mL of the *Sample preparation* separately into three appropriately labeled, sterile 15-mm × 100-mm Petri plates, and then pour about 10–15 mL of the *Lactobacilli MRS agar* at about 45° onto each plate, flaming the lip of the bottle between pours. Place the lid on each plate after adding the agar medium, and then gently swirl the plates to mix the *Sample preparation* and the agar medium. Avoid spillage onto the lid of the dish when swirling the plates. Repeat this procedure for additional dilutions of the *Sample preparation*. Prepare a blank plate containing only *Lactobacilli MRS agar* and a second blank plate containing 1.0 mL of *Peptone diluent* mixed with *Lactobacilli MRS agar*. Allow the plates to sit at room temperature on a level surface until the agar solidifies, and then incubate the plates at 36°–40° for 3–5 days under anaerobic conditions.[10] After the incubation period, count the colonies and record the results as viable colony-forming units (cfu) per gram, taking into account the appropriate dilution factor of the *Sample preparation*. Only count plates containing 25–250 colonies. Determine the average plate count, in colony-forming units per gram.

Acceptance criteria: Refer to the acceptance criteria in the individual monograph.

PERFORMANCE TESTS

Performance tests are recommended for any dosage forms of probiotics, including tablets, capsules, softgels, gelcaps, powders, or chewable gels, etc.

- **DISINTEGRATION AND DISSOLUTION** ⟨2040⟩, *Disintegration*: Meet the requirements
- **WEIGHT VARIATION** ⟨2091⟩: Meet the requirements

CONTAMINANTS

The level of the standard contaminant-indicator organisms as well as the absence of pathogenic microorganisms specified in the individual monographs must be determined to ensure the quality of probiotics. Recommended standard indicator organisms and specified microorganisms are listed in *Table 3* and *Table 4*. Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence has been demonstrated. Principles of validation of alternative microbiological methods are also described in *Validation of Alternative Microbiological Methods* ⟨1223⟩. Methods (e.g., dilution, buffer, or membrane filtration) may be suitably modified to overcome potential interferences such as acid production resulting from growth of probiotic organisms during the enrichment step, leading to possible false-negative results. Method suitability to detect the presence of microorganisms in the product to be tested must be established. Method suitability must be confirmed if changes in the testing performance occur, or changes in the product composition that may affect the outcome of the test are introduced.

- **CONTAMINANT MICROORGANISMS**

Unless otherwise specified in the individual monograph, the recommended tests and acceptance criteria for contaminant microorganisms in probiotic ingredients or finished products for oral use are listed in *Table 3*.

Table 3. Recommended Tests for Contaminant Microorganisms in Probiotic Ingredients or Finished Products for Oral Use

Probiotic Classification	Test	Test Methods	Acceptance Criteria (cfu/g)
Non-spore-forming bacteria	Non-lactic acid bacteria	ISO 13559 (IDF 153)[a]	NMT 5×10^3
	Total yeasts and molds	*Microbial Enumeration Tests* ⟨2021⟩	NMT 100
Spore-forming bacteria	Total yeasts and molds	*Microbial Enumeration Tests* ⟨2021⟩	NMT 100
Yeasts and molds	Total aerobic microbial count	*Microbial Enumeration Tests* ⟨2021⟩	NMT 1×10^3

[a] Available from the International Organization for Standardization (www.iso.org).

[8] Suitable peptone for microbiological analysis is available from BD Bacto (www.bd.com).
[9] Suitable stomacher (e.g., Stomacher 400 Circulator) for mechanical sample blending is available from Seward (www.seward.co.uk).
[10] Suitable anaerobic systems are available from BD GasPak EZ Container System (www.bd.com).

• **Specified Microorganisms**

Unless otherwise specified in the individual monograph, the recommended tests and acceptance criteria for specified microorganisms in probiotics or finished products for oral use are listed in *Table 4*.

Table 4. Recommended Tests for Specified Microorganisms in Probiotic Ingredients or Finished Products for Oral Use

Probiotic Strain	Test	Test Methods	Acceptance Criteria
Non-spore-forming bacteria, spore-forming bacteria, or yeasts and molds	*Escherichia coli*	*Absence of Specified Microorganisms* ⟨2022⟩	None detected in 10 g
	Salmonella species	*Absence of Specified Microorganisms* ⟨2022⟩	None detected in 10 g

The absence of *Listeria monocytogenes*, *Staphylococcus aureus*, or *Pseudomonas aeruginosa* in addition to the absence of the specified microorganisms listed in *Table 4* should be tested and confirmed if a probiotic ingredient or a finished product containing a probiotic ingredient(s) poses a risk associated with the contamination of the above microorganisms based on formal risk assessment programs such as Hazard Analysis and Critical Control Points (HACCP). The absence of *Clostridium perfringens* and *Cronobacter sakazakii* in addition to the absence of the listed specified microorganisms should be tested and confirmed if a probiotic ingredient or finished product is intended for infant use. The methods for *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Clostridium perfringens*, and *Cronobacter sakazakii* must be officially accepted methods or appropriately validated methods.

ADDITIONAL REQUIREMENTS

• **Packaging and Storage**

Shipping and storage conditions will necessarily be adapted to the particular strains being sold. Unless otherwise specified in the individual monograph, probiotic ingredients should be stored in high-barrier foil-laminate bags and kept at or below 4° for long-term storage. Dosage forms of probiotics should be stored in tight containers in a cool, dry place.

• **Labeling**

For probiotic ingredients, the strain designation should be listed on the label for each strain. An ingredient or a dosage form of probiotics should be labeled with the genus, species, and strain names. In cases where there is suitable scientific rationale, such as scientific substantiation of health benefits that are not strain specific, a dosage form of probiotics may be labeled with the genus and species names. A total formulated enumeration of all probiotic ingredients throughout the product shelf life should be included at minimum in cfu/g or cfu/serving.▲ (USP 1-Aug-2019)