monella Enrichment Broth, mix, and incubate at 30° to 35° for 18 to 24 hours. Streak a loopful from both incubated media onto individual surfaces of one or more of following media: *Brilliant Green Agar Medium (BG-Agar), Xylose–Lysine–Desoxycholate–Agar Medium (XLDC-Agar),* and *Hektoen Enteric Agar Medium (HE Agar).* Cover, invert the plates, and incubate at 30° to 35° for 24 to 48 hours. Examine the inoculated plates of *BG-Agar, XLDC-Agar,* and/or *HE Agar,* and interpret the results with reference to *Table 2:* if no colonies having the characteristics described are observed, the test specimen meets the requirement for the absence of *Salmonella* species. If colonies with characteristics described in *Table 2* are present, the suspect colonies are transferred to a slant of *Triple Sugar–Iron–Agar Medium (TSI)* using an inoculating wire, by first streaking the surface of the slant, and then stabbing the wire well beneath the surface. Incubate at 30° to 35° for 24 to 48 hours. If the tubes do not have red alkaline slants and yellow acid butts, with or without concomitant blackening of the butts from hydrogen sulfide production, the test specimen meets the requirement for the absence of *Salmonella* species.

Table 2. Characteristics of *Salmonella* Species on Specified Agar Media

Agar Medium	Colonial Morphology	Gram Stain
Brilliant Green	Small, transparent and colorless; or opaque, pink or white (often surrounded by pink to red zone)	(–), rods
Xylose–Lysine–Desoxycholate	Red, with or without black centers	(–), rods
Hektoen Enteric	Blue-green, with or without black centers	(–), rods

Test for Absence of *Escherichia coli*

Incubate at 30° to 35° for 24 to 48 hours. From *FSCD,* pipet a 1-mL aliquot into a container containing 10 mL of *MacConkey Broth,* mix, and incubate at 42° to 44° for 24 to 48 hours. Streak a loopful from both incubated media onto individual surfaces of *MacConkey Agar Medium (MC Agar),* and incubate at 30° to 35° for 18 to 24 hours. Examine the inoculated *MC Agar* plate, and interpret the results with reference to *Table 3:* if no colonies having the characteristics described are observed, the test specimen meets the requirement for the absence of *Escherichia coli.* Suspect colonies showing the characteristics described in *Table 3* are transferred individually, using an inoculating loop, to the surface of a plate with *Levine Eosin–Methylene Blue–Agar Medium (LEMB-Agar).* If a large number of suspect colonies are to be transferred, divide the surface of each plate into quadrants, each quadrant being inoculated with a different colony. Cover the plates, invert, and incubate at 30° to 35° for 24 to 48 hours. If none of the colonies exhibit a characteristic metallic sheen under reflected light, and if none exhibit a blue-black appearance under transmitted light, the test specimen meets the requirement for the absence of *Escherichia coli.*

Table 3. Characteristics of *Escherichia coli* on *MacConkey Agar Medium*

Colonial Morphology	Gram Stain
Brick red, may have surrounding zone of precipitated bile	(–), rods

Test for Absence of *Clostridium* Species

Test Preparation—Prepare as directed for *Sampling.* [NOTE—On the basis of results for *Preparatory Testing,* modify the *Test Preparation* as appropriate.]

Procedure—Take two equal portions of the *Test Preparation,* heat one to 80° for 10 minutes, and cool rapidly. Transfer 10 mL of each portion to separate containers, each containing 100 mL of *Reinforced Medium for Clostridia,* and incubate under anaerobic conditions at 35° to 37° for 48 hours. After incubation, subculture each specimen on *Columbia Agar Medium* to which gentamicin has been added, and incubate under anaerobic conditions at 35° to 37° for 48 hours. Examine the plates, and interpret with reference to *Table 4:* if no growth of microorganisms is detected, the test specimen meets the requirement for the absence of *Clostridium* species.

Table 4. Characteristics of *Clostridium* Species on Specified Media

Medium	Gram Stain	Catalase
Reinforced Medium for Clostridia	(+), rods	
Columbia Agar	(+), rods	Negative

If growth occurs, subculture each distinct colony on *Columbia Agar Medium,* and separately incubate in aerobic and in anaerobic conditions at 35° to 37° for 48 hours. The occurrence of only anaerobic growth of gram-positive bacilli, giving a negative catalase reaction, indicates the presence of *Clostridium sporogenes.* To perform the catalase test, transfer discrete colonies to glass slides, and apply a drop of dilute hydrogen peroxide solution: the reaction is negative if no gas bubbles evolve. If the test specimen exhibits none of these characteristics, it meets the requirement for the absence of *Clostridium* species.

Retest

For the purpose of confirming a doubtful result by any of the procedures outlined in the foregoing tests following their application to a 10 g specimen, a retest on a 25 g specimen of the nutritional or dietary supplement may be conducted. Proceed as directed under *Procedure,* but make allowances for the larger specimen size.

⟨2023⟩ MICROBIOLOGICAL ATTRIBUTES OF NONSTERILE NUTRITIONAL AND DIETARY SUPPLEMENTS

The raw materials, pharmaceutical ingredients, and active ingredients used in the manufacture of nutritional and dietary articles may range from chemically synthesized vitamins to plant extracts and animal byproducts, and these ingredients are typically not sterile. Considerable experience has accrued with these highly refined plant- and animal-derived pharmaceutical ingredients, such as microcrystalline cellulose, modified starch, lactose, and magnesium stearate, and their microbiological attributes are well established. Botanicals may be microbiologically contaminated at any point during cultivation, harvesting, processing, packing, and distribution. Major sources of microbial contamination are associated with human or animal feces used as plant manure,

contaminated irrigation water and/or process water, and poor worker hygiene and sanitation practices during harvesting, sorting, processing, packaging, and transportation. Furthermore, it is essential that microbiological contamination be minimized during the manufacture of nonsterile dietary supplements. To achieve this, Good Manufacturing Practices are employed and adequate microbiological specifications are established.

Microbiological process control, control of the bioburden of raw materials, and control of the manufacturing process to minimize cross-contamination are necessary to guarantee acceptable microbial quality in the final dosage forms. Because nonaqueous or dry dosage forms do not support microbial growth because of low water activity, the microbial quality of such articles is a function of the microorganisms introduced through ingredients or during processing. In addition to considering the intended use of the product, the frequency of microbial testing for the finished nonsterile dietary supplement would be a function of the historical microbial testing database of that product, knowledge of the manufacturing processes, the susceptibility of the formulation to microbial proliferation, and the demonstrated effectiveness of programs controlling the raw materials.

FORMULATION AND PROCESS DESIGN

From a microbiological perspective, the development of the formulation of nutritional or dietary supplements includes an evaluation of raw materials and their suppliers and the contribution made to the products by each ingredient and the manufacturing processes. Characterization of these elements allows the adequacy of the manufacturing process to be demonstrated. For example, if a product is formulated with an ingredient of botanical or animal origin known to possess a high, variable, or unpredictable level of microbiological contamination, it is necessary to ensure that the microbiological monitoring identifies ingredients that have an inappropriate bioburden level and that a premanufacturing process such as drying, extraction, heat treatment, irradiation, or gaseous sterilization treatment will inactivate or remove any objectionable contaminant possibly present.

However, the selected treatment technique should not have any adverse effects. The treatment of raw materials by irradiation and ethylene oxide may cause unwanted changes affecting the safety and efficacy of the raw material. For instance, when treated by ethylene oxide, crude extracts containing alkaloids have shown reduced contents of alkaloids. Dry heat treatment has been used for inactivation as well, but it requires further evaluation because it may adversely affect stability and degradation of the raw material. With regard to the design of the manufacturing process, appropriate consideration should be given to the microbiological effect of wet granulation manufacturing processes. Wetting of a dry powder can result in increased levels of microorganisms if the granulation is stored prior to drying. However, it is recognized that the pressure and temperature associated with compression of tablets will decrease microbial counts. Antimicrobial activity is also achieved, especially with aqueous preparations, by the addition of chemicals that have known antimicrobial properties and that are compatible with the formulation.

However, antimicrobial preservation is not a substitute for Good Manufacturing Practices. A process has to be designed to minimize the microbiological population. Operating procedures and temperatures and time limits, including holding times, are established to protect the product from microbiological contamination and growth. All processes have to be validated for their intended purposes. Moreover, in-process manufacturing and testing controls necessary for microbiological quality should be identified and implemented.

FACILITIES, EQUIPMENT, WATER, AND SANITIZATION

Facilities—The facilities, including the building and the heating, ventilation, and air-conditioning (HVAC) systems, should be designed to minimize microbiological contamination. The design of facilities used for the manufacture of supplements and their operating parameters should be documented, and the documentation should include, when appropriate, HVAC filter types, space pressure differentials, temperature, and relative humidity and air changes. Dry products processed in a dry environment do not possess a high potential for increased microbial levels. However, some control is warranted to minimize microbiological and chemical contamination. Potentially problematic areas are those that utilize *Purified Water* for wet granulation, batching liquid products, and film-coating tablets, because water encourages microbial growth.

Equipment—Equipment used for the processing of semisolid and dry supplements should be designed to promote sanitary conditions, to be self-drying, and to be easy to clean. Dryers, ovens, wet granulation equipment, bulk tanks, and equipment for preparation of coating solutions are periodically evaluated to ensure that cleaning procedures are adequate.

Water—As one of the major components in nutritional and dietary supplement manufacturing processes, water deserves a special consideration in the microbiological control of these articles. It is a growth medium for a variety of microorganisms that present a threat to product quality, safety, preservation, and stability. Water may even act as a carrier of objectionable microorganisms. In view of this, water used in manufacturing is *Purified Water.* For the manufacture of raw materials, process water that meets specific microbiological objectives and U.S. Environmental Protection Agency National Drinking Water standards or equivalent European and Japanese standards may be used.

Cleaning and Sanitization—Detailed and specific cleaning and sanitization procedures should be evaluated, developed, and validated, with special attention given to product contact surfaces. Personnel should possess sufficient knowledge of these procedures.

SUPPLEMENT COMPONENTS

Raw materials, excipients, and active substances as components of nutritional and dietary supplements can be a primary source of microbiological contamination. Specifications should be developed and sampling plans and test procedures should be employed to guarantee the desired microbiological attributes of these materials. The nature and extent of microbiological testing should be based upon a knowledge of the material's origin, its manufacturing process, its use, and historical data and experience. For instance, materials of animal or botanical origin that are not highly refined might require special, more frequent testing than synthetic products.

Since members of the family *Enterobacteriaceae* are a major component of the normal epiphytic and endophytic microflora (e.g., members of genera *Klebsiella, Enterobacter,* and *Erwinia*) and have been associated with the seeds, pods, roots, leaves, and stems of plants of economic importance, *Coliform* or *Enterobacteriaceae* counts will not be an appropriate general microbiological criterion for botanicals. However, when it is considered advantageous, *Coliform* or *Enterobacteriaceae* counts may be included in the individual monographs. Typically on new leaves, bacteria predominate in the microflora, while yeast and filamentous fungi succeed bacteria and become dominant late in the growing season. With dried botanicals, the bacterial population will tend to change from Gram-negative bacteria to Gram-positive spore formers and fungi. Refinement of botanicals from chopped or powdered plant material to powdered extracts using alcoholic, alkaline, acid hydro-alcoholic, or aqueous extracting materials will reduce the likelihood of vegetative microorganisms within the botanical material. The classification of botanical materials is contained in *Table 1.*

MICROBIOLOGICAL TESTING

Frequency of Sampling and Testing

Microbiological attribute sampling and testing plans vary widely. In some cases no sampling or testing is necessary; in other cases periodic monitoring is warranted; and yet for some articles each batch requires sampling and testing. The design of the sampling and testing plans and the kind of attributes examined depend on the application and the type of the product, the potential for contamination from components and processing, the growth promotion or inhibition properties of the formulation, and the target population for the supplement. For example, a powdered botanical may have highly variable microbiological attributes so that an incoming batch would be sampled and composite testing would not be advised, while a highly refined botanical extract may not require routine microbial testing. Similarly, products with a low water activity will not be susceptible to microbial growth during their shelf life provided they are protected from elevated humidity by their containers.

Microbial Enumeration Tests

See the *Introduction* under *Microbial Enumeration Tests—Nutritional and Dietary Supplements* ⟨2021⟩. These tests provide meaningful information regarding the microbiological acceptability of excipients, active substances, and nonsterile supplement formulations. If the individual monograph does not specify microbial enumeration limits, the guidance provided in this chapter is used. Acceptable general limits of microbial levels for raw materials, excipients, and botanical products are shown in *Table 2;* and those for raw materials, excipients, active ingredients, and other nonsterile finished articles that are nutritional supplements but do not contain botanicals are shown in *Table 3*.

Table 1. Definitions of a Range of Botanical Materials

Botanical Preparation	Definition
Chopped or Powdered Botanicals	Hand-picked portions of the botanical (e.g., leaves, flowers, roots, tubers, etc.) that are air dried, and chopped, flaked, sectioned, ground, or pulverized to the consistency of a powder.
Botanical Extracts	Extracts are solids or semisolid preparations of a botanical that are prepared by percolation, filtration, and concentration by evaporation of the percolate. The extracting material may by alcoholic, alkaline, acid hydro-alcoholic, or aqueous in nature. Typically an extract is 4 to 10 times as strong as the original botanical. The extracts may be semisolids or dry powders termed powdered extracts.
Tinctures	Tinctures are solutions of botanical substances in alcohol obtained by extraction of the powdered, flaked, or sectioned botanical.
Infusions	Infusions are solutions of botanical principles obtained by soaking the powdered botanical in hot or cold water for a specified time and straining. Typically infusions are 5% in strength.
Decoctions	Decoctions are solutions of botanicals prepared by boiling the material in water for at least 15 minutes and straining. Typically decoctions are 5% in strength.
Fluidextracts	A fluidextract is an alcoholic liquid extract made by percolation of a botanical so that 1 mL of the fluidextract represents 1 g of the botanical.
Botanicals to be treated with boiling water before use	Dried botanicals to which boiling water is added immediately prior to consumption.

Table 2. Recommended Microbial Limits for Botanical Ingredients and Products

Material	Recommended Microbial Limit Requirements (cfu/g or mL)
Dried or Powdered Botanicals	Total Aerobic Microbial Count NMT 10^5
	Total Combined Yeast & Mold Count NMT 10^3
	Bile-tolerant Gram-negative Bacteria NMT 10^3
	Absence of *Salmonella* spp. & *E. coli* in 10 g
Powdered Botanical Extracts	Total Aerobic Microbial Count NMT 10^4
	Total Combined Yeast & Mold Count NMT 10^3
	Absence of *Salmonella* spp. & *E. coli* in 10 g
Tinctures	Total Aerobic Microbial Count NMT 10^4
	Total Combined Yeast & Mold Count NMT 10^3
Fluidextracts	Total Aerobic Microbial Count NMT 10^4
	Total Combined Yeast & Mold Count NMT 10^3

Table 2. Recommended Microbial Limits for Botanical Ingredients and Products *(Continued)*

Material	Recommended Microbial Limit Requirements (cfu/g or mL)
Infusions/Decoctions	Total Aerobic Microbial Count NMT 10^2
	Total Combined Yeast & Mold Count NMT 10
Nutritional Supplements with Botanicals	Total Aerobic Microbial Count NMT 10^4
	Total Combined Yeast & Mold Count NMT 10^3
	Absence of *Salmonella* spp & *E. coli* in 10 g
Botanicals to be treated with boiling water before use	Total Aerobic Microbial Count NMT 10^5
	Total Combined Yeast & Mold Count NMT 10^3
	Absence of *E. coli* in 10 g

Table 3. Recommended Microbial Limits for Dietary Supplement Ingredients and Products

Material	Recommended Microbial Limit Requirements (cfu/g or mL)
Other raw materials and dietary supplement ingredients	Total Aerobic Microbial Count NMT 10^3
	Total Combined Yeast & Mold Count NMT 10^2
	Absence of *E. coli* in 10 g
Nutritional supplements with synthetic or highly refined ingredients	Total Aerobic Microbial Count NMT 10^3
	Total Combined Yeast & Mold Count NMT 10^2
	Absence of *E. coli* in 10 g

Absence of Objectionable Microorganisms

See *Introduction* under *Microbiological Procedures for Absence of Specified Microorganisms—Nutritional and Dietary Supplements* ⟨2022⟩. Absence of one or more species of objectionable microorganisms is required in some individual monographs.

Test for Aflatoxins—Dietary and nutritional articles containing botanical products with a history of mycotoxin contamination are also typically tested for aflatoxins, especially if the material is obtained from roots or rhizomes. See *Articles of Botanical Origin* ⟨561⟩ for the details of a test for aflatoxins. Where necessary, this test is included in the individual monograph.

Solid Oral Dosage Forms—Among all dosage forms, solid oral dosage forms present the lowest microbiological risk because of their method of manufacture, low water activity, and route of administration. When justified, reduced microbiological testing may be appropriate.

Other Concerns—The presence of some microorganisms in articles can be an indicator of processes that are not under microbiological control. For example, *Purified Water* used at some stage of the manufacture of these products might contain a typical flora of Gram-negative microorganisms. As with pharmaceutical products, inadequate processing of water and poor maintenance of water systems may result in the contamination of processed formulations by Gram-negative microorganisms.

⟨2030⟩ SUPPLEMENTAL INFORMATION FOR ARTICLES OF BOTANICAL ORIGIN

This general chapter provides information about several aspects of botanical articles not covered in *USP* standards monographs. Although the standards in the monographs address the quality issues associated with botanical plant materials, extracts, and preparations of Pharmacopeial articles, there is a need to develop appropriate information to optimize the pre-harvest conditions for appropriate growth and the post-harvest handling to achieve consistent quality with minimum variations in the composition of chemical constituents.

PROTOCOL CONTENTS

Black Cohosh (*Actaea racemosa* L.)
Ginger (*Zingiber officinale* Roscoe)
Valerian (*Valeriana officinalis* L.)
Elm (*Ulmus rubra* Muhlenberg)

GENERAL GUIDANCES

It is recommended that, at a minimum, growers and others involved in the handling and distribution of botanical products should become familiar with and follow the WHO Guidelines for Good Agricultural and Collection Practices (GACP) for Medicinal Plants (found at http://www.who.int/medicinedocs/collect/edmweb/pdf/s4928e/s4928e.pdf .

Commercial trade in natural products occurs in a global market. Material of domestic origin must be produced in compliance with all federal laws of the United States. Material of foreign origin, imported into the U.S., must be produced and transported in compliance with the laws of the U.S., the country of origin, and relevant international treaties. These include, but may not be limited to, the following:

1. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) is an international agreement between governments. Its aim is to ensure that international trade in specimens of wild animals and plants does not threaten their survival. Information about CITES is available at http://www.cites.org.
2. The Convention on Biological Diversity (CBD) establishes three main goals: the conservation of biological diversity, the sustainable use of its components, and the fair and equitable sharing of the benefits from the use of genetic resources. Each country that has ratified and is a party to the Convention is responsible for implementation by means of national enabling legislation that can differ from country to country.
3. The Endangered Species Act (ESA) was originally adopted in 1973. The ESA is a law that aims to protect species of fish, wildlife, and plants believed to be threatened with extinction. The ESA is administered