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Microbiological Quality of Probiotic Products

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Abstract

Microorganisms used as probiotics should meet elementary safety aspects (non-toxicity, absence of antibiotic resistance genes and translocation) and functional/technological aspects (resistance and survival in the acid gastric environment, adhesiveness, stability, and cell viability). Probiotics with the health claim of being a dietary product or a pharmabiotic (drug category) should be clinically tested, validated, documented, and continuously controlled for quality. Important quality parameters include the identification of declared probiotic strains, the number of viable microorganisms (probiotic bacteria and/or fungi), and microbiological purity (absence of specified pathogenic/opportunistic pathogenic bacteria and fungi, and limitation of total unspecified contaminants such as aerobic bacteria, yeasts, and molds). Due to numerous reports of low-quality commercial probiotics marketed for human use, this review discusses the methods used to test the probiotic microorganism content, safety for the intended use, and proven health benefits of those probiotics whose microbiological quality deviates from the manufacturer's stated content, as well as the maintenance of cell viability, i.e., stability of the probiotic during the shelf life. In addition, the adverse effects of probiotics and the potential hazards to the health of the user are addressed.

Key words: probiotic, microbiological quality, safety, stability

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Introduction

According to the Food and Agriculture Organization (FAO)/World Health Organization (WHO), probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (1-3). Their main health benefits are in maintaining a healthy microbiota or improving microbiota resistance, maintaining gut integrity and barrier function, improving lactose intolerance, gut function and gastrointestinal comfort, preventing diarrhea and relieving symptoms, and regulating the immune response (1). Probiotics are used in the form of foodstuffs, dietary supplements, foods for special medical purposes, medicinal products, and medical devices in humans, as well as feed supplements in farm animals.

Main characteristics of probiotics

Probiotics intended for human use must first undergo clinical trials approved by the relevant agencies and controlled by official control laboratories to confirm their quality, efficacy, and safety. They target specific host species (humans and animals), populations (children, adults) and sites (gut, oral cavity, etc.), have different routes of administration, efficacy endpoints and regulatory categories, and must have strain- or group-specific benefits and be safe for the intended use. Although many microorganisms affect the microbial balance in the intestines, only those species and strains with confirmed beneficial effects on the host can be selected as probiotics. Probiotic products usually contain bacteria from the *Lactobacillaceae*, especially *L. acidophilus* and *L. rhamnosus*, *L. plantarum*, *L. casei*, *L. paracasei*, and *L. salivarius*, as well as bacteria from the genera *Bifidobacterium* (*B. longum*, *B. lactis*, *B. bifidum*, *B. breve*), *Lactococcus*, *Bacillus* or strains of *Streptococcus thermophilus*, less commonly *Enterococcus* spp, and from fungi the yeast *Saccharomyces cerevisiae* var. boulardii (4). Probiotic microorganisms must be nonpathogenic, i.e., they must be Generally Recognized as Safe (GRAS) or have Qualified Presumption of Safety (QPS) status, and there must be no probiotic strains-host microbiota interactions, i.e., probiotics and their metabolites (especially bacteriocins) must not have an inhibitory or bactericidal effect on the host microbiota (5-9). To achieve health benefits, the effective daily dose in terms of the number of viable microorganisms is 10^8-10^{11} CFU (Colony-Forming Units) of bacterial or yeast cells (4). The criteria that must be met for certain strains of microorganisms to be classified as probiotics are: sufficiently characterized and identified by scientifically based methods, safe for the intended use, supported by at least one human clinical study (clinical trials), and live at an effective dose throughout the product's shelf life (retains the declared dose – CFU until the end of the period of use) (10). Probiotics may contain one strain of a specific species, multiple strains of the same species, and strains belonging to two or more species, even to different genera, and are accordingly designated as mono-strain or single-strain product (SSP), multi-strain product (MSP), and mixed-species product (11). MSP has been shown to be more effective than SSP, which has host-specific effects and one or several health benefits (12). Regulatory aspects for probiotics and probiotic categories vary widely among countries (13, 14). Potential next-generation probiotics (NGPs) have been sought among gut bacteria of the genera *Bacteroides*,

Clostridium, *Faecalibacterium*, and *Akkermansia*. Studies with *C. butyricum*, a strain commercially available as a supplement, are the most advanced among NGPs (15).

Possible mechanisms of action and distribution of probiotics

The main mechanisms of action of probiotics include maintaining normal intestinal microbiota, competitive exclusion by bacteriocin production, modulation of the activity of fecal enzymes associated with bile salt metabolism, lowering blood glucose and serum lipids, inactivation of carcinogens and other xenobiotics, short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs) production, cell adhesion and mucin production, immunomodulation, impact on the brain-gut axis and consequently neurological and endocrine functions. Common species-level effects include direct antagonism (16-19), enzymatic activity, bile salt metabolism, vitamin synthesis, gut barrier reinforcement, and neutralization of carcinogens. Hyperlipidemia is a potential risk factor for cardiovascular disease, so research and application of probiotics to reduce serum lipid levels are increasingly important (20-22). Rare strain-specific effects are neurological, immunological, and endocrinological, as well as the production of specific bioactive substances. Not only live, but also inactivated, most often heat-killed cells ("non-living probiotics"), i.e. parabiotics (23) and various metabolites of probiotics known as postbiotics have also shown health benefits. Postbiotics are soluble factors secreted by live probiotic bacteria or released after cell death (bacterial lysis), such as SCFAs, enzymes, peptides, teichoic acids, muropeptides derived from peptidoglycan, endo- and exo-polysaccharides, cell surface proteins, vitamins, and organic acids and exhibit antimicrobial, antioxidant, and immunomodulatory properties (24-26). According to the International Scientific Association for Probiotics and Prebiotics (ISAPP), a new definition of postbiotics as "preparations of inanimate microorganisms and/or their components that provide a health benefit to the host" means that the term postbiotics also includes parabiotics (27). Potential mechanisms of their action are prevention of infections, immunomodulation, influence on lipid/cholesterol metabolism, and antitumor/antioxidant activity (28). Postbiotics, unlike probiotics, do not contain live microorganisms, which is an advantage because it reduces the risks of their intake. As for adverse effects, there is no risk of bacteremia and sepsis in the case of postbiotic translocation.

Health safety requirements for probiotics

A probiotic product may be regulated by the U.S. Food and Drug Administration (FDA) as a dietary supplement, food ingredient, or drug. Many probiotics are used as dietary supplements and don't need to be approved by the FDA before marketing. A probiotic intended for the treatment, alleviation, therapy, or prevention of human disease is classified as a drug and must be subject to more stringent requirements, namely clinical evidence of safety and efficacy for the intended use and FDA approval. Different categories of health claims can be legally made for foods and dietary supplements. The FDA has issued regulations defining each claim and specifying the requirements for evidence. The categories of live microorganisms for human consumption can be defined as a probiotic in

food or supplement without a health claim, a probiotic in food or supplement with a specific health claim, and a probiotic drug. A probiotic in food or supplement without a health claim must meet the criterion of being a member(s) of a safe species (29, 30), which is supported by evidence of a beneficial effect on human health or safe microbe(s). The evidence does not need to be generated for the specific strain included in the product and can be based on taxonomical or functional comparisons. A probiotic in food or supplement with a specific health claim, such as “helps strengthen the body’s natural defenses in children” or “helps reduce the risk of antibiotic-associated diarrhea”, may be used with the following criteria: defined content and a sufficient number of viable microorganisms in an effective dose at the end of the shelf life (2). The minimum requirement for a health claim is a confirmed benefit of the specific strain(s) or strain combination(s) in well-conducted human studies, such as positive meta-analyses, according to the principles outlined by Cochrane (31), randomized controlled trials (RCTs), or large observational studies useful for determining the health effects of products in “real life” i.e., outside the controlled setting of RCTs. (32). A probiotic drug is a probiotic with a health claim that implies a specific indication for the treatment or prevention of disease, such as “useful for preventing relapse of ulcerative colitis”, and meets the criteria of being a defined strain(s) of live microbes, evidence that a viable probiotic is delivered at an effective dose at the end of the shelf life, and that a risk-benefit assessment justifies its use. Drug claims vary from country to country (2), but the minimum requirement for a claim is appropriate trials to meet regulatory standards for drugs. The term “pharmabiotics” has been used for some time to distinguish drugs from dietary supplements or foods, which until recently were collectively called probiotics. It refers to biotherapeutic products that contain live microorganisms and are used to prevent or treat disease, but, unlike probiotics, as dietary supplements or foods for special medical purposes in healthy people (33).

In the European Union, the European Food Safety Authority (EFSA) plays a central role in the regulation of probiotics by providing guidance for the evaluation of the safety aspects of probiotic (food) products, control systems to ensure the integrity, purity, and stability of the culture method for determining the number of viable cells, and control procedures for determining viability during storage (7). Since 2007, EFSA has maintained a list of probiotic species considered safe for human consumption in food under the QPS concept (29) based on an assessment of published data for each strain regarding its taxonomic identity, relevant knowledge, safety concerns, and occurrence of antimicrobial resistance. Safety concerns identified for a taxonomic unit are confirmed at the species/strain or product level where possible and supported by qualifications (10). In many Member States, the term “probiotic” is generally used to refer to a product category, especially in Italy, as one of the largest markets in Europe. Since the entry of Regulation (EC) 1924/2006 on nutrition and health claims made on foods into force, products claiming to be probiotics or to have specific health effects may no longer be sold in the European Union, as those are considered to be unauthorised health claims.

Probiotic microorganisms that have a potential benefit to host health are commercially available in a wide variety of products, but not all strains of

microorganisms contained therein have been shown to have beneficial health properties. The effectiveness of probiotics depends on the specific strain, the number of viable cells in an effective dose, and the absence of contaminants, so their quality control is of great importance. It is crucial to accurately identify declared microorganisms and determine the number of viable cells, as well as the presence of contaminants and their number by microbiological purity tests. In a marketed product, the probiotic formulation should be based on dose-response relationships established by human studies, which can depend on the strain viability during the shelf life of the product and its survival in the gastrointestinal tract. The presence of low-quality probiotic products on the market (inadequate number of probiotic bacteria/fungi, increased number of contaminants above the permitted limit, presence of non-permitted specified microorganisms, and others; see review article 4 for details) imposes the need for standardized testing and increased inspection frequency of randomly sampled products. In the absence of specific and stringent regulations currently governing the marketing of probiotics, the interests of manufacturers whose goal is to identify, research, and market new probiotic products beneficial to human health are not adequately protected. The interests of end users are also at risk, as they may be misled by product labeling and deprived of information about the true nature of the product they are using. To protect both manufacturers and users, stricter regulations specifically addressing medically useful probiotics that cannot be classified as drugs would be welcome (14).

Biosafety challenges

Probiotics have long been considered safe dietary supplements, and accordingly, many have been granted GRAS status. Regarding translocation, it is generally believed that probiotics cannot translocate (34). However, cases of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, and *Bifidobacterium* isolated from infection sites are not consistent with this assumption (35). Bacterial translocation is the transfer of viable indigenous bacteria from the gastrointestinal tract to extraintestinal sites, triggered by a defective intestinal barrier (decreased integrity and increased permeability), immunodeficiency and immunosuppression, or prematurity of the gut, potentially causing bacteremia, septicemia, and multiple organ failure. The exact mechanisms of bacterial translocation are not yet known but are thought to be based on the complex interplay of host defense and bacterial pathogenicity (36, 37).

In the healthy adult host, probiotic bacteria can translocate across the intestinal mucosa, but these “spontaneously” translocating bacteria are usually killed by the host’s innate immune defenses. Given the critical role of host defenses, bacterial translocation is more common in patients with intestinal obstruction and a weakened immune system. Recently, a case of bacteremia due to *L. rhamnosus* translocation was reported in a patient with a central venous catheter (38). In addition, the adverse effects of bacterial translocation have been documented in individuals of neonatal age and/or with certain clinical conditions such as malignancies, leaky gut, diabetes mellitus, and convalescence after organ transplantation. To make matters worse, some probiotic

strains can exploit the weak immunity of these susceptible individuals and transform into opportunistic pathogens that lead to life-threatening pneumonia, endocarditis, and sepsis (39). In addition, uncontrolled and extensive use of probiotics potentially carries the risk of plasmid-mediated antibiotic resistance transfer to infectious gut pathogens (40). For bacterial species associated with more frequent opportunistic infections, translocation testing should be part of the safety assessment. The FDA does not have standards for the testing required to evaluate the safety of new probiotic strains. Thus, some GRAS or NDI (New Dietary Ingredient) reports may contain undesirable or complicated data. There are numerous probiotics on the market that have not been fully tested for safety. The FDA should require all manufacturers of probiotics not to market their products until they have passed a series of safety tests based on the specific strain(s) (13).

Microbiological testing of probiotic products

All products, including probiotics, must undergo quality control and quality assurance, as well as comply with national/regional regulations for their intended use. In Serbia, they are regulated by the Rulebook on Dietary Supplements (41). Quality control of probiotic cultures relies exclusively on tests to ensure that sufficient numbers of viable bacteria are present in the products throughout their shelf life. In the context of microbiological quality control of probiotics, there are three groups of tests: determination of the total number of viable cells of probiotic microorganisms, identification of declared probiotic bacteria and/or fungi, and microbiological purity testing (determination of the total number of aerobic microorganisms – TAMC, total number of yeasts and molds – TYMC, and detection of the absence/presence of specified microorganisms). This paper is primarily concerned with the microbiological purity of probiotic products randomly taken from the market.

Microbiological purity and acceptance criteria of probiotic products

The microbiological criteria and required tests for probiotics are not uniform and depend on the type of product and applicable regulations. In any case, the probiotic must contain the declared number of viable bacteria/fungi until the end of the shelf life and the bacteria/fungi must be identified at the species, strain, or group level. One of the safety criteria for live biotherapeutic products (LBPs) used in clinical trials, as well as ready-made probiotic products from regular production, is microbiological purity, which determines the presence and abundance of foreign, undesirable microorganisms-contaminants. LBPs are medicinal products containing living microorganisms such as bacteria or yeasts. The most common species are the bacteria *Lactobacillus* spp, *Bifidobacterium* spp, some *Streptococcus* spp, *B. clausii*, and the yeast *S. cerevisiae* var. boulardii. The European Pharmacopoeia Commission has approved a general monograph laying down harmonized requirements for LBPs for human use and two general chapters addressing the microbiological contamination of LBPs (2018). The quality standards include a general monograph on LBPs for human use and two general

chapters (microbial testing of LBPs): test for enumeration of microbial contaminants and test for specific microorganisms (42-44). The problem with detecting undesirable microbial contaminants is that detection sensitivity decreases in the presence of desirable probiotic microorganisms, which are always present in large numbers (most commonly 10^9-10^{10} CFU/dose). The main strategy to increase the sensitivity of the detection of contaminating microorganisms is to reduce or eliminate the growth of probiotic bacteria in the product so that the contaminating bacteria present can grow and be detected (45). For product quality, the development of rapid and convenient tests to determine harmful contaminants that may be introduced into LBPs during various stages of production or product handling is essential. To this end, FDA scientists have developed and used recombinant phage lysins (hydrolytic enzymes) to lyse and kill lactobacilli (45). In an in vitro study, lysine was used as a reagent against *L. jensenii* cells (a probiotic strain). The study was conducted by inoculating a small number of live microorganisms of potential bacterial pathogens (either *Escherichia coli* or *Staphylococcus aureus*) into the culture of *L. jensenii*. Reduction or elimination of *L. jensenii* allowed the growth and detection of contaminating cells of *E. coli* or *S. aureus*. Regardless of the product's classification as food or medicine, it should not be contaminated with pathogenic bacteria, such as *E. coli* or *Salmonella* spp. These products should also exclude the presence of other pathogens such as *S. aureus*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Clostridioides* or *Cronobacter sakazakii* in infant products. The requirements for each product group are regulated by the European Pharmacopoeia (42-44; Table I), the United States Pharmacopoeia (USP, 46-48; Table II), as well as the FDA and ISO 17516:2014 (49-51; Table III).

Table I Acceptance criteria for microbiological quality for medicinal products containing probiotic microorganisms according to the European Pharmacopoeia (42-44).

Tabela I Kriterijumi prihvatljivosti mikrobiološkog kvaliteta za medicinske proizvode koji sadrže probiotičke mikroorganizme prema Evropskoj farmakopeji (42-44).

Route of administration	AMCC	YMCC	Specified microorganisms
Non-aqueous preparations for oral use	10^3 CFU/g/ml	10^2 CFU/g/ml	Absence: *E. coli*/g/ml
Aqueous preparations for oral use	10^2 CFU/g/ml	10^1 CFU/g/ml	Absence: *E. coli*/g/ml
Vaginal use	10^2 CFU/g/ml		Absence: *P. aeruginosa* *S. aureus* *C. albicans*/g/ml

AMCC – aerobic microbial contamination count; YMCC – yeast and mold contamination count

Table II Acceptance criteria for microbiological quality for medicinal products containing probiotic microorganisms according to the USP (46-48).

Tabela II Kriterijumi prihvatljivosti mikrobiološkog kvaliteta za medicinske proizvode koji sadrže probiotičke mikroorganizme, prema USP (46-48).

Probiotic products for oral use	TAMC	TYMC	Specified microorganisms
Non-spore-forming bacteria	$<5\times10^3$ CFU/g (except lactic acid bacteria)	$<10^2$ CFU/g	Absence: *E. coli*/g *Salmonella spp*/10g *L. monocytogenes*/g *S. aureus*/g *P. aeruginosa* if there is a risk of product contamination *C. perfringens* and *C. sakazakii* in infant products
Spore-forming bacteria	Not applicable		
Yeasts and molds	$<1\times10^3$ CFU/g	Not applicable	

TAMC – total aerobic microbial count; TYMC – total yeast and mold count

Table III Acceptance criteria of dietary supplements, food for special medical purposes, according to the FDA (49), USP (46-48), FDA (50) and ISO 17516-only for cosmetics (51).

Tabela III Kriterijumi prihvatljivosti za dijetetske suplemente, hranu za posebne medicinske namene, prema FDA (49), USP (46-48), FDA (50) i ISO 17516-samo za kozmetiku (51).

Dietary supplements, food for special medical purposes			
Documents	TAMC	TYMC	Specified microorganisms
FDA	5×10^2	nd	Absence: *Cronobacter* spp./10 g, *Salmonella* spp./25 g, *E. coli*/ g, *Enterobacteriaceae*/10 g
USP	5×10^3	10^2	Absence: *E. coli*/10 g, *Salmonella* spp./10 g, *L. monocytogenes*, *S. aureus*, *P. aeruginosa* if there is a risk of contamination of the product *C. perfringens* and *C. sakazakii* in infant products

Cosmetics		
Documents	TAMC	Specified microorganisms
FDA	≤5×10^2 CFU/g – cosmetics applied around the eyes ≤1×10^3 CFU/g – other cosmetic products	Absence/g/ml: *S. aureus*, *S. pyogenes*, *P. aeruginosa*, *K. pneumoniae*
ISO 17516	≤1×10^2 CFU/g/ml cosmetics intended for children under three years of age, applied around the eyes or on mucous membranes ≤1×10^3 CFU/g/ml other cosmetic products	Absence/g/ml: *E. coli*, *S. aureus*, *P. aeruginosa*, *C. albicans*

TAMC – total aerobic microbial count; TYMC – total yeast and mold count; nd – no data

The microbial content in probiotic products can be determined by different methods. To determine LBPs contamination in the presence of probiotic strains (lactic acid bacteria (LAB), spores of *B. clausii*, yeast *S. cerevisiae* var. boulardii) by the cultivation method, different nutrient media, and incubation conditions adapted to the specifics of the product under study should be used. Although there are various techniques for determining the number and detecting the presence of certain microorganisms, the cultivation method is the most commonly used one. To detect the presence of microorganisms, it is necessary to test the suitability of the method in advance. Practically, this means that the probiotic product is inoculated with an accurate number of live cells of the indicated microorganism (≤100 CFU), i.e., the product is well homogenized and the homogenized product is immediately spread on differential and selective nutrient media for the indicated microorganism (e.g. Baird Parker (BP) Agar and Mannitol Salt Agar (MSA) for *S. aureus*, MacConkey Agar (MCA) for *E. coli*, Cetrimide Agar (CA) for *P. aeruginosa*, Sabouraud-Dextrose Agar (SDA) supplemented with chloramphenicol and cycloheximide for *C. albicans*). The method is suitable if the recovery is 100±50 CFU. Low quality of probiotic products is usually due to an incorrect number of probiotic microorganisms compared to the manufacturer's declaration, very high contamination of tested products, and improper labeling of the strains in the products (4). Microorganisms can be identified by different methods (API 50 CHL, API 20A, MALDI-TOF MS, PCR) and the results obtained are often inconsistent. The biochemical test API 50 CHL did not correctly identify *L. rhamnosus* strain and often recognized this species as *L. paracasei*, the test API 20A recognized bifidobacteria only for genera, MALDI-TOF MS was used to confirm biochemical identification, and PCR for the most reliable identification of the strain. Not all identification results confirmed the strain species declared by the manufacturers (4).

Methods for enumeration of cells in probiotic products

The number of bacterial cells is currently considered the most important parameter for the efficiency and quality of finished probiotic products or LBPs. Cell

enumeration is generally performed using culture-based methods such as colony counting, which determine the number of viable cells able to replicate and form a colony without providing information on the heterogeneity of the bacterial culture. It can also be performed by flow cytometry, fluorescence in situ hybridization (FISH), and nucleic acid amplification methods, which can be used to count both culturable and viable but non-culturable cells (52-54). Detailed methods and procedures for microbial content testing are described in the USP, European Pharmacopoeia, and Russian Pharmacopoeia (55-57). The total number of viable probiotic bacteria/fungi is indirectly determined by colony count (CFU). CFU/ml is calculated using the following formula: CFU/ml = number of colonies (N)/dilution factor (R) × volume of sample plated (V). The number of live probiotic bacteria/fungi and the microbiological purity of the product are determined as a mandatory part of the quality control of each batch produced. The results obtained are compared with the requirements given in the product specification, and if the results are in accordance with the specifications, the product is released for the market (4, 41).

Identification of microorganisms in probiotic products

In the context of biosafety assessment, it is now widely recognized that proper identification at the species and even strain level is essential for understanding health benefits. Inappropriate use of identification methods is a major cause of inaccurate species designations of probiotic strains and mislabelling of probiotic products. Inconsistencies in the microbiological identification of commercial products with probiotic claims affect their potential efficacy and safety. It is recommended to perform phenotypic testing and genetic typing (molecular genetic methods, e.g., PCR) be used. Phenotypic methods include microscopic examination of microorganisms (cell morphology) and determination of the following: culture, physiological and biochemical features, sensitivity to antibiotics, other drugs, and bacteriophages. However, it is impossible to identify species or strains of the genus *Lactobacillus* based solely on the colony appearance or cell morphology. These features provide an initial differentiation of the bacteria present in a product before they are identified by other phenotypic or genotypic methods. Tests, such as cell motility assessment, Gram stain, and tests for oxidase or catalase are screening tests that can be used to determine whether a particular microorganism belongs to a large group of LABs. FAO/WHO recommends testing phenotypic traits as a complement to more advanced identification methods (58). Biochemical methods are based on determining the organisms' ability to assimilate, ferment, or decompose certain compounds. They are not only used for identification, but also provide data on the number of microorganisms present in the test product, can only be used to identify living organisms, and their speed depends largely on the ability of the microorganisms to multiply. Many researchers emphasize their high sensitivity and low cost of implementation. The main disadvantage of the applied method is the time required to obtain results. The problem of substrate preparation for many biochemical tests is

solved by rapid tests in the form of ready-made kits, and 24 h is sufficient to obtain data for organism identification. Biophysical methods are based on the study of the chemical composition of microbial cells using physical techniques, such as electrophoresis, chromatography, or spectroscopy (Raman spectroscopy with Fourier transform, infrared spectroscopy (IR) with Fourier transform (FT-IR), and matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF)). The basic phenomenon used in immunological methods is to establish a stable link between antigens and specific antibodies. The most commonly used technique for immunological identification is enzyme-linked immunosorbent assay (ELISA). Molecular biology methods based on PCR for identification are based on the study of the genome of microorganisms. Genotyping methods based on hybridization allow complex and automated identification techniques to be performed. Fluorescent in situ hybridization (FISH), microarrays, and Southern blot are also among the most popular methods.

Stability study

According to the International Probiotics Association (IPA) guidance (59), stability testing is performed to ensure that the stated shelf life of a given probiotic product is scientifically supported. The protocols of storage recommendations for the probiotic product are listed in Tables IV and V. Stability testing is conducted under the same temperature conditions as the recommended storage conditions on the finished product label. The use of accelerated or other testing to support product release should be scientifically justified and documented. Product and packaging conditions used in stability testing are supported by science-based evidence. Stability testing should be conducted under conditions representative of the finished product in the final packaging intended for the market. All stability testing methods should be scientifically sound, repeatable, reproducible, and documented (59).

Table IV ICH guideline for Stability Testing Q1A(R2) storage conditions for long-term stability studies.

Tabela IV ICH smernica za ispitivanje stabilnosti Q1A(R2) uslova skladištenja za dugoročne studije stabilnosti.

Storage	Storage condition
Products intended for storage in a freezer	-20±5°C
Products intended for storage in a refrigerator	5±3°C
General case*	25±2°C / 60±5% relative humidity or 30±2°C /65±5% relative humidity

*Only if the product is not specifically covered by other conditions listed in the guideline.

Table V USP General Chapter storage condition definitions.

Tabela V Definisani uslovi skladištenja dati u USP opštem poglavlju.

Storage	Storage condition
Refrigerated	2°C to 8°C
Cold	Not exceeding 8°C
Cool	8°C to 15°C
Controlled room temperature	20°C to 25°C

Labeling and storage conditions

Labeling guidelines were developed to provide information about the identity and quantity of probiotics contained in a product to help consumers make informed choices. The label should indicate the genus, species, and strain of each microorganism in the product. The amount (viable cells/dose) of probiotics indicated on the label should reflect the amount of live microorganisms at the end of the indicated shelf life, not at the time of manufacture. According to the FAO/WHO (58), the following information should be included on the label of a probiotic: genus, species, and strain name for each probiotic strain in the product; minimum viable numbers of each probiotic strain at the end of the shelf life expressed in CFU, the effective dose of probiotics in relation to the indicated health benefit, health claim(s) allowed by law and supported by studies, proper storage conditions, and company contact information (58).

Storage is an important aspect of probiotic products before their use, as storage conditions directly affect the biological viability and efficacy of the products. Factors such as temperature, water activity, oxygen content, content of the probiotic product, storage time, and pH level are critical during the storage process (59). Probiotics are extremely sensitive to temperature and are therefore usually stored at 4°C, as storage at room temperature shortens their shelf life.

Safety evaluation of probiotics for human use

The safety of probiotics depends on the potential susceptibility of the end user, the dose and duration of consumption, and the route and frequency of administration. It also depends on the type of microorganism used, so the evaluation of the safety of probiotics must take into account a large number of probiotic strains, the risks associated with using strains that do not have GRAS or QPS status, and the possibility of probiotic-human microbiota interactions. In essence, it is proposed that a safety assessment of a defined taxonomic group (e.g., a genus or a group of related species) can be made based on four pillars (identification, knowledge, potential pathogenicity, and end use). If a taxonomic group does not raise safety concerns, or safety concerns exist but can be defined and excluded (the qualification), the group could be granted QPS status. Thereafter, any strain

of a microorganism whose identity can be clearly established and assigned to a QPS group would be exempt from further safety assessment other than meeting specific qualifications (29). Probiotics may be responsible for systemic infections, excessive immune stimulation, especially in immunocompromised individuals, adverse metabolic effects, gene transfer, and gastrointestinal side effects (60). Concerns have been raised about *Enterococcus* strains, namely *E. durans*, *E. faecium*, and *E. faecalis*, which are classified as probiotic bacteria (single strains only), although it is known that probiotic microorganisms can cause opportunistic infections. In addition, numerous studies indicate the increasing importance of multidrug-resistant *Enterococcus* sp., especially those resistant to vancomycin and those that can transfer resistance genes to other bacterial genera by horizontal gene transfer (61). Therefore, due to safety concerns and a lack of safety information and regulations, only a limited number of probiotics containing enterococci are on the market. Although EFSA has approved enterococci as additives in silage and feed supplements, it does not recommend their use in probiotic products for human consumption (6). In Germany, live *E. faecalis* DSM 16431 is a probiotic drug called Symbioflor 1 and is used for acute and recurrent sinusitis and bronchitis (62). *E. faecium* M74 and *E. faecium* SF-68 strains are also included in several probiotic drugs and have been shown to be effective and safe (63).

Most probiotic cosmetic products on the market (creams, serums, masks, gels) do not contain living microorganisms, but their metabolites (bacterial lysates, extracts, products from the fermentation process) (64). Probiotic products applied to the skin surface are usually poorly controlled. The mechanism of probiotic-based cosmetics action is mainly based on improving the barrier function of the epithelial layer and inhibiting the growth of pathogenic microbes. The efficacy of this group of products has been demonstrated in the treatment of acne and atopic dermatitis (65). Research is also underway to develop bandages and plasters containing probiotic bacteria (*S. salivarius* K-12, *S. salivarius* M-18, and *L. plantarum* 8P-A3) that could inhibit the growth of bacteria on the skin surface and pathogens that cause wound infections (e.g., *Cutibacterium acnes*, *S. aureus*, *P. aeruginosa*) by producing bacteriocins (66).

Microbiological quality of probiotics randomly sampled from the, market

The use of low-quality probiotic products, especially those contaminated with pathogenic microorganisms, can be associated with serious health risks. Thus, it is very important to constantly control their quality. Some reports indicate that the microbiological quality of probiotic products and manufacturers' label claims are inconsistent (4, 67-69). The main discrepancies are a lower number of probiotic bacteria, misidentification, presence of unlabelled microbes, poor survival of microorganisms, absence of viable bacteria, high content of contaminants such as *E. faecium*, *Acinetobacter baumannii*, *Lysinibacillus fusiformis*, *B. cereus*, *B. leantus*, and *Staphylococcus* spp, lower survival of isolated bacteria in gastric juice simulated in vitro, and reduced antimicrobial activity.

Conclusion

Probiotic products available on the market should be subjected to routine and thorough control by the competent authorities to ensure good product quality and indicated health benefits. This requires a good knowledge of the legal requirements for the marketing of products containing probiotics and the methods for testing their microbiological quality and stability.

References

1. Food and Agriculture Organization/World Health Organisation. Evaluation of Health and Nutritional Properties of Probiotics in Food. Córdoba, 2001.
2. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11(8):506-14.
3. Kong Q, Liu T, Xiao H. Editorial: Effects of probiotics and prebiotics on gut pathogens and toxins. Front Microbiol. 2022;13:856779.
4. Zawistowska-Rojek A, Zaręba T, Tyski S. Microbiological testing of probiotic preparations. Int J Environ Res Public Health. 2022;19(9):5701.
5. Patra F, Duary RK. Determination and safety aspects of probiotic cultures. In: Panesar PS, Anal AK, editors. Probiotics, Prebiotics and Synbiotics. New York: John Wiley & Sons, Inc.; 2022; p. 122-60.
6. Suvorov A. What is wrong with enterococcal probiotics? Probiotics Antimicrob Proteins. 2020;12(1):1-4.
7. EFSA on Dietetic Products, Nutrition and Allergies (NDA). Guidance on the scientific requirements for health claims related to gut and immune function. EFSA Journal. 2011;9(4):1984.
8. EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA). Scientific Opinion on Dietary reference values for water. EFSA Journal. 2010;8(3):1459.
9. EFSA Panel on Biological Hazards (BIOHAZ). Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 14: suitability of taxonomic units notified to EFSA until March 2021. EFSA Journal. 2021;19(7):6689.
10. Binda S, Hill C, Johansen E, Obis D, Pot B, Sanders ME, et al. Criteria to qualify microorganisms as "probiotic" in foods and dietary supplements. Front Microbiol. 2020;11:1662.
11. Timmerman HM, Koning CJ, Mulder L, Rombouts FM, Beynen AC. Monostrain, multistrain and multispecies probiotics – A comparison of functionality and efficacy. Int J Food Microbiol. 2004;96(3):219-33.
12. Puvanasundram P, Chong CM, Sabri S, Yusoff MS, Karim M. Multi-strain probiotics: Functions, effectiveness and formulations for aquaculture applications. Aquac Rep. 2021;21:100905.
13. Arora M, Baldi A. Regulatory categories of probiotics across the globe: A review representing existing and recommended categorization. Indian J Med Microbiol. 2015;33(Suppl):2-10.
14. de Simone C. The unregulated probiotic market. Clin Gastroenterol Hepatol. 2019;17(5):809-17.

15. Saarela MH. Safety aspects of next generation probiotics. Curr Opin Food Sci. 2019;30:8-13.
16. Zavišić G, Radulović Ž, Vranić V, Begović J, Topisirović L, Strahinić I. Characterization and antimicrobial activity of vaginal Lactobacillus isolate. Arch Biol Sci. 2011;63(1):29-35.
17. Zavišić G, Petričević S, Radulović Ž, Begović J, Golić N, Topisirović L, et al. Probiotic features of two oral Lactobacillus isolates. Braz J Microbiol. 2012;43(1):418-28.
18. Prabhurajeshwar C, Chandrakanth RK. Probiotic potential of Lactobacilli with antagonistic activity against pathogenic strains: An in vitro validation for the production of inhibitory substances. Biomed J. 2017;40(5):270-83.
19. Lau LYJ, Chye FY. Antagonistic effects of Lactobacillus plantarum 0612 on the adhesion of selected foodborne enteropathogens in various colonic environments. Food Control. 2018;91:237-47.
20. Kim SJ, Park SH, Sin HS, Jang SH, Lee SW, Kim SY, et al. Hypocholesterolemic effects of probiotic mixture on diet-induced hypercholesterolemic rats. Nutrients. 2017;9(3):293.
21. Zavišić G, Ristić S, Petričević S, Novaković Jovanović J, Radulović Ž, Janać Petković B, et al. Characterisation and preliminary lipid-lowering evaluation of Lactobacillus isolated from a traditional Serbian dairy product. Benef Microbes. 2015;6(1):119-28.
22. Zavišić G, Ristić S, Rikalović M, Petković B, Janković D, Vukadinović A, Petričević S. Beneficial effects of probiotic supplementation on glucose and triglycerides in a mouse model of metabolic syndrome. J Funct Foods. 2022;95:105167.
23. de Almada CN, Almada CN, Martinez RC, Sant'Ana AS. Paraprobiotics: Evidences on their ability to modify biological responses, inactivation methods and perspectives on their application in foods. Trends Food Sci Technol. 2016;58:96-114.
24. Maria Carmen C, Seppo S, Vinderola G. Postbiotics: Defining the impact of inactivated microbes and their metabolites on promotion of health. In: Koren O, Rautava S, editors. The Human Microbiome in Early Life. Cambridge, CA, USA: Academic Press; 2021; p. 257-68.
25. Aggarwal S, Sabharwal V, Kaushik P, Joshi A, Aayushi A, Suri M. Postbiotics: From emerging concept to application. Front Sustain Food Syst. 2022;6:887642.
26. Thorakkattu P, Khanashyam AC, Shah K, Babu KS, Mundanat AS, Deliephan A, et al. Postbiotics: Current trends in food and pharmaceutical industry. Foods. 2022;11(19):3094.
27. Salminen S, Collado MC, Endo A, Hill C, Lebeer S, Quigley EMM, et al. The International Scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. Nat Rev Gastroenterol Hepatol. 2021;18(9):649-67.
28. Żółkiewicz J, Marzec A, Ruszczyński M, Feleszko W. Postbiotics – A step beyond pre- and probiotics. Nutrients. 2020;12(8):2189.
29. Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA Journal. 2007;587:1-16.
30. U.S. Food and Drug Administration [Internet]. Generally Recognized as Safe (GRAS) [cited 2023 Feb 11]. Available from: http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS /default.htm.
31. Higgins JPT, Thomas J, Chandler J, Cumpston M, Li T, Page MJ, et al. Cochrane Handbook for Systematic Reviews of Interventions. 2nd Edition. Chichester (UK): John Wiley & Sons; 2019.

32. van Loveren H, Sanz Y, Salminen S. Health claims in Europe: probiotics and prebiotics as case examples. Annu Rev Food Sci Technol. 2012;3:247-61.
33. Cordaillat-Simmons M, Rouanet A, Pot B. Live biotherapeutic products: the importance of a defined regulatory framework. Exp Mol Med. 2020;52(9):1397-1406.
34. Hempel S, Newberry S, Ruelaz A, Wang Z, Miles JN, Suttorp MJ, et al. Safety of probiotics used to reduce risk and prevent or treat disease. Evid Rep Technol Assess (Full Rep). 2011;(200):1-645.
35. Salminen MK, Salminen M. Lactobacillus bacteremia, with special focus on the safety of probiotic Lactobacillus rhamnosus GG. Espoo: Minna K. Salminen; 2006. 95 p.
36. Berg RD. Bacterial translocation from the gastrointestinal tract. Adv Exp Med Biol. 1999;473:11-30.
37. Potruch A, Schwartz A, Ilan Y. The role of bacterial translocation in sepsis: a new target for therapy. Therap Adv Gastroenterol. 2022;15:17562848221094214.
38. Rubin IMC, Stevnsborg L, Mollerup S, Petersen AM, Pinholt M. Bacteraemia caused by Lactobacillus rhamnosus given as a probiotic in a patient with a central venous catheter: a WGS case report. Infect Prev Pract. 2022;4(1):100200.
39. Rossi F, Amadoro C, Colavita G. Members of the Lactobacillus Genus Complex (LGC) as opportunistic pathogens: A review. Microorganisms. 2019;7(5):126.
40. Kothari D, Patel S, Kim SK. Probiotic supplements might not be universally-effective and safe: A review. Biomed Pharmacother. 2019;111:537-47.
41. Pravilnik o dodacima ishrani (dijetetski suplementi). Službeni glasnik RS 45/2022.
42. European Pharmacopoeia. 10th ed., Strasbourg, France: EDQM, 2019; 876-878.
43. European Pharmacopoeia. 10th ed., Strasbourg, France: EDQM, 2019; 250-254.
44. European Pharmacopoeia. 10th ed., Strasbourg, France: EDQM, 2019; 255-258.
45. Dreher-Lesnick SM, Schreier JE, Stibitz S. Development of phage lysin LysA2 for use in improved purity assays for live biotherapeutic products. Viruses. 2015;7(12):6675-88.
46. United States Pharmacopeia. General Tests and Assays: Probiotic Tests, USP42-NF37, Rockville, MD, USA: United States Pharmacopeial Convention, 2019.
47. United States Pharmacopeia. General Chapter: Microbial Enumeration Tests –Nutritional and Dietary Supplements, USP42-NF37, Rockville, MD, USA: United States Pharmacopeia, 2019.
48. United States Pharmacopeia. General Chapter: Microbiological Attributes of Nonsterile Nutritional and Dietary Supplements, USP42-NF37, Rockville, MD, USA: United States Pharmacopeial Convention, 2019.
49. Food and Drug Administration [Internet]. Revised Guidelines for the Assessment of Microbiological Quality of Processed Foods. No. 2013-010; 2013 [cited 2021 Sep 22]. Available from: https://www.fda.gov.ph/wp-content/uploads/2021/03/FDA-Circular-No.-2013-010.pdf.
50. Food and Drug Administration [Internet]. Bacteriological Analytical Manual Chapter 23: Methods for Cosmetics; 2021 [cited 2023 Feb 11]. Available from: https://www.fda.gov/food/laboratory-methods-food/bam-chapter-23-methods-cosmetics.
51. ISO 17516:2014. Cosmetics-Microbiology-Microbiological Limits. Geneva, Switzerland: International Organization for Standardization (ISO), 2014.
52. Foglia C, Allesina S, Amoruso A, De Prisco A, Pane M. New insights in enumeration methodologies of probiotic cells in finished products. J Microbiol Methods. 2020;175:105993.

53. Pasulka AL, Howes AL, Kallet JG, VanderKelen J, Villars C. Visualization of probiotics via epifluorescence microscopy and fluorescence in situ hybridization (FISH). J Microbiol Methods. 2021;182:106151.
54. Davis C. Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria. J Microbiol Methods. 2014;103:9-17.
55. United States Pharmacopeia, General Chapter: Microbiological Procedures for Absence of Specified Microorganisms – Nutritional and dietary supplements, USP42-NF37, Rockville, MD, USA: United States Pharmacopeial Convention, 2019.
56. European Pharmacopoeia, 10th ed., Strasbourg, France: EDQM, 2019; 6297-6301.
57. Russian Pharmacopoeia [Internet]. 13th ed. [cited 2021 Sep 17]. Available from: https://pharmacopoeia.ru/en/gosudarstvennaya-farmakopeya-xiii-online-gf-13-online/.
58. FAO/WHO. Working Group on Guidelines for the Evaluation of Probiotics in Food, 2002.
59. Best Practices Guidelines for Probiotics. The Council for Responsible Nutrition. The International Probiotics Association (IPA), 2017.
60. Doron S, Snydman DR. Risk and safety of probiotics. Clin Infect Dis. 2015;60(Suppl 2):S129-34.
61. Ben Braïek O, Smaoui S. Enterococci: Between emerging pathogens and potential probiotics. Biomed Res Int. 2019;2019:5938210.
62. Krawczyk B, Wityk P, Gałęcka M, Michalik M. The many faces of Enterococcus spp. – commensal, probiotic and opportunistic pathogen. Microorganisms. 2021;9(9):1900.
63. Hanchi H, Mottawea W, Sebei K, Hammami R. The genus Enterococcus: Between probiotic potential and safety concerns – An update. Front Microbiol. 2018;9:1791.
64. Huang M-CJ, Tang J. Probiotics in personal care products. Microbiol Discov. 2015;3:5.
65. França K. Topical probiotics in dermatological therapy and skincare: A concise review. Dermatol Ther (Heidelb). 2021;11(1):71-7.
66. Khalfallah G, Gartzen R, Möller M, Heine E, Lütticken R. A new approach to harness probiotics against common bacterial skin pathogens: Towards living antimicrobials. Probiotics Antimicrob Proteins. 2021;13(6):1557-71.
67. Zawistowska-Rojek A, Zareba T, Mrówka A, Tyski S. Assessment of the microbiological status of probiotic products. Pol J Microbiol. 2016;65(1):97-104.
68. Mohar Lorbeg P, Golob M, Kramer M, Treven P, Bogovič Matijašić B. Evaluation of dietary supplements containing viable bacteria by cultivation/MALDI-TOF mass spectrometry and PCR identification. Front Microbiol. 2021;12:700138.
69. Mazzantini D, Calvigioni M, Celandroni F, Lupetti A, Ghelardi E. Spotlight on the compositional quality of probiotic formulations marketed worldwide. Front Microbiol. 2021;12:693973.

Mikrobiološki kvalitet probiotičkih proizvoda

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Kratak sadržaj

Mikroorganizmi koji se koriste kao probiotici treba da zadovolje elementarne aspekte bezbednosti (netoksičnost, odsustvo gena rezistencije na antibiotike i translokacije) i funkcionalne/tehnološke aspekte (otpornost i preživljavanje u kiselom gastričnom okruženju, adhezivnost, stabilnost i vijabilnost ćelija). Probiotici sa zdravstvenom tvrdnjom, bilo da su dijetetski proizvod ili farmabiotik (kategorija lekova), treba da budu klinički testirani, validirani, dokumentovani i stalno kontrolisani u pogledu kvaliteta. Važni parametri kvaliteta su identifikacija deklarisanih probiotičkih sojeva, broj živih mikroorganizama (probiotičke bakterije i/ili gljivice) i mikrobiološka čistoća (odsustvo specifičnih patogenih/uslovno patogenih bakterija i gljivica, kao i ograničavanje ukupnog broja kontaminanata kao što su aerobne bakterije, kvasci i plesni). Zbog brojnih izveštaja o komercijalnim probioticima niskog kvaliteta koji se prodaju za ljudsku upotrebu, ovaj pregled razmatra metode koje se koriste za testiranje sadržaja probiotičkih mikroorganizama, bezbednost za nameravanu upotrebu i dokazane zdravstvene koristi onih probiotika čiji mikrobiološki kvalitet odstupa od sadržaja koji tvrde proizvođači i održavanja vitalnosti ćelija, tj. stabilnosti probiotika tokom roka trajanja. Pored toga, razmatraju se i neželjeni efekti probiotika i potencijalne opasnosti po zdravlje korisnika.

Ključne reči: probiotik, mikrobiološki kvalitet, bezbednost, stabilnost