Preclinical Testing in the Development of Probiotics: A Regulatory Perspective with *Bacillus* Strains as an Example

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Preclinical testing of the microbial strains is the first important step in the development of probiotics. Requirements for the set of tests can vary depending on the bacterial species and the expected mechanism of action in the organism. Common approaches to preclinical testing of probiotic strains include strain identification (i.e., determination of phenotypic and genotypic properties), safety evaluation (i.e., characterization of history of use [safety contact], assessment of resistance to antibiotics, and evaluation of pathogenic properties in vitro and in animal models), and efficacy testing (i.e., functional characterization). Future progress in probiotics requires more studies to determine the mechanisms of their action, as well as an understanding of the basis and mechanisms of pathogenicity for different probiotic strains. Special attention should be given to recombinant probiotics, particularly in the formulation of criteria for selection of the host strain, for assessment of environmental safety, and for tracing the fate of recombinant DNA in vitro and in vivo.

Different microorganisms are claimed to be probiotics and are widely used in foods and drugs. An issue arises regarding the standard criteria for selection and characterization of strains to be termed “probiotic.”

The World Health Organization developed guidelines for the evaluation of probiotics in food, which indicate parameters for preclinical testing of probiotics, clinical trials, and labeling [1]. According to these guidelines, preclinical testing of probiotics includes strain identification, safety evaluation, and efficacy testing in vitro and in animal models.

Why is it so important to know the exact taxonomic status of the probiotic strain? It is well-known that different species belonging to the same genus may have different biological characteristics. Often, commercial probiotics have been shown to contain bacterial species other than those claimed. Phenotypic characterization of bacteria, by use of classic microbiological approaches, is very important for species identification. For closely related species that occupy similar ecological niches, phenotype characterization alone is insufficient. Genotypic 16S ribosomal DNA–based identification of bacteria can be a useful supplement to phenotypic characterization. Phenotypic characterization and analysis of 16S ribosomal DNA sequences result in species-level identification of strains. Probiotic activity, however, is strain specific [2]. For probiotics, strain-specific characteristics are important not only for quality control but also for understanding the mechanism of their action. Different approaches, based on biochemical tests, bacteriophage typing, analysis of DNA, and protein-based methods, have been used to obtain a unique characterization of bacterial strains.

We used random amplification of polymorphic DNA (RAPD) for analysis of 31 *Bacillus subtilis* strains isolated from the environment [3]. Patterns obtained with primer OPA3 allowed the differentiation of isolates into 11 groups. Applying the RAPD approach, we demonstrated strain-specific characteristics for different *Bacillus* probiotic strains and showed unique patterns for them by using primers OPA3 and OPH8.
A requirement for further development of a bacterial strain as a probiotic is the deposition of the strain into an internationally recognized collection of cultures. Currently, 522 culture collections in 66 countries are registered with the World Data Centre for Microorganisms [4].

Safety of probiotics is paramount. Microbial products recommended for human use should be safe. Today, however, there are no established criteria for testing the safety of probiotic strains. Since most probiotics are used in foods, the requirements for safety of *Lactobacillus* and *Bifidobacteria* strains should include their history of use by humans. However, many other bacteria may hold promise as probiotics. What safety criteria should be required for such bacteria? From this point of view, let us examine *Bacillus* probiotic strains. Humans have been in contact with these bacteria during their entire existence as a species. Evidence of this is isolation of *Bacillus* species from various probes dated millions of years ago. The phylogenetic relatedness of bacilli with lactobacilli and bifidobacteria is an additional proof of their safety record. It is well-known that phylogenetic relatedness is the indication of genetic relatedness.

Bacteria of the *Bacillus* genus are among the most widespread microorganisms in nature. *Bacillus* species are predominant in soil, and these bacteria have also been isolated frequently from water and air. Because these bacteria are everywhere in the environment, they find their way easily into food products, being the component of common microflora in meat, milk, bread, and so forth. Thus, large amounts of bacilli (10^5–10^8 cfu) consistently enter the gastrointestinal and respiratory tracts of healthy people together with air, water, and food and exist in the gut microflora. Some researchers believe that *Bacillus* organisms are a normal component of human intestinal microflora. The amount of bacilli in the gut is less than that of, for example, bifidobacteria, but they enter the human organism regularly.

One of the safety requirements for live bacteria directly consumed by humans is the absence of any acquired resistance to antibiotics of importance in medicine. Study of different *Bacillus* probiotic strains showed species-specific antibiograms [5]. The results of additional experiments confirmed the absence of any plasmids in the strains examined. Therefore, these studies of resistance to antibiotics show the peculiarities of *Bacillus* species [6]. The same results were obtained for *Lactobacillus* and *Bifidobacterium* probiotic strains, showing that the antibiotic-resistance profile is species specific [7, 8]. Thus, it is important to analyze antibiotic resistance in probiotic strains and to distinguish the acquired (i.e., transferable) resistance and the natural resistance, which is one of the phenotypic characteristics of a species. Intrinsic resistance can be distinguished from acquired resistance by a testing scheme, as recommended by the Scientific Committee on Animal Nutrition [9].

Some probiotic strains (e.g., *Enterococcus faecium* SF6) possess intrinsic tolerance against several antimicrobial agents but may be potential recipients of vancomycin resistance genes [10], which can be transferred to *Listeria* species [11] and methicillin-resistant *Staphylococcus aureus* [12]. These findings cast some doubt on the safety of enterococci as probiotics [13] and indicate the necessity of additional approaches to analyze the problem of antibiotic resistance in potentially probiotic strains. Currently, the safety analysis for probiotics includes only species characterization and testing of antibiotic resistance. Many strains are used in humans without preliminary examination of their pathogenicity. Available studies on probiotic safety are limited, although pathogenicity is known to be a strain-specific feature [14]. It is, therefore, unacceptable to extrapolate pathogenicity data from closely related strains, because each strain should be studied independently.

The basis of pathogenicity of some bifidobacteria [15] and lactobacilli [16] remains unclear; however, the pathogenic potential of several *Bacillus* species is known. The European Scientific Committee on Animal Nutrition proposed a scheme for the testing of toxin production in *Bacillus* species intended for use as feed additives or other purposes [17]; this scheme included use of commercially available kits, primers, and cytotoxicity assays with Vero and Hep2 cells.

But the proposed methods have some limitations.

1. Not all *Bacillus cereus* strains produce toxins detected by PCR [18].
2. There are insufficient data regarding the toxicity of *B. subtilis* [19].
3. Some enterotoxigenic strains do not show cytotoxicity in assays of Vero cells [20].
4. Not all cytotoxic Bacillus species resulted in toxicity in Hep2 cells [19].
5. Only 36% of *Bacillus* species that have positive results of the cytotoxicity assay show positive results of immunological tests [21].

Until the molecular basis for pathogenicity of probiotics is well understood, animal studies still provide the most valuable methods of evaluating safety. Although acute and chronic oral-toxicity studies are considered useful, it is well-known that it is difficult to establish infection after oral administration of bacteria, even with strongly infective species [22]. Different *Bacillus* probiotic strains (*B. cereus* IP 5832, which showed production of hemolysins, and *B. subtilis* 3 and *Bacillus licheniformis* 31, which were without hemolytic properties) were compared in studies of acute and chronic toxicity in mice [23]. Only in testing acute toxicity by intraperitoneal administration did we find a significant difference in the safety of these probiotic strains. The median lethal dose was 10^9 cfu/mouse for the probiotic strain *B. cereus* IP 5832, which corresponds to the amount of bacteria in 1 therapeutic dose for this probiotic. For the strains *B. subtilis* 3 and *B. licheniformis* 31, the median lethal dose was >10^10 cfu/mouse, which is equal to >10 therapeutic doses. No pathological changes were detected, either...
macroscopically or histologically, in the mice treated with the probiotic strains \( B. \text{subtilis} \) and \( B. \text{licheniformis} \). Results of the toxicity study of \( B. \text{subtilis} \) and \( B. \text{licheniformis} \) are in accordance with the safety data obtained for \( Lactobacillus \) and \( Bifidobacterium \) strains. Therefore, safety of probiotic strains can be obtained from animal toxicity studies using various types of animals and routes of administration.

The beneficial effects of bacteria that show promise for being probiotics should have to be demonstrated in studies in vitro. Criteria for these effects differ among the various bacteria, depending on the expected activity of the organism. However, some criteria are common for all promising probiotics: viability in the gut, resistance to bile and acid, and antagonistic activity to potentially pathogenic microorganisms.

Species of the \( Bacillus \) genus possess the following features common to probiotic cultures: gut viability [26], resistance to bile and acid [27], high biological activity, and the ability to synthesize different substances useful to humans. Different probiotic effects reported for \( Bacillus \) species include the following.

1. They produce antibiotics that differ in their structure, as well as their spectrum of activity [28].
2. They produce enzymes, including digestive and lytic enzymes [29].
3. They produce essential amino acids and vitamins [30, 31].
4. They decrease the levels of cholesterol in blood [32].
5. They have antimutagenic effects [33].
6. They stimulate nonspecific immunoreactivity of microorganisms [34].

These findings show the possibility of selecting probiotic \( Bacillus \) strains with a wide spectrum of biological activity. However, probiotic activity is strain specific. We compared various \( Bacillus \) probiotic strains in their antimicrobial activity against multiresistant strains of pathogens isolated from humans [35]. Only \( B. \text{subtilis} \) was effective during in vitro studies; it showed a wide spectrum of antagonistic activity toward the tested pathogens and did not inhibit normal microflora. In vitro activity of \( B. \text{subtilis} \) was confirmed by the results obtained in animal models. This strain showed efficacy against pathogenic cultures of \( \text{Escherichia coli} \) and \( \text{Campylobacter} \) species during treatment of experimental infections in mice and maintained normal microflora in the animals during receipt of antibiotic therapy [36, 37].

Various probiotics are on the market in different countries, and they vary in both strain type and composition. Despite the fact that these comprise a large number of probiotic bacteria with different mechanisms of action and a spectrum of specific activity, they cannot resolve many human health problems. With novel genetic-engineering methods, it is possible to modify probiotics both to strengthen their existing properties and to create new strains with desired properties. Recombinant probiotic bacteria have been designed for treatment of colitis, diarrhea, and cholera and for antiviral activity, and so forth [38, 39]. Probiotic bacteria are also being developed as vaccine-delivery vehicles [40]. Currently, 2 recombinant probiotic strains have been approved by national authorities as therapeutic agents: \( \text{Lactococcus lactis} \) that produces human IL-10 (in The Netherlands) and \( B. \text{subtilis} \) that produces human \( \alpha-2 \) IFN (in Ukraine). Further studies will lead to new genetically created strains with desired properties.

More studies are needed to determine the mechanisms of action, as well as to understand the basis and mechanisms of pathogenicity of different probiotics. Special attention should be given to recombinant probiotics, particularly with regard to selection criteria for the bacterial host strain. A key concern is that recombinant bacteria that colonize body cavities and mucosal surfaces may persist for a long time in the organism. Therefore, it could be problematic to control the quantity of heterologous proteins released, which is very important during therapy with recombinant bacteria. For genetically modified probiotics, establishing the criteria for assessment of environmental safety and tracing the fate of recombinant DNA in vitro and in vivo are important tasks for future progress.

Special attention should be paid to preclinical testing of microbial strains that show promise for probiotic development. Testing requirements can vary depending on the bacterial species and the expected mechanism of action in the organism. However, the common approaches outlined below should be considered.

1. Probiotic strain identification and description
   A. Strain identification by phenotypic and genotypic methods (to determine strain-specific features, if possible)
   B. Deposit in international collection of cultures
2. Safety evaluation
   A. History of use (safety contact)
   B. Assessment of resistance to antibiotics (e.g., distinguishing between intrinsic and transmissible resistance)
   C. Evaluation of pathogenic properties (e.g., toxin production) in vitro
   D. Testing of acute toxicity (in rats and mice)
   E. Testing of chronic toxicity (in rats and mice)
3. Efficacy testing (functional characterization)
   A. Testing in vitro
   B. Testing in vivo (i.e., in animal models)

Each strain should have complete characterization without the application of data from one strain to another of the same species. Adequate preclinical testing will result in effective and safe products for further clinical trials and eventually for use in humans.
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References


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