HANDBOOK OF PROBIOTICS AND PREBIOTICS

Second Edition

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PREFACE

The first edition of the *Handbook of Probiotics* was published in 1999 when probiotics was still a relatively new scientific discipline. The idea of compiling a handbook came from our review article, “The coming of age of probiotics,” published in the *Trends in Food Science and Technology* (61: 241–245, 1995), confirming probiotics to be a scientific discipline. The handbook was meant to serve as a source book for aspiring scientists, and it was the first handbook of its kind.

Probiotics have since developed into a major research focus area. Product applications include several commercially successful functional foods, health supplements, and therapeutic components and preparations. Cutting-edge methodologies, such as molecular approaches for the identification and quantification of intestinal probiotics, viability of probiotics under processing and storage conditions, and markers for host immune modulation, have been developed. Therefore, it is timely to update the scientific research and clinical trial data and to review and compile advances in methodology for easy reference.

At the time of publication of the first edition of the handbook, prebiotics were only at a concept level. Substantial research and clinical interventions on specific prebiotics have since been published to provide scientific basis for their reported effects. It is timely to include prebiotics in this updated handbook.

The aim of this updated handbook is to put together information and technology required in the development of a successful probiotic and prebiotic product from the laboratory to the marketplace. The book would continue to serve as a resource material for students, researchers, and company product development technologists.
This second edition of the *Handbook of Probiotics and Prebiotics* includes the following changes:

1. New chapters on methods for the analysis (enumeration, identification) of gastrointestinal microbiota.
2. The safety issue in novel probiotic bacteria is expanded, in view of the new regulation requirements for novel food products in Asia, Europe, and North America.
3. Understanding on probiotic mechanisms is incorporated in a new chapter.
4. A new chapter on commercially available human probiotic microorganisms covers in detail most of the early and new strains and preparations as well as the scientific information.
5. The chapter on “Enhancement of Indigenous Probiotic Organisms” is renamed as “Prebiotics” and expanded to accommodate the most recent findings.

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PART I

PROBIOTICS
1

PROBIOTIC MICROORGANISMS

1.1 DEFINITIONS

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“Probiotics” is derived from Greek and means “prolife.” It has been redefined throughout the years as more scientific knowledge and better understanding on its relationship between intestinal health and general well-being has been gained. The following are definitions of “probiotics” derived through times.

Lilly and Stillwell in 1965 (5) defined probiotics as “Growth promoting factors produced by microorganisms.”

Parker in 1974 (7) suggested an interaction between microorganisms with the host: “Organisms and substances with beneficial effects for animals by influencing the intestinal microflora.”

Fuller in 1989 (3) defined it as “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.”

Havenaar and Huis Int Veld in 1992 (4) said probiotics are “A mono- or mixed culture of live microorganisms which, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora.”

ILSI (International Life Sciences Institute) Europe Working Group (1998) (9): “A viable microbial food supplement which beneficially influences the health of the host.”
Diplock et al. in 1999 (1) puts it as
“Probiotic food is functional if they have been satisfactorily demonstrated to beneficially affect one or more target functions in the body beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction in the risk of diseases.”

Naidu et al. in 1999 (6) said “A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract.”

Tannock in 2000 (11) observed that long-term consumption of probiotics was not associated with any drastic change in the intestinal microbiota composition, and thus proposed an alternative definition: “Microbial cells which transit the GI tract and which, in doing so, benefit the health of consumer.”

Schrezenmeir and de Vrese in 2001 (10) defined probiotics as “A preparation of a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host.”

FAO/WHO (Food and Agriculture Organization and World Health Organization) (2001)(2) and Reid et al. (2003) (8) concentrated exclusively on its health purpose: “Live microorganisms which when administered in adequate amounts confer a health benefit on the host.”

1.2 SCREENING, IDENTIFICATION, AND CHARACTERIZATION OF Lactobacillus AND Bifidobacterium STRAINS

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Several genera of bacteria (and yeast) have been proposed as probiotic cultures, the most commonly used are Lactobacillus and Bifidobacterium species. However, the selection of a strain to be used as an effective probiotic is a complex process (Fig. 1.1). The work begins with the source of screening of strains, the most suitable approach being the natural intestinal environment.

According to FAO/WHO guidelines it is necessary to identify the microorganism to species/strain level given that the evidence suggests that the probiotic effects are strain specific (60). It is recommended to employ a combination of phenotypic and genetic techniques to accomplish the identification, classification, and typing. For the nomenclature of bacteria, scientifically recognized names must be employed and it is recommended to deposit the strains in an internationally recognized culture collection. Further characterization of strains must be undertaken taking into account the “functional” or probiotic aspects and safety assessment. In vitro tests, some of them summarized in Fig. 1.1, are useful to gain knowledge of both strains and mechanisms of the probiotic effect. In addition, even if these genera have a long history of safe consumption in traditionally fermented products and several species have been awarded a
“General Recognised As Safe” (GRAS) status by the American Food and Drug Association (63) or a qualified presumption of safety (QPS) consideration by the European Food Safety Authority (EFSA) (59), some characteristics (Fig. 1.1) must be studied to ensure the safety of the novel lactobacilli and bifidobacteria strains. Several of the \( \textit{in vitro} \) tests can be correlated with \( \textit{in vivo} \) studies with animal models, but probiotics for human use must be validated with human studies covering both safety (phase 1 trials) and efficacy (phase 2 trials) aspects. Phase 2 studies should be designed as double-blind, randomized, and placebo-controlled to measure the efficacy of the probiotic strain compared with a placebo and also to determine possible adverse effects (60).

This chapter focuses on the current techniques for bacterial identification, taxonomic classification, and typing of \textit{Lactobacillus} and \textit{Bifidobacterium} strains, and also reviews the \( \textit{in vitro} \) probiotic characterization of strains based on their functional aspects.

1.2.1 Sources of Screening for Probiotic Strains

Even though essentially all animals contain strains of both \textit{Lactobacillus} and \textit{Bifidobacterium} genera, it is well accepted that an effective human probiotic should
be of human origin. The underlying reason for this is that human intestines are sufficiently different from those of animals, such that the isolates suited to those environments would not necessarily be suited to the human intestine (121). The human gastrointestinal tract (GIT) is a very complex ecological niche and its bacteria inhabitants can achieve the highest cell densities recorded for any ecosystem. Nonetheless, diversity at a division level is among the lowest (19) and the lactobacilli and bifidobacteria comprise less than 5% of the total microbiota (92). A number of articles have been published in the last few years studying the diversity of the GIT ecosystem employing several culture-independent genetic tools. But, for the isolation of novel strains, classical cultivation techniques must be employed. Enrichment, selective media, and specific culture conditions are employed for the isolation of strains from human samples that are initially identified by morphological characterization under the microscope. Molecular tools, mainly based on the sequencing of the 16S rRNA gene, allow identification down to the species level. Using this basic scheme several collections of strains have been isolated from human (and other animal) samples. Commonly, fecal samples are donated by healthy adult or infant volunteers (49, 156). But other GIT sections obtained from healthy individuals and patients submitted to biopsies such as the terminal ileum (56) or colonic mucosa (49) can be screened. Also the oral cavity seems to be the origin of some allochthonous lactobacilli of the intestine (44). Recently, it has been indicated that the infant fecal microbiota reflects the bacterial composition of the breast milk (79, 101). Therefore, the natural microbiota of human milk could be proposed as a source for the isolation of novel probiotic bacteria.

Another approach to search for improved probiotic strains (Fig. 1.1) is the adaptation of wild types to the intestinal stressful conditions. After ingestion, the probiotic bacteria must survive the passage through the GIT and reach the colon in order to exert their beneficial effect. The low pH in the stomach and the high concentration of bile salts in the small intestine, which act as biological detergents disrupting the cell membrane, are the principal challenges that probiotics must overcome (21). Margolles and coworkers (100) obtained sodium-cholate-resistant Bifidobacterium derivatives by exposure to gradually increasing concentrations of this compound. The resistant phenotype remained stable and promoted some physiological changes that improved the survival of the adapted bacteria into the colon environment (52). Similarly, Collado and Sanz (39) developed a method for direct selection of acid-resistant Bifidobacterium strains by prolonged exposure of human feces to stressful conditions. The recovered strains were intrinsically resistant to acid gastric conditions (pH 2.0) and also showed good tolerance to high concentrations of bile salts and NaCl. This cross-resistance between low pH and bile salts was previously described in bile-adapted strains (118). Several strains with improved tolerance to these and other stressful factors have been described in literature (34, 111, 130, 146) as a method of selecting lactobacilli and bifidobacteria strains with improved viability to GIT and technological conditions.

Finally, taking advantage of the genome sequences, novel strains with improved or “designed” probiotic characteristics can be constructed toward specific therapies (157, 165). However, the use of recombinant strains is still far from being applied in
functional foods, at least in the European legal frame. Some *Bifidobacterium* strains have been genetically engineered for therapy against tumors after oral administration (74) and to fight against intestinal pathogens (114, 168).

Recombinant *Lactobacillus* strains are currently under study for the enhancement of the immune system (77, 78), treatment against *Helicobacter pylori* (41) and improvement of inflammatory colitis (76). Although the species *Lactococcus lactis* is generally not considered as a probiotic, recombinant strains have been constructed for the oral delivery of therapeutic molecules (87) for the treatment or alleviation of diverse diseases such as allergies (12) and colitis (164).

### 1.2.2 Identification, Classification, and Typing of *Bifidobacterium* Strains

#### 1.2.2.1 Taxonomy

Microorganisms of the genus *Bifidobacterium* are nonspore-forming, nonmotile, and nonfilamentous rods, which can display various shapes, with slight bends or with a large variety of branchings, from which the most typical ones are slightly bifurcated club-shaped or spatulated extremities. They can be found singularly, in chains, in aggregates, in “V” or palisade arrangements when grown under laboratory conditions. They are strictly anaerobic, although some species can tolerate low oxygen concentrations, and they have a fermentative metabolism (151). Tissier described these bacteria at the beginning of the twentieth century (173). They were first included among the family *Lactobacillaceae*, but in 1924 Orla-Jensen proposed the reclassification of the species *Lactobacillus bifidum* into the new genus *Bifidobacterium* (151).

The species of the genus *Bifidobacterium* form a coherent phylogenetic group and show over 93% similarity to the 16S rRNA sequences among them (150). This genus is clustered in the subdivision of high G+C Gram-positive bacteria, and it is included in the phylum *Actinobacteria*, class *Actinobacteria*, subclass *Actinobacteridae*, order *Bifidobacteriales*, and family *Bifidobacteriaceae*. According to the DSMZ Bacterial Nomenclature database (http://www.dsmz.de/microorganisms/bacterial_nomenclature), the species included in the genus *Bifidobacterium* are 29: *B. adolescentis*, *B. angulatum*, *B. animalis*, *B. asteroides*, *B. bifidum*, *B. boum*, *B. breve*, *B. catenulatum*, *B. choerinum*, *B. coryneforme*, *B. cuniculi*, *B. dentium*, *B. gallicum*, *B. gallinarum*, *B. indicum*, *B. longum*, *B. magnus*, *B. merycicum*, *B. minimum*, *B. pseudocatenulatum*, *B. pseudolongum*, *B. psychraerophilum*, *B. pullorum*, *B. ruminantium*, *B. saeculare*, *B. scardovii*, *B. subtilis*, *B. thermaeidophilum*, and *B. thermophilum*. In turn two subspecies constitute the species *B. animalis* (subsp. *animalis* and *lactis*), *B. pseudolongum* (subsp. *globosum* and *pseudolongum*), and *B. thermaeidophilum* (subsp. *thermoacidophilum* and *porcinum*), and the species *B. longum* is subdivided in three different biotypes (longum, infantis, and suis).

All the currently known *Bifidobacterium* isolates are from a very limited number of habitats, that is human and animal GITs, food, insect intestine, and sewage (65, 196). Among the strains most commonly found in human intestines and feces are those belonging to the species *catenulatum*, *pseudocatenulatum*, *adolescentis*, *longum*, *breve*, *angulatum*, *bifidum*, and *dentium*, and the typical species isolated from functional foods is *B. animalis* subsp. *lactis* (104); therefore, strains belonging to these species are the first target for health-promoting studies.
A number of phylogenetic studies carried out during the last few years (108, 148, 196, 200), mainly based on sequence comparison of total or partial sequences of the 16S rRNA genes and other housekeeping genes, have grouped the bifidobacterial species in six groups, \(B. \) longum group, \(B. \) pullorum group, \(B. \) adolescentis group, \(B. \) pullarum group, \(B. \) longum group, and \(B. \) pseudologum group (Fig. 1.2).

**FIGURE 1.2** Evolutionary relationships of *Bifidobacterium* strains obtained using 16S rDNA sequences. The evolutionary distances were inferred using the neighbor-joining method and were computed using the maximum composite likelihood method. Units indicate the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset.

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**Identification and Typing** Currently, there is great concern that the correct identification of a probiotic strain is the first prerequisite to be able to state its microbiological safety. Many studies have revealed deep deficiencies in the microbiological quality and labeling of currently marketed probiotic products for human and animal use. The incorporation of incorrectly identified probiotic bacteria in functional