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."

Handbook of Probiotics and Prebiotics, Second Edition Edited by Yuan Kun Lee and Seppo Salminen

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

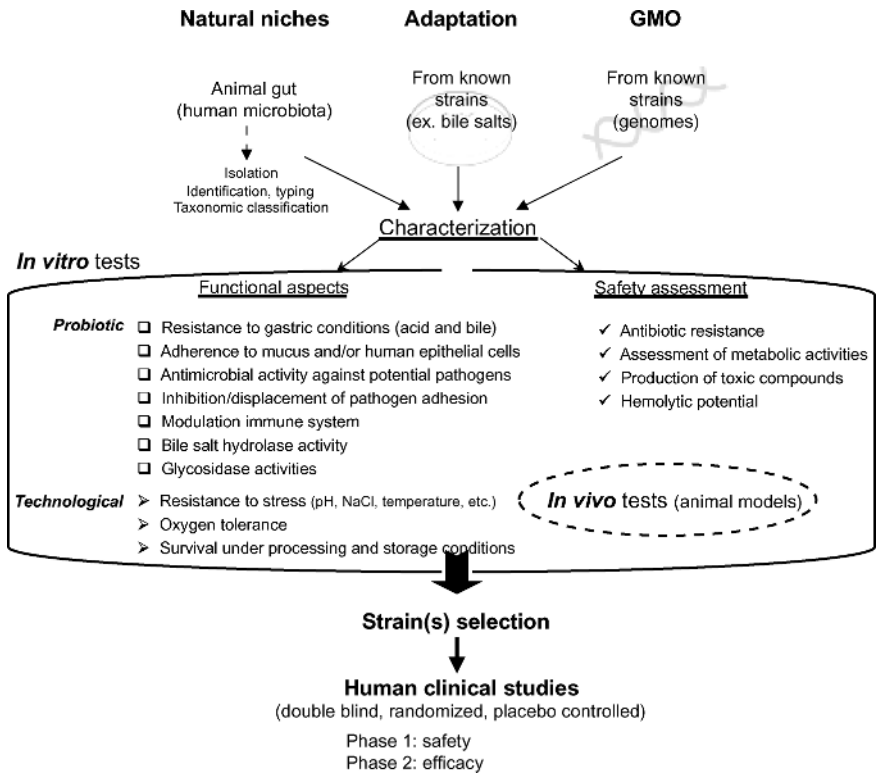

FIGURE 1.1 Procedure for the isolation and characterization of novel strains with putative probiotic status.

"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 *in vitro* tests can be correlated with *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 *Lactobacillus* and *Bifidobacterium* strains, and also reviews the *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 *Lactobacillus* and *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 probitics 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. magnum, B. merycicum, B. minimum, B. pseudocatenulatum, B. pseudolongum, B. psychraerophilum, B. pullorum, B. ruminantium, B. saeculare, B. scardovii, B. subtile, B. thermacidophilum, and B. thermophilum.* In turn two subspecies constitute the species *B. animalis* (subsp. *animalis* and *lactis*), *B. pseudolongum* (subsp. *globosum* and *pseudolongum*), and *B. thermacidophilum* (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*, *pseudocatenolatum*, *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.

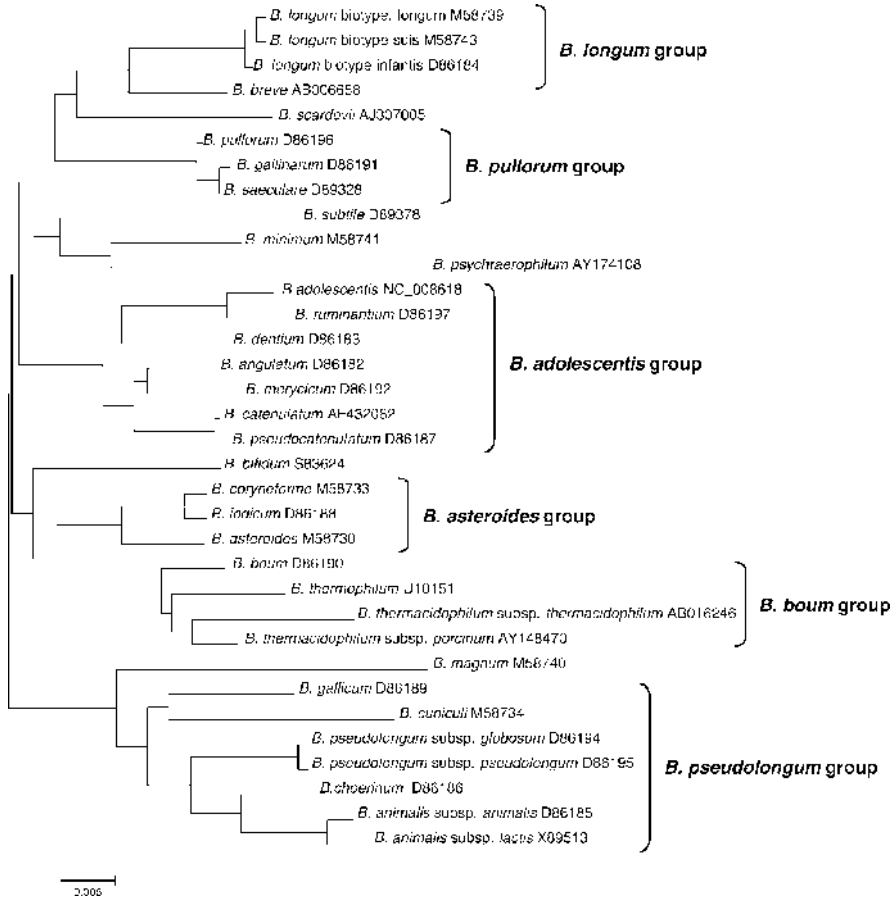

FIGURE 1.2 Evolutionary relationships of *Bifidobacterium* strains obtained using 16S rDNA sequences. The evolutionary distances were inferred using the neighbor-joining method and were computed using the maximum composite likelihood method. Units indicate the number of base substitutions per site. All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

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. boum* group, *B. asteroides* group, *B. adolescentis* group, *B. pullarum* group, *B. longum* group, and *B. pseudologum* group (Fig. 1.2).

1.2.2.2 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

food products clearly has public health implications, by undermining the efficiency of probiotics and by affecting public confidence in functional foods (83). Thus, the use of adequate tools to provide proper strain identification for legal and good manufacturing practices, and to track probiotics during food production, as well as during their intestinal transit, are strictly necessary.

Traditionally, bifidobacteria have been identified on the basis of phenotype investigations. The host from which the bifidobacteria was isolated (e.g. *animalis, adolescentis, pullorum, dentium*, etc.) often represented the first identification criteria for many of these bacteria. Cell morphology, determination of metabolites, enzyme activities, and the ability to utilize sugars are the most commonly analyzed phenotypic characteristics for this genus, and until the 1960s the only identification criteria used. Specifically, the association of a branched shape with the presence of fructose-6-phosphate phosphoketolase (F6PPK) activity in a strain indicates that it belongs to the genus *Bifidobacterium* (20, 170, 196). However, several problems become apparent when the identification is carried out at species level, and the classical phenotyping, such as sugar fermentation profiles, transaldolase serotyping, cell-wall composition, and the study of the F6PPK isoforms, is clearly not discriminative enough to reach species, subspecies, and biotype level identification with confidence. Furthermore, these phenotypic methods suffer from a certain lack of reproducibility due to the culture conditions, metabolic status of the cells, and sometimes the lack of stability of the genetic determinants responsible for such phenotypes. As a matter of fact, most cases of probiotic misidentifications stem from the use of inappropriate phenotypic methods (83).

Mainly in the last decade molecular tools have been developed for identifying probiotics, based on the analysis of nucleic acids and other macromolecules because of the high potential provided by using polymerase chain reaction (PCR) amplification and hybridization with DNA and RNA (22). A summary of the molecular techniques used for identification and typing of potential probiotic bacteria is presented in Table 1.1.

The study of ribosomal rRNA genes (rDNA) is the most common methodology for bifidobacteria identification up to date. Bacterial ribosomes are formed from proteins and three ribonucleic acids: 5S RNA, 16S RNA, and 23S RNA. The rRNA genes are organized in *rrn* operons, bifidobacteria harboring from two to six depending on the species (152, 196). The 16S rDNA has nine variable regions (V1 to V9), and the three genes are separated by variable spacer regions. The detailed analysis of the 16S rDNA, as well as the 16S–23S spacer region (intergenic transcribed sequence, ITS), showed nucleotide fingerprints with different discriminatory levels. The 16S sequencing is being employed to discriminate all bifidobacterial species and their respective subspecies and biotypes (67, 106–108, 200), whereas the 16S-23S rRNA ITS sequence is much more variable than the 16S rRNA structural gene both in size and sequence, even within closely related taxonomic groups, which makes it a suitable target for both identification and typing by using species-specific primers (72); its analysis has a higher discriminatory capacity and allows the differentiation of different *Bifidobacterium* strains among the same species (94).

TABLE 1.1 Main Methods for Identification and Typing of *Bifidobacterium* and *Lactobacillus* Species

Methods[a]	Target	Outcome	Advantages	Limitations
Phenotyping	Cell metabolism	Identification and biotyping	Special equipment not required	Phenotypic variability, standardization of culturing and reading conditions
Protein profiles of whole cells	Whole-cell proteins	Identification	Easy to perform, high repeatability	Laborious, need of reference strains
FAME profiles	Fatty acids	Identification	Cheap	Special equipment and technical skill needed, mathematical treatment of the results
Species-specific PCR/ multiplex PCR	rRNA genes, end intergenic regions, other genes	Identification of single/ multiple strains, detection of microbial species/strains	Simple, highly reproducible, fast and sensitive, availability of primer sequences for most species, possibility of sequencing	Only few species can be detected at a time; unknown members are not identified; requires sequence data for specific primer design
ARDRA	rRNA operons	Identification, typing	Simple, digestion with many restriction enzymes	Availability of rRNA gene sequences
rRNA 16S sequencing	rRNA genes	Identification, phylogenetic relationships	Simple, sequencing of PCR fragments and sequence comparison, availability of many "universal" and group-specific primers	Absence of a clear definition of genetic species

PCR amplification and sequencing of housekeeping genes	*recA, groESL, dnaK, hsp60, tuf*, etc.	Identification and typing	More resolution for closely related bacteria than the 16S rRNA sequences	Availability of sequences
Ribotyping	rRNA genes	Typing and identification	Automated (Riboprinter, Qualicon, DuPont)	Laborious except automated, high conservation of rRNA operons, expensive equipment
DGGE, TGGE	16S rRNA gene, other genes	Identification of bacteria, typing of microbial communities	Large numbers of samples can be analyzed simultaneously; superior taxonomic information regarding unknown bands	PCR biases; co-migration of different species; dominant species; heterogeneous copies of rDNA operons; lysis and extraction efficiency
PFGE	Whole genome	Typing	High resolution and reproducibility, use of many restriction enzymes	Laborious, time-consuming, analysis of small number of samples
AFLP	Whole genome	Typing	High reproducibility	Laborious, time-consuming, technical skill and high standardization needed, expensive
AP-PCR, RAPD	Whole genome	Typing and identification	Simple, easy to perform, many primers, cheap	Low repeatability, small number of bands
ERIC-PCR	Whole genome	Typing	Sensitivity and specificity, simple, simultaneous comparison of many samples	PCR biases, careful standardization of the technique
REP-PCR	Whole genome	Typing	Simple, PCR-based technique	PCR biases

(*continued*)

TABLE 1.1 (*Continued*)

Methods[a]	Target	Outcome	Advantages	Limitations
TAP-PCR	Whole genome	Typing	RADP variant, 18 mer oligonucleotide with a degenerated position at the 3′ end, more reproducibility	PCR biases
RISA, ARISA	rRNA intergenic spacer region	Typing of bacteria, fingerprinting of microbial community structure	Highly reproducible; automated	PCR biases; requires large quantities of DNA
Microarray hybridisation	16S rRNA genes, whole genome, specific genes	Bacterial detection and community analysis in complex environments	High power of identification; thousands of genes can be analyzed, automated analysis	Laborious; expensive; in early stage of development

[a] FAME: fatty acids methyl esther; ARDRA: amplified ribosomal DNA restriction analysis; DGGE, TGGE: denaturing gradient gel electrophoresis, termal gradient gel electrophoresis; PFGE: pulsed field gel electrophoresis; AFLP: amplified fragment length polymorphism; AP-PCR, RAPD: arbitrarily primed PCR, also know as random amplification of polymorphic DNA; ERIC: enterobacterial repetitive intergenic consencus sequence; REP-PCR: repetitive extragenic palindromic elements-PCR; TAP-PCR: triple arbitrary primed-PCR; RISA, ARISA: ribosomal intergenic spacer analysis, automated ribosomal intergenic spacer analysis.

Recently, more robust and powerful typing methods have been applied to *Bifidobacterium* species and strains, such as the multilocus sequence typing (MLST) scheme. The MLST method was first utilized for bacteria in 1998, and it made use of an automated DNA sequencing procedure to characterize the alleles present at different housekeeping gene loci (95). As it is based on nucleotide sequences, it is highly discriminatory and provides unambiguous results that are directly compared between laboratories. Several authors (103, 191, 192, 194, 195, 197–200, 208, 211), analyzed several gene sequences for detailed identification and classification purposes (*tuf, recA, xfp, atpD, groEL, groES, dnaK, hsp60, clpC, dnaB, dnaG, dnaJ1, purF, rpoC*). Other gene sequences (*pyk, tal*) have also been studied proving to be valuable for species and subspecies identification (137, 183).

Methods based on the PCR are widely used and allow the differentiation between strains of the same species and to some extent, also between species. By examining fingerprint patterns generated by amplification of DNA fragments these methods offer considerable potential for probiotic strain typing. The random amplification of polymorphic DNA (RAPD) technique uses short random sequence primers that are able to bind under low stringency to partially or perfectly complementary sequences of unknown location along the genome. Fingerprint patterns generated with this technique were useful to differentiate *Bifidobacterium* strains from human and food origin (50, 110, 201). Amplified ribosomal DNA restriction analysis (ARDRA) consists of the amplification of rDNA genes (totally or partially) and subsequent digestion with restriction enzymes, thus the choice of the enzyme(s) is critical for the discriminatory power. Species-specific identification is usually achieved with this technique (89, 143, 185, 188, 190), although *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis* can also be distinguished (191). ERIC (enterobacterial repetitive intergenic consensus sequence)-PCR, and REP (repetitive extrogenic palindromic)-PCR examine specific patterns of repetitive DNA elements. ERIC sequences are 126-bp inverted repeats and REP sequences are short DNA fragments (between 21 and 65 bases) detected in the extragenic space; both are dispersed throughout the bacterial genomes (193, 174). The application of ERIC-PCR for bifidobacterial identification at species and subspecies level has been reported (158, 189), and REP-PCR can be considered as a promising genotypic tool for the identification of bifidobacteria potentially up to strain level (102, 103).

Although the aforementioned PCR techniques are the most common methods for identification and typing *Bifidobacterium* strains, other PCR-based approaches include TGGE/DGGE (temperature gradient gel electrophoresis/denaturing gradient gel electrophoresis) (62, 99, 172), Amplified fragment length polymorphism (AFLP), PCR coupled to enzyme-linked immunosorbent assay (ELISA), triplicate arbitrarily primer (TAP)-PCR, restriction fragment length polymorphism (RFLP)-PCR (48, 147), and multiplex PCR (26, 55, 91, 116, 187) to some extent have been utilized to type bifidobacteria to species, subspecies or strain level (Table 1.1).

Some methods for bifidobacteria identification and typing using total (or partial) DNA profiles, including plasmid analysis and RFLP of total DNA, have been used (14, 27, 142). However, pulsed-field gel electrophoresis (PFGE), which involves the digestion of genomic DNA with rare-cutting restriction enzymes and

the subsequent separation of the macrofragments through a continuously reorienting electric field, is often considered by microbiologists the best technique for strain-specific typification. PFGE protocols have been established for different *Bifidobacterium* species (29, 110, 159, 207) and have shown a high discriminatory power to differentiate, for example, *B. animalis* subsp. *lactis* strains, which are often not discriminated using other methodologies due to the close genetic background among strains (66).

The ribotyping is the most popular and widespread hybridization method for bacterial typing. It combines southern hybridization of genomic DNA restriction patterns with rDNA probes. Furthermore, the availability of commercial systems allows the analysis of a wide range of bacteria in an automated manner. Although it is generally believed that ribotyping has a lower discriminatory power than the PFGE analysis (150), it has been extensively used for bifidobacterial typing (88, 97, 98, 110, 148).

Among these techniques, southern blot and microplate blot have also been used to type *Bifidobacterium* strains (97, 205), and microarray hybridization has arisen during the recent years as a valid alternative to discriminate between *Bifidobacterium* species (203), although the need for specific equipment and specialized personnel for the analyses severely limits its current applicability as an ordinary method of probiotic identification.

Finally, it is worthwhile pointing out that other methods have also been applied to the identification and typing of *Bifidobacterium*. The chromatographic analysis of organic acids (93) appears to be a useful tool for rapid identification of *Bifidobacterium* spp. at the genus level. Also, the analysis of the intrinsic fluorescence of aromatic amino acids (16) was shown to be an inexpensive and convenient means of rapidly identifying intestinal bifidobacteria, which could be of help for large probiotic surveys.

1.2.3 Identification, Classification, and Typing of *Lactobacillus* Strains

1.2.3.1 Taxonomy The genus *Lactobacillus* is the largest group among the lactic acid bacteria (LAB) containing, at present, more than 120 species and 20 subspecies (http://www.dsmz.de/microorganisms/bacterial_nomenclature_info.php?genus=LACTOBACILLUS (65, 150)); though its number increases every year (13 new species have been proposed in 2005, 9 in 2006, and 7 in 2007 up to the time of writing; http://www.ncbi.nlm.nih.go/sites/entrez). The lactobacilli are a broad, morphologically defined group of Gram-positives, nonspore-forming rods or coccobacilli with a G+C content usually below 50 mol% (86). Lactobacilli are clustered in the subdivision of low G+C Gram-positive bacteria, and are included in the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and family *Lactobacillaceae.* They are strictly fermentative (either homo- or heterofermenters), aerotolerant or anaerobic, aciduric or acidophilic having complex nutritional requirements (carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, vitamins) (86). They are naturally associated with a large variety of nutritive-rich plant- and animal-derived environments, and many species are involved in the manufacture and preservation of fermented foods and feed from raw agricultural materials (such as milk,

meat, vegetables, and cereals) in which they are present as contaminants (166). Moreover, some species and strains are broadly used as starters and adjunct cultures to drive food and feed fermentations; notably dairy products (yogurt and cheese), fermented vegetables (olives, pickles, and sauerkraut), fermented meats (salami, sausages), and sourdough bread and other cereal-based food commodities. Although less numerous than bifidobacteria, lactobacilli are natural inhabitants of the GIT and genitourinary (GUrT) tracts of animals and humans, where they are thought to play pivotal roles in the maintenance and recovery of a healthy state (136, 182). Not surprisingly, a number of strains have been used as probiotics for more than 70 years (138). Beneficial effects attributed to indigenous and probiotic lactobacilli include colonization of intestinal and genital mucosa (85), inhibition of pathogens (36, 81), immunomodulation (88), and cholesterol assimilation (132).

1.2.3.2 Identification and Typing Reliable identification of bacterial species and strains and correct naming are primary aims of taxonomic studies, but it also has important consequences for industrial application of bacteria. Morphology, Gram staining, and biochemical tests (fermentation of carbohydrates, growth at different temperatures, salt concentration, etc.) have traditionally been used as the primary methods for classifying *Lactobacillus* species; these methods are still in use. Based on phenotypic and biochemical characteristics, lactobacilli were divided into three groups according to the type of sugar fermentation (86). Obligate homofermentative lactobacilli ferment hexose sugars by glycolysis and produce mainly lactic acid, while obligatory heterofermentative species use the 6-phospho-gluconate/phosphoketolase (6PG/PK) pathway and produce other end products (CO_2, ethanol) in addition to lactic acid (18). A third group includes the facultative heterofermentative lactobacilli that ferment hexoses via the glycolysis and pentoses via the 6PG/PK pathways, respectively. Phenotypic analyses are time consuming and require technical skill and standardized assays and reading conditions, in order to avoid subjective results. Furthermore, it has been widely recognized that *Lactobacillus* species and strains display an inherent high level of phenotypic variability (86). Thus, phenotypic heterogeneity makes classical microbiological methods ambiguous and unreliable. In fact, many studies emphasize that the phenotypic classification of lactobacilli is unsatisfactory (13, 40, 113, 133, 162). As a recent example, Boyd et al. (28) have reported that the API 50 CH identification system failed to identify the seven *Lactobacillus* reference strains utilized in their study, and 86 out of 90 vaginal isolates, as compared to the identification obtained by hybridization using whole-chromosomal DNA probes. Of particular complexity are the phenotypically and genetically closely related species belonging, among others, to the *Lactobacillus casei* group (115) or to the *Lactobacillus acidophilus* complex (131, 144). Moreover, the phenotypic identification does not reflect the phylogenetic relation of the different species (51).

The taxonomy of the *Lactobacillus* species has changed considerably with the increasing knowledge of their genomic structure and phylogenetic relationships gathered with molecular methods (65). The DNA–DNA hybridization technique is still of reference, although this technique is labor intensive and time consuming. Fatty

acid methyl ester (FAME) analysis has also been applied to the identification of lactobacilli from dairy and probiotic sources (68, 206). This is an inexpensive procedure that is also of help to study diversity, composition and dynamics of microbial communities, but FAME profiles are rather difficult to interpret and have to be subjected to mathematical treatments. Identification and classification of *Lactobacillus* species has also been accomplished by analysis of whole-cell protein patterns (133, 204). Highly standardized SDS-PAGE conditions allow a rapid and precise identification of a large number of strains. Profiles of unknown strains are compared to a pattern database of known species. In spite of all these techniques, at present, a majority of the molecular identification methods of *Lactobacillus* strains rely on the analysis of rRNA genes, mostly after their partial or complete amplification by the PCR technique (Table 1.1).

rRNA genes have been generally accepted as the potential target for identification and phylogenetic analysis of bacteria (15). Consequently, PCR amplification and sequencing of 16S rDNA- or 23S rDNA-targeted primers have successfully been used for the detection and identification of *Lactobacillus* species (115, 171, 186). Amplicons are usually digested with restriction enzymes for some techniques (such as ARDRA), and, more frequently, subjected to double-stranded sequencing. It has been experimentally determined that species having 70% or greater DNA similarity (at the DNA–DNA hybridization or re-association level) share, in fact, more than 97% of 16S rDNA sequence identity (127, 161). Isolates having such a percentage of identity belong to what has been called an operational taxonomic unit (OTU). Comparison of the rRNA gene sequences (mainly 16S rRNA) allows a precise identification and, at the same time, tracking of the evolutionary relationships among the distinct species. The analysis of 16S rDNA sequences has shown that the division of lactobacilli species in three groups is not in accordance with their natural relationships (18). In fact, *Lactobacillus* species branch into several groups and do not form a coherent phylogenetic unit (65, 150) (Fig. 1.3). At present, specific primers are available for targeting most *Lactobacillus* species (24, 90, 150, 154). Besides genes of both rRNA molecules, the analysis of ITS has also been utilized for identification purposes (24, 75, 119). Based on either the genes or the ITS regions, some authors have developed multiplex PCR of species-specific primer pairs for the detection of up to eleven different LAB species (90, 154, 160). In the same way as oligonucleotide primers, oligonucleotide probes can also be used in hybridization experiments for specific detection, identification, and quantification of *Lactobacillus* species (80, 128, 133, 155). Nucleotide differences in the 16S rRNA genes can also be exploited for the electrophoresis separation of PCR-derived amplicons by DGGE technique or its relative temporal temperature gradient electrophoresis (TTGE). These techniques can either be used for the identification of individual strains (61, 184) or for the analysis of the diversity and evolution of whole populations in complex bacterial mixtures (80, 120, 135).

Coding genes of highly conserved proteins such as RecA (64, 175), GroESL (202), and the elongation factor (EF) Tu (33, 192) have all been used to identify lactobacilli species and to determine their phylogenetic relationships. These gene sequences provide phylogenetic resolutions comparable to that of the 16S rRNA gene at all

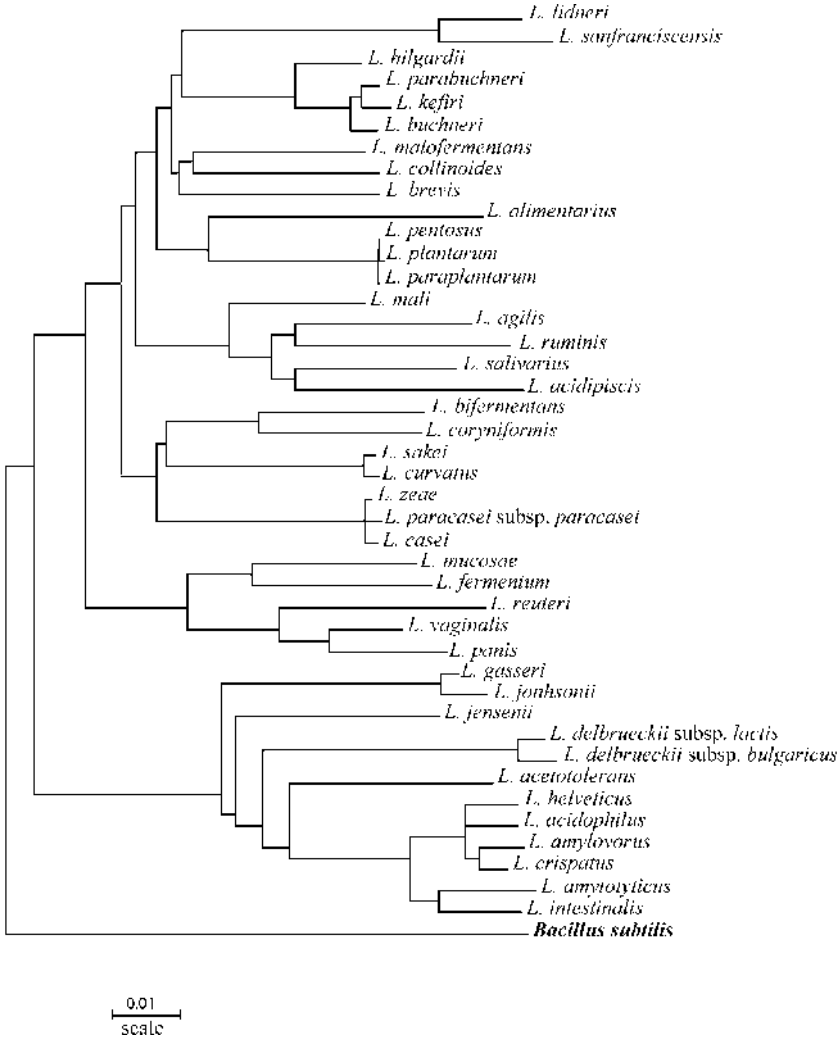

FIGURE 1.3 Phylogenetic tree showing the relationships of the 16S rDNA sequences of type strains of selected *Lactobacillus* species. Sequences were obtained from the Ribosomal Database Project (http://rdp.cme.msu.edu/) and the phylogenetic tree was constructed by an online tree builder resource that uses the Weighbor-weighted neighbor-joining algorithm. The 16S rDNA sequence from the *Bacillus subtilis* type strain was selected as an outgroup.

taxonomic levels, and better resolution between closely related organisms, as rates of evolutionary substitution in protein-coding genes are one order of magnitude higher than those for 16S rRNA genes. The use of protein-coding sequences further avoids the biases due to multicopy and intragenomic heterogeneity associated to rRNA sequences The comparison of the sequences of the fructose-1,6-bifosphatase (*fbp*) gene

has been recently used for identifying and typing food borne and clinical strains of *Lactobacillus rhamnosus* (145). Beyond sequence data, the polyphasic approach, which integrates phenotypic, genotypic, and phylogenetic information, has been recognized by the International Committee on Systematic Bacteriology as a new tool for the description of species and for the revision of the present nomenclature of some bacterial groups (181).

Intraspecific differentiation of bacteria is highly relevant for the selection of starter and probiotic cultures, because technological, sensorial, antimicrobial, and probiotic attributes are strain specific. Typing methods are very helpful in distinguishing patent protected strains, as well as the distinction of starter, adjunct, and probiotic cultures (strain tracking) from natural isolates. As for the safety aspects, it is crucial to be able to compare clinical (pathogenic) isolates with biotechnological strains in use. Besides phenotypic methods, many PCR-based typing methods have been used for the typing of lactobacilli strains, such as ribotyping (141, 210), RAPD (46, 58, 117, 153, 175), PFGE (144, 179, 190), TAP-PCR (43), AFLP (176), REP elements PRC amplification (REP-PCR), ERIC-PCR (190), etc. PCR-RFLP of intragenic DNA fragments of protein-coding genes involved in primary metabolism (β-galactosidase, lactose permease, and proline dipeptidase) has also been used as a typing method for dairy *Lactobacillus delbrueckii* strains (69). Chromosome typing (restriction endonuclease fingerprinting of chromosomal DNA, chromotyping) has been applied to the discrimination of strains of lactobacilli and found to be specific and highly reproducible (163, 210); although the large number of bands requires careful standardized electrophoretic conditions.

The powerful MLST technique has recently been applied for species identification and phylogenetic studies of *Lactobacillus* strains. A MSLT method based on the analysis of six loci (*pgm, ddl, gyrB, purK1, gdh*, and *mutS*) has been developed for the analysis of *L. plantarum* strains (47). Even more recently, Diancourt et al. (54) developed and applied a MLST variant, called multilocus variable-number tandem repeats (VNTR) analysis (MLVA), for the fine subtyping of *L. casei/L. paracasei* strains. A high concordance between the profiles obtained by MLVA and those obtained by AFLP and MLST was observed.

Whole-genome sequencing and comparative genomics providing insights on bacterial evolution will surely influence bacterial taxonomy in the near future. In fact, gene and genomic sequence information has recently been proposed as a tool for defining a new genomic–phylogenetic species concept for prokaryotes (163). Comparative genomics has further strengthened the idea that the lactobacilli as a whole do not form a coherent phylogenetic group, supporting the recognition of new subgeneric divisions (32, 96). In fact, it seems that some species (*L. salivarius, L. plantarum*) are more closely related to *Enterococcus faecalis* than they are to other lactobacilli (32), and other non-lactobacilli such as *Pediococcus pentosaceus* will likely cluster within or close to some species of the genus (32, 96). Genome techniques such as comparative genome hybridization (CGH) can quickly be used to determine the genome content of a bacterial strain whose genome sequence is not known (112). CGH has also been found to be valuable for clarifying controversial taxonomical issues. It has already been used for comparison of members within the *L. acidophilus* group, addressing

both intra- and interspecies diversity (23). Microarrays based on the *L. johnsonii* NCC533 genome were hybridized with total DNA from strains of this and other species of the *L. acidophilus* complex. A clear stepwise decrease in similarity between members of the complex was found, suggesting that these species belong to a natural phylogenetic unit. Exhaustive phylogenetic analyses based on genome data will be performed when more genome sequences are completed and analyzed. To date, 10 *Lactobacillus* genomes have been published, and at least 11 more sequencing projects are ongoing (35, 96).

1.2.4 Characterization of Probiotic Properties in *Bifidobacterium* and *Lactobacillus* Strains

Several criteria have been used for the selection of probiotic strains (Fig. 1.1), the most commonly employed being the survival of the stressful GIT conditions (low pH and high bile salts concentrations), the ability to transitory colonize the GIT, which is related with the adhesion to mucus and/or intestinal epithelium and the antimicrobial activity through the production of antimicrobial molecules or the ability to inhibit/displace the adhesion of pathogens. Several *in vitro* and *in vivo* tests are employed for the screening of these characteristics (45, 57, 178, 209), although there is a lack of standardized or unified methodology for the assessment of probiotic functionality. Table 1.2 summarizes some works that report the screening of the most common probiotic characteristics within collections of *Lactobacillus, Bifidobacterium*, and other LAB strains, mainly isolated from human samples but also from other sources.

1.2.4.1 Survival to GIT Stressing Conditions The transit of probiotics included in foods through different sections of the GIT takes variable times and is submitted to different stressful conditions. After mastication, the first barrier that bacteria must overcome is the low pH values of the stomach with values ranging from 1 to 3 and mean exposure times of 90 min. Into the duodenum the pH value rises to 6–6.5, but bile salts are poured from the gallbladder to reach concentrations ranging from 1.5 to 2% during the first hour of digestion and decreasing afterwards to 0.3% w/v or lower (118). The residence period in the small intestine until 50% emptying oscillate between 2.5 and 3 h and the transit through the colon could take up to 40 h (31). In this location pH values are close to neutral (from 5.5 to 7) and the physiological concentration of bile salts is lower. For the screening of putative probiotic bacteria most works (Table 1.2) simulate *in vitro* these GIT conditions. Several pH values and bile concentrations are tested for variable times in order to determine the survival of the strain(s) under test. Bacteria are enumerated by culture dependent and/or independent techniques, such as those employing fluorescent probes that allow knowledge of the population of dead and live bacteria. The results of viability obtained are strain dependent and, in general, bifidobacteria strains are less tolerant to acidic conditions than lactobacilli, whereas the first seems to be more tolerant to bile challenge. Few studies have been carried out employing human samples of gastric juice and bile and interestingly, the source of bile (bovine, porcine, or human) modifies the tolerance pattern (56). Therefore, it would be

TABLE 1.2 Screening of Probiotic Characteristics within Collections of *Lactobacillus* and *Bifidobacterium* Isolated from Different Sources

Strains Tested (number)	Strain Origin	Probiotic Character Studied	Observations	References
Bifidobacterium (25)	Infant feces	Survival GIT conditions Adhesion/colonization GIT Antimicrobial activity	Not all desirable characteristics were present in a single strain; most adherent and inhibitory were less resistant	(209)
Bifidobacterium (40)	Human, dairy	Survival GIT conditions	One strain (*B. lactis*), survive the simulated GIT conditions	(42)
Bifidobacterium (280)	Human feces	Antimicrobial activity	Inhibition ability varied according to pathogen tested	(25)
Bifidobacterium (11)	Human, animals	Adhesion/colonization GIT Antimicrobial activity	Adhesion and antimicrobial ability varied according to strain and its origin	(38)
Bifidobacterium (15)	Human, culture collection (70)	Adhesion/colonization GIT	The acquisition of bile resistance modify the adhesion ability of strains	(70)
Bifidobacterium (8)	Human, culture collection	Adhesion/colonization GIT	Adhesion is strain-dependent and is modified by pH and pathogens presence	(139)
Lactobacillus (12)	Culture collections	Adhesion/colonization GIT	Adhesion was strain-dependent	(177)
Lactobacillus (47)	Human, fermented foods, unknown	Survival GIT conditions Adhesion/colonization GIT Antimicrobial activity	The survival of three strains seemed to be linked to adhesion ability and tolerance to pH 2.5.	(84)

L. acidophilus group (35)	Human, animals, dairy	Adhesion/colonization GIT	The adhesion to rat colonic mucin ability was strain dependent	(109)
Lactobacillus (7)	Culture collection	Adhesion/colonization GIT Antimicrobial activity	The growth media and the food matrix affect de adhesion properties	(125)
Lactobacillus (35)	Infant feces	Antimicrobial activity	More effective against Gram- and inhibitory activity was strain-dependent	(17)
Lactobacillus (88)	Dairy	Survival GIT conditions Adhesion/colonization GIT Antimicrobial activity In vitro and *in vivo* safety assessment	One strain (*L. plantarum*) showed probiotic characteristic and survive incorporated in cheese	(37)
Lactobacillus (65)	Dairy	Antigenotoxic properties	High number of strains showed antigenotoxic and antimutagenic properties	(30)
Lactobacillus (4)	Culture collection	Adhesion/colonization GIT Antimicrobial activity	High specificity in the inhibition/ displacement of pathogens by lactobacilli	(71)
Lactobacillus (22)	Dairy	Survival GIT conditions Antimicrobial activity Bile salt deconjugation	Most strains were resistant to biological barriers. Only 3 strains were able to deconjugate bile salts.	(180)
Lactobacillus (26)	Human oral cavity	Survival GIT conditions Antimicrobial activity	Two strains (*L. salivarius* and *L. gasseri*) as putative probiotics	(167)
L. plantarum (31)	Animals, dairy, fermented foods	Adhesion/colonization GIT	Adhesion depends on both the adhesion model employed and the strain tested	(169)

(*continued*)

TABLE 1.2 (*Continued*)

Strains Tested (number)	Strain Origin	Probiotic Character Studied	Observations	References
Lactobacillus and *Bifidobacterium* (200)	Human, dairy	Survival GIT conditions	Three lactobacilli and 1 bifidobacteria strains survive to GIT conditions	(134)
Lactobacillus and *Bifidobacterium* (7)	Culture collections	Antimicrobial activity	Antagonistic activity variable according to strain tested	(82)
LAB (120)	Human feces	Survival GIT conditions Antimicrobial activity Bile salt hydrolase activity (BSH) *In vivo* cholesterol reduction	One strain (*L. plantarum*) showed high *in vitro* BSH activity and after oral administration was able to reduce cholesterol levels	(73)

recommendable that probiotic strains intended for human consumption are tested in the presence of human intestinal fluids.

1.2.4.2 Adhesion/Colonization to/of GIT Some of the health effects attributed to probiotics are related to their capability to adhere to the intestinal mucosa. Adhesion is a prerequisite for intestinal colonization, stimulation of the immune system, and for antagonistic activity against enteropathogens through competitive exclusion (57). The intestinal mucosa is covered by a layer of different types of epithelial cells, which are distinctly different in the different regions of GIT, and is in contact with the lumen, the outside of the body. In addition to secretory and absorption cells, an important part of the immune system is placed in this location and it is collectively referee to the GALT (gut-associated lymphoid tissue). The intestinal epithelium is almost completely covered by a protective mucus gel composed predominantly of mucin, glycoproteins acting as the anatomical GIT site in which the host first encounters gut bacteria (53). Genomic information of some probiotic strains revealed the presence of several molecules able to adhere to different components of the intestinal mucosa and to exchange signals with the intestinal immune system (149), which indicate a good adaptation of probiotics to the gut environment.

Several models have been employed to study the ability of putative probiotic strains to adhere to the intestinal epithelium. Studies have often been carried out with cellular lines obtained from human colon adenocarcinomas such as Caco-2 (ATCC HTB-37) and HT-29 (ATCC HTB-38) the last one being able to produce mucin (37, 84, 139, 169, 177). Frequently, the adhesion ability of putative probiotics from different collections has been extensively tested against mucus obtained from human (38, 70, 123, 125) or animal origin (109). Interestingly, some strains of *Bifidobacterium* adhere better to human mucus than to porcine mucus indicating that adhesion is property strain dependent (124), because mucus from different origins (human, canine, possum, bird, and fish) did not modify the adhesion of probiotic strains (140). In addition, bacterial adhesion to human mucus decreased with the age of the donor of the mucus sample, which could be one of the reasons for low bifidobacteria colonization in elderly subjects (122). A good correlation between the human mucus model and the adhesion to Caco-2 has been demonstrated by Gueimonde and collaborators (71) employing three *Lactobacillus* strains. Both methods are adequate for *in vitro* adhesion studies but some *ex vivo* models employing resected tissue of the intestinal mucosa from human or animals have also been shown to be useful (105, 126). In the human intestinal mucus model proposed by Ouwehand and coworkers (126) the material is obtained from patients with colon cancer submitted to surgery. The healthy sections of resected tissue obtained from different sites of the colon are employed in these studies. In general, the strains tested showed higher adhesion to mucus than to colonic tissue and, depending on the strain, the location of the colonic tissue but not that of mucus, also influenced the adhesion properties of the probiotics tested. This is a good model for the assessment of the adhesion of LAB to GIT epithelium and to mucus.

1.2.4.3 Antimicrobial Activity In the complex GIT ecosystem probiotics have developed mechanisms to survive in competition with other microorganisms. Essentially, the antagonism is exerted by competition for nutrients and for physical location, but also through the production of antimicrobial substances. In connection with the previous paragraph, the ability of probiotics to produce antimicrobials is one mechanism to inhibit, exclude or compete with adherent enteropathogens for the ecological niche. Several works (Table 1.2) have been carried out to test *in vitro* the interference on adhesion between probiotics and pathogens such as *Salmonella enterica* serovar Typhimurium, *Escherichia coli, Clostridium difficile, Enterobacter sakazakii*, and *Listeria monocytogenes*. Using human intestinal mucus it has been demonstrated that the adhesion antagonism is clearly both, probiotic- and pathogen-strain dependent (38, 71). This specific interaction indicates the need for a case-by-case assessment in order to select probiotics with the ability to inhibit or displace certain pathogens. Most often, cocultures probiotic/enteropathogen are carried out to test the antimicrobial ability of probiotic strains (17, 25, 37, 82, 84, 167). Viability of both types of bacteria is determined and in some cases the antimicrobial activity is tentatively assigned to the production of substances such as organic acids, ethanol, H_2O_2, or proteinaceus components bacteriocin-like. The general conclusion that arises from these *in vitro* studies suggests again that the inhibition ability is strain- and culture-condition- dependent and that several molecules and mechanisms are involved in the interrelationship between probiotics and pathogens.

1.2.4.4 Other Probiotic Properties In addition to the previously reviewed properties, other characteristics could be tested to consider a strain as putative probiotic. From these screenings it has been reported that some strains are able to modulate the immune system (129), to produce antigenotoxic compounds (30), to deconjugate bile salts (73, 180), and to decrease cholesterol levels (73).

1.2.5 Conclusion

The selection of a strain to be used as an effective probiotic is a complex process. For human consumption, it is widely accepted that an effective human probiotic should be of human origin the most suitable source being the human GIT. A vast array of specific and reproducible molecular techniques is now available for identification and typing of lactobacilli and bifidobacteria. Molecular techniques have allowed the precise and rapid identification and typing of novel probiotic stains, providing new ways to check for their presence and monitor their development. Nevertheless, for microbial characterization, a polyphasic combination of phenotypic assays and molecular techniques is preferred, since these approaches may provide complementary results. On the other hand, several *in vitro* and *in vivo* tests have been found to be useful for the screening of novel strains with putative probiotic properties. In general, the probiotic characteristics are strain-dependent and properties are not all simultaneously present in a single strain. Of note is the realization that the efficacy and safety of a probiotic should be validated in phase 2 clinical trials, a pending subject for most current-in-use probiotic strains.

1.3 DETECTION AND ENUMERATION OF GASTROINTESTINAL MICROORGANISMS

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1.3.1 Methods for Intestinal Microbiota Assessment

Understanding the cross talk that occurs between intestinal microbiota and its host promises to expand our views about the relationship between intestinal microbiota and well-being. Unfortunately, we are still far from knowing the qualitative and quantitative composition of the intestinal microbiota and the factors governing its composition in an individual. Several different methodologies, culture dependent and culture independent, have been used for intestinal microbiota assessment (Table 1.3). The aim of this chapter is to review these methodologies, which are divided into two main groups: the culture-dependent and culture-independent methods.

1.3.1.1 Culture-Dependent Methods

1. Nonselective and selective culture media for intestinal microorganisms. The study of intestinal microbiota composition, both qualitatively and quantitatively, has been traditionally carried out by cultivation of feces. In some cases it has also been considered that there are mucosa-associated intestinal microbiota in biopsies of healthy individuals (229, 293) or patients (225, 252, 347). The classical method has been culturing fecal samples on suitable growth media, the sample generally being handled in anaerobic cabinets and processed immediately or within a few hours after collection. The bacterial counts of a given microbial group are determined after incubation in the appropriate conditions. Both nonselective- and selective-differential media have been used for growth and counting and the choice for one or the other was dependent on the microbial group being screened and on the method used for subsequent identification. Some of the more widely used media in recent years includes Wilkins-Chalgren (257, 330, 331, 341) and Columbia blood agar (225, 296) as general media for total anaerobic bacteria. The same media were also employed as selective and/or differential after the addition of the appropriate antibiotics and selective agents for the enumeration of Gram-positives including *Clostridium,* as well as *Bacteroides*, *Prevotella*, and other Gram-negatives (257, 341). MRS, Rogosa, and trypticase phytone yeast extract (TPY) agar were frequently used as base media for counting *Bifidobacterium* with or without the addition of selective agents (231, 257, 322, 323, 330, 331, 341), and MRS and Rogosa agar were also employed for the enumeration of *Lactobacillus* (212, 231, 269, 293, 322, 323, 330, 331). Bile–Esculin agar and derived supplemented media were among the most frequently employed for the isolation and enumeration of enterococci (225, 269, 323) and *Bacteroides* (229, 231, 296, 323). Enterococci have been also counted and isolated

TABLE 1.3 Advantages and Disadvantages of Some Techniques Widely Used for Intestinal Microbiota Research

Techniques	Advantages	Disadvantages
	Culture-Dependent Techniques	
All	Widely used	Provides information only on culturable microorganisms
	Quantitative	Time consuming
	Possibility to isolate strains for further study and as source of potential probiotics	Complicated sample manipulation (e.g. anaerobes)
		Sublethally injured or dormant cells not detected
Culture in selective or differential media	Direct quantification of the microorganisms of interest	The presence of selective and/or inhibitory agents may inhibit the growth of part of the population (e.g. injured cells)
Culture in nonselective media followed by specific counting	Avoids the use of selective and/or inhibitory agents in the media	Requires an extra step for specific counting
	Culture-Independent Techniques	
All	Provides information on both culturable and non-culturable microorganisms	Bias due to the different methodologies
	High throughput, rapid, and sensitive	Difficult standardization
	Possibility to use frozen samples	
Detection by PCR	Fast and easy to perform	Not quantitative
	Versatile (primers with different specificities can be used)	Previous knowledge required on the target microorganisms (to design specific primers instead of using universal primers)
	Different degrees of specificity possible (from group to strain specific)	

Sequence analyses of randomly amplified 16S rRNA genes	Provides information on previously unknown bacteria	Not quantitative
	No previous knowledge of the microbiota present is needed (when using universal primers)	Bias due to different cell lysis/PCR/Cloning efficiencies
	Provides important information at population level (e.g. diversity)	Methodologically difficult
TGGE/DGGE	Versatile (primers with different specificities can be used)	Not quantitative
	No previous knowledge of the microbiota present is needed (when using universal primers)	Bias due to different cell lysis/PCR efficiencies
	Provides information at population level (if using appropriate primers)	Methodologically difficult.
T-RFLP	Versatile (primers with different specificities can be used)	Not quantitative
	Provides information at population level (if using appropriate primers)	Bias due to different cell lysis/PCR efficiencies
		Methodologically difficult and expensive
DNA-Arrays	Very high throughput. Allows detection of thousands of sequences in a single assay	Not quantitative
		Bias due to different cell lysis/PCR efficiencies
		Methodologically difficult and expensive
FISH	Quantitative	Bias due to different cell permeability to probe
	Possibility to observe bacteria *in situ* (e.g. position in the mucosa)	Difficult visual counting (e.g. cell aggregates)
	Possibility to couple with automatic image analysis or flow cytometry	Previous knowledge of the target microorganisms required for probe design (if not universal)

(*continued*)

TABLE 1.3 (*Continued*)

Techniques	Advantages	Disadvantages
	Fast and easy to perform	Previous knowledge of the target microorganisms required for primer design (if not universal)
RT quantitative PCR	Allows to determine *in situ* metabolic activity or specific gene expression	Previous knowledge of the target genes required for primer design Does not provide information on cell numbers
Metagenomics	No previous knowledge on the microbiota present is needed Allows identification of novel genetic features in the ecosystem Provides important information at population level (e.g. diversity)	Not quantitative Time consuming Bias due to different cell lysis/PCR/cloning efficiencies Methodologically difficult and expensive
Metaproteomics	No previous knowledge on the microbiota present is needed Provides information on protein coding genes that are being expressed in the ecosystem	Time consuming Methodologically difficult

frequently in Slanetz-Bartley (230, 330) and KF media (296). It is also worthy to note the extensive use of the selective and differential McConkey agar for counting coliforms and enterobacteria (225, 293, 231, 232). Finally, for heterogeneous and complex groups of intestinal bacteria such as clostridia, a great diversity of culture media has been used provided that a unique medium is not appropriate for cultivation of all microbial clusters (257, 269, 296, 322, 331).

2. Identification and typing of intestinal strains. Traditionally, intestinal strains isolated from solid media were identified by means of some general phenotypic characteristics such as carbohydrate fermentation profiles, enzymatic tests, cell morphology, and colony appearance, which have lead to numerous misidentifications. The development of new and different phenotypic methods contributed to the improvement of the accuracy of identification and it is currently possible to identify not only colonies isolated from solid culture media but also nonisolated bacteria present in mucosa and feces. The genus *Bifidobacterium* is the only intestinal Gram-positive displaying fructose-6-phospate phosphoketolase (F6PPK) activity and its determination constitutes a reliable test for this genus among bacteria from the intestinal environment (255, 330). Vlková and coworkers (330) used F6PPK, α-galactosidase, and α-glucosidase as enzymatic methods for detection of the abundance of bifidobacteria directly in infant feces. Determination of catabolic end products was also useful for the identification of some particular and characteristic groups of microorganisms (257). One of the most accurate phenotypic methods currently used for identification at the species level is based on the determination of the cellular fatty acid composition (234) with the help of the chromatographic MIDI system (http://www.midi-inc.com/pages/literature.html) both directly in feces (269, 324) and in previously isolated cultures (263, 341).

The development of genetic methods for the identification and typification of the bacterial isolates greatly contributed to the improvement our knowledge of intestinal microbiota, although its level of sensitivity is obviously limited by the accuracy and sensitivity of the previous culture media on which microorganisms were isolated. Partial amplification and sequencing of the 16S rRNA gene from previously isolated colonies has been extensively used for identification at the genus and species level (212, 228, 323, 330, 331). Monoclonal antibodies have been used for selective enumeration of *Bacteroides vulgatus* and *Bacteroides distasonis* on fecal samples after dilution and plating in a nonselective medium, avoiding the use of selective agents to which part of the *Bacteroides* population could be sensitive (226). Oligonucleotide probes have also been used for the identification and the quantification of intestinal microbiota by means of colony hybridization (232, 271). Typing of the strains from a given species of intestinal microorganism has often been carried out by pulsed field gel electrophoresis (PFGE) (322, 323) or RAPD by PCR (212, 228) among other genotypic techniques.

Several different culture-dependent techniques have been used for quantitative and qualitative characterization of human intestinal microbiota although large differences

in species composition and quantitative contents can be found among the results obtained by different authors. These differences could be attributed to the different culture media employed, different methodologies used for the subsequent identification, etc. The high variability of the intestinal microbiota among individuals and the analysis of a low number of individuals in some studies, could also have contributed to the different results observed.

1.3.1.2 Culture-Independent Methods It is estimated that less than 25% of the intestinal bacteria have been cultivated so far, suggesting that many bacteria in the human gut have not been cultured yet and that classical culture based methods have not provided an accurate representation of this community (233, 259, 319). Consequently, the study of intestinal microorganisms has been restricted to the cultivable species and from these, only to the cultivable fraction of each population. These facts led to the overestimation of some species and the underestimation of others, limiting our understanding of intestinal microbiota composition and function. By using different bifidobacterial selective culture media it has been shown that they differ in their selectivity and some media even fail in the recovery of certain species, which could lead to a biased representation of the population (213). These results clearly show the limitations of the culture-based approaches for the study of complex communities. Therefore, more rapid, accurate and specific methods of detection and quantification have been developed.

During the last few years, developments in molecular biology have led to alternative culture-independent methods in addition to the traditional culture. One of the most widely applied approaches deals with the use of 16S rRNA and its encoding genes as target molecules. The 16S rDNA gene contains highly conserved regions, present in all bacteria, and highly variable ones that are specific for certain microbes. Specific PCR primers and probes can thus be designed based on these variable regions to detect certain species or groups of bacteria. These culture-independent approaches include 16S rRNA measurements, PCR amplification with specific primers of 16S rDNA extracted from fecal or mucosal samples, universal or group 16S rDNA PCR amplification followed by cloning and sequencing, TGGE, DGGE, terminal restriction fragment length polymorphism (T-RFLP) analysis, fluorescence *in situ* hybridization (FISH), real-time quantitative PCR, and oligonucleotide-microarrays. In more recent years metagenomic and metaproteomic approaches have also been applied to the intestinal microbiota assessment.

1. *Design of PCR Primers for DNA Amplification*. Several authors have developed species or group-specific primers for the detection of different microorganisms in the GIT including members of the genera *Bacteroides, Clostridium, Fusobacterium, Peptostreptococcus, Eubacterium, Bacteroides, Prevotella, Lactobacillus*, and *Bifidobacterium* (251, 254, 289, 291, 335).

   Nowadays, rDNA-targeted PCR primers enable a rapid and specific detection of a wide range of bacterial species. Therefore, procedures in which these primers are used have a widespread use in intestinal microbiota assessment. By means of PCR amplification with species-specific primers Wang and

collaborators (335) analyzed the fecal microbiota, showing that *Fusobacterium prausnitzii, Peptostreptococcus productus*, and *Clostridium clostridiforme* had the highest PCR titters followed by *Bacteroides thetaiotaomicron, Bac. vulgatus* and *Eubacterium limosum*. Matsuki et al. (292) studied the bifidobacterial microbiota in adults' fecal samples and found that *Bifidobacterium catenulatum* group *Bifidobacterium longum* and *Bifidobacterium adolescentis* were the most common species whereas *Bifidobacterium breve, Bifidobacterium infantis*, and *B. longum* were the predominant species in infants. Other DNA sequences, such as ERIC sequences, have also been used as targets of PCR primers to fingerprint the microbial community of the human gut (337).

2. *Design of Hybridization Probes*. Several probes have been developed for the assessment of intestinal microbiota. There are probes for specific detection of *Bifidobacterium* (271, 280), some *Clostridium* groups (241, 338), *Bacteroides/Porphyromonas/Prevotella* group (232), *Bacteroides fragilis* group, *Bac. distasonis* or *Streptococcus/Lactococcus* group (241). Also probes for some species of *Bifidobacterium* (343) and *Eubacterium* (313) have been developed. Some other probes for specific intestinal groups such as *Phascolarctobacterium* group, *Veillonella*, *Eubacterium hallii* and relatives, *Lachnospira* group, *Eubacterium cylindroides* and relatives and *Ruminococcus* and relatives are also available (258).

   These probes have been used for specific culture-independent detection and quantification of different intestinal microorganisms by means of FISH (241, 258, 280, 313, 338) or dot blot hybridization (264, 287, 314).

   Combinations of PCR amplification and hybridization have also been reported. In a study by Wei and coworkers (337) ERIC-PCR amplicons from a sample were labeled and used to hybridized against other samples in order to identify those amplicons common to different fecal samples. This approach may be helpful for the identification of specific microbiota aberrancies related to different diseases by comparing healthy and ill individuals.

3. *Polymerase Chain Reaction – Enzyme-Linked Immunosorbent Assay (PCR-ELISA)*. This technique combines PCR amplification of DNA and ELISA. The amplified DNA is labeled, commonly with digoxigenin, and hybridized with the specific detection probe that is immobilized in microtiter plate wells. The presence of hybridized DNA is determined by using digoxigenin-targeted antibodies. This methodology has not been extensively used, but it has been applied to the analysis of *Bifidobacterium* species composition in human feces during a feeding trial (285).

4. *Sequence Analysis of Randomly Amplified 16S RNA Genes*. Another procedure that has been used in intestinal microbiota research is the PCR amplification of 16S rRNA genes in a sample, using universal or group-specific primers followed by cloning and sequencing of the amplified DNAs.

   By using universal primers different studies (259, 261, 319) have shown that the predominantly cloned sequences from fecal samples belonged to

Clostridium coccoides group (*Clostridium* rRNA cluster XIVa), *Clostridium leptum* group (*Clostridium* rRNA cluster IV), and *Bacteroides,* which is in agreement with the results recently reported by Eckburg and coworkers (233). By using this approach it was found that, according to the results obtained by culture, even though clostridia tends to increase with age, the *Clostridium* rRNA cluster XIVa tends to decrease in elderly individuals. This is probably due to a decrease in the number of *Ruminococcus obeum* and related phylotypes, indicating a possible relation between *R. obeum* and aging (261). Regarding the species composition, it was found that the predominant fecal species in the *Bacteroides* group are *Bacteroides uniformis* and *Bac. vulgatus* (319). In the *Clostridium coccoides* group, *Eubacterium eligens, Eubacterium rectale*, and *Eubacterium hadrum* were the predominant species. *Ruminococcus bromii, Eubacterium siraeum*, and *F. praustnizii* were the unique species detected in the *C. leptum* group, being *F. praustnizii* one of the most frequent and numerous species detected by 16S rDNA analysis of human fecal samples (259, 261, 319). Sequencing of amplified 16S rRNA genes has also been used to characterize the differences between the fecal and mucosal microbiota. Eckburg and coworkers (233) analyzed over 11,800 bacterial 16S rDNA sequences from different intestinal locations of three subjects and found that 62% of the phylotypes were novel (244 out of 395) and 80% represented noncultivable species. On the other hand, the same authors indicated that different phylotypes were present in fecal and mucosal samples.

Surprisingly, in some studies carried out using this methodology (261, 319) no sequences belonging to the genus *Bifidobacterium* were detected, probably indicating some problems during the amplification. In this regard, the number of PCR cycles can significantly distort the representation of some organisms in the ecosystem due to preferential amplification of some rDNAs (339).

5. *Temperature Gradient Gel Electrophoresis (TGGE) or Denaturing Gradient Gel Electrophoresis (DGGE) Analyses of 16S rRNA Genes*. This technique has been one of the most widely used for intestinal microbiota assessment. It consists of the PCR amplification of the 16S rRNA genes with universal or group-specific primer pairs, one of which has a GC clamp attached to the 5′ end in order to avoid a complete dissociation of the two DNA strands of the amplified product. Then, amplification products are separated by denaturing gel electrophoresis, through a gradient of temperature (TGGE) or denaturant agent (DGGE), in which the double-stranded DNA will migrate until it reaches its denaturing conditions in the gradient. This method has been shown to be a powerful tool for monitoring bacterial succession phenomena. In addition, the predominant bands obtained can be sequenced in order to know the identity of the most abundant microorganisms. Using PCR-TGGE, Zoetendal et al. (348) studied the diversity of predominant bacteria in fecal samples from adults. A remarkable stability of the profiles over time was observed and *E. hallii,*

R. obeum, and *F. prausnitzii* were the most commonly encountered species. In spite of the stability of the predominant fecal microbiota, it was possible to detect variations in some subpopulations over time by using these techniques (327).

Satokari and collaborators (311) studied the bifidobacteria microbiota in fecal samples from adults by means of PCR amplification with genus-specific primers and DGGE. Their results highlighted *B. adolescentis* as the most common species in feces from adults. Other species also found were *B. catenulatum, Bifidobacterium pseudocatenulatum, Bifidobacterium dentium*, and *Bifidobacterium ruminatum*. By using these techniques *Bifidobacterium* and *Ruminococcus* were reported to be the dominant groups in the intestinal microbiota of babies (236). Favier and coworkers (237) studied the establishment and development of gut microbiota in babies during the first 4 months of life, finding that *Escherichia coli* and *Clostridium* spp. were the initial colonizers followed by the appearance of other microorganisms, such as *Bifidobacterium* and *Bacteroides* after 2–5 days. On the other hand, comparison of the babies PCR-DGGE profiles with those of their parents suggested a vertical transmission of some microorganisms.

With regard to the lactobacilli population, PCR-DGGE results showed that there is a relatively stable *Lactobacillus* population in each individual (262). *Lactobacillus ruminis* and *Lactobacillus salivarius* have been reported to be the true autochthonous lactobacilli whereas other species frequently used in food manufacture can be also detected in feces of individuals (332). In addition, by using PCR-DGGE Zoetendal and coworkers (350) showed that the mucosa-associated bacteria in the colon differ from those recovered from feces, and found host-specific profiles of the mucosa-associated microorganisms. This suggests that the intestinal microbiota composition is influenced by some host factors.

Most of the studies carried out using these techniques are aimed at the assessment of microbiota composition by targeting the rDNA genes. However, the rRNA (RT-PCR DGGE/TGGE) has also been used instead of rDNA allowing the identification of the metabolically active microorganisms in the gastrointestinal ecosystem (294, 348).

6. *Denaturing High-Performance Liquid Chromatography.* This recently developed technique (342) has also been applied to the study of the intestinal microbiota (246). It consists of the PCR amplification of the 16S rRNA genes followed by the separation of the amplification products by means of denaturing high-performance liquid chromatography. Separated PCR products are fluorescent dyed and detected using a fluorescence detector.
7. *Terminal-Restriction Fragment Length Polymorphism* (*T-RFLP*) *Analysis*. The 16S rDNA T-RFLP analysis consists of the amplification of the 16S rDNA with a primer fluorescent labeled and an unlabeled primer so that the PCR product is labeled at only one end. After digestion of the PCR

products with one or more endonucleases the length of the labeled terminal restriction fragments is determined by capillary electrophoresis. A rapid assessment and identification of predominant human intestinal bacteria can be accomplished with this method using the appropriate restriction enzymes (160, 260, 272, 289).

Sakamoto and coworkers (289) assessed the fecal microbiota in adults using T-RFLP analysis showing that the patterns are host specific. These results are in agreement with previous results obtained by DGGE. This technique has also been used for assessment of fecal microbiota in elderly people (261) and to study the effect of a vegetarian diet on intestinal microbiota (50). Interestingly, differences have been found between the results obtained by T-RFLP and cloned 16S rDNA analysis, indicating a possible bias related with a large number of cycles in PCR amplification (260, 261).

8. *Oligonucleotide Arrays*. Wang and coworkers (333, 334) developed an oligonucleotide-microarray using species-specific probes for the detection of the predominant human intestinal bacteria in fecal samples. Microarray technology can be used for simultaneous detection of thousands of target DNA sequences at one time. Thus, its use could permit the detection of many bacterial species in a sample in a rapid and accurate manner. In order to avoid the use of the expensive microarray equipment needed for this technique, a membrane-array procedure has also been reported (336). Recently, Palmer and coworkers (303) developed a microarray containing over 10,000 16S rDNA probes and applied it to the assessment of the colonic mucosa, which allowed the detection and the determination of the relative abundance of species present at levels of 0.03% or greater.

   In the next few years, with the increasing availability of genome sequences from intestinal bacteria, microarrays analysis will become a powerful and valuable tool to assess microbial composition of the human intestinal tract and to study how different members of the intestinal microbiota modulate the expression of genes from both intestinal cells and other intestinal bacteria.

   The culture-independent approaches discussed earlier has led to a better understanding of the qualitative content and the predominant species of the intestinal microbiota. Unfortunately, they have failed to provide reliable data on its quantitative content or on the less abundant groups or species that are also present in the GIT. Thus, some quantitative culture-independent methods have been recently developed and are discussed next.

9. *Relative Amount of Group or Specie-rRNA*. One approach for the quantitative study of the intestinal microbiota is the quantification of the relative amounts of 16S rRNA of each group or species with regard to the total amount of 16S rRNA in the sample by using specific probes and, for example, dot blot hybridization. The amount of 16S rRNA provides not so much a measure of cell numbers as a measure of the metabolic status of each microbial group. By using this procedure it was shown that six bacterial groups represented up to 70% of the total fecal rRNA, *Bacteroides-Prevotella* being the dominant

group with 37% of the total 16S rRNA (314). In addition, by means of this procedure Marteau et al. (287) showed that the human cecal and fecal microbiota differs quantitatively and qualitatively.

10. *Fluorescence In Situ Hybridization (FISH).* Using FISH with different group-specific probes around 90% of the total fecal bacteria can be detected; *Bacteriodes/Prevotella* and the *Clostridium coccoides/E. rectale* groups being the microorganisms present at higher numbers (10^{10}) (241, 258), followed by *Eubacterium* low G+C group (258). Other bacterial groups present at high levels (over 10^9 cells/g feces) included *Ruminococcus* (258) and *Bifidobacterium* (241, 258, 280). *Enterobacteriaceae, Veillonella*, and the group *Lactobacillus/Enterococcus* showed counts under 10^8 cells/g feces.

    FISH has also been used for the assessment of changes in levels of the predominant groups of intestinal bacteria as a result of the consumption of prebiotics or probiotics (273, 325) or to assess the influence of the mode of delivery on intestinal microbiota (309). Also the effect of breast-feeding was studied by means of FISH, and it has been shown to be related to the predominance of bifidobacteria, whereas formula-fed infants showed similar amounts of *Bacteroides* and *Bifidobacterium*. In addition, changes in the minor components of the fecal microbiota were also observed (256). By means of FISH it has also been shown that there are differences in the gut microbiota between infants who later do or do not develop atopy (269).

    Although FISH has been widely used for intestinal microbiota assessment this technique is laborious, there is some difficulties for the visual counting of the samples and is extremely time consuming, thus limiting its further applicability. Multi-color FISH would allow the detection of a few microorganisms by a single hybridization reaction. This approach has been applied to the analysis of seven bifidobacterial species in human feces (320), but still the visual counting is very laborious. Because of that, alternative methods have been developed in order to solve difficulties in manual-visual counting, such as automated image analysis (267) or flow cytometry (349).

    In FISH results can be influenced by differences in the availability of the target region, cell permeability or by the ribosome content of the cells. Low fluorescence levels in positively hybridized cells can also significantly overlap signals of the negative controls (280). Coaggregation of bacteria rests of broken cells or contaminating compounds make the counting difficult. Therefore, more rapid and accurate procedures have been developed and are commented on next.

11. *Quantitative Real-Time PCR*. Quantitative real-time PCR is a promising tool to study the composition of complex communities such as the GIT. This procedure has attracted the attention of researchers in recent years as a consequence of the need for new rapid and accurate quantitative culture-independent techniques for intestinal microbiota analyses.

    Different real-time quantitative PCR assays have been developed. By using the SYBR Green dye both total fecal bifidobacteria and specific

bifidobacterial species or groups have been quantified (290, 305). In addition, 5′nuclease assays have also been developed for *Bifidobacterium* and *Lactobacillus* quantification by using TAQMAN probes (253, 254, 305) or probes labeled with fluorescent lanthanide quelates (248, 251).

Real-time quantitative PCR has also been applied to quantification of other intestinal microorganisms, such as *Clostridium difficile* in feces by using molecular beacons (217), *Escherichia coli* and *Bac. vulgatus* in gastrointestinal mucosa by means of the 5′ nuclease assay with TAQMAN probes (265) or *Desulfovibrio* in feces and mucosa by using the SYBR Green assay (239). Real-time PCR has been employed to characterize and compare the fecal microbiota between healthy and hospitalized elderly subjects (216), proving to be a useful tool for quantitative microbiota monitoring. This procedure has also been used for the quantification of total bacteria and some characteristic species of dental plaque and caries dentine (284, 299).

Similarly to other techniques based on the PCR, the use of 16S rRNA instead of DNA (reverse-transcriptase quantitative PCR) would provide data on the activity/viability of the microorganisms rather than on cellular levels. This methodology may also be applied to monitor *in situ* the expression of specific genes by targeting the corresponding mRNA, as has been demonstrated by Fitzsimons and coworkers (240) using the gene *slp*A of *Lactobacillus acidophilus*. In this regard reverse-transcriptase quantitative PCR provides a very useful tool for monitoring bacterial activity and gene expression in gastrointestinal conditions.

Nowadays, the 16S rRNA genes are being used as target molecules, but as more bacterial sequences are becoming available, new specific primers and probes targeting other genes will also be available in the near future to be used in cases in which the 16S rDNA is not an adequate target. In this regard, it must be taken into account that the bacterial quantification by real-time PCR can be influenced by differences in the number of rRNA operons among the quantified species or groups, sequence heterogeneity among different operons within the same species or by differential amplification of different DNA molecules (312, 340).

12. "*Omics*." During recent years the so-called "omics" revolution (genomics, proteomics, metabolomics) has provided an impressive amount of new information allowing the development of new very powerful molecular techniques. The genome information about some gut microbiota members has increased our understanding on the adaptation of these microorganisms to the intestinal environment (310). Metagenomic and metaproteomic approaches have been applied to the study of the intestinal microbiota. These approaches consist of the procurement and study of a genetic library containing all the genetic material present in a sample (metagenomics) or the study of all the proteins present (metaproteomics). Metagenomic analyses have been used to study the microbiota of the large intestine (245) or to assess the diversity of fecal microbiota in Crohn's disease (286). Klaassens and

coworkers (274) applied metaproteomics for the first time to the study of the intestinal microbiota in infants.

When using omics, as with any other techniques, the possible bias due to the methodologies used must be considered. In this regard, in metagenomic studies, possible biases due to differences in bacterial lysis or cloning efficiencies among different bacteria or DNA sequences should be taken into account (244).

13. *Other Methods*. There are also other methods that have been applied to the assessment of intestinal microbiota without the need for cell culture. The analysis of cellular fatty acids profiles in fecal samples (264) has been frequently used. Metabolic activities (bile acids deconjugation or dehydroxilation, vitamin K production, some enzymatic activities, etc.) can be also used as a crude signature of the microbiota and compositional changes may be tracked by noting changes in these metabolic activities (295). Flow cytometry coupled with fluorescent labeling of live–dead bacteria has been applied to the identification of the viable and active populations in the gut (219). Another interesting approach is the rRNA-stable isotope probing to identify the specific microorganisms responsible for the utilization of a substrate among those present in the complex intestinal ecosystem (235). Two-dimensional polyacrylamide gel electrophoresis of the amplified rRNA genes from a population has also been found to be a high performing technique for the study of complex microbial populations (268) although it has not yet been used to assess gut microbiota composition.

1.3.2 Detection and Enumeration in Dairy Products

Fermented dairy products are considered as one of the most suitable vehicles for the administration of probiotic bacteria. *Lactobacillus* and *Bifidobacterium* species are the most commonly used probiotics, which are often implemented in dairy products in combination with other LAB. In spite of the availability of culture-independent molecular tools for quantification of probiotics in commercial products, most manufacturers still use conventional culture techniques for enumeration purposes. In addition, culture-dependent methods are crucial to determine possible physiological or biochemical changes in the population of probiotic bacteria during the refrigerated storage of the product (329).

From a practical point of view, differential enumeration of probiotic and starter bacteria in food products is rather difficult due to the presence of several closely related species of LAB. The majority of media currently available for the selective enumeration of probiotics and LAB included in dairy products are based on differentiation by colony appearance (223, 300, 307, 321, 328). However, this is not always a stable phenotypic feature and, in addition, it is largely dependent on the subjectivity of each one. Therefore, for a more conclusive identification and enumeration of probiotic bacteria, some selective media for each targeted species have also been developed (326). The disadvantage of these media is that they can underestimate counts of the microbial group selected.

Among the great variety of general, modified, selective, and differential media, only a few of them have proven in comparative studies to be suitable for quantification of a given probiotic species on the basis of their high recovery, and clear differentiation from or inhibition to other LAB also present. In spite of that, authors in general agree that no unique, selective or differential medium provides reliable counts of probiotic bacteria in all dairy products available and the most representative for fermented milks are indicated in Table 1.4.

It is worthy to mention an enzyme-based most probable number (MPN) method for the enumeration of *Bifidobacterium* in dairy products developed by Bibiloni et al. (220). It is based on the selectivity for bifidobacteria of MRS broth containing 0.3% bile and subsequent analysis of the F6PPK activity in grown tubes.

Several studies have been performed using selective and differential media for the correct identification of bacterial species claimed in the product label and for following the viability of probiotics and starter cultures during the refrigerated storage of fermented milks. Probiotics often show poor viability in market preparations (217, 249, 304, 316). Several factors could be involved in affecting the viability of probiotic cultures in fermented milks such as fat content (329), temperature, oxygen content, acidity, pH, and the presence of other LAB, among others (315).

1.3.3 Detection and Enumeration of Specific Probiotics in the Gut

To detect or enumerate a specific probiotic strain among the vast array of microorganisms present in the intestinal environment is often a challenging issue. However, it is essential in order to study the survival in the gut or the colonization ability of probiotic strains. Several different methods, both culture-dependent and culture-independent, have been used to this end (Table 1.5).

Traditionally, culture followed by morphological colony characteristics or strain isolation for genotypic or phenotypic characterization has been used. Nevertheless, this approach shows all the limitations of culture-dependent techniques and if the probiotic strain is outnumbered by similar microorganisms present in the gut, the proper isolation and further identification of the specific strain is difficult to achieve. In some cases antibiotics are used as selective agents in the media. After culture in appropriate media the identity of the isolated strains is confirmed by a highly discriminatory technique such as RAPD (242, 247, 288), ARDRA (224, 297), or REA-PFGE (275, 283). Fluorescent hybridization has also been used for this purpose (283). Antibiotic-resistant variants of the probiotic strains may also be used to allow specific enumeration by using media supplemented with the appropriate antibiotic (242, 302). The combination of selective culture media with monoclonal antibodies has also been applied (346).

PCR primers have been developed for some probiotic strains (222, 243, 318) and then used to confirm colony identity (243, 250, 318, 344) or for direct detection in the samples (250). Molecular biology offers also the possibility to label the strain by transforming it with a plasmid containing a gene marker (238). However, it must be taken into consideration that the use of GMOs may imply certain limitations, especially in the setting of clinical studies.

TABLE 1.4 Media for Viable Cell Counts of Probiotic *Lactobacillus* and *Bifidobacterium* in Fermented Milks Containing the Yogurt Starters *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*

Agar Medium	Basic Medium	Microorganisms Counted	Supplements Added	Type of Medium	Differential Count Based on	Oxygen Conditions for Incubation	References
G-MRS	MRS	*S. thermophilus* *L. bulgaricus* *L. acidophilus* *B. bifidum*	Galactose (carbon source)	Modified	Colony appearance	Anaerobiosis	(317)
T-MRS	MRS	*L. acidophilus*	Trehalose (carbon source)	Modified	No growth of other LAB	Anaerobiosis	(266)
Bile-MRS	MRS	*L. acidophilus*	Bile	Selective	Inhibition of other LAB	Aerobiosis	(266)
MRS-clindamycin	MRS	*L. acidophilus*	Clindamycin	Selective	Inhibition of other LAB	Anaerobiosis	
LC medium	Basic medium	*L. casei* *L. paracasei* *L. rhamnosus*	HCl until pH 5.1 Bromocresol green Ribose	Selective and modified	Inhibition/no growth of other LAB	Anaerobiosis	(304)
MRS-AC	MRS	*L. rhamnosus* *L. paracasei*	Acetic acid until pH 5.2	Selective	Inhibition of other LAB	Anaerobiosis	
NA-salicin	Nutrient agar	*L. acidophilus*	Salicin	Selective/ differential	Inhibition/ colony appearance	Anaerobiosis	(281)
LP-MRS	MRS	*B. bifidum*	Lithium chloride Sodium propionate	Selective	Inhibition of other LAB	Anaerobiosis	(282)

(*continued*)

TABLE 1.4 (*Continued*)

Agar Medium	Basic Medium	Microorganisms Counted	Supplements Added	Type of Medium	Differential Count Based on	Oxygen Conditions for Incubation	References
MRS-NPLN	MRS	*Bifidobacterium* sp.	Neomycin sulfate Paromomycin sulfate Nalidixic acid Lithium chloride	Selective	Inhibition of other LAB	Anaerobiosis	(227)
AMC	Reinforced clostridial medium	*Bifidobacterium* sp.	Nalidixic acid Polymyxin B Iodoacetate 2,3,5-triphenyltetrazolim chloride Lithium propionate	Selective	Inhibition of other LAB	Anaerobiosis	(214)
DP	Columbia agar base	*Bifidobacterium* sp.	Dicloxacillin Propionic acid	Selective	Inhibition of other LAB	Anaerobiosis	(220)
BFM	–	*Bifidobacterium* sp.	Lactulose (carbon source) Propionic acid Methylene blue Lithium chloride	Selective	Inhibition of other LAB	Anaerobiosis	(300)

TABLE 1.5 Methods Used for Detection of Some Probiotic Strains in Human Fecal/Intestinal Samples

Strains	Culture Step	Identification Techniques	References
B. animalis DN173010	Yes	ARDRA	(224)
B. animalis BB-12	Yes	PFGE or fluorescent hybridization	(283)
L. johnsonii La1	Yes	PCR	(243)
L. rhamnosus GG	Yes	PCR	(250)
L. plantarum 299v	Yes	RAPD	(247)
L. gasseri SBT2055SR	Yes	Use of streptomycin-rifampicin resistant mutant and RAPD	(242)
L. casei DN114001	Yes	Use of rifampicin-resistant spontaneous mutant	(302)
L. casei Shirota	Yes	Monoclonal antibodies	(346)
L. paracasei B21060	Yes	ARDRA	(297)
L. paracasei CRL-341	Yes	PFGE	(283)
L. rhamnosus GG	No	PCR	(250)

1.3.4 The Problem of the Viability and Physiological State of Intestinal Bacteria

Microorganisms in different ecological niches, including the GIT and acidic food products, may exist in several physiological states of viability. Traditionally, microorganisms were considered viable if they were capable of multiplying in an appropriate medium, being culture-based methods such as plate counts and MPN counts largely used to enumerate viable cells. However, certain microorganisms, which are readily cultivable can also exist in other states where the cell fails to replicate, but retains some metabolic activities typical of viable cells and may return to be cultivable under certain conditions. This is the case of the so-called viable but uncultivable cells (306), starved cells (301), dormant cells (270), or sublethally injured cells (345). While in pathogenic and environmental bacteria the phenomenon of the different states of viability has received quite a lot of attention, these studies are considerably less developed in probiotics and intestinal bacteria.

Bacteria under stressful conditions may modify their viability maintaining equilibrium between multiplication and survival activities (301). Recently, strains of *Bifidobacterium* used as health-promoting probiotic bacteria, have been shown to become dormant during storage of fermented products (277) or sublethally injured following stress treatment (218). Moreover, Ben-Amor et al. (219) demonstrated a great physiological heterogeneity within separated populations of viable, injured, and dead fecal bacteria.

Methods measuring multiplication as the sole criterion of viability have been extensively used although temporally uncultivable cells fail to be detected. Therefore, other viability assays apart from those based on multiplication in culture media have been developed. For example, using the antibiotic ciprofloxacin as an inhibitor of

cellular division, Barcina et al. (215) used changes occurring in cell morphology and elongation of cells to determine microscopically "direct viable counts." Nevertheless, most currently available methods for measuring the viability are based on the employment of fluorescence techniques that generally use two fluorochromes with different emission wavelengths in combination to discriminate between intact or viable cells, injured or damaged cells, and dead cells. Membrane integrity has been employed as a criterion of viability for intestinal bacteria and probiotics (278–280, 299). The commercial LIVE/DEAD® BacLight™ kit contains two nucleic acid stains: the green fluorochrome SYTO 9 is a small molecule that can penetrate all membranes whereas the larger red fluorochrome propidium iodide can penetrate only compromised membranes, thus rendering cells green when they are viable or red when they are dead. Other criteria that have been used as markers for viability and different cell states of probiotic and intestinal microbiota include the assessment of intracellular esterase activity (279), the maintenance of intracellular pH (279), and quantification of the 16S rRNA (276).

1.3.5 Conclusions

The intestinal microbiota is a complex ecosystem showing great variations among individuals and which is influenced by environmental and physiological factors of the host, making its study difficult. Culture-dependent methodologies have been traditionally used for intestinal microbiota assessment, allowing the isolation of some cultivable intestinal microorganisms for their further characterization. The development of culture-independent methods provides more rapid and accurate tools for the study of complex microbial intestinal populations, which has lead in recent years to a significant increase in our understanding of intestinal microbiota composition and its interaction with the host. Most culture-independent techniques target the rDNA, although the rRNA has also been used instead of rDNA, allowing the identification of the metabolically active microorganisms. Techniques such as the PCR-DGGE/PCR-TGGE and especially DNA microarrays that use the information of genome sequences available, greatly contributed to the study of the qualitative content and the predominant species of the intestinal microbiota. However, they failed to provide reliable data on its quantitative content or on the less abundant groups. Among the quantitative culture-independent methods, FISH has been widely used for the assessment of changes in the levels of predominant and minor components of the intestinal microbiota. However, it is laborious and time consuming, which is limiting its further applicability. Real-time quantitative PCR is becoming very promising for studies of intestinal microbiota composition and when it is targeted to the rRNA also provides a very useful tool for monitoring bacterial activity and gene expression in gastrointestinal conditions. The recent development of metagenomics and metaproteomics allows the study, at the same time, of all the genetic material or the proteins present in a sample. In spite of all that is indicated here, since the GIT is a very stressful environment the possibility that microorganisms may exist in several physiological states that could condition their metabolic activity should be taken into consideration and

investigated further. All the classical and molecular techniques currently available enhance our understanding of microbial ecology in the gut but at the same time have evidenced that our current knowledge of intestinal microbiota composition and interactions with the host is still limited.

1.4 ENTERIC MICROBIAL COMMUNITY PROFILING IN GASTROINTESTINAL TRACT BY TERMINAL-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP)

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Traditional culture-dependent methods of analyzing complex microbial communities such as those found in the GIT of all life forms have been limited because of cultural bias when selective culture medium is used for bacteria isolations. This is almost always associated with the tedium of having to conduct colony enumerations followed by characterization and identification based on metabolic chemistries. Since the primary objective in many community studies is aimed at understanding the diversity and richness of bacteria species that have colonized various niche compartments in the GIT, culture-independent methods of enumeration with minimal cultural bias would be desirable, especially if these methods also enabled identification of OTU or phylotypes. With a rapidly burgeoning database of 16S rDNA sequences as well as a suite of software tools to query alignment homologies and primer design, molecular PCR-based techniques such as denaturing/thermal gradient gel electrophoresis (DGGE/TGGE) (369)), single-stranded site conformational polymorphism (SSCP) (357), and terminal-restriction fragment length polymorphism (T-RFLP) (366) are becoming viable alternative tools for dissecting and analyzing complex microbial communities.

1.4.1 T-RFLP

T-RFLP is a quantitative molecular technique for the analysis of microbial communities and is based on the use of common or universal primers where one of them (usually the forward or *f*-primer) has been fluorescent labeled at the 5′ end (364) with a DNA dye. PCR products amplified in this way from source DNA are then subjected to carefully selected restriction enzyme (usually 4-base cutters) digestion. DNA fragments or digestion products from generated amplicons representing various OTUs are identified by variations in the length of the fluorescent and terminally labeled restriction fragments (*T*RFs). The entire mix of *T*RFs is analyzed by sequencing capillary electrophoresis. Only fluorescent peaks are visualized and profiled based on the length of the nucleotide sequence. Restriction fragments (RF) that are not terminally labeled by the fluorescent primer remain as invisible debris.

1.4.2 Universal and Group-Specific Primers

A review of recent publications on T-RFLP describes more than 95% of citations base microbial diversity analysis on polymorphisms around the small subunit-rRNA gene (SSU 16S rDNA) sequence. The ribosomal database project (RDP) (365) has available an unaligned SSU rDNA sequences of 14,870 nucleotides from which primer sequences can be designed depending upon degeneracy or conservation. The first key requirement of T-RFLP is the selection of "universal" primers to amplify the targeted region of 16S rRNA that is representative of the domain *Bacteria*. The most commonly used universal primer is the 8f-926r domain primer pair proposed by the original developers of T-RFLP (364). Depending upon the needs of the investigator, primer pairs can be universal but relatively specific such as the detection of *Bacteroides/ Prevotella* group in feces (352) or pathogens in prosthetic joints (372). Table 1.6 provides a list of some of the more commonly used primer/probes in T-RFLP. It is important that various applications require more rigorous scrutiny of the universality of universal primers as different combinations of primer pairs can provide quite different levels of diversity coverage for Gram-positive and -negative lineages to division level (358). With time, as the microbial genome database is built from the gold standard approach of assessing sequence polymorphisms and phylogenetic diversity via clone libraries and high-throughput sequencing, it can be anticipated that intergenic spacer (IGS) (371) or internal transcribed spacer (ITS) (356) regions between or within ribosomal operons will be deployed in community profiling analyses. Intending users of T-RFLP are advised to consult the T-RFLP analysis program (TAP) located at the RDP website (367) for guidance in primer designing.

1.4.3 Fluorescent Dyes

The most common fluorophore used in terminal labeling of the forward primer is the blue dye FAM (http://docs.appliedbiosystems.com/pebiodocs/00115046.pdf). It is theoretically possible to use a number of other colored dyes such as HEX, VIC, JOE, and TET (green); TAMRA, NED (yellow); ROX, PET (red), and LIZ (orange). It is possible to increase the interrogative potential of T-RFLP by combining various universal primer pairs in a multiplex PCR (MPX). In this case, different dyes have been tagged to the forward primer of each universal primer pair or different color combinations for different forward primer pairs. We have observed an effect of dyes in shifting the *T*RF size of OTUs when the same universal primer is being evaluated in different dye configurations. The reason for testing different dyes is the potential to combine one-colored universal primer pair with a differently colored group specific primer pair or various other combinations thereof in a MPX reaction. The resolution is dependent on certain dye combinations for each MPX and great care must be taken to optimize the PCR reaction for each specific application to ensure that artifacts are not generated in the TRF profiles. Despite this limitation, the added versatility of different colored TRFs generated from the use of dual-labeled forward and reverse primers increases the complexity of analysis as there will now be two sets of terminally labeled restriction fragments. In this case, one color can provide matching confirmation or not

TABLE 1.6 List of Universal and Specific Primer/Probes Used in T-RFLP[a]

Application (Reference)	16S rDNA Position	Primer or Probe	5′–3′ Sequence
Domain primers bacteria (364)		8f	*f*-AGAGTTTGATCCTGGCTCAG
		926r	*r*-CCGTCAATTCCTTTRAGTTT
Domain primers archaea (355)		Ar109F	*f*- ACKGCTCAGTAACACGT
		Ar912R	*r*- CTCCCCCGCCAATTCCTTTA
Environmental bacteria (379)	349–365	Bac349F	*f*-AGGCAGCAGTDRGGAAT
	787–806	Bac806R	*r*-GGACTACYVGGGTATCTAAT
	516–540	Bac516F	*p*-TGCCAGCAGCCGCGGTAATACRDAG
Intestinal microflora (375)	46–65	ANA1F	*f*-GCCTAACACATGCAAGTCGA
	518–536	K2R	*r*-GTATTACCGCGGCTGCTGG
Human feces (377)	516–532	516f	*f*-TGCCAGCAGCCGCGGTA
	1510–1492	1510r	*r*-GGTTACCTTGTTACGACTT
Dental bacteria (376)	331–349		*f*-TCCTACGGGAGGCAGCAGT
	772–797		*r*-GGACTACCAGGGTATCTAATCCTGTT
	506–528		*p*-CGTATTACCGCGGCTGCTGGCAC
Eubacteria (378)		27F	*f*–AGAGTTTGATCCTGGCTCAG
		1492R	*r*-GGTTACCTTGTTACGACTT
This article universal primer		7f	*f*- AGAGTTTGAT(C/T)(A/C)TGGCTCAG
		1510r	*r*- ACGG(C/T)TACCTTGTTACGACTT
This article LAB group specific *r*-primer		7f	*f*- AGAGTTTGAT(C/T)(A/C)TGGCTCAG
		LbLMA1*r*	*r*- CTCAAAACTAAACAAAGTTTC

[a] Modified from Horz (358).

of community diversity and richness by the other. By using different restriction enzymes, an entire series of profiles can be generated from a simple reaction. Even more complex diversity analysis will become feasible if current T-RFLP protocols can be overlaid with MPX-enabled fluorescent primer pairs for IGS or ITS. These strategies will offset the limitation that more than one OTU may be associated with each *T*RF (368).

1.4.4 DNA Extraction

Various protocols have been used to extract total community DNA. Intestinal washings and fecal suspensions are frequently particulate in texture and can be further disrupted by homogenization in stomacher bags in tryptone–salt solution. Aliquots can be removed at this stage for culture-dependent enumeration. Otherwise, the uniformly dispersed suspension is pelleted by centrifugation, resuspended, and washed in buffer or water, and the bacteria lysed mechanically in the presence of glass or ceramic beads with a bead beater (374). Mechanical disruption of DNA should be limited to 30–60 s to avoid excessive DNA shearing (363). Final selection for DNA extraction methods should be decided upon by trialing different protocols dependent upon the samples undergoing evaluation (363). Washed bacteria cell pellets can also be ground in a mortar/pestle in the presence of 4 M guanidine thiocyanate–150 mM Tris-HCl (pH 7.5)–1% *N*-lauroyl-sarcosine; de-proteinized in phenol–chloroform and precipitated with isopropanol. Occasionally, substances present in feces can be inhibitory to PCR polymerase. Further purification of DNA is then required and an excellent protocol involves the use of benzyl chloride – sodium dodecyl sulfate (373). Genomic DNA is then captured on a membrane in spin column format, and eluted and rehydrated for PCR analysis. Alternatively a number of commercial kits such as UltraClean soil DNA isolation kit by MO BIO Laboratories (354, 359, 360) and Fast DNA kit by QBiogene (363) can be used. The concentration (μg/mL) of purified genomic DNA can be determined by spectrometry as follows: $[-36(A_{280}–A_{320})] + [62.9(A_{260}–A_{320})]$ and its integrity evaluated by agarose gel electrophoresis.

1.4.5 PCR Amplification

Conditions of PCR amplification vary with different applications. In particular, the number of amplification cycles must be optimized to reduce artifacts such as the formation of chimeric amplicons. This bias can be minimized by limiting the number of PCR cycles to 20–35 (370). The pooling of multiple PCR reactions from a single sample can also ensure the minimization of random artifacts (361). With some samples, the presence of "interfering" agents that inhibit PCR reactions can be eliminated by the addition of aluminum ammonium bisulfate (353).

1.4.6 Generation of Terminal Restriction Fragments (TRF) by Digestion of Amplicons with Restriction Enzymes

The selection of restriction enzyme is very important to generate fluorescent-labeled *T*RFs following community DNA amplification with universal primers. Most T-RFLP

applications utilize 4-base cutters and depending upon the GC content of the community, restriction enzymes like CfoI (GCG/C); HaeIII (GG/CC); HpaII and MspI (C/CGG) are commonly used for GC-rich communities while others such as AfaI or RsaI (GT/AC) AluI (AG/CT) and MseI (T/TAA) can provide phylotype discrimination for less GC-rich communities. A number of websites are available without subscription fees to assess what restriction enzyme to use against different universal primers under evaluation. One of these, microbial community analysis III or MiCA3 can be accessed at http://mica.ibest.uidaho.edu/trflp.php. *In silico* modeling of *T*RFs can be quite useful in trying to establish actual and theoretical phylotype diversity. On a precautionary note, secondary *T*RF can be a complicating artifact, generating "pseudo" T-RFLP peaks. These can be eliminated by digestion of amplicons with single-strand-specific mung bean nuclease prior to analysis of *T*RF (355).

1.4.7 Software and Data Processing

*T*RFs can be resolved by capillary electrophoresis using systems such as the Applied Biosystems 3730 DNA Analyzer. The 48-capillary analyzer is fitted with argon–ion multi-line, single-mode laser with primary excitation lines of 488 and 514.5 nm. The machine utilizes in-capillary detection by dual-side illumination. Multiple filter sets allow for the reading of five dyes in a single run. Community profiles can be further processed using the STRand software developed by the Davis' Veterinary Genetics Lab at University of California. This software is freeware available at http://www.vgl.ucdavis.edu/informatics/STRand/. Sequencer data files generated by ABI 3730; ABI 377, ABI 373, and MJ GeneSys Base Station are supported by this software. Readers interested in statistical methods for processing and comparing *T*RF data sets including binning, clustering, and statistical analysis should refer to Abdo (351).

1.4.8 Microbial Diversity in Different Intestinal Compartments of Pigs

To illustrate the enteric microbial community profiling capabilities of T-RFLP, we show in Fig. 1.4 the distribution of phylotypes based on *T*RFs in different gastrointestinal compartments of subclinical pigs (see Section 5.4.3). Amplicons were generated using the universal primers 7*f* and 1510*r* (Table 1.6). Two restriction enzymes—Hha1 and MspI—were used on different aliquots of the same PCR reaction. In profiles (Fig. 1.4, Table 1.7) representing the duodenal contents, HhaI resolved a very strong *E. coli* peak (*T*RF 370, >2000 FI) while this is not visible in Msp1 digests. Clostridium and Corynebacterium complex are seen as smaller peaks at *T*RF 231 and 358 respectively in HhaI digests while the second most prominent peak in MspI digests is represented by Campylobacter species. Both restriction enzymes generate increased richness of phylotypes in the small intestine (ileum) but the relative abundance in terms of fluorescence intensity (FI) is decreased by about fourfold compared to the duodenum or colon (500 FI vs. 2000 FI). In general, MspI produced a richer OTU profile in the cecum and colon compared to HhaI. These results highlight the importance of using different restriction enzymes to generate more information about community diversity. If this were combined with the use of universal primers and group-specific primers, then it would be possible to mine a plethora of information on

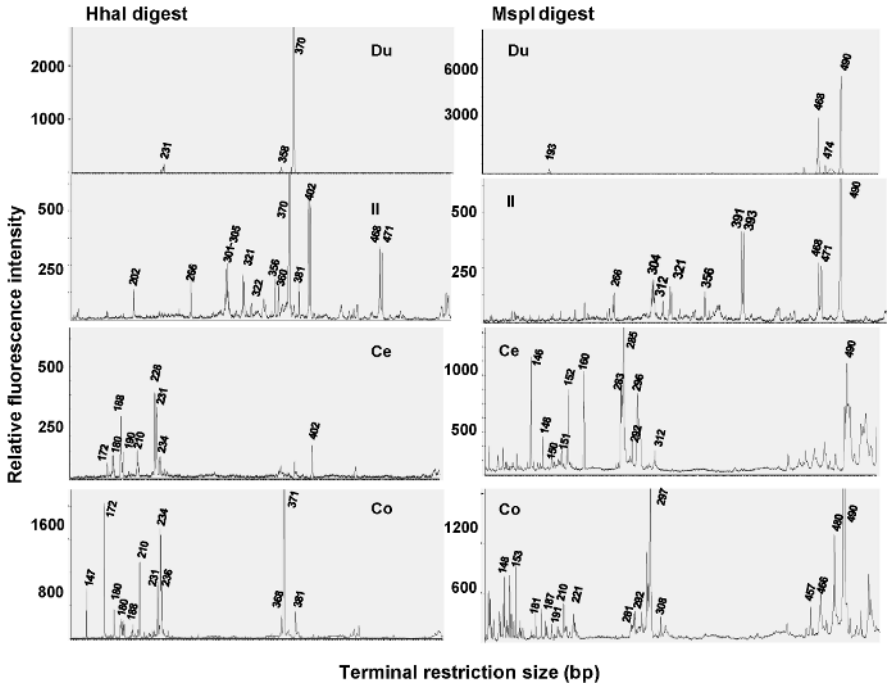

FIGURE 1.4 Enteric microbial community profiles of genomic DNA extracted from different intestinal compartments of pig. HhaI and MspI digests are profiled on the left and right columns respectively, showing profiles from duodenum (Du), ileum (Il), caecum (Ce), and colon (Co) from top to bottom.

the microbial community. We have found the EMCoP profiles very reproducible within assays and between animals in any one treatment group. This enables temporal comparisons to be conducted with treatment protocols such as growth promotants, dietary changes, and therapeutic antibiotics.

1.4.9 Tracking the Fate of Orally Delivered Probiotics in Feces

In Chapter 5, Section 5.4.3, we described a LAB formulation consisting of *L. salivarius, L. casei, L. plantarum*, and *L. acidophilus* that had been developed specifically to target enterotoxigenic *Escherichia coli* (ETECs) strains responsible for neonatal and postweaning diarrhea in pigs. In one on-farm trial, this LAB formulation (ColiGuard) was administered (10^9 cfu/g weaner mash) to newly weaned pigs for 10 days. At this time, rectal swabs or feces can be taken from control and probiotic-supplemented pigs, DNA extracted, and T-RFLP analysis carried out with VIC-LAB-specific group primer pairs 7*f* and LbLMA1*r* (see Table 1.6). Under normalized PCR conditions, the profiles (Fig. 1.5) of pigs in the control group show the presence of only *L. acidophilus* at a comparatively low intensity (738 FI). After 10 days of ColiGuard,

TABLE 1.7 List of Operational Taxonomic Unit Identities Based on Terminally Labeled Restriction Fragments (*T*RF) Generated Following HhaI (A) or MspI (B) Restriction Digestion of Amplicons

Fragment Size (bp)	Organism Name
2A HhaI Digest	
147	*Fibrobacter intestinalis*
172	*Enterococcus saccharolyticus*
180	*Bifidobacterium thermophilum*
188	*Eubacterium hallii*
190	*Clostridium symbiosum, Clostridium clostridiiforme*
202	*Eubacterium ruminantium*
210	*Fusobacterium simiae*
231	*Clostridium botulinum, Clostridium scatologenes, Clostridium tetani, Clostridium sporogenes, Clostridium collagenovoransm, Clostridium ljungdahlii, Clostridium algidicarnis*
234	*Lactobacillus* sp.
236	*Clostridium subterminal, Clostridium* sp.
266	*Lactobacillus mucosae*
301–305	*Flexibacter filiformis*
321–322	Unidentified
356	*Corynebacterium variabilis*
358	*Corynebacterium genitalium, Corynebacterium pseudogenitalium*
360	*Clostridium tetanomorphum*
368	*Desulfovibrio desulfuricans*
370	*E. coli*
381	*Desulfotomaculum thermosapovorans*
402	*Lactobacillus fermentum* *Lactobacillus reuteri*
468	*Streptomyces tendae, Streptomyces diastatochromogenes, Streptomyces bottropensis, Streptomyces scabiei, Streptomyces coelicolor, Streptomyces ambofaciens, Streptomyces ornatus, Streptomyces nodosus, Streptomyces caelestis*
471	*Streptomyces bluensis, Streptomyces mashuensis, Streptomyces vellosus*
2B MspI Digest	
132	*Fibrobacter intestinalis*
134	*Desulfovibrio gigas*
141	*Eubacterium dolichum*
148	*Bacillus badius,* Bacillus *firmus*
150	*Fusobacterium russii*
151	*Fusobacterium varium*
152	*Fusobacterium moriferum, Fusobacterium gonidiaformans*

(*continued*)

TABLE 1.7 (*Continued*)

Fragment Size (bp)	Organism Name
153	*Bacillus macroides,* Bacillus *benzoevorans,* Bacillus *sphaericus,* Bacillus *fusiformis*
160	*Desulfotoaculum halophilum*
181	*Lactobacillus gallinarum*
187	*Leptotrichia* sp.
191	*Lactobacillus gasseri, Lactobacillus crispatus*
193	*Clostridium irregularis, Clostridium bifermentans*
210	*Desulfotomaculum thermobenzoicum*
221	*Eubacterium hallii, Eubacterium barkeri, Eubacterium limosum*
266	*Clostridium filamentosum*
281	*Fusobacterium simiae*
282	*Ruminococcus flavefaciens*
283	*R. flavefaciens*
285	*Bifidobacterium inopinatum*
292	*Clostridium ramosum*
296	*Eubacterium desmolans, Eubacterium yurii* subsp.
304	*Eubacterium* sp.
308	*Eubacterium* sp.
312	*Butryvibrio fibrisolvens*
321	*B. fibrisolvens*
356, 391, and 393	Unidentified
468	*Camplyobacter* sp., *Camplyobacter showae, Camplyobacter concisus, Camplyobacter rectus*
471	*Camplyobacter* sp.
474	*Peptostreptococcus anaerobius*
480	*Flexibacter flexilis*
490	*E. coli*

the probiotic-treated group shows significant increases in the *L. acidophilus*, *L. casei*, and *L. plantarum* peaks (note log increase in FI scale). *L. acidophilus* is located at position TRF247 and has an increased FI of 29.3-fold relative to nonprobiotic supplemented pigs. Due to redundancy in the 16S rDNA sequence for the LAB primer pair, *L. plantarum* and *L. casei* are phylotyped at positions TRF 327 and 328 respectively and therefore appear as two very close proximity peaks with fluorescent intensities of 8061 and 32,088 respectively. Since each animal would consume on the average about 300 g of feed per day, one would expect delivery of about 7.5×10^{11} cfu of each of the four LAB strains per day. This would contribute to the significant increases observed in FI of all LAB phylotypes with the possible exception of *L. salivarius* (projected location at TRF 279 and 281). Pending further analysis, the fate of *L. salivarius* in the GIT of probiotic-treated pigs can only be speculated at this point. It may be that *L. salivarius* is a very efficient colonizer with most community members domiciled in as yet undefined intestinal compartments. Alternatively,

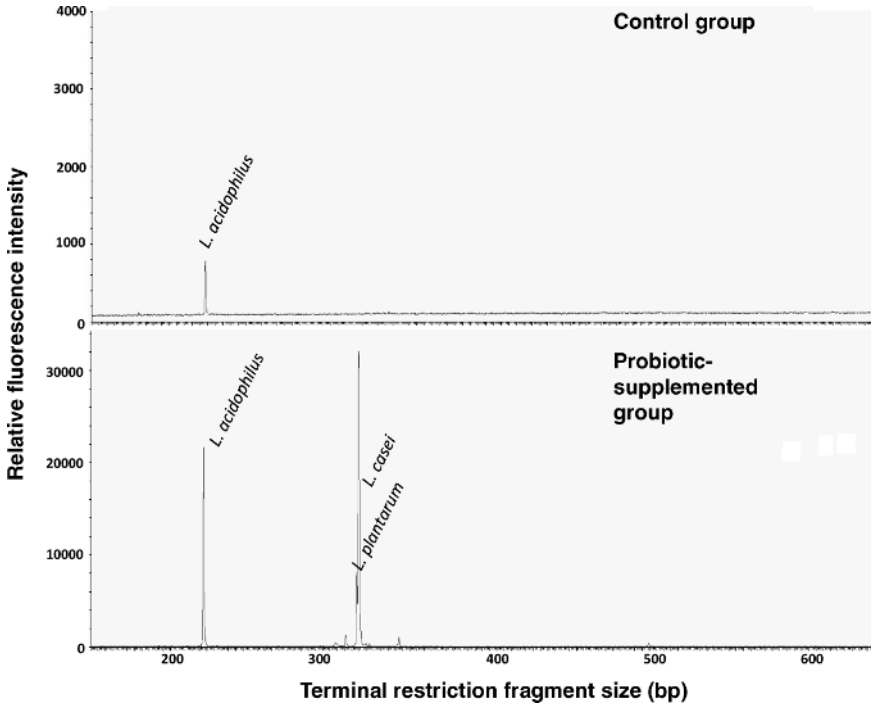

FIGURE 1.5 EMCoP profile of LAB in DNA extracted from the feces of pigs treated with ColiGuard probiotic and nonprobiotic-supplemented animals (control).

L. salivarius may have limited viability and may not be as competitive as other strains, dying off rapidly after it has delivered its impact in altering the gene signatures of ETECs in the GIT of subclinical pigs (Section 5.5.2). In any event, this example clearly documents the usefulness of T-RFLP in tracking the LAB community and if combined with time course sampling, will provide a temporal estimate of persistence of orally delivered probiotics.

1.4.10 Conclusion

Even though TRFLP was first developed for the analysis of community diversity in soil and environmental samples, its adaptation to gut microflora has provided a strong impetus in its use as a tool to unmask the impact of pre- and probiotics in the GIT. It is a far simpler procedure to use than DGGE, highly reproducible, amenable to fine-tuning at the level of primer design, and restriction fragment generation. T-RFLP also does not suffer from the disadvantage of longer primer sequences needed to design GC clamps for the DGGE procedure that can in turn cause artifacts during the annealing step; as well as the production of heteroduplexes that are innately unstable under the denaturing conditions of a DGGE run (362). We anticipate that T-RFLP will become a very important analytical protocol in enteric microbial community profiling.

Acknowledgments

The authors acknowledge Jannine Patterson who established the T-RFLP protocol in the IMDRU laboratory and Kent Wu for contributions of group-specific LAB T-RFLP. The expertise and input of Bernadette Turner merits special mention for technical work well done. This program was funded substantively by a Commonwealth Research and Development Start Grant awarded to International Animal Health (C. Lawlor and K. Healey) in conjunction with CSIRO and NSW DPI. ColiGuard® probiotic strains were provided by International Animal Health, Sydney, Australia.

1.5 EFFECTIVE DOSAGE FOR PROBIOTIC EFFECTS

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Among the human clinical trials cited in Section 4.3 (Effects on human health and diseases), the probiotics were administered in great variation in accordance to the following:

- Type of probiotics (lactobacilli, bifidobacteria, yeasts, enterococci);
- Daily dose (10^7–10^{10} cfu);
- Daily frequency of administration (1–4 times);
- Timing of administration (before, during, and after meal);
- Duration of administration (1 day to several months);
- Method of delivery (fermented food, beverage, capsule, tablet, or powder);
- Viability.

To achieve probiotic effects, the probiotics and their products need to be delivered to the desired gastrointestinal site in sufficient quantity. The importance of viability depends on the mechanism of the probiotic effect and each probiotic bacteria needs to be evaluated respectively. The method of delivery appears to have minimal effect on probiotic efficacy, as different preparations of the same dose were reported to achieve the same preventive or therapeutic efficacy. An example is the treatment of diarrhea (Section 4.3.3). The duration of administration would depend largely on the needs and nature of the diseases; for example treatment for diarrhea is short term whereas cancer prevention is of longer term.

There is no information as to when is the best time to administer probiotic preparation. It is logical to assume that probiotics administered orally before meal should have the capability to tolerate the extreme pH condition and digestive enzymes and bile present in the intestinal tract. Probiotics taken together with meal would be diluted by food materials, which could reduce the chances and frequency of physical encounter between the probiotic organisms and the mucosal receptors. Moreover, food

matrix may compete with mucosal receptors for probiotic and product binding. Hence it is reasonable to assume that the best period for the administration of probiotics is between meals, and be carried in liquid media.

It is not so clear if the frequency of administration has any effect on probiotic efficacy. Microbiologically, 1×10^{10} cfu administered four times daily has little different from 4×10^{10} cfu administered once a day. It could nevertheless be assumed that a probiotic strain, which does not adhere well on the mucosal receptors and is unable to colonize temporarily would need to be administered more frequently. And probiotic strains that are denatured readily by the gastrointestinal conditions should be administered in larger dosage to counter the wastage.

This leaves us with the remaining factors of dosage and strain of probiotics. How much a probiotic needs to be consumed to achieve the probiotic effects is crucial information in the formulation of probiotic functional foods as well as the therapeutic products. So far, there is no systematic study on the effective dosage of the respective probiotics for specific applications. We could nevertheless obtain a glimpse from the human clinical studies available. The dose effect of probiotics on specific disease also shed light on the mechanisms of probiotic effects and the interaction between probiotic organisms and the host.

1.5.1 Acute (Rotavirus) Diarrhea in Children

Probiotics used as adjunctive therapy appeared to improve the treatment of acute diarrhea in 18 out of the 23 clinical trials conducted among children (Section 4.3.3.1). The trials involved more than 1800 children. When the mean days of reduction in diarrhea in the probiotic group in comparison to that of the placebo group were plotted against the dosage of the respective probiotic administered per day, no direct correlation between the treatment efficacy and the dosage could be recognized, as shown in Fig. 1.6. No direct correlation was observed even among the same probiotic of different dosage.

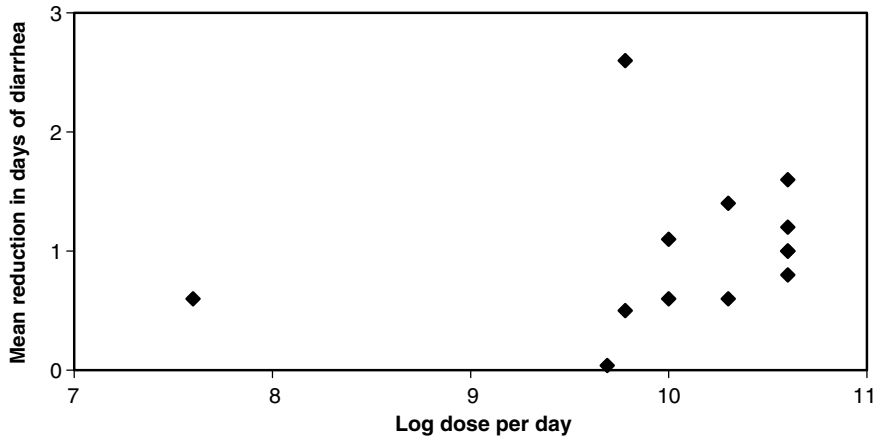

FIGURE 1.6 The efficacy of daily probiotic dose on the recovery from acute diarrhea among children. The data were extracted from Section 4.3.3.1.

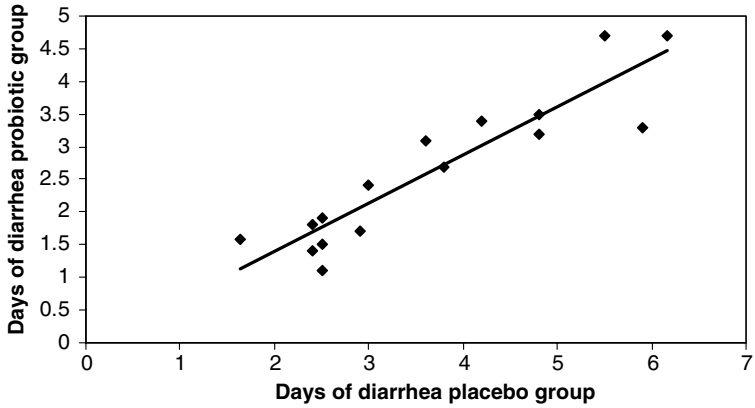

FIGURE 1.7 Correlation between days of diarrhea in probiotic group and corresponding placebo group among children suffering from acute diarrhea. The data were extracted from Section 4.3.3.1.

When the days of diarrhea in the probiotic group was plotted against the corresponding placebo group, a linear correlation was obtained (Fig. 1.7) with a slope of 0.78, which intercepts the origin. This implies that a constant reduction of 22% in the number of days of diarrhea was achieved through the consumption of all the probiotics tested, namely *L. acidophilus, L. bulgaricus, L. reuteri, L. rhamnosus, Streptococcus thermophilus, B. infantis*, and *Saccharomyces boulardii,* applied singly or in various combination. The efficacy was the same with daily dose of 4×10^7 to 6×10^{10} cfu, suggesting that the probiotics have a common mechanism in relieving acute diarrhea, which contributes to 22% of the cure of diarrhea. It can be concluded that probiotics could assist in the speedy recovery of acute diarrhea among children but are not able to prevent and cure diarrhea.

1.5.2 Antibiotic-Associated Diarrhea

Probiotics were widely reported to reduce the incidence of antibiotic-associated diarrhea (Section 4.3.3.2). The probiotics were used singly (*L. rhamnosus GG, Saccharomyces boulardii*) or in combination (*L. acidophilus* + bifidobacteria or *Streptococcus thermophilus*).

1.5.2.1 Combination of L. acidophilus + bifidobacteria or Streptococcus thermophilus When the probiotics were used in combination involving 194 subjects, a 0.5 risk ratio was achieved at a daily dose of 6×10^8 cfu (Fig. 1.8A). The risk factor of the combined probiotics decreased with the daily dose in a hyperbolic manner to reach a value of 0.3 in antibiotic-associated diarrhea at a daily dose of 1×10^{11} cfu. The interaction could be described as the competition for specific receptor binding between the probiotics, pathogens and the host surface (380).

Let us assume that (a) diarrhea is caused by the adhesion of diarrheic microbes on the intestinal surface and the biochemical reactions initiated, (b) the protective effect

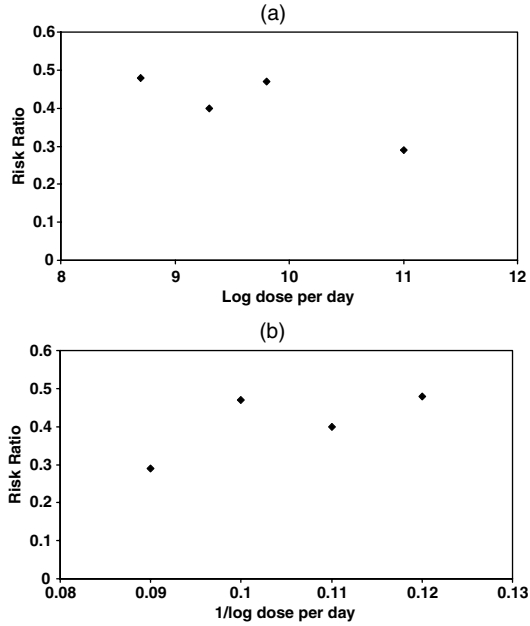

FIGURE 1.8 Dose-dependent efficacy of combination of *L. acidophilus* + bifidobacteria or *Streptococcus thermophilus* in the prevention of antibiotic-associated diarrhea. The data were extracted from Section 4.3.3.2.

of probiotic bacteria is due to the competition for binding onto intestinal surface and it is a simple dissociation process:

$$\text{Microbial cell} + \text{Intestinal cell} \underset{K-1}{\overset{K+1}{\rightleftharpoons}} \text{Microbe}-\text{Intestinal Cell Complex}$$

Where $K+1$ and $K-1$ represents the association and dissociation constant of the reaction, respectively. The process is similar to the interaction between a substrate and the receptor on an enzyme that forms a substrate–enzyme complex, but without the subsequent formation of products.

There are three assumptions in the relationship:

1. The interaction between the microbial cells and the intestinal cell surface receptor remains in equilibrium. This condition should be achieved if the microbial cells do not penetrate the intestinal cells.
2. Microbial concentration remained essentially unchanged throughout the clinical studies, so that the concentrations of the microbial cells can be considered equal to the initial microbial concentrations. This condition could be achieved when the total number of microbial cells present is much greater than the number of microbial cells adhering to the intestinal surface. This is probably the case, where the concentration of the probiotic bacterial cells consumed is

usually in the range of 10^5–10^8 per mL, whereas the number of bacterial cells adhering to the intestinal cells is fewer than 10 per cell.

3. In the simple dissociation equation described above, if X is the concentration of the microbial cell suspension, e is the epithelial cell or mucus concentration, and e_x is the concentration of the microbe–intestinal cell–mucus complex, then the concentration of free epithelial cells or mucus will be $(e - e_x)$.

Since the process is in equilibrium, the dissociation constant for the process (K_x) can be defined as

$$K_x = \frac{K-1}{K+1} = (e-e_x)\frac{X}{e_x}.$$

This equation can be rearranged to give an expression for the concentration of the microbe–intestinal cell–mucus complex,

$$e_x = \frac{eX}{K_x+X}.$$

When X is very much larger than K_x, the intestinal cells or mucus is saturated with microbial cells (i.e., e_x approaches e), and the maximum value of e_x, e_m is obtained. As it is technically easier to estimate the maximum concentration of adhered microbial cells (e_m) than the epithelial cells/mucus concentration (e), the equation could thus be re-written as

$$e_x = \frac{e_m X}{K_x+X} \quad . \tag{1.1}$$

The equation could be further re-arranged to give a linear relationship,

$$\frac{1}{e_x} = \frac{1}{e_m} + \frac{K_x}{e_m X}. \tag{1.2}$$

The values of e_x and K_x are independent of each other. That is, a microbe that adheres on intestinal surface in large number could have a low affinity for the intestinal surface receptors and vice versa.

In the case where the probiotic bacteria and the diarrheic microbes are present at the same time and compete for the same receptors on the intestinal surface, the competition for adhesion of each of the microorganisms is determined by the affinity of the competing organisms to the intestinal surface (K_x) and the concentration of the microbial cells (X). Thus, the ratio of e_x for probiotic bacteria (p) and diarrheic microbes (d) in the mixed microbial system can be described as

$$\frac{e_{xp}}{e_{xd}} = \frac{e_{mp}}{e_{md}}\frac{X_p}{X_d}\frac{K_{xd}+X_d}{K_{xp}+X_p}. \tag{1.3}$$

The relationship in Equation 1.3 above suggests that the outcome of competition between two microorganisms for adhesion on the same receptors on intestinal surface is determined by the ratio of the respective microbial concentrations around the receptors and the affinity of the respective microbes for the receptors.

If the concentrations of the diarrheic microbes were of comparable magnitude in the clinical trials reported, the values of e_{xd}, (e_{mp}/e_{md}), X_d, K_{xd}, K_{xp} were constant values.

$$\frac{e_{xd}}{e_{xp}} = \frac{1}{e_{mp}/e_{md}} \frac{1}{X_d} (K_{xd}+X_d) \frac{K_{xp}+X_p}{X_p} \frac{e_{xd}}{e_{xp}} = \frac{K' K_{xp}}{X_p+K'}. \quad (1.4)$$

Since e_{xd}/e_{xp} determines the risk ratio in the clinical studies, the plot of the risk ratio versus the 1/(daily dose of probiotic, X_p) should yield a linear relationship as shown in Fig. 1.8B. The data points in Fig. 1.8B are too few to obtain a statistically meaningful linear plot. Nevertheless the dosage where risk ratio is 1 (total prevention of diarrhea) is estimated at 10^{13} probiotic bacteria per day.

1.5.2.2 L. rhamnosus GG* or *Saccharomyces boulardii Applied Singly In the cases of *L. rhamnosus* GG involving 281 subjects or *S. boulardii* involving 888 subjects, applied singly, near 90% of preventive efficacy was achieved at a daily dose of about 1×10^{10} cfu (Fig. 1.9). At above a daily dose of 1×10^{10} cfu, the diarrhea-preventing efficacy dropped precipitously. Negative corporative effect, where the binding of a probiotic on the mucosal surface receptor resulted in a reduction in affinity for the subsequent bacterial binding could have occurred (380). It is a demonstration that maximal probiotic efficacy is achieved at the optimal probiotic dose, which needs to be determined for respective probiotic strains.

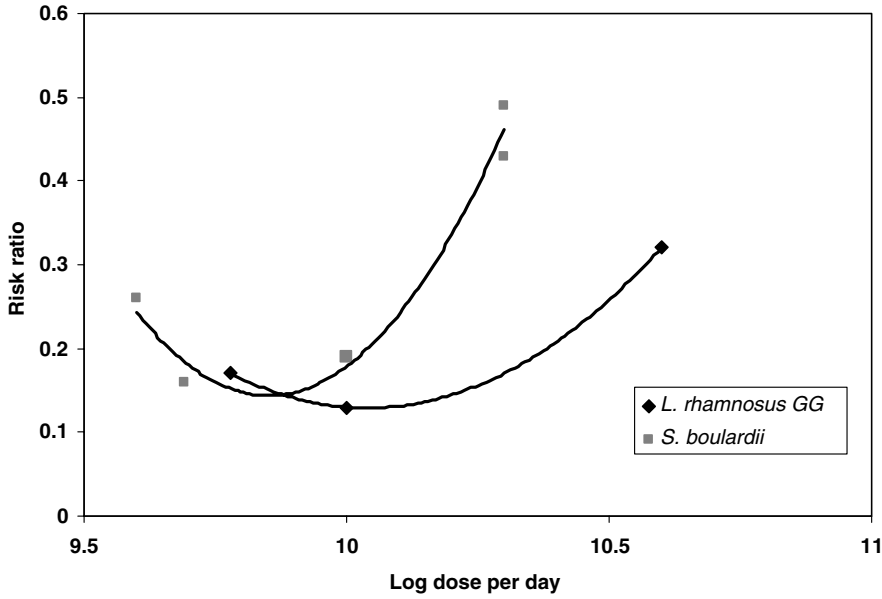

FIGURE 1.9 Dose-dependent efficacy of *L. rhamnosus* GG and *S. boulardii* in the prevention of antibiotic-associated diarrhea. The data were extracted from Section 4.3.3.2.

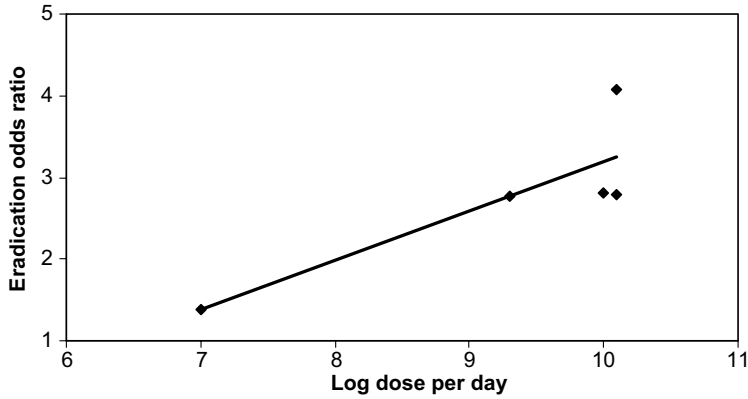

FIGURE 1.10 Dose-dependent efficacy of probiotics (*L. acidophilus, L. casei, L. rhamnosus, Propionibacterium freudenreichii, B. breve, B. animalis* singly or in combination) in enhancing the eradication of *H. pyroli* during antibiotic treatment. The data were extracted from Section 4.3.3.2.

1.5.3 *Helicobacter pyroli*

Some probiotics have been demonstrated to enhance the eradication of *H. pyroli* during antibiotic treatment and reduce the occurrence of side effects. The eradication efficacy involving 478 subjects was dose dependent (Fig. 1.10), and the minimal effective dose is estimated to be about 5×10^6 cfu per day, when the graph in Fig. 1.10 intercepts the eradication odds ratio of 1.

1.6 INCORPORATING PROBIOTICS INTO FOODS

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Food Science Australia, Australia

Probiotics have for decades been used in fermented dairy products such as yogurts and fermented milks. The techniques and technologies to incorporate these organisms into fresh, refrigerated dairy products are now relatively mature. The continuing emergence of clinical evidence for benefits to consumers and the subsequent marketing power these ingredients bring have now seen probiotics become the fastest growing category of functional food ingredients (458). Food companies worldwide are seeking ways to incorporate these ingredients into a much broader range of foods and beverages. However, incorporating live probiotic microorganisms into foods and then keeping them alive throughout shelf life is a significant challenge for food technologists. Indeed, it is an anathema to usual food-processing methods and matrices that have always been designed to minimize the survival of microorganisms with food safety considerations foremost in mind.

Although the need for probiotic viability for some health impacts, such as immunomodulation (390, 402), may not require the bacteria to be alive, viability may still be an essential property of probiotics for some health effects (421). Indeed, probiotics remain defined as *live* microorganisms that when administered in adequate amounts confer a health benefit on the host (407). Viability (in fact, more accurately defined as cultivability) is in reality only a convenient surrogate marker of probiotic activity. However, since the ability of probiotic organisms to impart benefits to the health of the host are usually only quantifiable through animal or clinical studies, viability remains the only really practical quality assurance measure for probiotics. Health benefits have usually been attributed in clinical studies to doses of probiotics in excess of 10^8–0^9 viable cells per day (427). Therefore, food regulatory/advisory bodies generally stipulate that foods containing probiotic organisms need to have $>10^6$–10^7 cfu/g at the time of consumption (e.g., International Standard of Fédération Internationale de Laiterie/International Dairy Federation (403)).

The viable count of probiotic organisms generally declines during product storage (10–100-fold or more) (433). An acceptable viable count can sometimes be achieved by introducing higher numbers of probiotics during manufacture (called overage). The consumption of probiotic organisms at high doses is safe (440, 452, 463), and so oversupplying consumers does not appear to pose a health risk. However, in practice the addition of considerable overage can be an expensive proposition given the relatively high cost per weight of probiotic cultures as ingredients. There may also be organoleptic limitations to the amount of a probiotic that can be acceptably added to foods. Therefore, there is a strong imperative to maintain the viability of probiotics in foods during production and shelf storage.

Overall, there are five main points to address when incorporating probiotics into foods:

1. Select a compatible probiotic strain/food type combination.
2. Use food-processing conditions that are compatible with probiotic survival.
3. If fermentation is required, ensure that the food matrix will support probiotic growth.
4. Select a product matrix, packaging, and environmental conditions to ensure adequate probiotic survival over the product's supply chain and during shelf storage.
5. Ensure that addition of the probiotic does not adversely impact on the taste and texture of the product.

This chapter summarizes the main parameters that affect probiotic survival during manufacture and storage of foods and provides examples of successful incorporation of probiotics into a range of shelf-stable foods. It aims to provide food technologists with the knowledge to select and incorporate suitable probiotics into foods beyond the traditional fermented dairy food sector, and to maximize their survival over extended shelf lives.

1.6.1 Probiotic Ingredients

Probiotic organisms are predominantly bacteria selected from the genera *Lactobacillus* and *Bifidobacterium*, which are normal constituents of the human intestinal microbiota. A range of different species within each genus is commonly used and within each species there are particular strains that have been shown to have probiotic attributes (Chapter 6). Considerable strain-to-strain differences have been observed within species and probiotics are generally defined down to the strain level (for example, *Lactobacillus rhamnosus* GG and *Lactobacillus rhamnosus* LC-705) (406). Probiotic organisms have typically been selected via screening regimes to perform well technologically, to survive intestinal transit, and to impart health benefits on consumers. Good probiotic strains have demonstrated health and safety data from randomized, controlled clinical trials.

Probiotics organisms are usually supplied by manufacturers of these ingredients as either dry powders (freeze-dried or spray-dried) at 10^{10}–10^{12} cfu/g or as frozen "direct vat set" concentrates at 10^{9}–10^{10} cfu/g (430). When received as ingredients, it is important that the probiotic be correctly stored as per the manufacturer's instructions in order to avoid rapid losses in probiotic viable counts. For dried powders, this means storing the probiotics cold and avoiding moisture or humidity, while for frozen cultures is it important to maintain constant temperatures and to avoid repeated freeze–thawing.

Probiotics can be incorporated into foods and beverages in a variety of ways.

- Dry blended into foods and powders such as infant formulas.
- Dispersed into liquid or semiliquid products such as juice or ice-cream.
- Inoculated into fermented products such as yogurts and fermented milks.

In the first two cases, the probiotics do not multiply in the product and are generally added at doses in the order of 10^{7}–10^{8} cfu/g. For a standard probiotic freeze-dried powder at 10^{11} cfu/g, this represents addition of the probiotic at 0.01–0.1% (w/w) of the final product. In fermented products there may be some growth and increase in probiotic numbers during fermentation, allowing a lower number of organisms to be initially added (for example 10^{6} cfu/g). The number of viable probiotic organisms then usually declines during product storage, with the rate of decline dependent on a range of factors as discussed in the following sections. Ensuring losses in probiotic viability are minimized is the one of the main goals for food technologists developing foods containing probiotics.

1.6.2 Factors Affecting the Viability of Probiotics in Foods

A number of intrinsic and extrinsic factors influence the survival of probiotics in foods. It is important to consider these factors at all stages between addition of the probiotic to the food and delivery of the probiotic to the gut of the consumer. These include manufacturing processes, food formulations and matrices, packaging materials, and environmental conditions in the supply chain and during self-storage. The main factors to be considered that may influence the ability of the probiotics to survive in food products include:

1. the physiological state of the added probiotic;
2. the physical and chemical conditions of food processing;
3. the physical conditions of product storage (e.g. temperature);
4. the chemical composition of the product (acidity, nutrients, moisture, oxygen);
5. interactions with other product components (inhibitory or protective).

The first stage of product development is to align a compatible combination of probiotic strain(s) and food product(s).

1.6.2.1 Choice of Probiotic Organism/Food Combinations Probiotic organisms are generally selected from constituent intestinal lactobacilli and bifidobacteria, which have evolved to grow and survive in environmental conditions within the human intestinal tract. In the small intestine and colon, the pH is generally close to neutral, the temperature is constant (37–39°C), a complex nutrient supply is constantly available and there is little oxygen. These conditions are of course very different to those found in food processes and food matrices. Nonetheless, bacteria show a remarkable ability to survive in adverse environments and probiotics can survive in food environments, to a point.

Probiotic ingredients are not all the same. Differences extend from the genus to the species and even strain level, and apply both to their physiological impacts on the consumer and to their technological attributes in foods (406, 410). The closer probiotic organisms are related, usually the more similarly they will perform. However, considerable strain-to-strain differences are still apparent that can significantly impact on the performance of probiotics in foods (Fig. 1.11).

The differences in the technological characteristics of different probiotic species and strains means that care must be taken in selecting the most appropriate strain for a particular food application. Indeed, the first step in incorporating a probiotic into a food is identifying compatibilities between the attributes of the selected strains and the food production steps, food matrix and storage conditions. This may involve a compromise between the desired health attributes and technological capabilities of particular strains for particular food applications. When developing new products some research may be required to ensure that the selected strain is able to survive well in the food, provide the appropriate technological properties (e.g. acidification during fermentation, if required) and importantly, that the added probiotic does not adversely affect the taste, smell, and texture of the food or beverage.

While emphasizing the importance of strain specificity of technological attributes of probiotics, some generalizations can still be made on the robustness of probiotic organisms. Generally, lactobacilli are more robust than bifidobacteria (406, 431, 449). There is a wider range of probiotic *Lactobacillus* species that are technologically suitable for food applications than bifidobacteria. Common examples include *L. acidophilus, L. johnsonii, L. rhamnosus, L. casei, L. paracasei, L. fermentum, L. reuterii* and *L. plantarum.* Often, the *L. acidophilus* group of organisms, while resistant to low pH, prove less robust than other lactobacillus

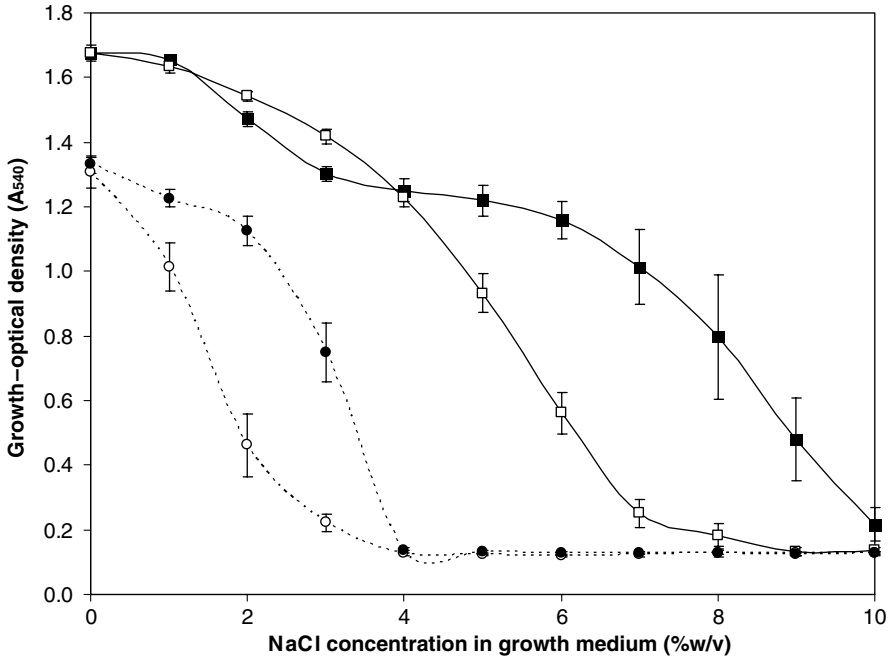

FIGURE 1.11 Inter- and intraspecies variation in the ability of probiotic lactobacilli to grow in the presence of salt. This characteristic is important, for example, in meat fermentation. The figure demonstrates the importance of selecting probiotic species and strains that are compatible with a particular food. The lactobacilli were grown in MRS broth containing various concentrations of NaCl, at 37°C, for 24 h. Error bars represent 1 standard deviation from the mean of three biological replicates. *Lactobacillus rhamnosus* GG (■); *Lactobacillus rhamnosus* CSCC 5277 (□); *Lactobacillus acidophilus* MJLA1 (●) *Lactobacillus acidophilus* CSCC 2401 (○). From Crittenden R, Morris L, and Playne MJ. Unpublished data, Food Science Australia.

species in non-traditional probiotic food applications (391, 413, 415, 445, 453, 461). The *Bifidobacterium* species most commonly used in foods is *B. animalis* subsp. *lactis* (398). This species is significantly more robust than human intestinal species such as *B. longum* (*infantis*), *B. breve*, and *B. bifidum,* although certain strains of these species are able to survive well in some foods (395, 398, 409). *B. adolescentis* is a common species in the intestinal tract of adult humans, but tends to be sensitive to environmental conditions in foods (395, 457, 462) and is rarely used commercially as a probiotic.

The metabolism of the probiotic organism is an important consideration in fermented probiotic foods, not only for probiotic growth and survival, but also for food quality. For example, heterofermentative lactobacilli that produce CO_2 as a metabolic end product are not suitable where gas formation adversely impacts on food quality (384). Bifidobacteria produce acetate and lactate as end products of carbohydrate fermentation, and a more vinegar-like taste profile if they are actively fermenting in food products (395, 404).

The ability to utilize the available carbon and nitrogen substrates in a product may be required for probiotic growth and acidification (455). Lactobacilli and bifidobacteria can generally utilize a wide range of carbon substrates, with differences in the carbon substrate profiles occurring between species and strains. Probiotic strains may also be selected on the basis that they metabolize desirable bioconversions, such as deconjugation of isoflavones in soy (375), or indeed, because they do not metabolize other ingredients in the food. For some fermented foods where LAB form part of the native microbiota (e.g. fermented meats), an approach has been taken where the probiotic attributes of cultures isolated from these foods (and therefore known to survive well) have been examined to select new probiotic strains (422, 426, 443).

1.6.2.2 Physiologic State of the Probiotic An important factor in probiotic survival is the physiological state of the bacteria when prepared, and the physiological state of the bacteria in the product itself. If the food product is dry (e.g., a powdered infant formula) the probiotic will also be dried and in a quiescent state during storage. However, when included in a wet product such as a yogurt, the bacteria will be in a vegetative state and potentially metabolically active (albeit slowly at refrigeration temperatures). The state of the bacteria will have a large bearing on the possible shelf life of the bacteria, with long-term survival of vegetative cells only possible at low temperatures (403). In comparison, dried, quiescent cells may have longer shelf lives at ambient temperatures, though they too will be more stable at lower temperatures.

Bacteria are able to respond to stressful environments through the induction of various stress tolerance mechanisms. The induction of stress proteins by exposure of the cells to sublethal stresses such as heat, cold, starvation, low pH, and osmotic tension can condition probiotics to better tolerate environmental stresses in food production, storage, and gastrointestinal transit (449, 456). Cross-protection has often been observed, where exposure to one stress provides protection against other stresses (403, 449, 456). The main point to emphasize is that while different probiotic strains have their own intrinsic tolerances to environmental conditions, tolerance can also be influenced by how the culture is prepared. Stress responses can be exploited to make probiotic strains more resilient and likely to survive in food matrices.

1.6.2.3 Temperature The temperature at which probiotic organisms grow is important in food applications where fermentation is required. The optimum temperature for growth of most probiotics is between 37°C and 43°C (395, 403). Species of bifidobacteria isolated from the human intestinal tract such as *B. longum (infantis), B. breve, B. bifidum,* and *B. adolescentis* have optimum growth temperatures in the range of 36–38°C, whereas *B. animalis* subsp. *lactis* can grow at higher temperatures of 41–43°C (398, 403). Usually no growth is observed for bifidobacteria at temperatures below 20°C or above 46°C (403). Probiotic lactobacilli can grow well over a similar temperature range though some can grow at up to 44°C and at mesophilic temperatures down to 15°C (454).

Temperature is also a critical factor influencing probiotic survival during manufacture and storage. In practical terms, the lower the temperature the more stable probiotic viability in the food product will be. During processing, temperatures above 45–50°C will be detrimental to probiotic survival. The higher the temperature, the shorter the time period of exposure required to severely decrease the numbers of viable bacteria, ranging from hours or minutes at 45–55°C to seconds at higher temperatures. It is obvious that probiotics should be added downstream of heating/cooking/pasteurization processes in food manufacture.

Elevated temperature also has a detrimental effect on stability during product shipping and storage. Again, the cooler a product can be maintained, the better probiotic survival will be. For vegetative probiotic cells in liquid products, refrigerated storage is usually essential (403). In dried products containing quiescent bacterial cells, acceptable probiotic viability can be maintained in products stored at ambient temperatures for 12 months or more. As discussed later, there is a substantial interaction between temperature and water activity. Therefore, producing and maintaining low water activities in the foods is the key to maintaining probiotic viability during nonrefrigerated storage.

1.6.2.4 pH Lactobacilli and bifidobacteria produce organic acid end products from carbohydrate metabolism. Hence, these genera can tolerate lower pH levels than many bacteria. Indeed, numerous *in vitro* and *in vivo* studies have demonstrated that probiotic organisms can survive gastric transit where the cells are exposed to pH values as low as 2.0, though the time of exposure (1–2 h) is relatively short (398, 452). Adapted vegetative cells are usually able to survive better in acidic environments compared to quiescent cells (448).

In food products, lactobacilli are able to grow and survive in fermented milks and yogurts with pH values between 3.7 and 4.3 (395). Bifidobacteria tend to be less acid tolerant, with most species surviving poorly in fermented products at pH levels below 4.6 (395, 449). Again, *B. animalis* subsp. *lactis* is more acid tolerant than human intestinal species and, hence, is the species of *Bifidobacterium* most commonly used in acidic foods (398). A recently described phenotypic group, *B. thermoacidophilum* is even more tolerant to low pH (and heat) (398, 403), but has not been characterized thoroughly for probiotic traits and is not used commercially, at least so far.

Survival in low pH beverages such as fruit juices (pH 3.5–4.5) posses a significant challenge to probiotic survival, but commercially successful products have been produced, such as Gefilus (Valio Ltd, Finland), which contains *Lactobacillus rhamnosus* GG. Carriers such as dietary fibers have been shown to improve viability at low pH (451). Survival of lactobacilli in acidic environments has also been enhanced in the presence of metabolizable sugars that allow cell membrane proton pumps to operate and prevent lowering of intracellular pH (397). This can improve survival during gastric transit, but may not be applicable to improving probiotic survival over the time frames of shelf-storage.

1.6.2.5 Water Activity For quiescent, dried, probiotic bacteria water activity is a crucial determinant of survival in food products during storage (430, 434, 436, 444). As

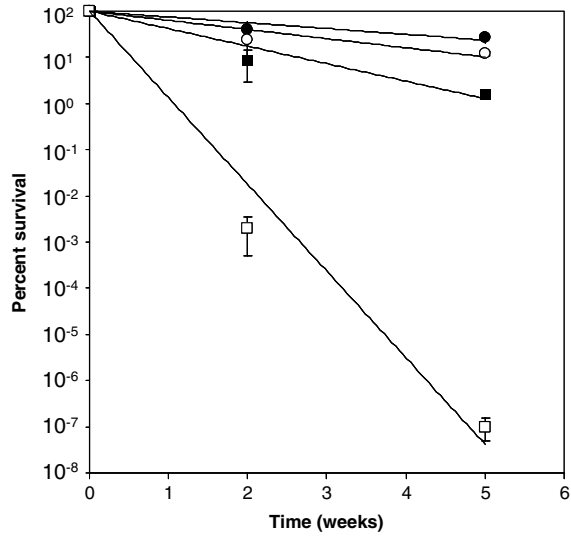

FIGURE 1.12 The impact of water activity on the survival of a freeze-dried probotic *Lactobacillus rhamnosus* strain stored at 38–40°C. The probiotic was stored in the same type of food matrix, but the water activity controlled at various levels: $a_w = 0.1$ (●); $a_w = 0.15$ (○); $a_w = 0.22$ (■); $a_w = 0.25$ (□). Error bars represent the standard deviation from two to three biological replicates. From Crittenden R, Weerakkody R, and Sanguansri L. Unpublished data, Food Science Australia.

moisture levels and water activity are increased the survival of probiotics is substantially decreased (Fig. 1.12). Probiotics can survive well over long shelf lives (12 months or more) at ambient temperatures in dried products as long as the low moisture levels in the products can be maintained (at least below a_w 0.2–0.3). In general, the lower the water activity, the better the bacterial survival will be (434). There is a substantial interaction between water activity and temperature with respect to their impact on the survival of quiescent probiotics. As the storage temperature is increased the detrimental impact of moisture is magnified (Fig. 1.13). Although the precise mechanisms of cell death remain unclear, osmotic stresses appear to play a role, with the presence of smaller molecules resulting in poorer bacterial survival (434).

Despite the clear evidence that very low water activities improve probiotic survival there may be technological limitations to reducing water activity to very low levels. These include the energy costs of drying, adverse impacts on the palatability of foods and difficulties in wetting and dispersing powders. Moisture barrier packaging may be applied to prevent the egress of moisture from the environment during storage. Maintaining probiotic viability in moderate water activity foods (0.4–0.7) is a major challenge and solutions such as microencapsulation or incorporation of probiotics into fat phases of products can provide improved survival.

1.6.2.6 Oxygen Both bifidobacteria and lactobacilli are considered strict anaerobes and oxygen can be detrimental to probiotic growth and survival (419). However,

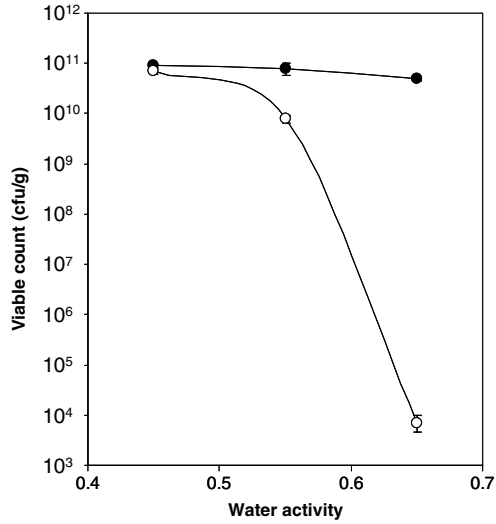

FIGURE 1.13 The viable count of a freeze-dried probiotic *Lactobacillus acidophilus* strain after 3 weeks of storage at moderate water activities at either 20°C (●) or 30°C (○). Increased temperature magnified the detrimental impact of water activity on the survival of the dried probiotic. Error bars represent the standard deviation from triplicate biological replicates. From Crittenden R, Weerakkody R, and Sanguansri L. Unpublished data, Food Science Australia.

the degree of oxygen sensitivity varies considerably between different species and strains (419). In general, lactobacilli, which are mostly microaerophilic, are more tolerant of oxygen than bifidobacteria, to the point where oxygen levels are rarely an important consideration in maintaining the survival of lactobacilli. Most probiotic bifidobacteria do not grow well in the presence of oxygen (398). However, many bifidobacteria have enzymatic mechanisms (via NADH-oxidase and NADH-peroxidase) to limit, oxygen toxicity (457). *B. animalis* subsp. *lactis* is relatively resistant to oxygen stress, and *B. longum* (*infantis*) and *B. breve* are more resistant to oxygen than *B. adolescentis* (395, 432, 457).

For oxygen sensitive strains, some strategies are available to prevent oxygen toxicity in food products. Antioxidant ingredients such as ascorbic acid or cysteine have been shown to improve probiotic survival (394, 401), as well as the use of oxygen barrier or modified atmosphere packaging (459). Since oxygen toxicity can sometimes influence probiotic survival in foods, it is advisable to minimize processes that are highly aerating, particularly when using bifidobacteria.

1.6.2.7 Toxicity of Ingredients The compatibility of probiotics with other ingredients within food formulations can have a significant impact on bacterial survival. Interactions between probiotics and other ingredients can be protective, neutral, or detrimental to probiotic stability (430). Obviously, the inclusion of antimicrobial preservatives can inhibit probiotic survival. Elevated levels of ingredients such as salt, organic acids, and nitrates can inhibit probiotics during storage (387, 395, 423), while

starter cultures can sometimes inhibit the growth of probiotics during fermentation through the production of specific bacteriocins (384, 395).

1.6.2.8 Growth Factors, Protective, and Synergistic Ingredients Probiotic lactobacilli and, in particular, bifidobacteria are only weakly proteolytic and grow relatively slowly or poorly in milk (395, 423, 455). The growth of bifidobacteria can be enhanced by the presence of suitable companion cultures, including starter cultures, which can aid in protein hydrolysis and through the production of growth factors (430, 455). Alternatively, growth substrates such as carbon sources (e.g. glucose), nitrogen sources, and growth factors (e.g. yeast extract or protein hydrolysates) or antioxidants, minerals, and vitamins can be added to improve growth (430, 455). Other ingredients can protect the viability of probiotics in foods by acting as carriers (449–451). Finally, the food matrix itself can be protective. An example is cheese, where the anaerobic environment, high fat content and buffering capacity of the matrix helps to protect the probiotic cells both in the product and during intestinal transit (395).

1.6.2.9 Freeze–Thawing Freezing probiotic cells damages cell membranes and is detrimental to survival (388, 410). Protectants are usually added to cultures to be frozen or dried in order to prevent, or at least mitigate, cell injury. The most common protectants used at industrial scale are lactose or sucrose, monosodium glutamate, milk powders, and ascorbate (430). Once frozen, probiotics can survive well over long shelf lives in products such as frozen yogurts and ice-cream. Use of slow cooling rates, or conditioning cells with prefreezing stress, can significantly improve cell survival (388). Repeated freeze–thawing cycles are highly detrimental to cell survival and should be avoided. The cell membrane damage caused by freezing can also render probiotic cells more vulnerable to environmental stresses (410). In the example shown in Fig. 1.14, the survival of a probiotic *Lactobacillus paracasei* in a low pH fermented whey drink was studied during shelf storage. In one sample the culture was frozen during supply chain transport and then thawed. A parallel sample was only refrigerated during transport. It was evident that freeze–thawing increased the sensitivity of the cells to the acidic environment.

1.6.2.10 Sheer Forces Probiotic lactobacilli and bifidobacteria are Gram-positive bacteria with thick cell walls that are able to tolerate the sheer forces generated in most standard food production processes. Some high-sheer processes such as high-speed blending or homogenization may result in cell disruption and losses in viability.

1.6.3 Synbiotics

Probiotics are not the only functional food ingredients developed to improve human health by modulating the intestinal microbiota. Prebiotic ingredients represent an alternative and potentially synergistic approach. These nondigestible carbohydrates pass through to the colon where they selectively stimulate the proliferation and/or activity of beneficial microorganisms within the intestinal microbiota (412). Ingredients and foods that contain both prebiotics and probiotic are called synbiotics. A

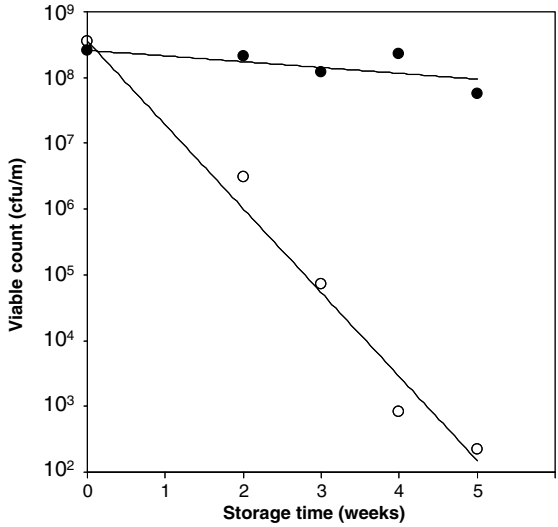

FIGURE 1.14 The impact of freeze–thawing on the subsequent refrigerated storage stability of a probiotic strain in a low-pH (3.8) whey drink. A culture of *Lactobacillus paracasei* was divided into two aliquots. One was frozen at −20°C for 72 h (○) while the other was stored refrigerated at 4°C for 72 h (●). The frozen culture was then thawed and the viability of the probiotic in both aliquots was monitored during subsequent storage at 4°C for 5 weeks. A single freeze–thaw cycle proved highly detrimental to the storage stability of the probiotic. From Crittenden R. Unpublished data, Food Science Australia.

range of nondigestible sugars, oligosaccharides, and polysaccharides can act as prebiotics, and possess a range of physiological and physicochemical properties that make them attractive food ingredients. Prebiotics are discussed in more detail in Chapter 7.

1.6.4 Delivery Systems

1.6.4.1 Microencapsulation Providing probiotics with a physical barrier to environmental conditions by microencapsulating the bacteria is an approach that has been trialed using a range of materials and techniques. Microencapsulation of probiotics, though, is not a simple undertaking. There are many demands for a successful probiotic microencapsulant:

- The materials have to be food grade, inexpensive, and compatible with the food into which the probiotic will be encapsulated.
- The microencapsulation process must be simple, inexpensive and must not reduce probiotic viability.
- The encapsulation efficiency must be high (i.e. close to 100% of the bacteria in a suspension should be encapsulated).

- The microcapsules must contain a high loading (%v/v) of probiotics.
- The microcapsules must not adversely impact on the taste and texture of food and beverages (small capsules <30 μm).
- The microcapsules must protect the probiotics against a range of environmental stresses during manufacture and storage. Protection against moisture and low pH are two of the most common stresses to protect against.
- It is also an advantage for the microcapsules to protect the probiotics during gastrointestinal transit.
- The microcapsule must be able to release the probiotic bacteria in the gut at the required site of action.

A range of experimental microencapsulation technologies have been reported, including entrapment in polymers such as alginate, carrageenan, and starch; coating in emulsions or fat; or dry impacting of prebiotics and enteric coats (385, 418, 424). Commercial microencapsulation systems for probiotics available currently include Priobiocap™, a fat-coating system developed by Institut Rosell, Canada; Micro-MAX™, an emulsion-based, synbiotic-coating system developed by Food Science Australia (399, 400), and EnCoate™, a biopolymer system produced by EnCoate Ltd, New Zealand.

1.6.4.2 Delivery Devices Another approach to maintaining the viability of probiotics for long periods at ambient temperatures is to physically separate the probiotics from the food and atmosphere. This can most simply be achieved by keeping dried probiotics in sealed sachets with the food or beverage to be mixed immediately prior to consumption. More innovative packaging and delivery systems have been developed to deliver dried probiotic ingredients into beverages. These include drinking straws that contain dried probiotics, which are released into the beverage as it passes through the straw (438) and a drink cap that contains the dried probiotics, which are released into the drink as the cap is opened (439).

1.6.5 Probiotic Foods

Probiotics have been successfully incorporated in a number of foods including the traditional vehicles of fermented milks and yogurts, and increasingly in other fermented and nonfermented foods beyond the dairy sector. Examples of the major product classes that have successfully incorporated probiotics are listed in Tables 1.8–1.10 along with the main technological points involved in incorporating probiotics and maintaining their viability.

1.6.6 Conclusions

Industry demand to include probiotics as functional ingredients in foods will no doubt continue to grow as the clinical evidence of health benefits builds. Indeed, the number of randomized, controlled trials reported that involve probiotic interventions has

TABLE 1.8 Examples of Fermented Dairy Products Containing Probiotics and the Major Technological Considerations Affecting Probiotic Survival

Products	Probiotic Species	Main Technological Points	References
Yogurt/ fermented milk	Wide range of lactobacilli and bifidobacteria	Probiotics can be inoculated together with traditional starters or, in the case of stirred yogurts, added after the fermentation.	(395, 403, 417, 430, 455)
	B. animalis subsp. *lactis* is the most robust and commonly used *Bifidobacterium* species used	The combination of starter culture and probiotic must work technologically. Probiotic lactobacilli and especially bifidobacteria are not highly proteolytic and do not grow well in milk alone. The addition of growth factors can improve probiotic growth. Starter cultures can both improve growth or inhibit probiotic cultures. Relative proportions of starter and probiotic inoculation and/or timing of inoculations can be manipulated to maximize probiotic survival. Mild (pH 4.3–4.6) yogurts improve survival of bifidobacteia compared to traditional yogurts (pH 3.7–4.3). Mild yogurts can be manufactured with the substitution of *L. delbrueckii* subsp. *bulgaricus* with the probiotic cultures, though the inclusion of this traditional starter is mandatory for yogurts in some jurisdictions.	
Matured cheeses	Strains of *L. casei*, *L. paracasei*, *L. rhamnosus* and *B. animalis* subsp. *lactis* usually survive well	Probiotics can be inoculated with the traditional starters to ferment and grow or to the curd after scalding.	(391, 392, 395, 396, 411, 414, 417, 441, 442, 445, 464)

	L. acidophilus survival is strain variable *B. adolescentis*, *B. breve*, and *B. longum* (*infantis*) usually survive poorly	The action of rennet releases peptides, which aid the growth of the typically weakly proteolytic probiotics. The probiotics are usually added at approximately the same dose as the starter cultures and then increase 10-fold in numbers during fermentation. Probiotic viability then declines slightly during ripening. The pH value of cheeses (4.8–5.6) is markedly higher than the pH of yogurts and fermented milks, which aids long-term survival of probiotics. High salt levels can inhibit the survival of bifidobacteria. The metabolism of starter and nonstarter bacteria within the cheese results in an almost anaerobic environment within a few weeks of ripening. Probiotics can sometimes cause flavor defects such as bitterness and acidity. Relative to the starter organisms, the probiotic inoculum size, time of addition, and growth conditions must be balanced to promote probiotic viability without excessive acid production. The inclusion of probiotics can increase secondary proteolysis and accelerate ripening.	
Fresh cheeses	*L. rhamnosus*, *L. casei* survive well Bifidobacteria including *B. infantis* and *B. bifidum. B. animalis* subsp. *lactis* survive poorly	Probiotics can be added with traditional starters or added with the cream. Addition with the starters can increase probiotic numbers but may have an adverse impact on taste by increasing acidity Synergies with or antagonism by the starter cultures	(395, 396, 417, 461)

TABLE 1.9 Examples of Nonfermented Dairy Products Containing Probiotics and the Major Technological Considerations Affecting Probiotic Survival

Products	Probiotic Species	Main Technological Points	References
Ice-cream	*L. johnsonii, L. casei, L. rhamnosus.*	Ice-cream is an excellent matrix for the long-term stability of probiotics	(381, 382, 389, 409, 416, 429)
	B. longum, B. bifidum, B. animalis subsp. *lactis*	Even relatively sensitive species of probiotics can remain stable for many months	
	L. acidophilus survives, but not as well as other species.	Can be made by direct addition of probiotics or with addition of fermented milk	
		Losses in viability occur mainly in ice-cream preparation during addition of overrun (aeration) and cell damage during freezing.	
		The added sugar acts as a cryoprotectant.	
		The higher pH of ice-cream compared to fermented products probably aids survival of probiotics.	
		Occasional impacts on sensory properties, but very good sensory properties achievable	
Dairy desserts	*L. paracasei* generally survives well	Neutral pH and refrigerated storage of these products is conducive to probiotic survival	(383)
	Survival of *L. acidophilus* is variable	No fermentation by probiotics desirable since acid production will lead to undesirable flavors	
		Absence of preservatives, neutral pH, and high water activity means spoilage organisms are a risk and may limit shelf life	
		Important to find compatible strains and products	
Dry products such as infant formula and nutritional powders	*L. rhamnosus, L. acidophilus,*	Probiotics usually dry blended into infant formula powder	(430, 444)
		Mixing probiotic with formula blend prior to spray drying may improve survival if the strain is robust enough to survive spray drying	
	B. animalis subsp. *lactis*	Water activity is a major determinant of survival and should be as low as possible	

TABLE 1.10 Examples of Nondairy Products Containing Probiotics and the Major Technological Considerations Affecting Probiotic Survival

Products	Probiotic Species	Main Technological Points	References
Fermented soymilk	*L. acidophilus, L. casei, L. rhamnosus, L. johnsonii.*	Bifidobacteria and lactobacilli often grow better in soymilk than in cows' milk	(404, 4408, 460)
	B. animalis subsp. *lactis.*	Able to use the soy oligosaccharide substrates raffinose and stachyose	
		Approx. 10-fold increase in probiotic numbers during fermentation which then decline over shelf storage	
		Deconjugation of soy isoflavone phytoestrogens may increase bioavailability	
		Some manipulation of inoculation timing required to achieve correct sensory balance	
		Minimize acetate formation which has undesirable sensory attributes	
Fermented cereal products	*L. acidophilus*, *L. plantarum, L. paracasei*	Fermented-oat products such as YOSA® (Bioferme Oy, Finland)	(386, 413)
	B. animalis subsp. *lactis*	Growth nutrients such as simple sugars and proteins often needed for fermentation	
		No requirement for additional starter bacteria	
Juice	*L. rhamnosus*	Low pH value of juice (3.5–4.5) means only acid-tolerant strains survive well	(428, 450, 451)
	B. animalis subsp. *lactis*	Probiotic survival is achievable (e.g. *Gefilus* produced by Valio Ltd, Finland)	
		Probiotics added after pasteurization/UHT of juice	
		Probiotics are particulate and tend to settle, so not applicable for clarified juices	
		Can slightly reduce palatability, but can be masked	
		Carriers such as dietary fibers can improve stability	

(*continued*)

TABLE 1.10 (*Continued*)

Products	Probiotic Species	Main Technological Points	References
Fermented meats	*L. rhamnosus, L. casei, L. paracasei, L. plantarum.*	Probiotics inoculated along with starter cultures Numbers increase during fermentation and then decline slowly during ripening and storage	(384, 387, 405, 420, 422, 435, 443, 446, 453)
	B. animalis subsp. *lactis* Survival of *L. acidophilus* sometimes poor	Some inhibit pathogens such as *Listeria monocytogenes* and enterotoxigenic *E. coli* or spoilage organisms Should not inhibit rapid acidification by starter bacteria and should not produce excessive levels of lactic acid which can affect sensory quality	
	Heterofementative lactobacilli are not suitable due to gas formation	Need to tolerate high salt and nitrite/nitrate levels	
Confectionary	*L. casei, L. paracasei*	Probiotics mixed with confectionary (e.g. chocolate) at approximately 40°C	(425, 436, 437)
	B. longum	No growth of probiotics in confectionary Only dried probiotics can be added Can be added to chocolate without impact on tempering conditions, texture, hardness, and flavor Crucial factors affecting the viability of probiotics in confectionary are water activity, osmotic tension, and temperature Entrapment in fat provides protection against oxygen, moisture and gastric transit	
Food service products	*L. acidophilus*, *B. animalis* subsp. *lactis*	Probiotics can be added to fresh service products such as sandwiches, sushi, and smoothies stored refrigerated Short shelf life required Frozen cultures survive better than freeze-dried cultures	(447, 448)

grown rapidly in recent years. Not only is the evidence of specific health benefits emerging, but some potential mechanisms of action are also beginning to be elucidated (393). Such knowledge may allow the development of specific quality assurance tests for probiotic potency in foods that go beyond simply viability. However, today, viability remains the only practical measure of the quality of probiotic ingredients in foods and the measure employed by regulatory bodies.

It is already possible to include probiotics into a variety of foods beyond traditional fermented milks and yogurts. Numerous examples exist where probiotics have been successfully incorporated without adversely impacting sensory quality of the food and stability maintained above 10^6–10^7 cfu/g over long shelf lives. However, the maintenance of adequate viability in many food types remains a major challenge. Moderate water activity foods (0.4–0.8), in particular, are difficult matrices in which to maintain the viability of probiotics in shelf-stable products. For many foods, adjunct technologies such as microencapsulation are likely to be required in order to stabilize probiotics. As "off-the-shelf" probiotic microencapsulation technologies that work in all food matrices are not yet available, these generally have to be adapted to suit the particular food matrix.

The five critical points to address when incorporating probiotics into foods remain the following:

1. Select a compatible probiotic strain/food type combination
2. Use food-processing conditions that are compatible with probiotic survival.
3. If fermentation is required, ensure that the food matrix will support probiotic growth.
4. Select the product formulation, packaging and environmental conditions to ensure adequate probiotic survival over the product's supply chain and during shelf storage.
5. Ensure that addition of the probiotic does not adversely impact on the taste and texture of the product.

1.7 SAFETY OF PROBIOTIC ORGANISMS

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Consumers searching on the Internet can readily find a multitude of probiotic products. Among them is one stated to be a prize-winning preparation constituted by a celebrated microbiologist. It contains multiple strains of natural live LAB, including an award-winning strain proven to be an order of magnitude stronger than any other probiotic. This mixture, consisting of *Enterococcus faecalis*, *Streptococcus thermophilus*, three species of *Bifidobacterium*, and seven *Lactobacillus* species, is guaranteed to colonize the colon with good microorganisms essential for a healthy immune system and general good health.

We are informed that scientific studies conclude that this preparation strongly enhances immunity and is effective against deadly bacteria such as methicillin- and vancomycin-resistant *Staphylococcus aureus*, *Helicobacter pylori*, food poisoning *Escherichia coli*, and *Bacillus cereus*. It is suggested that in the age of bioterrorism the product may have the potential to protect against anthrax as it is effective against *Bacillus cereus*, a member of the same genus.

The recommended dose is two to four capsules daily, at a cost of US$52 for 60 capsules. Regular consumption is strongly recommended for a plethora of conditions including irritable bowel syndrome, leaky gut, peptic ulcers, bloating, heartburn, ulcerative colitis, Crohn's disease, constipation, or diarrhea. Consumers of natural products such as psyllium, which are purported to remove good intestinal bacteria, are similarly advised. It is suggested that the product may be useful in treating diseases as diverse as asthma, cystic fibrosis, diabetes, Epstein-Barr virus, acne, psoriasis, eczema, arthritis, multiple sclerosis, human immunodeficiency virus (HIV), acquired immune deficiency syndrome (AIDS), and hepatitis B and C.

A search of five scientific and medical databases however was unable to identify a single scientific publication for the product mentioned above.

In a community, which is often suspicious of chemicals, probiotics are marketed, and seen by many, as a natural and appealing alternative to maintain and promote good health. In contrast to pharmaceuticals, traditional probiotics appear safe and serious adverse effects appear rare. Each year over 20 billion doses of probiotics are used by both healthy people and those with medical conditions. New species and more specific strains of organisms are constantly being sought and their probiotic attributes characterized to generate an expanded range of probiotic foods and targeted therapies (466–470). The demonstration of efficacy in probiotics offers untold opportunities for the development and marketing of human and veterinary products. Probiotics consumed in foods and dietary supplements do not have to comply with more rigorous guidelines for probiotics that claim amelioration or prevention of disease in clinical use. Probiotics that claim specific nutritional, functional, or therapeutic characteristics blur the boundaries between food, dietary supplement, or medicine, posing challenges for regulators. Their safety cannot be assumed. There is currently a lack of standard safety requirements for new probiotic organisms (471–474).

In November 2006, a Google search on the Internet for probiotics returned 2,810,000 hits. In contrast five scientific databases located 4447 journal papers, of which 345 related to aspects of probiotic safety. It is apparent that there are significant discrepancies between health claims made for what are being promoted as probiotics and verifiable scientific evidence to substantiate these claims. The probiotic concept has been appropriated for commercial exploitation, to the detriment of those probiotics tested to be safe and beneficial by rigorous science. When confronted with inflated and dubious claims the consumer cannot be expected to judge what a proven probiotic is and what the twenty-first-century equivalent of snake oil is. As efficacy is inextricably linked to safety, any claims of health benefits for a probiotic require substantiation by scientific evidence. What is a "probiotic" and who is entitled to use the term? What standards should be attained to ensure a product labeled "probiotic" is safe and effective?

The use of "history of safe use" as a criterion for the safety of food organisms is an arbitrary classification. Lactic acid bacteria and yeasts intrinsic to the production of traditional foods have been accepted as safe without any real scientific criteria, partly because they are normal commensal flora, and because of their consumption through centuries presumably without adverse effect. Evidence for the safety and efficacy of probiotic organisms has until recently been largely anecdotal or based on relatively little, and often poorly designed research.

The initial concept of probiotics has shifted from one of traditional dairy bacteria that ferment milk to "promote gut health" to that of a complex range of bacteria potentially capable of colonizing any human mucosal surface, not only the gut (477, 478). Probiotics have expanded to include nontraditional bacteria, with applications targeted to clinical conditions and not limited to oral therapy (477). The change from traditional bacteria in food to designer probiotic has led to considerably increased numbers of organisms per dose to achieve health effects (472). Where previously a limited number of food products existed, now there are single or multiple species of organisms in a range of quasi or proven therapeutic products.

The drift from dairy foods to complementary or prescribed medicines with therapeutic claims has elevated probiotics to a class that was once the sole province of pharmaceuticals. How to assess the safety of new probiotic products needs to be re-evaluated in these altered circumstances. Regulators must judge whether a probiotic is a food, a supplement or a clinical therapy and develop enforceable safety standards accordingly. If probiotics are intended for therapeutic use they must be evaluated for quality, safety, and efficacy in the same manner as any other therapeutics–with documented and verifiable characterization of the active ingredient, dose, efficacy, safety, and adverse effects (479). This raises a quality control issue for food and therapeutic manufacturers, who have the responsibility to market accurately characterized, stable, and viable organisms, in appropriate doses and formulations, with clearly defined applications and attendant health benefits. Consumers need to have accurate information from a credible source to ascertain whether a product is safe and the purported health benefits are genuine.

1.7.1 Current Proposals for Probiotic Safety

Conventional toxicology and safety evaluation has limitations for the safety assessment of probiotic bacteria. Vigorous debate continues on what constitutes appropriate safety testing for novel probiotic strains proposed for human use. In recent years several organizations have formulated approaches to assess the safety of probiotics. For the most part these have been predicated on oral applications but it should be remembered that probiotics are not limited to oral products, but may be applied vaginally, topically, and intranasally (478).

The Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food (475) proposed a framework consisting of strain identification and functional characterization, followed by safety assessment and Phase 1, 2, and 3 human trials. It recommended that probiotic foods be properly labeled with the strain designation, minimum numbers of viable bacteria at the end of shelf life, storage

conditions, and manufacturer's contact details. Importantly, the Working Group further proposed that the use and adoption of the guidelines should be a prerequisite for calling a bacterial strain "probiotic."

The Working Group considered the minimum tests required to characterize safety are

- determination of antibiotic resistance patterns;
- assessment of metabolic activities (e.g. D-lactate production, bile salt deconjugation);
- assessment of side effects during human studies;
- postmarket epidemiological surveillance of adverse incidents in consumers.

If the strain being evaluated belongs to a species known to produce a mammalian toxin or to have hemolytic potential, it must be tested for these characteristics.

The EFSA has proposed a scheme based on the concept of QPS, defined as "an assumption based on reasonable evidence" and qualified to allow certain restrictions to apply (480). The scheme aims to have consistent generic safety assessment of microorganisms through the food chain without compromising safety standards. Individual evaluations would be limited to aspects particular to the organism, such as acquired antibiotic resistance determinants in LAB. QPS status would not apply to a microorganism that commonly causes pathogenicity. A microorganism would not necessarily be considered a potential pathogen where there are infrequent reports of clinical isolates from severely ill people.

Broadly the characteristics to be evaluated for QPS approval are

- unambiguous identification at the claimed taxonomic level;
- relationship of taxonomic identity to existing or historic nomenclature;
- degree of familiarity with organism, based on weight of evidence;
- potential for pathogenicity to humans and animals;
- the end use of the microorganism.

The latter would influence any qualifications imposed, depending on whether the organism is to be directly consumed; is a component of a food product not intended to enter the food chain, but which may adventitiously; or is used as a production strain in a product intended to be free of live organisms.

Bernardeau et al. (481) consider this generic approach to safety assessment of microorganisms is not relevant to the *Lactobacillus* genus and have proposed modifications. They contend that LAB are not a homogeneous group as some species are pathogens, and that the rarely pathogenic *Lactobacillus* genus should undergo its own limited safety assessment. The genus should be accorded the status of Long Standing Presumption of Safety based on its long history of safe use in fermented foods. Individual species could then be assessed for safety based on one, two, or a full suite of tests, depending on the intended use. The first safety test would be to demonstrate an absence of antibiotic resistance and its ability for transference. The second, a high dose tolerance test in animals, would be required if the organism was not

resistant to antibiotics and was a known lactobacillus for which a new application was being proposed.

A full safety assessment would be required if the body of knowledge was insufficient. In the event of risks being identified an experimental presumption of safety (EPS) status would be given for 10 years as the body of knowledge accumulated. If specified risks were identified the organism would be granted restricted EPS status (EPSr) with conditions placed on its use for 10 years. If the body of knowledge had increased after 10 years a restricted QPSr status could be granted, with the restrictions on use standing. Bernardeau et al. (481) consider this approach would be preferable to granting non-QPS status, as it allows the community at large to benefit while simultaneously protecting the small proportion of the population at risk.

A more stringent perspective is evident in the regulations of the Canadian National Health Product Directorate, where products can be considered "traditional" or "nontraditional." To be considered traditional a product must have at least 50 consecutive years of use, two independent references to traditional use in support of health claims, and safety reports on adverse reactions and interactions (482).

Gueimonde et al. (483) propose a sequence to determine safety of new bacteria of nondairy origin sought specifically for their probiotic effect and frequently isolated from the human or animal intestinal tract. While the scheme has many features in common with that of the Working Group, its emphasis is on the absolute necessity for correct strain identification to allow comparisons of potential risk with taxonomically related organisms, to avoid use of potential pathogens, and for continuous quality control in postmarker surveillance of bacteremia. Gueimonde et al. (483) suggest that the increasing availability of probiotic genome sequences will facilitate identification of potential risk factors.

While guidelines for probiotics have been proposed and refinements suggested, as yet agreement has not been arrived at on a universal or enforceable standard. There is general consensus on the need for standards, enforced regulations, and improved quality control over products (483, 472, 477, 478, 482).

1.7.2 Taxonomic Identification

The safety of a putative novel probiotic is contingent on its unequivocal identification at the genus, species, and strain level, as probiotic effects are strain specific. Sophisticated phenotypic and molecular techniques enable species identification and discriminate between closely related strains. Reliable taxonomic identification of both species and strain is a safety issue for quality control of the product, consumer or prescriber information, diagnosis, and appropriate treatment of suspected clinical cases and epidemiological surveillance of the exposed population.

The taxonomy of lactic acid and other bacteria has changed significantly with the advent of genetic methods of classification. Strains previously thought to be dissimilar have merged, while other strains have been added or reassigned to different genera. The persistent use of incorrect or nonexistent species names on product labels despite taxonomic re-assignation is a significant issue for the credibility and safety of probiotics.

Yeung et al. (484) used partial 16S rDNA sequencing to identify named commercial strains obtained directly from the manufacturer and found discrepancies in 14 of 29 species designations. Lourens-Hattingh and Viljoen (485) concluded that probiotic cultures in South African yogurt were little more than a marketing tool upon finding the initial counts of *Bifidobacterium bifidum* in three different sources of commercial yogurts were lower than the therapeutic minimum. Weese (486) identified isolates from eight veterinary and five human probiotics to find accurate descriptions of organisms and concentrations for only 2 of the 13 products.

Temmerman et al. (487) found that of isolates from 55 European probiotic products, 47% of food supplements and 40% of dairy products were mislabeled. The food supplements yielded either no viable bacteria (37%) or significantly lower counts than the dairy products, contradicting the concept that health benefits derive from the presence of a minimum concentration of live probiotic bacteria.

In six products, all species isolated conformed to the label description; in 19 products they differed from those listed. *Enterococcus faecium* was isolated in such high numbers that contamination was unlikely to be the source. Only 2 of the 22 food supplements purporting to contain *Lactobacillus acidophilus* did. Bifidobacteria were isolated from 5 of 27 products claiming to contain them, despite the use of different selective media. The organism most frequently claimed to be in, and isolated from dairy products was *L. acidophilus*, though it was not necessarily found where claimed.

Huys et al. (488) used a suite of validated and standardized molecular methods to taxonomically re-identify 213 cultures of LAB and propionibacteria obtained for the PROSAFE project, a European Commission project into safety of probiotic LAB for human use. Probiotic strains, candidate probiotic strains, and nutritional strains, with their identity, were submitted from international culture collections, commercial manufacturers, and a research institute. The genus was confirmed correct for 194 strains (91%), with the genus of 87% of probiotic strains being confirmed as accurate. Of the 186 cultures submitted with species identity, that identity was confirmed for 159 (86%) and for 83% of the probiotic strains.

More probiotic strains (28.1%) were misidentified than nutritional (11.4%) or research strains (14.0%). The 34 misidentified probiotic strains were submitted by 10 commercial companies, with 18 of the 34 being provided by two companies. The finding that 28% of probiotic strains were misidentified at either genus or species level corroborates the reports from these and other studies (489, 490) that inaccurate identification and mislabeling of probiotic products continue. The evidence from this study suggests mislabeling originates at the start of production with incorrect identification of strains. The authors suggest misidentification may in many instances result from use of methods that are technically inadequate for reliable taxonomic characterization of a bacterial species (488).

A new probiotic culture must be at least as safe as its conventional counterparts. Inaccurate nomenclature provides no scientific or regulatory validity, misinforms or confuses the consumer, and compromises quality and safety of the product. Consumers are entitled to expect that the label on a probiotic product accurately reflects its contents: the organism is what it purports to be, it is present alive in a specified

concentration range for a stated period, and the suggested serving size contains sufficient organisms to achieve the proven benefit.

1.7.3 Pathogenicity

It is an obvious requirement that a probiotic should not cause infection. This is a significant issue where the intestinal barrier is immature as in infants; where its integrity is impaired from radiotherapy, antibiotic treatment or disease; and in immunocompromized states, such as HIV infection. With advances in medical care, an increasing proportion of the community may at some time be immunocompromized, or at risk of opportunistic infection.

Lactobacillus species are commonly used probiotics and considered nonpathogenic in most situations. Vesterlund et al. (491) tested 44 fecal, 52 blood, and 15 probiotic isolates to compare the presence of properties that are known virulence factors in recognized pathogens. No significant differences in adhesion to collagen, fibrinogen, or mucus were observed between blood, fecal and probiotic isolates, although blood isolates had a higher tendency to adhere to mucus than probiotic strains. No lactobacilli tested positive for α- or β-hemolysis. Probiotic strains induced a respiratory burst activity lower than, but close to, that of the blood isolates. Although probiotic strains showed a higher resistance to the bactericidal effect of complement-activated serum than did fecal strains, no significant differences were seen in serum resistance between fecal, clinical, or probiotic isolates. In summary, the tested properties varied greatly between strains and no unequivocal virulence factors for lactobacilli were identified. Of the three groups of isolates the probiotic strains induced the lowest respiratory burst in polymorphonucleocytes and showed the highest serum resistance, an observation that warrants further examination (491).

Lactobacillus species in general are thought to have low pathogenicity or be opportunistic pathogens in immunocompromized individuals or those with serious underlying disease. It has been suggested that *Lactobacillus rhamnosus* in particular warrants surveillance because it is associated with more cases of bacteremia than other lactobacilli. *L. rhamnosus* is among the most common Lactobacillus species in the human intestine so the incidence of bacteremia may be relative to its extensive presence in the intestine (492).

Two clinical cases have been reported in which a lactobacillus indistinguishable from an ingested probiotic strain has been identified in association with infection. A 74-year-old woman with hypertension and diabetes mellitus developed a liver abscess in association with pneumonia and pleural empyema. She had a history of drinking probiotic milk containing *L. rhamnosus* GG and a strain indistinguishable from that was isolated from the abscess (493). A 67-year-old man with mild mitral regurgitation developed endocarditis after dental extractions. His blood cultures were positive for a strain of *L. rhamnosus* indistinguishable from that in probiotic capsules he chewed (494).

Wolf et al. (495) assessed the safety of probiotic *Lactobacillus reuteri* in a double-blind, placebo-controlled study in HIV adults, and found the organism to be well tolerated with no significant safety problems. A review of probiotic safety found no

published evidence that immunocompromized patients had an increased risk of opportunistic infection from probiotic lactobacilli or bifidobacteria (496).

Probiotic *L. acidophilus* has been identified as the cause of persistent bacteremia complicated by recurring pulmonary emboli associated with a catheter infection in an AIDS patient, post chemotherapy for Hodgkin's disease (497). The patient had undergone probiotic treatment three times daily for 3 weeks. Isolation of the probiotic from the catheter site suggested that, rather than gastrointestinal translocation, to be the origin of infection. The bacteremia resolved with clindamycin and gentamicin, the only antibiotics to which *L. acidophilus* was sensitive.

Land et al. (498) reported two instances of bacteremia attributable to therapy *with L. rhamnosus* GG in young children. A 6-week-old baby was treated enterally with *L. rhamnosus* GG to ameliorate diarrhea following prolonged postoperative complications of cardiac surgery and broad spectrum antibiotic therapy. Despite improvement in the diarrhea the baby remained ill. A thrombus was found adherent to the right atrial wall and cultures from blood and a central venous catheter yielded penicillin-sensitive isolates of *Lactobacilli*.

Another child aged 6 years received enteral *L. rhamnosus* GG therapy for antibiotic-associated diarrhea, following treatment for infections with *E. coli, S. aureus*, and enterococcal sepsis. Blood cultures taken after the child developed fever yielded a penicillin-sensitive *Lactobacillus* species.

The DNA fingerprint of the lactobacillus isolated from blood cultures of the two children was indistinguishable from that of the probiotic *L. rhamnosus* GG. The most likely mechanism propounded for the bacteremia was bacterial translocation following enteral administration, rather than contamination of the central venous catheter (498).

These first reports of probiotic bacteremia in children testify that this outcome is not limited to rare cases in severely compromised adults, but needs to be borne in mind in severely compromised patients regardless of their age.

Srinavasan et al. (499) studied the safety of *Lactobacillus casei* Shirota in a randomized controlled trial of pediatric patients admitted to intensive care with severe conditions such as meningococcal septicemia and respiratory failure. Known immunodeficiency and intolerance to cow's milk or lactose were exclusion criteria. *L. casei* Shirota (10^7 cfu/day in three divided doses) was given enterally to 28 children for up to 5 days. *L. casei* Shirota was cultured from five of the six stool samples produced, but not from any normal sterile body fluid or surface. Although the probiotic appeared to be tolerated without adverse effects, the small group size, short dosing period, and exclusion of vulnerable immunodeficient subjects limit the conclusions from the study, while its efficacy remains to be established.

It would appear that the general population is not at risk from exposure to probiotics. The rare cases of infection associated with probiotics or very similar organisms have occurred in groups of patients whose conditions predispose them to opportunistic infection. In contrast other patients with very serious underlying diseases have benefited from probiotics (471). Elucidation of the mechanisms underlying rare cases of probiotic bacteremia in immunocompromized or seriously ill patients will assist clinicians in identifying those patients for whom probiotics may be contraindicated. Until then a cautious use of probiotics in this group is suggested

until those individuals in whom probiotics are contraindicated can be identified with a greater degree of certainty (471, 477, 500).

Clearly the potential for infection in this group of patients must be kept in mind, but it should not prevent the use of probiotics in the general population. Case studies are isolated events particular to an individual's condition and need careful evaluation relative to the safety of the population as a whole. Rare adverse effects need to be interpreted in the context of relative risk, in a manner similar to pharmaceuticals. For the ill patient, the risk of forgoing the benefits of treatment needs to be assessed against the lesser risk of an adverse effect (482).

While hospitalized patients are monitored, those in the wider community who self-medicate with probiotics are without the benefit of clinical oversight. It has been proposed that individuals with serious gastrointestinal or blood conditions should inform their doctors if they are consuming probiotics and report symptoms such as fevers or chills (471).

Administration of a living probiotic differs from that of a pharmaceutical drug where a specific characterized chemical is given. The risk of infection from a probiotic could be eliminated if its active constituent was identified and isolated to design an inanimate equivalent. The likelihood of this will depend on elucidation of the mechanisms by which a probiotic modulates immune effects, and the constitutive functions of the probiotic as a carrier mechanism to intestinal sites. Probiotic candidates could be selected based on their active constituents and pharmacokinetic characteristics to enable delivery of the active constituent to a target site in the intestine (501).

1.7.4 Antibiotic Resistance and Susceptibility

Lactic acid bacteria are naturally resistant to many antibiotics by virtue of their structure or physiology. In most cases the resistance is not transferable and the species are also sensitive to antibiotics in clinical use. However it is possible for plasmid-associated antibiotic resistance to spread to other species and genera. The transmissible resistance of enterococci to glycopeptide antibiotics such as vancomycin and teicoplanin is of particular concern, as vancomycin is one of the remaining antibiotics effective in the treatment of multidrug-resistant pathogens (492).

Antibiotic resistance mechanisms, their genetic nature, and transfer characteristics of resistance determinants have been studied comparatively recently in anaerobic bacteria. It has been shown that the plasmid that encodes for macrolide resistance can be transferred from *L. reuteri* to *E. faecium* and from *E. faecium* to *E. faecalis* in the mouse GIT (502). The properties of enterococci render them not suitable both as probiotics and in the production of fermented foods. They are not infrequently associated with hospital-acquired infections such as endocarditis and bacteremia. Their superior survival properties coupled with innate and acquired antibiotic resistance make them difficult to eliminate once they have become pathogenic. Little is known of the mechanisms by which enterococci become pathogenic.

A study of virulence genes in 13 *E. faecalis* strains isolated from clinical, food, and animal sources found 8–13 virulence genes in all isolates (503). Study of two of the clinical isolates found patterns of virulence gene expression were dependent on

the growth phase, environmental conditions and the bacterial isolate, rather than the source of the isolate or the combination of virulence genes. The observation that gene expression of virulence in enterococci is modulated by environmental conditions such as temperature and pH has implications for manufacturers of foods and probiotics.

A study by Temmerman et al. (487) found 68.4% of probiotic isolates were resistant to two or more antibiotics. Strains of lactobacilli were found resistant to kanamycin (81%), tetracycline (29.5%), erythromycin (12%) and chloramphenicol (8.5%). The disc diffusion method showed 38% of *E. faecium* isolates were resistant to vancomycin, while the PCR-based van gene detection assay showed they were susceptible.

Salminen et al. (504) characterized 86 clinical lactobacillus blood isolates at species level and tested them for antimicrobial sensitivity. Of the eleven species identified, 46 isolates were *L. rhamnosus* ($n = 22$ *L. rhamnosus* GG type), *Lactobacillus fermentum* ($n = 12$) and *Lactobacillus casei* ($n = 12$). All lactobacillus isolates showed low minimum inhibitory concentrations (MICs) of imipenem, piperacillin-tazobactam, erythromycin and clindamycin. The range of MICs of cephalosporin varied widely with species while MICs of vancomycin were high except for *Lactobacillus gasseri* and *Lactobacillus jensenii*. The antimicrobial susceptibility pattern for probiotic *L. rhamnosus* GG was similar to those of *L. rhamnosus* GG type and other *L. rhamnosus* clinical isolates. This study of a large number of blood culture isolates of lactobacillus indicates their antimicrobial sensitivity to be species dependent.

Sullivan and Nord (505) characterized the Lactobacillus blood isolates from bacteremic patients in Stockholm, Sweden, between January 1998 and March 2004 to identify the possible presence of three probiotic strains of lactobacillus consumed in Sweden. The majority of the 59 isolates were *L. rhamnosus* ($n = 17$), *L. paracasei ssp. paracasei* ($n = 8$), and *L. plantarum* ($n = 8$). No isolates were identical to the probiotic strains. All isolates of *L. rhamnosus, L. paracasei ssp. Paracasei*, and *L. plantarum* were resistant to vancomycin and teicoplanin, while the majority of isolates were susceptible to clindamycin.

Opinions differ on the clinical significance of lactobacillus isolated in infections. In their retrospective review of 241 cases of lactobacillus-associated infections reported between 1950 and 2003; Cannon et al. (506) found the species most commonly isolated were *L. casei*, *rhamnosus, plantarum*, and *acidophilus*, at 35.7, 22.9, 10, and 10% respectively.

Bacteremia was identified in 129 cases, in association with conditions such as cancer, diabetes, broad-spectrum antibiotic treatment, transplantation, or abscesses. *L. rhamnosus* (32.1%) and *L. casei* (28.3%) were the most common species isolated. Over 90% of isolates were sensitive to clindamycin, erythromycin, and gentamicin, while 26.7% were sensitive to vancomycin.

Seventy-three cases of endocarditis were identified, 75% of whom had either existing structural heart disease or a previous episode of endocarditis. *L. casei* (40.6%), *L. rhamnosus* (17.2%), and *L. plantarum* (17.2%) were the most commonly identified species. Isolates were most sensitive to ciprofloxacin, erythromycin, and ampicillin (84.6–100% of isolates) with 26.7% sensitive to vancomycin.

Assessment of the clinical significance of lactobacillus isolated from blood is often confounded by the concomitant presence of other organisms. While Cannon et al. (506) found 38.8% of lactobacillus bacteremia cases were polymicrobial, it is interesting to note that in 61.25% of cases other organisms were not isolated. It is noteworthy that in 95.9% of endocarditis cases *Lactobacillus* was the only species isolated.

These data lend further support to recommendations that probiotics should be taken cautiously or not at all by people with specified serious conditions, in-dwelling devices, and prosthetic or abnormal heart valves (471, 472).

Danielson et al. (507) used molecular methods to characterize 23 *Lactobacillus* strains isolated from blood cultures of Danish patients between 1997 and 2004; prior to testing individual susceptibility to antibiotics. Their findings corroborated the high prevalence of *L. rhamnosus* (43%), *L. paracasei* (22%), and *L. plantarum* (17%) previously observed (506). Although lactobacilli are often regarded clinically as one group, Danielson et al. (507) found distinct variations in susceptibility patterns for the same antibiotic between species, with susceptibility varying widely between species.

Lactobacilli are ubiquitous commensals in humans and whether or not their presence is indicative of infection or contamination is a topic of contention. The source of lactobacilli is frequently unknown and they are present in supplements and dairy foods that may be consumed in high volume. It can be seen that rather than relying on a general recommendation for the genus, the selection of antibiotic treatment for lactobacillus infections should be based on sensitivity data for a species (504, 506, 507).

Organisms have intrinsic resistance to antibiotics or can acquire resistance, either naturally or deliberately. It is a significant reason to select strains lacking the potential to transfer genetic determinants of antibiotic resistance. There is little basis for scientific regulation of strains with intrinsic resistance, as little is known about the levels of intrinsic resistance in current probiotic and food strains.

Mathur and Singh (508) noted a lack of studies examining acquired antibiotic resistance in food LAB, and proposed that LAB used in probiotic or starter cultures may be a source of antibiotic-resistant genes, which if transferable have potential to be transferred to endogenous and pathogenic bacteria. The antibiotic resistance of *Enterococcus* species in particular has been studied because some strains cause serious infections in humans (509). In contrast less is known of the antibiotic resistance of other LAB that consists of numerous genera and species, with varied susceptibility to antibiotics.

Kastner et al. (510) questioned the extent of antibiotic resistance among desirable food bacteria so surveyed the antibiotic resistance of starter and probiotic cultures in Swiss foods by several molecular methods. For the first time the *lnu*(A) gene, which confers lincomycin and clindamycin resistance was found in an *L. reuteri* SD2112 isolate. Tetracycline resistance gene *tet*(W) was also detected in probiotic *B. lactis* DSM10140 and *L. reuteri* SD2112.

Hummel et al. (511) determined and verified the antibiotic resistance of 40 starter cultures and 5 probiotic cultures at the genetic level. Probiotic and starter strains of *Lactobacillus, Pediococcus, Leuconostoc*, and *Streptococcus* were found to be relatively sensitive to penicillin, ampicillin, tetracycline, erythromycin, and chloramphenicol, while more than 70% were resistant to gentamicin, streptomycin, and ciprofloxacin.

In the process of this study Hummel et al. (511) identified factors thought likely to hinder the implementation of the safety evaluation scheme proposed in EFSA's QPS system. The factors are listed below.

- There are no approved standards for phenotype and genotype evaluation of antibiotic resistance in food isolates.
- There is no optimal growth medium capable of growing the majority of *Lactobacillus* species.
- There are no approved standard MICs at which an organism is considered resistant or susceptible to an antibiotic, except for *Enterococcus* species.
- When the genetic basis for resistance is unknown, whether resistance is intrinsic or transferable is unable to be ascertained for many antibiotics.

Antibiotic treatment is known to modify the diversity of normal intestinal microbiota and cause other unwanted side effects. Probiotics are often given concomitantly with antibiotic therapy in attempts to reduce the side effects caused by the antibiotics. In these instances the probiotic should carry only the antibiotic resistance specific for that antibiotic.

Studies have examined the effect of antibiotic treatment combined with probiotic therapy on the fecal microflora. Conversely Saarela et al. (512) investigated the effect in patients of oral doxycycline on the gastrointestinal survival and tetracycline susceptibility of simultaneously administered probiotics *L. acidophilus* LaCH-5 and *Bifidobacterium animalis subsp. lactis* Bb-12. The gastrointestinal survival of both probiotics was similar in the control and probiotic groups. Doxycycline therapy increased tetracycline resistance in fecal anaerobic bacteria and the ingested probiotic *B. animalis subsp. lactis*. Consumption of the two probiotics with doxycycline did not increase transference of resistance genes in these two strains after 10 days of doxycycline therapy. *L. acidophilus* remained susceptible to tetracycline and no resistance genes were detected in *B. animalis* additional to preexisting *tet*(W).

Systematic screening for antibiotic resistance in probiotic strains is not undertaken at present. It is essential that probiotic organisms be sensitive to broad spectrum and commonly used antibiotics. The inability to transfer antibiotic resistance cannot be assumed for all members of a species and like many other probiotic properties this must be assessed on a strain-by-strain basis.

A decision strategy has been proposed (513) using molecular techniques to assess the risk of antibiotic resistance in bacterial strains. The steps are to

- identify the resistance gene;
- attempt to transfer resistance to normal gastrointestinal flora;
- characterize the biochemical mechanism of resistance;
- elucidate the genetic basis for resistance.

If after following this protocol it were shown that a resistance gene was not associated with a mobile genetic element then the risk of transfer of resistance would be assessed as low.

1.7.5 Immune Modulation

The relationships between host immune system and gut microflora and the many mechanisms underlying the beneficial effects of probiotics have yet to be elucidated.

It was originally thought that gut health was achieved by probiotic organisms binding to sites on the epithelial cells of the gut, thus excluding pathogens (477). Subsequent mechanistic studies have shown several possible mechanisms may be involved: stimulation of cell-mediated immune effects (514); altered immunity at mucosae distant from ingested probiotics mediated by Peyer's patches (515); and suppressing of IgE-mediated allergic hypersensitivity by oral probiotics (516).

Modulation of the immune system has a potential to ameliorate allergic, inflammatory, and autoimmune disorders. While an enhanced immune response is desirable in conditions such as infection and cancer, it may not be in allergic disorders where the response needs to be attenuated (473). Current evidence suggests regulation of effects on the immune system may differ between healthy and ill subjects (465, 517). It is thought that immunomodulation may depend not only on the dose of probiotics but also on the immune status of the host (518) and the probiotic strain.

In a murine study of the effect of oral probiotics on lymphocyte proliferation, responses were seen to vary from suppression of lymphocyte proliferation to enhanced T and B cell mitogenesis, depending on the strain (519).

It has been shown that probiotic bacteria can colonize and persist in the GIT of germ-free athymic probiotic-treated mice and their untreated progeny (520). Mortality associated with two of the tested strains (*L. reuteri* and *L. casei* GG) was observed in the immunodeficient-colonized pups. Athymic mice colonized with *Candida albicans* were also treated with the probiotics to test their ability to protect the immunodeficient mice from infection. Survival of the mice increased and dissemination of *Candida* decreased in athymic and control mice, but varied with probiotic strain (521). This paper is reportedly the first to describe an enhanced inflammatory infiltration by probiotics (*L. acidophilus* and *B. animalis)* in response to infected mucosal tissue.

The observation of mortality in probiotic-colonized immunodeficient pups appears to be the first report of neonate mortality associated with colonization by probiotics. It suggests that the safety of probiotic bacteria should be assessed cautiously in the immunodeficient, particularly in neonates (520).

The gut microflora are the major source of microbial stimulus in infancy. The initial colonization of the gut by microflora and their composition are pivotal to the development of immune responses and normal gut barrier function. Kalliomäki et al. (516) demonstrated that the composition of gut microflora differs between healthy and allergic infants. In a standardized double-blind placebo-controlled trial *L. rhamnosus* GG was given to mothers prenatally for 2 weeks before delivery and 6 months postnatally if breast feeding or to the infant if not. No adverse effects were observed in the mothers, and in infants the incidence of atopic eczema in the first 2 years of life was halved compared to that in infants given placebo.

The finding that a specific strain of probiotic bacteria strongly influences immune regulation in infants brings into question the use of probiotics in infancy. Several commercially available infant formulae contain probiotics. Long-term ingestion of

probiotics while the gut microbiota is being established in theory has the potential for the gut to be colonized with the probiotic organisms. The long-term effects of probiotics on the composition of the gut flora and gut immunity during maturation are unknown. It has been questioned whether probiotic safety can be assessed solely by an absence of adverse effects, and longer term endpoints have been proposed to determine whether there is increased risk of incurring diseases such as diabetes and inflammatory disorders (471).

The properties and effects of a probiotic are specific to genus, species, and strain, thus a single probiotic is unlikely or unable to elicit universal health benefits across a spectrum of diseases. In theory this could be overcome by combining several probiotics in one product, the notion being that an individual probiotic would either compensate for any inadequacies of another or neutralize its adverse effects. Synergistic or additive effects could potentially be gained by combining multiple species into a single probiotic product (522). Multispecies probiotics are available but at present no evidence base or criteria exist for selection of the optimal number of strains and their most desirable properties.

Timmerman et al. (522) attempted to resolve this by systematically testing 69 culture collection strains to design a multiplespecies probiotic for preventing infection in critically ill patients. Probiotic strains selected for high survival in the environment of the digestive tract were tested *in vitro* for their capacity to inhibit growth of gut pathogens and modulate immune responses. Candidate strains were tested and ranked on their ability to inhibit clinical isolates and induce high concentrations of anti-inflammatory cytokines or low concentrations of pro-inflammatory cytokines. Strains with negative selection criteria were then excluded, notably *L. rhamnosus* W71 and *Lactobacillus plantarum* W59 (from a species lacking a long history of safe use, and showing resistance to a range of antibiotics respectively). The resultant multispecies disease-specific probiotic consisted of *Bifidobacterium bifidum, Bifidobacterium infantis, L. acidophilus, L. casei, Lactobacillus salivarius*, and *Lactococcus lactis.* In *in vitro* tests the probiotic combination demonstrated a wider antimicrobial spectrum, greater induction of anti-inflammatory cytokines and suppression of pro-inflammatory cytokines than its constituent strains. Proof of efficacy in human clinical conditions remains to be demonstrated in randomized, double-blind, placebo-controlled trials.

Baken et al. (523) assessed the effects of oral doses of *L. casei* Shirota on T helper 1 (Th1) responses and development of autoimmunity in a panel of four assays and found varied effects between assays. In a modified local lymph node assay in BALB/c mice lymphocyte proliferation was significantly reduced only at the highest concentration of topical exposure to the Th1 cell-dependent antigen dinitrochlorobenzene. Treatment with *L. casei* Shirota exacerbated experimental autoimmune encephalomyelitis, a rat model of multiple sclerosis, in treated animals compared to controls, with increased incidence of disease, earlier appearance of neurological symptoms, longer duration of the disease and higher cumulative clinical scores. Neither inhibitory nor stimulatory effects on modulation were found in mitogen-induced cell proliferation and cytokine release assays in mesenteric lymph nodes of rats from treated and control groups. Gene expression profiling by microarray analysis in rat spleen, liver, thymus

and mesenteric lymph nodes found no clear changes in gene expression induced by *L. casei* Shirota. Inhibition or stimulation of Th1 mediated immune responses were found depending on the assay performed, an indication that probiotics can affect the Th1/Th2 balance in either direction. The observation of varying effects on Th1 responses indicates that probiotic consumption may have beneficial as well as harmful effects in immune-related conditions (523).

In conditions where acute immunosuppressive intervention is desirable, as in individuals suffering from conditions such as inflammatory bowel disorder (IBD), it has been proposed that treatment could be targeted through probiotics rendered more powerful through genetic engineering (524). The probiotic design could be customized for differing mechanisms of action and the specific end functions required. Examples already exist of organisms modified for functions as diverse as sequestration of toxins, competitive replacement of harmful bacteria, antibody production, correction of enzyme deficiencies, *in situ* production of detoxification enzymes, and *in situ* synthesis of cytokines (524, 525).

Additional criteria for safety characterization of a probiotic have been proposed to address concerns about immunomodulation (477).

- An infectivity test using high doses of viable organisms in immunocompromised animals.
- Measurement of changes in cytokine balance.
- Mixtures of probiotics to be assessed *in vitro*, to exclude an isolate that can inhibit the cytokine stimulation of another.

It is apparent that an evaluation of immunomodulatory effects should form part of the safety and efficacy assessment for a probiotic. Modifications to previous schemes have been proposed to include immunomodulatory effects for the evaluation of new products (473). As immune effects vary with the probiotic organism and the experimental model (523), a suite of tests is considered essential. Ezendam et al. (473) have initially proposed *in vitro* assays in monocytes and macrophages to determine cytokine profiles, and studies of dendritic cell maturation and activation. Subsequent experimental animal studies would include models for cellular immunity, allergy, autoimmunity, and contact hypersensitivity. Safety of candidate probiotics should then be evaluated in well-conducted clinical trials as evidence for safety and efficacy in humans is lacking. The comprehensive safety and efficacy data should finally be evaluated by an expert to consider the intended use, plausibility of health claims, possible adverse effects,and likelihood of high-risk groups in order to make a risk-benefit assessment (473). The effects of long-term consumption would be monitored by postmarketing surveillance.

The response of normal gut microflora to probiotic intervention varies with age and clinical status of the subject, so immunological effects need to be assessed in specific at-risk populations. Safety evaluation of long-term health effects will be important in the selection of, and characterization studies for a probiotic. The molecular factors modulating immunoregulation need to be elucidated. There are currently no agreed guidelines for the safe use of probiotics in immunocompromized

patients. Immunocompromized people undertaking treatment with probiotics should be observed clinically over a long period to assess the effects and safety of immunomodulation (471, 472).

Mass marketing of probiotics is directed at healthy individuals despite an absence of long-term studies to verify claims that long-term use of probiotics helps to maintain good health in this population. There are no studies measuring the effect of probiotics on the immune system of healthy individuals, or on their innate resistance to disease (465).

1.7.6 Clinical Studies

Clinical studies in humans have investigated the effect of oral administration of probiotics on the balance of intestinal microbiota and in a variety of disorders. Until recently many studies were of inadequate design and produced unreliable data. Inadequate studies have had an absence of a patient control group; small treatment groups; undefined treatment groups; a wide age range within a treatment group; a diversity of antibiotic treatments; an absence of dosing criteria such as dose and duration; or subjects with symptoms of concurrent disease with the potential to confound an observation of adverse effects. The gold standard remains a controlled study with randomized, blind assignation to treatment, placebo, and untreated groups.

Immunosuppressive therapy is considered a risk factor in bacteremia from opportunistic pathogens. Salminen et al. (526) evaluated the efficacy and safety of *L. rhamnosus* GG (LGG) in moderating gastrointestinal symptoms of HIV-positive patients on antiretroviral therapy, in a placebo-controlled double-blinded crossover study. Subjects with HIV infection and persistent noninfectious diarrhea taking highly active antiretroviral therapy were standardized to receive twice daily LGG (viable LGG 1–5 $\times 10^{10}$ cfu/dose) for 2 weeks and 2 weeks placebo in standardized order. No probiotic products were permitted during the washout periods before and after each treatment, to reduce the likelihood of a carryover effect from persistent probiotic. Although the LGG preparation was well tolerated it gave no significant reduction in gastrointestinal symptoms. No adverse events or clinical infections were observed in the subjects during the study or in the 6-month follow-up period. The evidence from this study suggests that LGG is unlikely to be a health risk in HIV patients.

Weizman et al. (527) conducted a 12-week double-blind, placebo-controlled, randomized trial of infant formula supplemented with either *Bifidobacterium lactis* (BB-12) or *L. reuteri* (ATCC 55730) and no probiotics, in healthy infants in child-care centers. The rate and duration of respiratory illness was unaffected by probiotic supplementation. In contrast, children supplemented with *B. lactis* and *L. reuteri* had fewer and shorter episodes of diarrhea compared with the placebo group, with *L. reuteri* showing a significant decrease. These probiotics are safe for infants.

Elderly hospitalized patients were treated with a commercial probiotic in a randomized double-blind placebo-controlled trial to determine the efficacy of the probiotic to prevent diarrhea associated with antibiotic use or caused by *Clostridium difficile* (528). One-hundred grams of probiotic (*L. casei* DN—–114001; 1.0×10^8 cfu/mL; *S. thermophilus*, 1.0×10^8 cfu/mL; *L. bulgaricus*, 1.0×10^7 cfu/mL) was consumed

twice daily during and for 1 week after antibiotic treatment. The reduction observed in the incidence of antibiotic-associated diarrhea and *C. difficile* associated diarrhea in the treated group was statistically significant, and the probiotic was well tolerated with no adverse effects. Criticisms of the study included highly selective inclusion and exclusion criteria such that subjects were unrepresentative of elderly hospital patients, giving rise to results with low generalizability to the wider hospital population (529).

It is thought that infants younger than 3 months may be at risk of acidosis from ingestion of high concentrations of D(−)-lactate-producing probiotic organisms (472). In a study reported by Connolly et al. (530) infants with a family history of allergy were supplemented daily from birth with *L. reuteri* ATCC55730 (SD2112) (10^8 cfu/day) in a double–blind, placebo-controlled clinical trial. *L. reuteri* ATCC55730 produces D(−)–lactic acid which it was thought may abnormally elevate levels in infants. Blood levels of D(−)-lactic acid measured in the infants after 6 and 12 months showed no differences between placebo and treated infants and no adverse effects from long-term supplementation, attesting to the safety of *L. reuteri* ATCC55730 in infants (530).

Severe acute pancreatitis is a serious illness associated with a significant mortality rate for a proportion of those patients who contract infections and necrozing pancreatitis. As antibiotic treatment has not proven effective in reducing infection it has been thought probiotic treatment may be beneficial.

Besselink et al. (531) recently addressed the deficiencies of earlier clinical studies in an elegant multicenter randomized, double-blind, placebo-controlled trial of a multispecies probiotic (522) in 298 patients with predicted severe acute pancreatitis, with unanticipated results.

The probiotic (total daily dose 10^{10} bacteria) or placebo was administered twice daily in enteral nutrition for a maximum of 28 days. The primary endpoint was a composite of several specified infectious complications, including infected pancreatic necrosis, bacteremia, and pneumonia.

Contrary to expectations the incidence of infectious complications was not significantly reduced in the probiotic group, being 30% compared to 28% in the placebo group. None of the infections were shown to be due to the probiotic strains.

Mortality was significantly higher ($p = 0.01$) in the probiotic group (16%) compared with the placebo group (6%). Most of the deaths resulted from multiorgan failure. Nine patients in the probiotic group developed bowel ischemia (eight with a fatal outcome) while no patient did in the placebo-treated group.

The focus on pathogenicity of probiotics has centered largely on the potential for bacteremia and immunomodulation. *In vitro* and animal studies were not predictive of the serious adverse effects seen in this study (531). The probiotic mixture consisted of *Lactobacilli* and *Bifidobacteria* strains that are common ingredients in probiotic supplements or dairy foods individually enjoy European Union QPS status and have not shown adverse effects in previous small clinical studies.

Whether the adverse effects resulted solely from probiotic administration or from this and other factors is unclear. The authors propose putative mechanisms for further investigation.

The evidence from this study is that therapy with this multispecies probiotic is contraindicated in patients with predicted severe acute pancreatitis. Importantly,

contrary to a hope for amelioration of the disease, the high mortality seen in the probiotic group raises doubts about the safety and efficacy of probiotics in such critically ill patients.

1.7.7 Postmarket Surveillance

Two Finnish studies have investigated the incidence of infections associated with LAB. In the first study 16S rRNA methods were used to characterize and identify LAB isolated from blood cultures of bacteremic patients in Southern Finland (532). The number of infections caused by lactobacilli was extremely low and the infections were not associated with the probiotic strain newly introduced in fermented milks.

In a subsequent study, lactobacilli isolated from bacteremic patients between 1989 and 1994 were compared to common dairy or pharmaceutical strains (533). From a total of 5192 blood cultures 12 were positive for lactobacilli, an incidence of 0.23%. None of the clinical cases could be related to lactobacilli strains used by the dairy industry. In both studies, patients with LAB bacterium had other severe underlying illnesses.

Salminen et al. (504) examined the incidence of lactobacilli bacteremia in the Finnish population for the period corresponding to a rapid increase in consumption of the probiotic strain *L. rhamnosus* GG (ATCC 53103). This strain was isolated from human intestinal flora and introduced into dairy products in 1990. By 1999 the annual per capita consumption was estimated at 6 L (3×10^{11} cfu) per person/year.

The Helsinki University Central Hospital collected all lactobacillus isolates from blood cultures and cerebrospinal fluid in its catchment area from 1990 to 2000. Blood culture isolates were also collected for all cases of lactobacillus bacteremia reported (and unreported) by mandatory notification to the National Infectious Disease Register, from its inception in 1995 to 2000. Species were characterized and compared to *L. rhamnosus* GG strain by molecular epidemiological methods.

Ninety cases of lactobacillus bacteremia were identified between 1995 and 2000, when the population in Finland was 5.2 million. Of the 66 isolates available for species-level identification 48 were lactobacillus isolates, with the most common species being *L. rhamnosus* (26, 54%), *L. fermentum* (9, 19%) and *L. casei* (7, 15%) respectively. In 35 cases more than one additional bacterial species other then *Lactobacillus* was also identified. Eighteen of the 66 isolates (27%) were organisms other than lactobacillus. Eleven of the 26 *L. rhamnosus* strains were indistinguishable by PFGE from the probiotic *L. rhamnosus* GG.

No increase in the incidence or proportion of lactobacillus bacteremia was observed, despite a clear increase in the number of cases of bacteremia over the period. Lactobacillus isolates as a proportion of all blood culture isolates was 0.24%, consistent with previous Finnish reports (533). The average annual national incidence of lactobacillus bacteremia was estimated to be 0.29 cases per 100,000 people per year. The study provides evidence that increased consumption of *L. rhamnosus* GG had not led to a corresponding increase in lactobacillus bacteremia.

Borriello et al. (496) was unable to find published medical literature regarding the consumption of viable probiotics by hospital patients, some of whom may be

predisposed to infection by probiotic bacteria. They suggested that because of the low incidence of probiotic bacteremia and the sophisticated methods and experience needed to confirm it, identification and confirmation of species and strain characteristics of suspect clinical isolates should be referred to national reference centers. National clinical and epidemiological databases could include identity of organism, status of the patient's underlying conditions, coexisting infections and outcomes, and data on the patient's use of probiotics.

1.7.8 GMO Probiotics

While the search continues for nonpathogenic organisms with therapeutic potential, genetic engineering of an organism to produce an identified desirable bioactive molecule may represent a technically more efficient and attractive approach (501). Administration of therapies by the systemic route is recognized to cause unwanted side effects at sites other than those of interest. The concept of localizing delivery to a region of the intestinal mucosa where synthesis of a bioactive molecule *in situ* may bring about the desired effect without the disadvantages of systemic side effects has appeal.

Genetic manipulation offers the potential to enhance the existing probiotic properties of an organism or to imbue an organism with probiotic properties (524). Elucidation of mechanisms of activity of a probiotic will enable manipulation of organisms to create specific and targeted probiotics. Consumer resistance to genetically modified organisms (GMO) is such that GMO probiotic foods are unlikely in the near future, but clinical applications to ameliorate or prevent chronic intractable diseases may be more readily accepted.

Steidler et al. (534) and Kaur et al. (535) treated mice models with GM bacteria to prevent colitis and enhance the efficacy of antitumor therapy respectively, demonstrating in principle that probiotics can be designed to produce potent bioactive chemicals. Having engineered *L. lactis* to deliver mouse cytokine IL-10 at the intestinal mucosa Steidler (524) then constructed a biologically contained *L. lactis* to produce human IL-10. In the first clinical trial of its kind Braat et al. [539] treated Crohn's disease patients with this GM *L. lactis* in a phase 1 placebo-uncontrolled trial. A decrease in disease activity was observed with minor adverse effects, and containment of the organism was achieved through its dependency on thymidine for growth and IL-10 production.

The incorporation of GMO bacterial strains into therapeutic products will necessitate stringent procedures for safety assessment. To treat an individual with a living recombinant microorganism is to release a GMO into the environment and in such instances safety is of paramount importance (476, 524, 537). Of no lesser importance introducing and exposing an individual to foreign protein in this manner has potential to provoke an immune response that may preclude clinical applications (524).

The organism will need to be "biologically contained" to prevent its undesirable release and accumulation into the environment, and to prevent transmission of the genetic modification to other bacteria (524). Methods for biological containment have been demonstrated previously. Control of the organism may be active through, for example, production of a bacterial toxin that is regulated through genetic

expression controlled by an environmental response. Passive control may be employed where growth of an organism depends on either the presence of an essential substrate or the gene facilitating its production (524).

1.7.9 Conclusion

Consumers are increasingly managing their health by self-medication, generating expanding market opportunities for the food and pharmaceutical industry. Food and drink manufacturers are adopting genomic and proteomic technologies previously the domain of the pharmaceutical industry to design more sophisticated and novel products.

Because exploitation of the probiotic concept is still associated with unsubstantiated claims reliable and proven products need to be readily distinguished from those of dubious quality. A significant proportion of consumers mistrust manufacturers' claims. Forty-five percent of Americans claim to largely or entirely disbelieve food and drink manufacturers' health claims, a figure similar to France and greater than in the Netherlands (538). The evolution of probiotic products thus necessitates changes in the regulations related to labeling, safety, and health claims.

The credibility of health claims for healthy individuals remains to be established. Viable probiotic bacteria have to be consumed in large quantities for a prolonged period to achieve a health benefit. The long-term effects of probiotic consumption on a healthy population are unknown, and yet the general population is being encouraged to consume probiotics regularly, to promote good health and well-being. Studies have yet to be undertaken to demonstrate what effect, if any, there is on the well-being of healthy individuals.

While probiotic cultures are incorporated into foods or dietary supplements without making specific health claims they avoid the need to conform to the more rigorous regulatory procedures for therapeutic products, which require demonstrated quality, safety and efficacy. There is evidence from well-conducted clinical trials of beneficial health effects from probiotics in a range of clinical conditions. The probiotic effects have been shown to be strain specific so health effects cannot be generalized between strains.

Standardized, verifiable clinical studies are needed to demonstrate the safety, efficacy, and limitations of a putative probiotic, and whether it is superior to existing therapies. Additional studies are needed to determine effects on the immune system in healthy and diseased individuals and effects of long-term consumption. More rigorous quality control, standards, and regulations have been called for (481, 482, 477, 483). The prospect of GM probiotics targeted for clinical conditions demands a rigorous safety strategy to prevent spread into the environment and dissemination of the genetic modification.

Permission to label a product probiotic should remain contingent on its compliance with the FAO/WHO definition of probiotic (475). Labels should include consumer or prescriber information about the identity of the organism(s); its GMO status; viability count and shelf life; dosing and duration; conditions for which its use is and is not appropriate; proven benefits; side effects, particularly symptoms that require clinical assessment; and a recommendation to advise health practitioners of probiotic use (471). Where an adverse reaction is suspected the facility to report it along with the

product details to a national database should be available, as it is for adverse effects of other therapies.

Guidelines have been proposed to assess the efficacy and safety of probiotics but international agreement on these has yet to be arrived at. Consensus on uniform regulations is desirable to ensure unequivocal identity, quality manufacturing processes, accurate labeling, proven safety and efficacy for a product that will then merit the label "probiotic."

1.8 LEGAL STATUS AND REGULATORY ISSUES

1.8.1 Human Probiotics

1.8.1.1 Asia

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Most of the early probiotic studies were related to fermented dairy products enriched in LAB. Items such as yogurt, sour milk, cottage cheese, etc. were in themselves regular foods with thousands of years of tradition. Beneficial microbes contained within these foods were identified and developed into specific products with health augmentation purpose. The market has been growing rapidly from 7% in the United States to 15% in China. (Business Communications Co. Ltd. (BCC) July 2005). Total value may reach US$1.1 billion 2010 (539). This does not include food items that already have live microorganisms as a part of their original production process, such as yogurt, Kefir, cheese, sauerkraut, Kimchi, etc. In the interest of public health protection, national governments tend to view probiotics as food rather than drug, unless specific claim on therapeutic effects were attempted. Regulatory attitudes also gradually change as more scientific information become available. This report intends to review the current status of regulatory policies regarding probiotics in Asian countries, to the extent that such information is publicly available.

The various definitions of probiotics are listed in Section 1.1.

Summarizing the common features of the definitions listed, probiotics is a term referring to products that

- are living organisms;
- requires sufficient dosage to be effective;
- confer health benefits.

Lactic acid bacteria are the most commonly mentioned example of probiotics. However, other microorganisms such as *Bacillus* sp., yeast, and algae (blue-green algae, *Spirulina*, *Chlorella*, etc.) were also grouped into the definition of probiotics, since the propagation and management of these organisms as human food seems to require similar technologies. Therefore, regulatory considerations usually include

these products into the same category. On the contrary, larger organisms such as mushrooms, and fungi, such as *Gonoderma* sp., *Cordyceps* sp., are usually not included in probiotics but are considered as regular foods. Therefore, the definition suggested by FAO/WHO (540) seems to have been accepted by most official organizations in Asia: "Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host."

Probiotics has been recognized as an important product due to numerous scientific publications that have become available in the past 20 years. The beneficial functions of probiotics are described in Section 4.3.

Probiotics has been demonstrated to be effective in relieving certain symptom of illness that has not been successfully addressed by conventional medical practice. As more data on the safety and successful performance of probiotics become available, traditional western health professionals have changed their suspicious attitude to welcome food supplement products that could help conventional health-care practice. The market of probiotics thus has grown significantly in the past 10 years. Products with clear label and proven functional properties, as well as stable specifications are more likely to succeed. Market survey of probiotic-related products varies depending on the scope of definition. Dairy products containing LAB was estimated to be worth over US$30 billion in 2005, with Japan alone accounting for US$5 billion (541). The market size for pure probiotics is smaller. Probiotics sold as food supplement in the USA was slightly over US$240 million (542). However, the annual growth rate was 14%, which is highly remarkable for any food item. The sale of probiotics in Europe was 12 million. The annual growth rate was also 15%, indicating an optimistic trend for the near future.

However, a series of legal incidences in recent years also caused concern for business development (543). Consumer advocates and insurance companies have also started demanding more guidelines when it comes to using probiotics as a part of regular health care portfolio. Some would insist that so long as probiotic products are used for therapeutic purposes, they should be regulated as any other conventional drugs, notwithstanding the fact that the product may have been consumed as a food item safely for many years. The others would argue that since the safety record of a probiotic product has been acceptable as a food item, the relevant requirement on safety tests should be rationally reduced, while emphasizing on the efficacy demonstration. The result of these tests, and the regulatory position pertaining such "therapeutic" products should be fully disclosed and inform the public aggressively to reduce confusion. These differences in attitude has been subject for debate in many forums, and resulted in legal action in some cases. Companies with major stakes in the matter have therefore stepped forward and tried to establish general consensus on product concepts based on strong scientific foundation that would encourage healthy market growth. Stabilization technology for microorganisms has been developed as a result. Food products with special functional purposes were also developed using probiotics. Items include baby formula, fruit juice, breakfast cereals, yogurts, even chocolates with over 1 year shelf life. It was felt that only with sound scientific data could a positive guideline be established for healthy development of probiotic

products that could benefit human health. This report intends to review the phenomenon in Asian countries, especially on the regulatory part.

Study of the Asian country's position regarding probiotics should start from the position of FAO/WHO of the United Nations (UN). In our survey, it was noticed that with a few exceptions, most of the Asian countries started their own official regulatory policy after the UN has established a relevant guideline. In fact a conference on functional food was organized by FAO/WHO in 2004 in Bangkok for the very reason of increasing awareness for the need of regulating such products. Probiotic was only part of the discussion during this conference. The two UN organizations have noticed the increased consumption of probiotic products in the world since 1990. Discrepancy among member countries, and even among regulatory authorities within the same country, has led to confusion in the general public. Inferior products competed under false pretense, and indeed may cause health risk to the consumer due to lack of accurate information. An expert panel on the subject of probiotic therefore was convened in 2001 at Cordoba, Argentina. Consensus was reached during the meeting regarding the importance to have a common guideline for probiotics regulation. A recommendation was drafted as a result and was approved in May 2002 during a follow-up meeting in Canada (544). This guideline recommends how a probiotic food product could be approved for marketing by the national regulatory authorities based on scientific principle. Member countries could establish their own national regulation based on this recommendation. The expert panel consciously excluded probiotic products designed for medical use, animal use, and microorganisms that have been modified through genetic modification, so as to limit the scope of discussion.

The principle involved in the guideline is that the probiotic product must first be qualified as a food; it can then be further regulated as microorganisms that confer beneficial effect to human health.

The leading issue in the guideline seems to be the identity of the microorganism within the product in question. It would be necessary to identify the culture at subspecies or strain level for most probiotic products, except some of the traditional LAB that are used for conventional fermentation foods. The microbiology associated with the safety, functional property and physiological effects cannot be studied until the identity of the bacteria has been confirmed.

The technology that was preferred in the FAO/WHO guideline for culture identification was DNA–DNA hybridization. However, the procedure involves complicated process and expensive equipment that may not be readily available. Therefore, 16sRNA sequence comparison was also recognized as a reliable technique to identify microbial taxonomy, in addition to biochemical reactions and phenotypic observations. Such data package was recommended to be the first step for probiotic product registration. The other review process and data required for safety, *in vitro* and *in vivo* trials, toxicity data, etc. are usually more lenient than those required for pharmaceuticals, more so if the product has been classified as GRAS, in lieu of Food and Drug Administration of USA. The recommended registration process for probiotic product is as shown in Fig. 1.15.

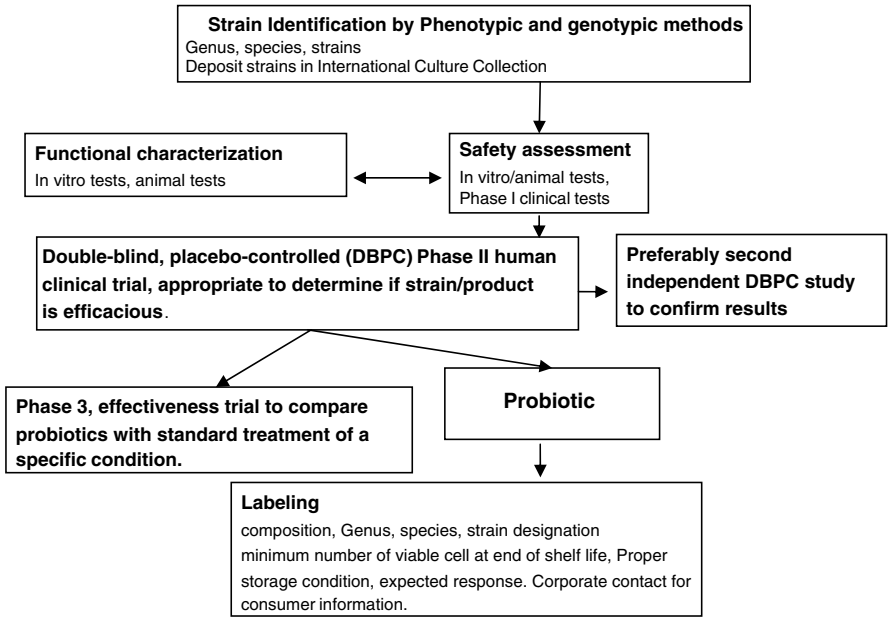

FIGURE 1.15 FAO/WHO recommended registration process for probiotic product (544).

United Nations has recommended a number of product specifications that should be considered when a probiotic product is to be approved. These include stability against pH, ability to colonize in the GIT, taxonomical identification, and proper dosage.

1. *Viability*: Probiotic should survive passage through the GIT. Since the most severe threat comes from acidity in the stomach, *in vitro* simulation in dilute HCl could be used. Bile acid and other digestive enzymes may also be tested *in vitro*.
2. *Colonization*: Good probiotic culture should be able to colonize and/or become attached in the GIT of the host, and multiply in the presence of bile acid. The growth should benefit the host by reproducible evidence. Different microbes may colonize at different locations within the GIT of different host.
3. *Probiotic Culture*: The culture should be able to compete successfully against pathogenic bacteria for the attachment on intestinal surface.
4. *Inhibition*: Probiotic may show inhibition of pathogenic bacteria by *in vitro* tests.
5. *Resistance*: Probiotics used for vaginal protection should show resistance against other sanitary agents or disinfectants.
6. *Identity*: Probiotic products should indicate on its label the genus and species name according to international nomenclature and expected viability during the shelf life of the product.

7. *Dosing*: The label should indicate recommended dosage and expiration date.
8. *Data*: It will be desirable if there are data indicating the product will not infect immunocompromized animal.

The FAO/WHO guideline constituted the background for each member nations to establish their own regulation. However, such is not always the case for every member nation. In Asian countries this is especially so. The following are some of the examples.

1. *Japan*. Japan is one of the few countries that actually have an endogenous regulation regarding probiotics before the UN guideline was published. One of the main reasons was that Japan had established its own microbiology industry almost simultaneously with those in western countries. Japanese scholars published widely in the field of pathogenic bacteriology in early 1900s and a robust fermentation industry had been established during early and mid-twentieth century. The most prominent companies in this industry are Yakult, Kyowa Hakko, Fujizawa, Tanabe, etc. As Japanese scholars involved in the early stage of guideline preparation for the FAO/WHO conference, Japanese regulatory policy on probiotics was friendly toward industrial manufacturers. In general, all producers of functional food in Japan may apply for a certification from the Ministry of Health, Labor and Welfare (MHLW) according to a "food for specialized health use" (FOSHU) system. The system was first initiated in 1984 by the Japanese government through a series of studies and recommendation from a group of scientists from the academics, industry, and government research organizations. It was suggested that with advanced age of Japanese population, the cost of health care could be controlled through proper intake of functional food, instead of offering therapeutic solutions after the person is ill. FOSHU system was therefore formally recognized and entered into Health Improvement Act, Article 26, and Food Sanitation Act, Article 11, in 1991. In fact, functional food was first coined in Japan. Probiotics were then included as part of functional food. Japan has further distinguished among functional foods so as to designate products that wish to make specific health claims against certain illness (special health protection food), general nutritional supplements, and special application food (for pregnant women, patients under recovery from surgery, or otherwise could not consume regular foods, etc.).

   There was no official requirement for the minimum viable cells in the final product. The government seemed to leave the issue to the discretion of industrial organizations such as Japanese Fermented Milks and Lactic Acid Bacteria Beverages Association. This organization stipulated that a product containing $\geq 1 \times 10^7$ viable bifidobacteria/g or ml is to be considered a probiotic food (545). Dosages also need to be considered alongside with frequency of intake, the strain involved, and the general health condition of the consumer. Food regulations in general are not concerned with such issue, as would a drug regulation. Industrial manufacturers are therefore leery of establishing a recommended dosage for fear

of being driven into drug category. It was generally agreed, however, the viability of the cells needs to be indicated for the duration of product's shelf life.

There were over 69 products formerly registered through FOSHU as probiotic products in Japan by December 2005, which were allowed to claim augmentation of gastrointestinal disorder out of a total of 569 registered functional food products (546). At the time of this writing there were a total of 680 functional food products registered through FOSHU system. It should be interesting to note that more than 57% of these foods were addressed to gastrointestinal disorder, and the remedies included not only probiotics, but also fiber, prebiotics such as oligosaccharides, etc.

2. *People's Republic of China*.The country with the largest populace in the world stands to gain advantage in health care cost saving by promoting probiotics. However, Chinese culture does not have a traditional role for LAB as most western countries would have in the form of fermented dairy products. Most of the Chinese physicians received their training with a microbiology curriculum that emphasized mainly on pathogenic bacteria. The benefit of probiotics has not been widely introduced in China until early 1980s. Nevertheless, with rapid economic growth, scientific data regarding probiotics from the developed countries soon received attention in China. While regulations were drafted, Chinese Ministry of Health took into consideration that the traditional Chinese Herbal Medicine actually included a number of fungal cultures that were believed to be beneficial to health. Thus, "Fungal Health Food Review Guidelines" and "Probiotic Health Food Review Guidelines"were both issued in March 2001 in accordance to Food Sanitation Law and Regulation on Health Food, with later revisions until 2004. The basic principle is more or less similar to those practiced in Japan and other western countries. The guidelines included specific instructions on the technical capability of the parties applying for the health food registration for either fungi or bacteria. The identification of the culture should be performed by officially sanctioned research institutes that are usually government-run to ensure accuracy. There are a number of clearly defined cultures (547), which are allowed to be included in health food category without the need for extensive safety studies due to historical reason. These culture lists were attached to each guideline, effectively discouraging companies from using cultures that has not been named in the list. In case a new culture that is not listed, companies would need to go through the expense and effort to register new cultures based on these guidelines, proving safety and efficacy.

   The PRC government however had been aware of the current regulation on health food were rather cumbersome, and a Provisional Health Food Registration and Management Directive went into effect on July 1, 2005. The new directive differs from the current system in the following manner:

   (a) Since companies tend to concentrate on using well-known ingredients and applying for health food registration only in the 27 sanctioned functions published by the State, for fear of uncertainty in the review

process if a new function is being applied, the current 27 functions will be abolished, so that new ingredients and functions will be encouraged. But benefits declared by health food for malignant tumor prevention will no longer be allowed.

(b) Parties who are allowed to apply for health food registration will now include not only corporation, but also individual persons. This is helpful for private individuals who are not attached to any official organizations to come forward with their invention.

(c) Once the documents that are required for health food registration are considered complete and accepted by the Ministry of Health, a decision must be made within 5 months by the reviewing committee, as compared to the average of 8 months at the present.

(d) Health Food License will be valid for only 5 years, and is renewable upon re-registration, which should be initiated no later than 3 months prior to the license expires. This way, the more than 8000 health food licenses already granted will be weeded out not only through marketing force but also by the economic consideration due to the cost of registration.

(e) Stricter limit was placed on the wording used for promotion of health foods. Several branded health foods are deemed as "negative examples" are named in the new regulation and will not be tolerated in the future.

(f) All advertisements relating to health foods will need to be approved first prior to publicity.

The regulation on probiotic health foods was also revised in the meantime, which clearly specified that any new culture applied for health food status would be approved by State Food and Drug Administration (SFDA) (548). The approved list of probiotic microorganisms at the time of this writing is shown in Table 1.11

There have been some arguments for the requirement of viability of probiotic products, citing examples that dead probiotics could also work in

TABLE 1.11 List of Probiotic Cultures Recommended by SFDA

Bacteria	Fungi
Bifidobacterium bifidum	*Saccharomyces cerevisiae*
Bifidobacterium infantis	*Candida utilis*
Bifidobacterium longum	*Kluyveromyces lactis*
Bifidobacterium breve	*Saccharomyces carlsbergensis*
Bifidobacterium adolescentis	*Paecilomyces hepiali* Chen et Dai, sp. Nov
Lactobacillus delbrueckii subsp. bulgaricus	*Hirsutella hepiali* Chen et Shen
Lactobacillus acidophilus	*Ganoderma lucidum*
Lactobacillus casei subsp. Casei	*Ganoderma sinensis*
Streptococcus thermophilus	*Ganoderma tsugae*
Lactobacillus reuteri	*Monacus anka*
Lactobacillus rhamnosus	*Monacus purpureus*

certain beneficial functions. Therefore, the official position is that dead organisms and/or their metabolites could also be considered as probiotics, as long as the functional ingredients and assay methods could be identified. Otherwise, a minimum of 10^6 cfu/g (mL) is required before the product expiration date.

3. *Korea*. Health/Functional Food Act of Korea was first published in August 2004. The regulation is unique in which it requires the products that come under this law must be sold in dosable form, that is, pills, tablets, capsules, etc. (549). There are also 37 categories that are recognized as generic health/functional foods that do not require special safety and efficacy studies, as shown in Table 1.12. Probiotics in the form of LAB, *Chlorella*, *Spirulina*, and *Monascus* species are also included in the table. However, specifications for a product to

TABLE 1.12 Categories of Functional Food in Korea

Health Food Product Category by KFDA			
1	Nutritional supplement products	20	Grape seed oil products
2	Ginseng products	21	Fermented vegetable extract Products
3	Red ginseng products	22	Muco-polysaccharide protein Products
4	Eel oil products	23	Chlorophyll-containing products
5	EPA/DHA fish oil products	24	Mushroom-processed products
6	Royal jelly products	25	Aloe products
7	Yeast products	26	Plum products
8	Pollen products	27	Turtle products
9	Squalene products	28	Beta-carotene products
10	Product of digestive enzyme	29	Chitosan products
11	Edible lactic acid forming bacteria products	30	Chito-oligo-saccharide products
12	Chlorella products	31	Glucosamine products
13	Spirulina products	32	Propolis extract products
14	Edible oil containing gama-linolenic acid products	33	Green tea extracts and its their products
15	Wheat germ/rice bran oil products	34	Soyprotein and its products
16	Products with wheat germ and/or others	35	Phytosterol, Phytosterolesters and their products
17	Egg and/or soybean lecithin products	36	Fructo-oligosaccharide and its products
18	Octacosanol products	37	Red rice and its products
19	Alkoxy-glycerol products	38	Product specific

be accepted into each category are rather strict. Ministry of Health and Welfare is the agency in charge of health food legislative changes on the Health/ Functional Food Act, while Korean FDA is in charge of all the other matter relating to food products. The 37 categories are listed in Table 1.12

Korea also has rather strict regulation regarding the safety and identity of probiotic products, requiring the industry to apply for a new evaluation by the Korea Food and Drug Administration each time a new culture combination is designed, even if individual cultures had been tested and approved previously. Probiotic products rank fourth in the functional food market, following nutritional supplements (such as vitamin mixes, etc.), ginseng and aloe. Furthermore, KFDA issues only health food license to companies with local registration. Overseas company can only register it health food products through an authorized domestic agent or its own subsidiary.

4. *Malaysia*. Malaysia government has been aware of the role of probiotics as a food item, and issued relative provisions in the Food Regulation (550) in 1985. Over the years Malaysia has adopted an attitude that seems closer to those being practiced in Commonwealth of United Kingdom. Lactic acid bacteria are recognized as a legitimate food ingredient, and *Bifidobacteria lactis* and *B. longum* are both mentioned specifically in the regulation with minimum of 10^6 cfu/g requirement if such cultures are to be labeled on any food item (551). The regulation also stipulates that the label must follow the following rules, which further emphasizes the requirement for viable cells.

   However, Malaysian regulation does not allow any functional claim that could be construed as having therapeutic benefits, as was stipulated in the same regulation (552):

   (a) In these regulations, "nutrient function claim" means a nutrition claim that describes the physiological role of the nutrient in the growth, development, and normal functions of the body.

   (b) A nutrient function claim shall not imply or include any statement to the effect that the nutrient would afford a cure or treatment for or protection from a disease.

   (c) Where a claim is made as to the presence of bifidobacteria in food, there shall be written in the label of a package containing such food, a statement setting out the viable bifido bacteria count present in a stated quantity of the food.

   (d) There shall be written in the label on the package of food containing bifido bacteria the words "Contains viable bacteria, require special storage condition" or "Contains viable bacteria, follow instruction for storage."

   The regulation specified 12 functions that could be allowed to print on the label relating to ingredients such as vitamins and minerals. But no probiotic culture was allowed to claim any benefits.

5. *Taiwan*. Taiwan has promulgated a Health Food Control Act in February 1999, focusing on the process of registration of health foods, claims, and penalties.

The Act has since been revised four times until 2002. The government also issued official guidelines regarding the efficacy and safety evaluation for certain human health conditions. At the time of writing, a total of 13 efficacy-testing protocols for health-improvement functions have been recommended. These include sero-cholesterol augmentation, osteoporosis improvement, immunity augmentation, dental health improvement, blood-sugar augmentation, liver function improvement, gastrointestinal function augmentation, antifatigue, antiaging, etc. The product may be labeled with specific claims that conform to recommended wordings by the Act, after the clinical trials have been successfully completed. The label is as follows:

The health care effects of health food shall be described in any of the following ways:

1. claiming the effect of preventing or alleviating the illness relating to nutrients when deficient in the human body if intake of the health food can make up said nutrients;
2. claiming the impact on human physiological structure and functions by the specified nutrients or specific ingredients contained in a health food or the food itself after the health food has been taken;
3. furnishing the scientific evidence to support the claim that the health food can maintain or affect human physiological structure and functions; and/or
4. describing the general advantages of taking the health food (553).

There exists specific regulation governing how a new health food registration should be applied, including requirements on the manufacturing facility, building, equipment, process control, labeling, ingredient listing, and even award for people who report to the government on violation of the specific Act. Mentioning of viable cells in the probiotic products is voluntary, with no requirement on the wording. Therefore most of the products on the market are content with claims on the viable cells at the time of the manufacturing, rather than at the end of stated shelf life, although such a requirement was recommended (not required) by the Health Food Control Act. By the end of 2005 there were 18 probiotic health foods officially registered by the Taiwan government.

6. *The Other Asian Countries*. A brief overview of other Asian countries such as India, Pakistan, Bangladesh, Nepal, Thailand, Vietnam, Philippines, Indonesia, etc. regarding functional food has been published by the UN (554). It seems that most of these countries, with the exception of India, are still in the development stage of policy and regulation regarding functional food, let alone probiotic products per se. Each country is aware of the need for special legislation for functional foods since most of them have traditional remedies that have been used for health care, but are not in the realm of western medicine. Probiotic is already recognized as having a special role in countries such as Thailand (555) and India.

7. *Impact of regulatory policy toward the development of probiotic industry.* Asia has the largest population among all of the world's continents. Economic development within Asian countries varies from one extreme to the other. It is apparent from this study that countries with endogenous microbiology industry would have more confidence in establishing more flexible guidelines and regulation on probiotic products, allowing such products to be sold as food rather than drugs. Japan is probably unique in that the industrial organizations would play important role in the initial review and certification, under the supervision and authorization of the government. Probiotic Industry has been careful in establishing the credibility of their products over the last 50 years, so that the sales of probiotic products has reached over US$5 billion in recent years, probably one of the highest per capita consumption in the world.

The world market of probiotic products has been growing at an annual rate of 15% until 2003; not only in the form of fermented dairy products but also in probiotic foods that mainly consists of viable LAB or other traditionally employed microorganisms such as *Bacillus natoensis* and *Monascus* sp.

It was remarkable that most of the countries studied in this article started to recognize the importance of regulating probiotics since 2004 probably as a response both to the market development, as well as the position taken by FAO/WHO of the UN. One of the important decision makers for purchasing probiotic foods who have been resistant to the idea of probiotics is the conventional medical service provider such as western physicians and pharmacists, especially in countries of underdevelopment economics. The main reason is probably due to the dubious reputation caused by inferior probiotic products in the past, when there was no regulation at all to control the quality of these products. It was only through the effort of major industrial players who collaborated with reputable academic institutes in various nations to perform scientific research and clinical trials, and demonstrated reproducible results through publication, were such negative attitudes among the physicians become soften. Advanced technology such as 16SRNA sequence analysis and other biochemical characterization also provided assurance of bacterial strain identification, and safety control during manufacturing. Researchers are now patenting specific cultures that have proactive physiological benefits such as allergy reduction LGG (556), cholesterol reduction *Bacillus coagulans* (557), cancer inhibition *L. acidophilus* 1–1492 (558), and cultures that have been developed in Asian countries such as LP33 (559). New patents on probiotic health functions are being filed at a rate of over 30 per year since 2002, indicating an important trend in the near future. The fact that most of the probiotic products are also qualified as food, as far as safety is concerned, provided an attractive alternative to sustain health in an aging population, with relatively low cost. Hence Asian governments have been aggressive in putting together a friendly environment to encourage the growth of the probiotic industry. Countries who have the most reasonable policy based on sound scientific knowledge would stand to gain the most advantage.

1.8.1.2 Europe

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Until now, health claims on functional foods have been regulated on a national level in the EU. Regulatory bodies like the Agence Française de Sécurité Sanitaire des Aliments in France, Joint Health Claims Initiative (now closed) in the United Kingdom, Voedings Centrum in the Netherlands, and the Swedish Nutrition Foundation in Sweden have evaluated health claims for functional foods. This situation is clearly not satisfactory as the nationally approved claims may interfere with the free movement of goods in the EU internal market; a product maybe sold with a health claim in one country but this claim might not be allowed in another country. A harmonization has therefore long been desired and a new regulation on nutrition and health claims was adopted by the European parliament on January 19th, 2007 (560). The new regulation deals with nutrition claims, health claims, and reduction in disease risk claims (561, 562).

1. *Definitions*. Within the new legislation, a number of terms are defined, some of them are mentioned below.

   A "claim" is defined as any message or representation in any form, which states, suggests or implies that a food has particular characteristics. "Nutrients" are protein, carbohydrate, fat, fiber, sodium, vitamins, and minerals. "Other substances" are defined as substances other than nutrients that have a nutritional or physiological effect. Nutrition claims refer to foods with particular beneficial nutritional properties such as

   (a) the energy the food provides or does not provide; the reduced or increased levels of it.

   (b) the nutrients or other substances it contains or does not contain or contains in different amounts or proportions.

   Health claims that are based on generally accepted scientific evidence fall under Article 13.1. Health claims based on newly developed scientific evidence and/or those claims that request protection of proprietary data fall under Article 13.5. Claims regarding a reduction of disease risk and claims referring to children's development and health fall under Article 14.

2. *Health claims* other than those referring to the reduction of disease risk, Article 13.1. Generally accepted claims that were submitted under Article 13.1 did not require a full scientific dossier. Instead, member states had until January 31st, 2008 to compile a list of claims and submitted them to the European Commission (EC). Although the member states were required to submit their list of claims by that date, many had indicated that they would have deadlines for submission well prior to the 31st of January 2008. This would allow the authorities to compile and finalize the list well in advance before submitting it to the EC. After this date, the EC will consolidate the lists and the EFSA will evaluate the claims. The final list with permitted and generally accepted claims and their conditions of use are to be

adopted by January 31st, 2010, at the latest. In the mean time, health statements can be made under the assumption that they would be approved by EFSA and hence have to have been submitted under Articles 13.1, 13.5, or 14.

The following area's of health claims have been indicated:

(a) Growth, development, and the functions of the body.

(b) Psychological and behavioral functions.

(c) Slimming or weight control or a reduction in the sense of hunger or an increase in sense of satiety or reduction of available energy from the diet.

The European trade associations representing the food and food supplement industry, Confederation of Food and Drink Industries in Europe (CIAA), European Federation of Associations of Health Product Manufacturers (EHPM), European Responsible Nutrition Alliance (ERNA), and the European Botanical Forum (EBF), developed in anticipation of the need to compile a list of claims a template for registration (Table 1.13). The layout of the list has been adopted by a substantial number of EU member states as a basis for the inventory of health claims.

3. *European Trade Associations Inventory for Article 13*. The CIAA, EHPM, ERNA, and EBF have jointly developed an inventory list for submission to the Commission. The trade associations' members have included their ingredients on the inventory following the layout suggested. The list will be used as an example here to describe the requirements for the application under Article 13.

The final list contains seven major categories: vitamins, minerals, proteins, carbohydrates, fats, fiber, and probiotic ingredients. Under "fiber," seven prebiotics have been included, while under "probiotic ingredients" more than 50 strains and combinations of probiotic strains have been included.

(a) *Food Category or Food Component*. The first column in the table is used to describe the food or food component. Here, an appropriate and correct description of the active component should be given. In particular for probiotics it is important that the correct taxonomic name is used, the strain designation is given and (when available) the code under which the strain

TABLE 1.13 For Registration of Health Claims, the European Food and Food Supplement Trade Associations (CIAA, EHPM, ERNA, and EBF) Have Developed a Template Table to Summaries the Claims

	No	Food or Food Component	Health Relationship	Conditions of Use	Nature of Evidence	Ref	Example wording
Vitamins							
Minerals							
Protein							
Carbohydrates							
Fats							
Fiber							
Probiotic ingredients							

has been deposited in a public culture collection for safe deposit. Examples for probiotics are *Lactobacillus acidophilus* NCFM (ATCC SD5221), *Lactobacillus johnsonii* NCC 533 (La1) (CNCM I-1225), or *Bifidobacterium animalis* ssp. *lactis* Bb-12®. Examples for prebiotics are inulin, fructo-oligosaccharides, galacto-oligosaccharides, and polydextrose. Although the production process of prebiotics may influence the structure, and thereby the functionality, origin and production methods are not mentioned in great detail; for example, fructo-oligosaccharides from chicory or sucrose.

(b) *Health Relationship*. In the past, much attention has been paid to the relation between foods and food components and health (563). A truthful documentation of the health claim and linking it to solid scientific evidence is the key. As mentioned earlier, three areas of health claims fall out side the Article 13.1: claims related to children's health and development, claims for which new scientific data is developed or which have requested proprietary protection, and claims related to reduction of disease risk. Claims in these areas will be discussed later.

The health relationship under Article 13.1 must relate to the maintenance or improvement of healthy body functions and should refer to the healthy state of those body functions, such as metabolism, immune function, intestinal health, digestion, etc. Specific physical or chemical properties of a food or a food ingredient (e.g. pre- or probiotic) may influence these physiological functions. If the effect is not shown in the final product, it would be essential to show that the bioactivity of the ingredients are not impaired by it's inclusion into a particular food matrix and thereby the scientific substantiation of the claim.

Examples of a health relationships for pre- and probiotics could be, immune function, intestinal health, bowel function, among others.

(c) *Conditions of Use*. The health claim must refer to the food as it is ready for consumption. The amount of food or food component that has to be consumed to obtain the claimed health benefit should correlate with the dose that a consumer reasonably could be expected to consume based on portion size and frequency with which it will be consumed. So, for prebiotics for example, the amount in grams per day and for probiotics the cfu per day and possibly the format (e.g. yogurt or fermented milk) should be taken into account. Scientific studies may use high doses of pre- or probiotics in order to ascertain the likelihood of observing a health benefit. Such studies may serve as proof of principle, but would have to be replicated with lower doses feasible in a final product.

(d) *Nature of Evidence*. The idea with the current legislation is to substantiate the health claim with sound scientific evidence. The evidence to support the health claim must be based on human studies. *In vitro* and animal studies may be useful as supporting evidence, but on their own are not sufficient to substantiate a claim. The human studies should be of sufficient quality and double-blind, randomized, placebo-controlled interventions provide the

strongest evidence. But, it is recognized that due to the nature of food it may not always be possible to perform such interventions, and other study designs may be sufficient to substantiate the claim. It is also recognized that not all studies give the same results. Therefore, the totality of available evidence needs to be taken into account. In this column on the nature of evidence, can also be indicated the type of documentation: individual studies, textbooks, meta-analyses, monographs, critical reviews, and opinions of authoritative bodies.

(e) *References.* The references mentioned in the claims table should be complete and demonstrate scientific justification of the proposed claim. There are no guidelines given concerning the number of references. But, the totality of the evidence should allow an objective evaluation.

(f) *Example Wording.* The example wording in the list is not an exhaustive list of all possible health claims. The examples should be consistent with the health relationship and the supporting evidence. They should not suggest health benefits that cannot be substantiated, are false, ambiguous, or misleading. Nor shall they doubt the safety and/or nutritional adequacy of other foods. Finally, they should be understandable by the average consumer.

4. *Article 13.5*. Any health claim based on newly developed scientific data and/or which include a request for the protection of proprietary data will have to be submitted separately to a member state. The application has to include the name of the applicant and the nutrition, substance, food or food category in question. Furthermore, references to the scientific studies with regard to the health claim and any other relevant studies, proposal of the wording of the health claim and a summary of the application have to be included. The member state will make the dossier available to EFSA. EFSA will evaluate the claim and make the summary publicly available.
5. *Article 14*. Reduction of disease risk claims and development of children. Claims on the reduction of disease risk may only be made when they have been specifically authorized. The application procedure is similar to the procedure described above for Article 13.5. When an authorization has been received for a reduction of disease risk claim, the presentation or advertising shall also include a statement indicating that the disease to which the claim is referring has multiple risk factors and that altering one of these may or may not have a beneficial effect.
6. *Labeling*. As mentioned with example wording, the health claim has to be supported by scientific evidence. Thus, labeling, presentation, and advertising should not mislead or deceive the consumer, nor should it suggest that a balanced and varied diet cannot provide appropriate quantities of the nutrient (564).
7. *Further information*. The new legislation has not been applied and, as has been mentioned, several uncertainties remain in the exact procedure that will be followed to evaluate the health claims. At the time of publication of this section, changes may have been introduced in the legislation. It is therefore advised to consult home pages of legislative and authorities in order to obtain the latest information (Table 1.14).

TABLE 1.14 Institutes for Further Information on Health Claim Legislation in the EU

Institutes	Country	Acronym	Homepage
European Food Safety Authority	EU	EFSA	http://www.efsa.europa.eu/en.html
Ministry of Health	Belgium		www.health.fgov.be/
Elintarvikevirasto	Finland	EVIRA	http://www.evira.fi/
Agence Française de Sécurité Sanitaire des Aliments	France	AFSSA	http://www.afssa.fr/
Direction Générale de la Concurrence, de la Consommation et de la Repression des Fraude	France	DGCCRF	http://www.finances.gouv.fr/DGCCRF/
Food Safety Authority of Ireland	Ireland	FSAI	http://www.fsai.ie/
Ministry of Health	Netherlands		www.minvws.nl
Agencia Española de Seguridad Alimentaria y Nutricion	Spain	AESA	www.aesa.msc.es
Swedish Nutrition Foundation	Sweden	SNF	http://www.snf.ideon.se/
Food Standards Agency	UK		http://www.food.gov.uk/foodlabelling/ull/claims/

1.8.1.3 The United States of America

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The regulation of microorganisms for human consumption in North America is guided by and is dependent on a number of multiple, complex, and interdependent factors. In the United States, the Federal Food Drug and Cosmetic Act (the FDC Act or the Act) (565) provides the Food and Drug Administration (FDA) with broad authority to classify and regulate an array of products for human consumption. Different regulatory standards govern the marketing of a product depending on how the product is classified under the Act, that is, as a "food," a "food additive," a "drug," a "new drug," a "dietary supplement" (to mention only a few possible classifications). As a result, the pathway under the Act to lawful marketing of a product—even a "probiotic" product—will be relatively easy for some products while, for others, arduous—depending on how the specific product is classified by FDA.

It is important however, to establish from the outset, that there is neither a legally recognized or regulatory definition for the term "probiotics" in North America, nor is there a standard of identity for "probiotics" for human consumption. Therefore, we will discuss the regulation of microorganisms for human consumption, some of which may be regarded by the scientific community as "probiotic" microorganisms, that is, they have been shown to provide a measurable benefit to the host when consumed.

The extent to which FDA is empowered to regulate the safety and claims of efficacy for a substance (including microorganisms) depends not only on the nature of the specific substance but also on how the specific substance is classified under the Act, that is, as a "food" or "food additive," as a "dietary supplement," as a "drug" or "new drug," etc. To determine what product classification applies, one must focus on the "intended use" of the product (most regulated products are defined in the Act according to their "intended use"). Thus, as a general rule, how a manufacturer or a purveyor of a product "intends" a product to be used will govern (565) how the product is classified under the Act and, even more importantly, (566) the data collection and substantiation requirements that must be met to achieve lawful marketing. To this end, the "intended use" of a product is generally determined by what a sponsor, manufacturer, or purveyor says about its product. As a result, "intended use" can be determined from a variety of sources, including product advertising and promotion, product labeling, representations on a company's website, and speeches or remarks by corporate officials. To help navigate through the complexity, a catalog of key statutory definitions and accompanying criteria is necessary.

1. Product classification: statutory definitions.
   Food. "Food" is defined in the Act (566) in a self-evident and circular manner as "articles used for food or drink for man or animals." The definition goes on to include chewing gum and articles "used" for components of food. Note that this least useful of the definitions focuses on actual "use," while for all the other relevant classifications, the key criterion is "intended use."

Drug. The term "drug" is defined in the Act (567) as an article "other than food" that is intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease or in other animals." This also provides that an article is a drug if it is "intended to affect the structure or function of the body of man or other animals." And, an article is also a drug if it is "intended for use as a component" of a "drug." A "new drug" is a statutory term indicating an article is not yet "generally recognized" as safe and effective for its "intended use" and must undergo extensive premarketing clearance requirements that are laid out in detail in Section 505 of the Act and FDA's implementing regulations. Product approval can, and does, take years of data collection and subsequent agency review.

Food Additive. The Act (568) broadly defines "food additive" as including any substance "the intended use of which" results or can "reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of food. . .." These substances intentionally added to food are generally subject to formal premarket review or approval by FDA. However, exempted from this requirement are "GRAS" (generally recognized as safe) substances. These are substances "generally recognized" by "qualified experts" as having been adequately shown through "scientific procedures" to be safe under "the conditions of intended use." Thus, importantly, a "GRAS" food substance or ingredient is *not* subject to the pre-market clearance requirements that accompany a "food additive" and the sponsor of a GRAS substance may proceed directly to market with it.

Dietary Supplement. The term "dietary supplement" is defined in the Act (569) as a product "other than tobacco" that contains a "dietary ingredient" (e.g., a vitamin, mineral, herb, amino acid, etc.) "intended" to "supplement the diet." Also, as defined, a dietary supplement must be intended for "ingestion," and must be in tablet, capsule, powder, softgel, gelcap, or liquid form *or*, if not intended for ingestion, in a form that is not represented as a conventional food and is not represented for use as a sole item, meal, or the diet.

Biological Product. Although not defined in the FDC Act, the term "biological product" is defined in the Public Health Service Act (PHS Act) (570) as, among other things, a "virus" "applicable" to the prevention, treatment, or cure of a disease or condition or "injuries to man." FDA is reported to be of the view that the term "virus" may, logically, include "microorganisms" and, thus, probiotics (571). Although various microorganisms, including probiotics are being studied for such type therapeutic applications, none have yet been commercialized or evaluated under this category.

Medical Food. A "medical food" is defined in an amendment to the FDC Act (572) as "a food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation. Currently, no foods containing microorganisms have been commercialized or recognized as medical foods.

2. FDA Authority to regulate food uses of probiotics.
   The agency not only has the authority to *differentiate among types* of products bearing microorganisms, including probiotic organisms, but also to consider and evaluate *different uses according to different safety standards*. And, in every case, the agency is empowered to regulate the labeling of such products and to demand, to *varying degrees of rigor, substantiation of the claims* made on behalf of such products
3. Safety considerations with respect to the food uses of probiotics.
   Food Additive. The FDC Act (573) establishes a system in which substances that meet the definition of "food additive" must be the subject of a premarket submission containing data and information documenting the safety of the intended use of the additive. A food additive approval cannot be issued unless a petition, submitted by a sponsor, contains convincing evidence establishing that the desired use of the additive is "safe," based on proof to a "reasonable certainty" that no harm will result from the proposed use of the additive (574). The regulation contains the important acknowledgement that it is impossible to establish the absolute harmlessness of any substance.

   GRAS Substances. As discussed earlier, a key exception to the definition of "food additive" is GRAS status: if a substance is GRAS for its intended use, the substance is, by definition, not a "food additive." On the basis of this GRAS exemption, many substances, including several microorganisms, are currently lawfully marketed without a food additive regulation and have achieved GRAS status without any formal FDA review given their very long history of use in the food supply (e.g., *Lactobacillus bulgaricus* in yogurt).

   The GRAS standard focuses on two key considerations: (a) whether the data and information concerning the desired use of the substance provides a scientific basis to conclude that there is consensus among qualified experts about the safety of the substance for the intended use and (b) whether the data and information relied upon to establish safety are generally available to the scientific community. For the purposes of a GRAS opinion, information in peer reviewed scientific journals can be supplemented by (a) publication of data and information in the secondary scientific literature, (b) documentation of an opinion of an experts convened in a panel charged to consider safety, and (c) the opinion or recommendations of an "authoritative body" (for example, another federal agency or a respected scientific entity like the National Academy of Sciences).

   It is the manufacturer's responsibility to assure a product or substance is GRAS, and it should neither be assumed that any microorganism currently in the food supply qualifies it as GRAS, nor that because a bacterium is appropriate for a supplement, it is GRAS for a food, since the determination is "for an intended use." Manufacturers can conduct a "self-determination" of GRAS status for a substance. The documentation of this determination, based on the criteria mentioned above maybe kept internally, and provided to FDA if the agency requests it. Although manufacturers are not required to provide

this self-determination to FDA prior to marketing a product, not working with FDA on GRAS issues before marketing a product risks adverse publicity and judicial action by FDA should the agency disagree with the self-GRAS determination and is concerned that consumers may be at risk. This is even more the case with respect to infant formulas, where FDA requires premarket notification and clearance.

The documentation of this determination, based on the criteria mentioned earlier maybe kept internally, and provided to FDA if the agency requests it. But manufacturers are not required to provide this self -determination to FDA prior to marketing a product. An exception to this is the use of a microorganism (as well as other substances) when intended for use in an infant formula, in which FDA explicitly requires premarket notification.

In some cases, the manufacturer may choose to notify FDA of its GRAS self-affirmation. To facilitate GRAS determinations, FDA for the last decade has followed a "notification" procedure under which the agency reviews a submission by a sponsor for the use (or the uses) of a given substance. The submission must contain a sufficient basis for a GRAS determination based on the criteria mentioned earlier. In reviewing a notification, FDA takes at face value the conclusions of the sponsor and the independent panel of experts. If the agency has questions with respect to the apparent adequacy of the cited data and information or the panel's conclusions, FDA will so notify the sponsor. Similarly, FDA will advise the sponsor if it has no questions. Although, a "no questions" letter is not the same as an agency conclusion that the use of a given substance is, in fact, GRAS, a "no questions" letter represents an agency acquiescence to the sponsor proceeding to market the product.

Probiotics have been the subject of at least two successful GRAS notifications to FDA. The agency has issued "no questions" letters with respect to the use of *Lactobacillus acidophilus* and *Lactobacillus lactis* for use in fresh meat for the control of pathogenic bacteria and to the use of *Streptococcus thermophilus*, and strain of *Bifidobacterium lactis* in infant formula (575). In the United States *B. lactis* is the only bacterium, which has shown probiotic effects and is currently GRAS for use in infant formula.

Dietary Supplements. Dietary supplements are not subject to the food additive provisions of the Act. As a consequence, dietary supplements not only are not subject to the demanding premarket approval requirements for food additives but also do not undergo "GRAS" scrutiny and evaluation.

From the safety point of view, a dietary supplement is "adulterated" (and thus unlawful) within the meaning of the Act if it "presents a significant or unreasonable risk of illness or injury" when used under its ordinary conditions of use (576). FDA can invoke numerous sections of the Act to declare food "adulterated" once it is on the market.

An additional safety standard applies if the dietary supplement contains a "new dietary ingredient" (NDI). An "NDI" is an ingredient "not marketed in the United

States before October 15, 1994, when the Dietary Supplement Health and Education Act (DSHEA) was passed" (577). For ingredients in supplements (including any microorganisms, with or without probiotic effects) commercialized before this date, no premarket approval is required by FDA. Thus, many microorganisms in the food supply used for a long time can be, and are neither considered NDIs nor have been subject of an NDI review by FDA. The FDA has no complete or authoritative list of dietary ingredients marketed before October 15, 1994, so it is up to the manufacturer to determine if a new product or ingredient is an NDI. If an NDI has been present in the general food supply as an article used for food "in a form in which the food has not been chemically altered," the NDI may be used in the food supply without first notifying FDA. But, if that is not the case, the NDI may only be used if (a) there is a history of use of the ingredient, or other evidence establishing that the proposed use of the ingredient "will reasonably be expected to be safe" *and* (b) the manufacturer files a premarket notification containing the information in support of safety at least 75 days before marketing the product. Once the 75-day period has expired, the sponsor is free to proceed to market. If at any time the agency disagrees with the sponsor's conclusions concerning safety, the agency may so notify the sponsor and, if need be, litigate to prevent the marketing of the product.

Good Manufacturing Practices. Regardless of whether a microorganism is in a food or a dietary supplement, and in addition to the establishment of the safety of the microorganism for its intended use, FDA will expect that both the microorganism and the food to which it is added are produced under good manufacturing practices designed to ensure safety, microbiological quality, and integrity of the probiotics.

Safety and Intended Use. It is a common misconception that "safety" evaluation is more stringent for a drug than for a supplement or a food. As mentioned earlier, from the regulatory point of view, safety depends on the intended use. While a drug may have specific documented adverse effects, its use may be approved as long as the safety of the drug has been rigorously investigated and the benefits (e.g. treating a disease) are established by "substantial evidence" and are found to outweigh the safety-related risks. On the contrary, for an infant formula, no adverse effects of any significance are permitted. Supplements, like drugs, may be labeled for use in particular subpopulations, such as adults, or children over a particular age. This is not the case for foods because, among other reasons, foods may be consumed in large amounts, by the population at large, and for very long periods of time, and may demonstrate cumulative effects that are not typical of supplement consumption patterns.

4. Labeling and claim substantiation for food uses of microorganisms.
   As mentioned at the outset, there is no regulatory or legally recognized definition for the term "probiotic" in the United States or worldwide. Thus, products containing the term "probiotic" on the label currently do not meet any specific requirement relative to the term. On the contrary, there is an increasing level of information and understanding by consumers and health care providers

that the term itself conveys "some benefit" and could be itself considered a claim. For now, the regulatory framework has dealt only with express or implied claims other than those conveyed by the term "probiotic."

Content Claims. Foods or dietary supplements containing microorganisms may mention the fact they contain certain microorganisms, and sometimes indicate certain amounts. Unfortunately many commercial products, particularly supplements do not contain numbers or types of viable probiotic microbes stated on label (578–580). This is likely a consequence of multiple factors. One is the evolving and continuous change in the taxonomy of these organisms, which has evolved in parallel with the technology available to identify and differentiate different genera, species, and strains. Unless current nomenclature is used, actual contents may not reflect the label. As an example, what used to be called *L. acidophilus* until recently could in fact be one or more of six different species (*L. acidophilus, L. gasseri, L. johnsonii, L. crispatus, L. gallinarum, and L. amylovoris*). A second factor is the lack of documentation of viability and quality control throughout shelf life, which is dependent on manufacturing conditions, humidity, storage temperature, etc.

When it comes to foods, similar issues are of concern. Many manufacturers do not mention content (number) of microorganisms at all, others do. Although more recently manufacturers have been more specific regarding genus, species and strain, and amount of a particular microbe in the product (and have used more aggressively claims associated to this organism), consumers are ultimately left to relying on manufacturer's statement regarding the presence, amount of microorganisms in these products. This becomes even more important given the fact that the potential probiotic effect of these products may relate to amount of microorganisms ingested.

Lastly, since similar to the term probiotic, the term "contains active live bacteria," "active live cultures," and other similar, simply imply "some" viability, or "some" microorganisms is present. At the consumer level, this also may imply these bacteria are of some benefit (a probiotic), which of course is not the case.

Infant formula is a particular product, which in the United States is governed by the same regulatory criteria mentioned above, as well as multiple other regulatory layers, and respond to a significantly greater level of care by manufacturers as well as oversight by FDA. Therefore both specific organism and amounts are better guaranteed.

Benefit or Efficacy Claims: FDA is empowered to comprehensively regulate the use of labeling and promotional material communicating health-related information or claims to the consumer. Under the Act (Sections 403(a) and 201 (n)) (581, 582), a food is misbranded if its label or labeling is false or misleading "in any particular." Under this standard, any claim regarding the value or benefit of a food or a dietary supplement must be demonstrated by reliable information. The responsibility for documenting the validity of label claims rests with the manufacturer and the oversight by FDA. The Federal Trade Commission (FTC) has oversight of claims made in advertising.

Health Claims. FDA has special authority with respect to claims made in the labeling of food and dietary supplements, which expressly or impliedly characterize the relationship of a substance to a disease or health-related condition (583). This type of claim is, in common parlance, referred to as a "health claim." Even an implied reference to an impact on disease can constitute a "health claim."

Before a health claim may be lawfully used on food, FDA must authorize the claim upon review of a petition for the claim: FDA can only approve a claim upon finding that the claim is supported by "significant scientific agreement among qualified experts." This is a rigorous statutory standard and only a handful of claims—*none involving probiotics*—has been found by FDA to meet the standard.

Qualified Health Claims. Judicial rulings over the last decade have led to an additional category of health claims—"qualified health claims"—for foods and dietary supplements. FDA has implemented a policy of reviewing and permitting health claims to appear on foods even if the data and information in the supporting health claim submission do not meet FDA's "significant scientific agreement" standard, but is satisfied that "qualifiers" can render the claim not misleading. For example, an appropriately qualified claim might provide guidance to the effect that although there is emerging scientific evidence supporting the claim, the evidence is "not conclusive." To secure FDA's acquiescence to the use of a "qualified health claim," a sponsor must submit a premarket petition for FDA and wait for the Agency's review and conclusions. No qualified health claims have been granted to any probiotic microorganism.

Structure or Function Claims. An additional category of claims that may lawfully appear on both foods and dietary supplements is the "structure or function" claim. This type of claim presents perhaps the most significant opportunity for the exercise of marketing creativity and, commensurately, for attracting regulatory concern. As noted above, the statutory definition of "drug" includes an article "(other than food)" intended to affect the structure or function of the body of man or other animals." The parenthetical expression reflects Congress' implicit recognition that a "food" may bear a claim with respect to the effect a food or food component may have on the body, for example, "Calcium builds strong bones." This reflects the fact that certain foods quite naturally affect the structure or function of the human body and that a claim with respect to such an effect should not be regulated under the rigorous standards that govern "drugs." Thus, coffee, without being considered a "drug," can lawfully bear a claim with respect to the mild alertness effect naturally occurring caffeine can impart.

In the 1980s, some tablet and capsule dietary supplement products could not meet the definition of food and, thus, were regulated by FDA as "drugs" if their label or labeling or bore or implied structure or function-related claims. So the U.S. Congress amended the FDC Act in 1994 to expressly authorize the use of structure or function claims in the labeling of dietary supplements. These amendments, however, require that any such statement of a dietary supplement

must bear the following disclaimer: "This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease." (584). Moreover, the sponsor of such a claim must notify FDA of the claim within 30 days after marketing a dietary supplement bearing the claim.

In spite of the amendment permitting supplements to bear a "structure or function" claim and not be considered a drug, FDA's primary enforcement tool against dietary supplements remains its authority (under the alternative prong of the "drug" definition found in the Act (Section 201(g)(1)(B)) to classify a supplement as a drug if product labeling and promotion imply that the product "is intended, for use in the. . .cure, mitigation, treatment, or prevention of disease. . .." Accordingly, FDA has issued detailed regulations that differentiate between (a) structure or function claims appropriate for dietary supplements and, by inference, appropriate for foods and (b) "disease" claims that may not be made on behalf of a dietary supplement or on behalf of a food, without the prior FDA authorization previously described for a "drug" or "health claim" (585).

At the heart of FDA's regulations is the concern that a structure or function claim carries the potential to imply a disease related benefit. When exactly a structure or function claim on a dietary supplement or on a food becomes a "drug" claim is not always clear. Thus, FDA's regulation contains guidance for how, in FDA's view, to assess when a structure or function claim will be treated by the agency as a "drug" claim.

With respect to probiotics, examples of appropriate structure/function claims include "helps maintain intestinal flora" and "helps replenish healthy microflora" or "helps support a healthy immune system." Notice how each claim avoids mentioning a disease or disease endpoint and each is clearly directed at healthy people who wish to remain healthy. On the contrary, attempted structure/function claims like "prevents adherence of *Candida albicans* to the intestinal mucosa" and "deters bacteria from adhering to the wall of the bladder and urinary tract" have been determined by FDA to be implied drug claims, presumably on the basis that the former claim may be viewed as suggesting the prevention of cancer and that the latter claim may be viewed as suggesting the prevention of bacterial infection of the bladder and urinary tract.

Publications as Claims. With respect to dietary supplements *but not* with respect to "foods," the FDC Act permits published scientific articles to be distributed in connection with the sale of the dietary supplement (586). The title of the publication may mention disease conditions and the relationship of the substance to such conditions. Such mentions will be exempt from any prohibition against unauthorized "disease claims" under the "health claim" or "drug" provisions of the Act, only as long as the publication (a) is not false and misleading, (b) does not promote a particular brand or manufacturer, (c) presents a balanced view of available scientific information, and (d) is reprinted in its entirety (summaries of published articles do not quality for the exemption).

Functional Foods. Although the term "functional food" is frequently employed to describe foods capable of imparting particular health benefits and is often used to describe probiotic-containing foods, the term is neither defined by FDA regulations nor to be found in the FDC Act. That said, "foods" or "dietary supplements" bearing "structure or function," "health," or "qualified health" claims—or even the entire classes of "medical foods," and "dietary supplements"—all can be regarded as "functional foods." Thus, this whole category of so-called "functional foods" is not governed by a single set of regulatory criteria, but is rather an array of possible product classifications, each with its own regulatory status and governing criteria.

5. Probiotic use as drugs.
   If a probiotic falls within the definition of "drug," that is, it is commercialized for the intended use of mitigating, treating, or preventing a disease, the probiotic would be subject to an array of data collection and submission requirements beginning with those concerning the conduct of "clinical" trials under FDA's "investigational new drug" (IND) (587) procedures and regulations and ending with the submission of a new drug application containing, with respect to the desired use, (a) data and information establishing safety and (b) substantial evidence, based on adequate and well-controlled investigations, conducted by qualified researchers, establishing effectiveness.

   IND regulations help guarantee the welfare of those participating in a clinical study, and include submission of data from laboratory and animal research, experience, or historical use of the drug in people, and detailed protocols of the planed trials as well as provisions for selecting qualified investigators, maintaining detailed study records, and keeping FDA informed of the progress of a given investigation and of any significant safety-related developments.

   Once a probiotic successfully runs the gauntlet of clinical testing under an IND, the sponsor must submit a "new drug application" (NDA) for the desired use of the product. Only if the application is approved by FDA may the product be lawfully marketed. It should be noted that, unlike the case with "food additive" or GRAS substances, FDA accords confidential status to data submitted in a new drug application, even the fact that an IND is in effect, or that an NDA has been submitted is privileged, confidential information.

   Currently, no specific probiotic product has been approved or commercialized as a drug for humans in the United States. The increase in "intended use" of specific bacteria with probiotic effects for prevention or treatment of disease will likely result ultimately in specific products marketed for this purpose, although the hurdles remain significant.

   That reality, in and of itself, is not troubling—there is a general agreement, at least in the scientific community, that claims of benefit should be put to the test and proven in an ethical manner consistent with sound principles of scientific methodology. The concern that can arise, however, is that although the distinction made in the FDC Act between "drug" and nondrug uses can, in some cases,

arguably be viewed as artificial or casuistic, the resulting demands on market entry for products whose "intended use" triggers regulation as a drug (or as a biological product) are stark from a product development perspective. Nevertheless, the distinction, however artificial it may in some cases be, has been and remains a touchstone of public health regulation in the United States and is ingrained in federal public health precedent and policy (588).

When a product is classified as a drug, there is an immediate and direct effect on the development of such a product, from complex and onerous investigational requirements for a "new drug" described earlier, all the way to its commercialization. As a consequence, there is little incentive for sponsors of probiotics to undertake the expense to develop probiotic products as true "biotherapeutics" subject to premarket approval. This lack of incentive has been reinforced by the fact that unlike investigational new drug studies, "food" or "dietary supplement" studies may be commenced, conducted, and terminated, without comparable FDA involvement (FDA's informed consent and IRB requirements attend any clinical trial regardless of the nature of the substance under test). The result is the understandable tendency of sponsors to clinically test "structure or function"-related indications or claims rather than indications likely to invoke new drug or biological product status.

6. Conclusions

The oral human consumption of specific microorganisms that provide health-related benefits, described elsewhere in this handbook, is considered safe and desirable by many—including researchers, food technologists, and, of course, food producers and purveyors.

The regulatory aspects of producing and commercializing such products, as summarily treated in this chapter, is complexly layered with increasingly demanding burdens of data collection and claim substantiation.

But with any decision to market probiotic products comes the understandable desire to tout the potential benefits of such products. It is in that context, that the focus of the FDC Act on "intended use" and the product classifications that derive from such a focus, force interested sponsors of such products to pause and confront the realities of public health regulation today in the United States.

Although benefit claims can trigger more demanding regulatory hurdles, common safety considerations apply to all probiotic products regardless of whether foods (including medical foods and foods for special dietary use, or dietary supplements), drugs, or biological products. Simply put, care must be taken to carefully and comprehensively document and establish the safety of any use. In the food area this is customarily accomplished in the context of (a) data results from adequately designed studies and (b) informed expert opinion. Not by coincidence, these elements square well with the key elements of any GRAS assessment.

With respect to showing the safety of the probiotic component of a dietary supplement, although food additive GRAS considerations do not apply,

TABLE 1.15 Differences Among Regulatory Categories Available for Marketing Probiotic Products in the United States

Approvals, Characteristics, and Claims	Regulatory Categories			
	Supplements	Food	Infant Formula	Drug
Premarket approval by the FDA	Not required for microorganisms used as "dietary ingredients before October 1994	Not required for GRAS microorganisms	Premarket notification and clearance required	Required
Disease claim Describes the effect of a drug on the diagnosis, treatment, mitigation, cure, or prevention of disease	Not allowed	Not allowed	Not allowed	Allowed if approved by the FDA
Health claim Describes the effect of a dietary substance on the reduction of risk of disease by the currently healthy population	Allowed if approved by the FDA (may be unqualified or qualified)	Allowed if approved by the FDA (may be unqualified or qualified)	Allowed if approved by the FDA (may be unqualified or qualified)	Not used, although can use stronger prevention claims
Structure function claim Describes the effect of a dietary substance on the structure or function of the body	Allowed if truthful and not misleading	Allowed if truthful and not misleading	Allowed if truthful and not misleading	Required for "new drugs."
	Commonly used	FDA takes the view that effect must derive from the "nutritive value" of the food (sensible, but not clearly established).	FDA takes the view that effect must derive from the "nutritive value" of the food.	

(*continued*)

TABLE 1.15 (*Continued*)

Approvals, Characteristics, and Claims	Regulatory Categories			
	Supplements	Food	Infant Formula	Drug
	Label must say "this statement has not been reviewed by the FDA Must notify the FDA of intent to use this claim within 30 days of marketing the product	No requirement for label disclaimer or FDA notification	No requirement for label disclaimer or FDA notification	
Safety standards	No significant or unreasonable risk of illness or injury	Reasonable certainty of no harm under the intended conditions of use or GRAS	Food additive approval or GRAS notification done prior to, or as part of, pre-market approval	The FDA assesses safety and effectiveness and determines if benefits are established by substantial evidence and if they outweigh risk
	Target consumer group can be stipulated on the label	GRAS status can be self-determined or submitted through GRAS notification process Must be safe for general population and all subgroups		
Product examples	Capsules	Yogurt	Infant formula	No probiotic products currently are regulated as drugs for human use in the United States
	Powder sachet	Dairy drink		

Adapted from Ref. 593.

approaching the substantiation of safety in a similar way is sensible and should guarantee that the safety standards, including those regarding new dietary ingredient notifications, of the Act are met.

In conclusion, FDA's regulatory authority over the use of probiotics is comprehensive. Different regulatory standards will govern the market entry of a probiotic product depending on how the product is classified under the Act, that is, as a "food," a "drug," a "dietary supplement," etc. (Table 1.15). Numerous alternative pathways to market are, as a result, available to probiotic manufacturers. Informed care and attention need to be taken if navigating these pathways is to be successful.

1.8.2 Animal Probiotics

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Probiotics can be defined as live microorganisms that have a beneficial effect on the animal health when used in animal nutrition. They are considered as feed additives in most countries and are therefore regulated separately from food. In some countries, they may also be considered as a veterinary chemical product and thus regulated accordingly. Probiotics used in animal nutrition comprise mainly of Gram-positive bacteria belonging to the genera of *Lactobacillus, Bifidobacterium, Pediococcus, Bacillus, Streptococcus*, and even *Enterococcus*. Others have included yeast or *Saccharomyces cerevisiae*. The approval of probiotics for use in animals follows essentially the same approach as that for humans, which is largely dependant on the efficacy and toxicity of the strains.

1.8.2.1 United States Regarded as the authority and reference on feed additive policy, the Association of American Feed Control Authority (AAFCO) published a list of microorganisms approved as direct-fed microbial products (590). Of the 45 microorganisms approved, about half belongs to the genus of *Lactobacillus* (14) and *Bifidobacterium* (16) (Table 1.16).

1.8.2.2 European Union Live microorganisms, together with enzymes and feed additives of biological origin were added to the list of feed additives regulated by the European Union in the 1980s due to the emerging market trends. The term "probiotics" have been rejected on the grounds of being too generic. In 2002, under the framework of establishing the European Food Safety Authority, a new draft regulation would group microorganisms as "zootechnical additives," defined as agents producing beneficial effect on gut microflora. This proposal was adopted in 2003, when the European Commission passed a new regulation (EC) No 1831/2003 of the European Parliament and of the Council on additives for use in animal nutrition. To be used in Europe, probiotics as additives must satisfy several criteria with regards to their identity, characteristics, and conditions for use of the additive; their safety of use in animals, humans, and environment

TABLE 1.16 Approved Direct-Fed Microbials in the United States

Genus	Species	Genus	Species
Aspergillus	*niger*	*Lactobacillus*	*acidophilus*
	oryzae		*brevis*
			bucheri[a]
Bacillus	*coagulans*		*bulgaricus*
	lentus		*casei*
	licheniformis		*cellobiosus*
	pumilus		*curvatus*
	subtilis		*fareiminis*[b]
			fermentum
Bacteroides	*amylophilus*		*delbruckii*
	capillosus		*helveticus*
	ruminocola		*lactis*
	suis		*pantarum*
			reuteri
Bifidobacterium	*adolescentis*		
	animalis	*Leuconostoc*	*mesenteroides*
	bifidum		
	infantis	*Pediococcus*	*acidilacticii*
	longum		*cerevisiae (damnosus)*
	thermophilum		*pentosaceus*
		Propionibacterium	*acidipropionici*[a]

[a]Cattle only.
[b]Swine only.

such as the lack of pathogenicity and production of antibiotics and antibiotic resistance; and their efficacy on animals or categories of the target animal species such as improved zootechnical performance, reduction of morbidity and mortality. To demonstrate efficacy effects, three trials conducted at a minimum of two separate sites presenting significant results ($p < 0.05$ or 0.1) are required. The main safety concerns regarding such probiotic strains, *Bacillus* and *Enterococcus* in particular, revolve around the potential production of toxins and virulence factors, detectable using modern techniques such as ELISA, cell line assays, and PCR amplification of toxin genes. Any harmful effects of overdosing are studied through administration of at least ten times the recommended maximum dose. Studies of genotoxicity and mutagenicity would also need to be carried out. Such an evaluation procedure and requirements have led to the reevaluation of some previously submitted product dossiers. Microorganisms are now regulated as zootechnical additives, which is one of the five categories of feed additives, viz:

- Technological additives (e.g. preservatives, antioxidants, emulsifiers, stabilizing agents, acidity regulators, silage additives).
- Sensory additives (e.g. flavors, colorants).
- Nutritional additives (e.g. vitamins, minerals, amino acids, trace elements).

- Zootechnical additives (e.g. digestibility enhancers, gut flora stabilizers).
- Coccidiostats and histomonostats.

Authorization of feed additives is granted by The European Food Safety Authority (EFSA), which evaluates the data submitted on efficacy, safety, and toxicology of the feed additive. Once the Commission is satisfied with the data, it prepares a draft regulation to grant authorization, following the procedure involving Member States within the Standing Committee on the Food Chain and Animal Health—Animal Nutrition. Authorizations are granted for specific animal species, specific conditions of use and for 10-year periods. Although the registration and approval can be interpreted as fairly complex, it can be argued that this is critical to ensure safety of probiotics used as feed additives that ultimately contributes to their efficacy. Approved feed additives are published in the Community Register of feed additive (591). A current list of microorganisms, together with the approved animal species, age, and dosage limits is summarized in Table 1.17.

1.8.2.3 China Feed additives are regulated by the Ministry of Agriculture of the People's Republic of China (592). A total of 16 microorganisms including bacteria and fungus are approved as probiotics in China, and they are to be used according to the application guidelines from the vendors (Table 1.18).

1.8.2.4 Japan The Food and Agricultural Materials Inspection Center (FAMIC) (593) approves a total of 11 bacteria strains for use as probiotics in animals (Table 1.18).

1.8.2.5 Korea A total of 16 microorganisms are approved for use in Korea as probiotics in animals, of which two are fungus and one is yeast (Table 1.18). In addition, there is a separate list of carriers, most of which are starch, derivatives of starch, or cereals and grains, to be used with each of the probiotics.

1.8.2.6 Thailand Feed additives including probiotics are regulated by Department of Livestock Development in Thailand (594). There are currently 42 strains of bacteria and 6 strains of fungus approved as probiotics for animals, which can be used singly or in combination, in producing finished feed at not more than 1×10^5 cfu/kg of feed (Table 1.18).

1.8.2.7 Australia Probiotics are considered a biological product, thus an import permit application must be filed with AQIS. In addition, as probiotic is considered a veterinary chemical product, registration with the Australian Pesticides and Veterinary Medicines Authority (APVMA) is required. The Australia APVMA (595) regulates probiotics as microbial agents, together with three other biological products:

- Group 1—biological chemicals (e.g., pheromones, hormones, growth regulators, enzymes and vitamins);

TABLE 1.17 Microorganisms Approved as Feed Additives in the EU

Genus	Species	Strains	Minimum Age	Species or Category of Animals	Composition in Complete Feedstuff (cfu/kg)	
					Min.	Max.
Aspergillus	*niger*					
	oryzae	AK 7001 DSM 1862				
Bacillus	*badius*					
	cereus	var. toyoi NCIMB 40112/CNCM I-1012		Cattles for fattening	0.2×10^9	0.2×10^9
	cereus	var. toyoi NCIMB 40112/CNCM I-1012		Rabbits for fattening	0.1×10^9	5×10^9
	cereus	var. toyoi NCIMB 40112/CNCM I-1012		Chickens for fattening	0.2×10^9	1×10^9
	cereus	var. toyoi NCIMB 40112/CNCM I-1012	From 2 to 4 months	Piglets	0.5×10^9	1×10^9
	cereus	var. toyoi NCIMB 40112/CNCM I-1012	From 4 months until slaughter	Pigs for fattening	0.2×10^9	1×10^9
	cereus	var. toyoi NCIMB 40112/CNCM I-1012	2 months	Piglets	1×10^9	1×10^9
	cereus	var. toyoi NCIMB 40112/CNCM I-1012	From service to weaning	Sows	0.5×10^9	2×10^9
	coagulans	CECT 7001				
	lentus	302				
	licheniformis	DSM 5749		Turkeys for fattening	1.28×10^9	1.28×10^9
	licheniformis	DSM 5749	3 months	Calves	1.28×10^9	1.28×10^9
	licheniformis	DSM 5749		Sows	1.28×10^9	1.28×10^9
	licheniformis	DSM 5749		Pigs for fattening	1.28×10^9	1.28×10^9
	licheniformis	DSM 5749		Piglets	1.28×10^9	1.28×10^9
	pumilus	BP288 ATCC 53682/CNCM I-3240 (NRRL B4064)/MBS-BP-01/Micron Bio-Systems Culture Collection				

	subtilis	DSM 5750		Turkeys for fattening	1.28×10^9	1.28×10^9
	subtilis toyoi	DSM 5750		Calves	1.28×10^9	1.28×10^9
Bacteroides	*amylophilus*					
	capillosus					
	ruminocola					
	suis					
Bifidobacterium	*adolescentis*					
	animalis	subsp. *lactis* CHCC5445 (DSM15954)				
	bifidum					
	infantis					
	longum	CNCM I-3241 (ATCC 15707)				
	pseudolongum					
	thermophilum					
Candida	*glabrata*	35120				
	pinolepessi					
	utilis					
Clostridium	*butyricum*					
	sporogenes	P				
	phage					
	tyrobutyricum					
	phage					
Enterococcus	*cremoris*					
	diacetylactis					
	faecalis					
	faecium	NCIMB 10415		Chickens for fattening	1×10^8	1×10^8
	faecium	NCIMB 10415	6 months	Calves	1×10^9	1×10^{10}
	faecium	NCIMB 10415		Chickens for fattening	0.3×10^9	2.8×10^9

(*continued*)

TABLE 1.17 (*Continued*)

Genus	Species	Strains	Minimum Age	Species or Category of Animals	Composition in Complete Feedstuff (cfu/kg)	
					Min.	Max.
	faecium	NCIMB 10415		Pigs for fattening	0.35×10^9	1×10^9
	faecium	NCIMB 10415		Piglets	0.35×10^9	1×10^9
	faecium	NCIMB 10415		Sows	0.7×10^9	1.25×10^9
	faecium	NCIMB 10415		Piglets	1×10^9	1×10^{10}
	faecium	NCIMB 10415	6 months	Calves	1×10^9	6.6×10^9
	faecium	DSM 7134	4 months	Calves	1×10^9	5×10^9
	faecium	DSM 7134		Piglets (weaned)	2.5×10^9	5×10^9
	faecium	DSM 10663/NCIMB 10415	6 months	Calves	1×10^9	1×10^{10}
	faecium	DSM 10663/NCIMB 10415		Piglets	1×10^9	1×10^{10}
	faecium	DSM 10663/NCIMB 10415		Chickens for fattening	1×10^9	1×10^9
	faecium	DSM 10663/NCIMB 10415		Dogs	1×10^9	1×10^{10}
	faecium	DSM 10663/NCIMB 10415		Turkeys for fattening	1×10^7	1×10^9
	faecium	DSM 10663/NCIMB 10415				
	faecium	NCIMB 11181	6 months	Calves	5×10^8	2×10^{10}
	faecium	NCIMB 11181		Piglets	5×10^8	2×10^{10}
	faecium	NCIMB 11181		Chickens for fattening	2.5×10^8	15×10^9
	faecium	1:1 mixture of ATCC 53519 and ATCC 55593		Chickens for fattening	1×10^8	1×10^8
	faecium	CECT 4515		Piglets (weaned)	1×10^9	1×10^9
	faecium	CECT 4515		Chickens for fattening	1×10^9	1×10^9
	faecium	NCIMB 10415		Dogs	4.5×10^6	2×10^9
	faecium	NCIMB 10415		Cats	5×10^6	8×10^9

	faecium	DSM 7134	Chickens for fattening	0.2×10^9	2×10^9
	faecium	DSM 7134	Piglets	0.5×10^9	4×10^9
	faecium	DSM 7134	Pigs for fattening	0.2×10^9	1×10^9
	mundtii	82760			
Kluyveromyces	*marxianus*	var. *lactis*K1 BCCM/MUCL 39434	Dairy cows	0.25×10^6	1×10^6
	marxianus-fragilis	B0399 MUCL 41579	Piglets (weaned)	6×10^6	6×10^6
Lactobacillus	*acidophilus*	36587/CHCC3777 (DSM13241)/ CNCM DALA I-1246/NCIMB 30067/NCAIM			
	acidophilus	DSM 13241	Dogs	6×10^9	2×10^{10}
	acidophilus	DSM 13241	Cats	3×10^9	2×10^{10}
	amyloliticus	CBS 116420			
	amylovorans	DSM 16251			
	brevis	DSM 12835/DSM 16570/IFA 92/KKP. 839/NCIMB 8038			
	bucheri	40177/71044/71065/BIO 73/CCM 1819/DSM 12856/DSM 13573/DSM 16774/KKP. 907/LN4637 ATCC PTA-2494/NCIMB 30137/NCIMB 30138/NCIMB 30139/NCIMB 40788/NCIMB 8007			
	bulgaricus	MA 547/3M			
	casei	32909/ATCC 7469/CCM 3775/ CHCC2115/CNCM DA LC I-1247/ MA 67/4U/NCIMB 11970/NCIMB 30007/*rhamnosus* LC 705 DSM 7061			
	cellobiosus	Q1			
	collinoides	DSMZ 16680			
	curvatus				

(*continued*)

TABLE 1.17 (*Continued*)

Genus	Species	Strains	Minimum Age	Species or Category of Animals	Composition in Complete Feedstuff (cfu/kg)	
					Min.	Max.
	farciminis	CNCM MA 67/4R		Chickens for fattening	5×10^8	1×10^9
	farciminis	CNCM MA 67/4R		Turkeys for fattening	5×10^8	1×10^9
	farciminis	CNCM MA 67/4R		Laying hens	5×10^8	1×10^9
	farciminis	CNCM MA 67/4R		Piglets (weaned)	1×10^9	1×10^{10}
	fareiminis	MA27/6B				
	fermentum	DSM 16250				
	delbruckii					
	helveticus					
	lactis					
	mucosae	DSM 16246				
	paracasei	30151/DSM 16245/DSM 16572/DSM 16773/NCIMB 30151/ssp. *paracasei* DSM 11394/ssp.*paracasei* DSM 11395/ssp. *paracasei* CNCM I-3292 (P4126)				
	pantarum					
	pentosus	DSM 14025				
	plantarum	16627/24001/24011/252/50050/88/ Aber F1 NCIMB 41028/AK 5106 DSM 20174/AMY LMG-P22548/ ATCC 8014/C KKP/783/p/C KKP/ 788/p/CCM 3769/CNCM DALP. I-1250/CNCM I-3235/				

		(ATCC 8014)/CNCM I-820/CNCM MA 18/5U/CNCM MA 27/5M/DSM 11520/DSM 11672/DSM 12187/ DSM 12836/DSM 12837/DSM 13367/DSM 13543/DSM 13544/ DSM 13545/DSM 13546/DSM 13547/DSM 13548/DSM 16247/ DSM 16565/DSM 16568/DSM 16571/DSM 16682/DSM 3676/DSM 3677/DSM 4748/DSM 4909/DSM 8427/DSM 8428/DSM 8862/DSM 8866/DSMZ 15683/DSMZ 16627/ EU/EEC 1/24476/IFA 96/K KKP/ 593/p/KKP/788/p/L-256 NCIMB 30084/L43 NCIMB 30146/L44 NCIMB 30147/L54 NCIMB 30148/ L58/LP286 DSM 4784 ATCC 53187/ LP287 DSM 5257 ATCC 55058/ LP318 DSM 4785/LP319 DSM 4786/ LP329 DSM 5258 ATCC 55942/ LP346 DSM 4787 ATCC 55943/ LP347 DSM 5284 ATCC 55944/LSI NCIMB 30083/MA 541/2E/MBS-LP-1/Micron Bio-Systems culture collection/MiLAB 393 LMG-21295/ NCIMB 12422/NCIMB 30004/ NCIMB 30094/NCIMB 301				
	reuteri	CNCM MA28/6E-g/CNCM MA28/6U-g/ DSM 16248/DSM 16249				
	rhamnosus	MA27/6R/NCIMB 30121				
	rhamnosus	DSM 7133	4 months	Calves	1×10^9	5×10^9

(*continued*)

TABLE 1.17 (*Continued*)

Genus	Species	Strains	Minimum Age	Species or Category of Animals	Composition in Complete Feedstuff (cfu/kg)	
					Min.	Max.
	rhamnosus	DSM 7133		Piglets (weaned)	2.5×10^9	5×10^9
	sakei	DSM 16564/subsp. Sakei AK 5115 DSM 20017				
	salivarius	CNCM I-3238 (ATCC 11741)				
Lactococcus	*lactis*	CCM 4754, NCIMB 30117/CNCM I-3291 (ATCC 7962)/*lactis* 30044/*lactis* NCIMB 30044/CCM 4754, NCIMB 30117/NCIMB 30149/NCIMB 30160/SR 3.54 NCIMB 30117/subsp. *Lactis biovar diacetylactis* CHCC2237/subsp. *Lactis* CHCC2871				
Leuconostoc	*mesenteroides*	DSM 8865				
	oeno LO1	LO1				
	pseudo-mesenteroides	CHCC2114				
Pediococcus	*acidilacticii*	30005/33-06 NCIMB 30086/33-11 NCIMB 30085/AK 5201 DSM 20284/CNCM I-3237 (ATCC 8042)/CNCM MA 151/5R/CNCM MA 18/5M/DSM 10313/DSM 11673/DSM 13946/DSM 16243/ET 6/NCIMB 30005				
	acidilacticii	CNCM MA18/5M		Chickens for fattening	1×10^9	1×10^{10}

	acidilacticii		Pigs for fattening	1×10^9	1×10^9
	cerevisiae (damnosus)				
	pentosaceus	69221/AP35/CCM 3770/CNCM MA 25/4J/DSM 12834/DSM 14021/DSM 16244/DSM 16566/DSM 16569/HTS LMG P-22549/MBSPP-1/Micron Bio-Systems culture collection/ NCIMB 12455/NCIMB 30068/ NCIMB 30089/NCIMB 30168/ NCIMB 30171			
Pediocuceus	sp				
Propinibacterium	*acidipropionici*	CNCM MA 26/4U			
	freudenreichii	shermanii JS DSM 7067/subsp. *shermanii* AK 5502 DSM 4902			
	globosum	CNCM DAPB I-1249			
	shermanii	ATCC 9614/MBSPS-1			
	sp.	DSM 9576/DSM 9577			
Rhodopseudomonas	*palustris*	ATTC 17001			
Scaccharomyces	*cerevisiae*	IFO 0203/37584/80566			
	cerevisiae	NCYC Sc47	Rabbits for fattening	2.5×10^9	2.5×10^9
	cerevisiae	NCYC Sc47	Rabbits for fattening	2.5×10^9	5×10^9
	cerevisiae	NCYC Sc47	Sows	5×10^9	2.5×10^{10}
	cerevisiae	NCYC Sc47	Sows	5×10^9	1×10^{10}
	cerevisiae	NCYC Sc47	Piglets (weaned)	5×10^9	1×10^{10}
	cerevisiae	NCYC Sc47	Lambs for fattening	1.4×10^9	1.4×10^{10}
	cerevisiae	NCYC Sc47	Dairy cows	4×10^8	2×10^9
	cerevisiae	CBS493.94	Dairy cows	5×10^7	3.5×10^8

(*continued*)

TABLE 1.17 (*Continued*)

Genus	Species	Strains	Minimum Age	Species or Category of Animals	Composition in Complete Feedstuff (cfu/kg)	
					Min.	Max.
	cerevisiae	CBS493.94		Horses	4×10^9	2.5×10^{10}
	cerevisiae	CBS493.94	6 months	Calves	2×10^8	2×10^9
	cerevisiae	CBS493.94		Cattle for fattening	1.7×10^8	1.7×10^8
	cerevisiae	CNCM I-1079		Sows	1×10^9	6×10^{10}
	cerevisiae	MUCL 39 885		Piglets (weaned)	3×10^9	3×10^9
	cerevisiae	MUCL 39 885		Cattle for fattening	9×10^9	9×10^9
	cerevisiae	MUCL 39 885		Dairy cows	1.23×10^9	2.33×10^9
	cerevisiae	CNCM I-1077		Dairy cows	4×10^8	2×10^9
	cerevisiae	CNCM I-1077		Cattle for fattening	5×10^8	1.6×10^9
Streptococcus	*cremoris*	CNCM DASC I-1244				
	diacetylactis					
	faecium	36 KKP.880				
	intermedius					
	lactis					
	thermophilus	CHCC3021/CNCM DAST I-1245				

- Group 2—extracts (e.g., plant extracts, oils);
- Group 3—microbial agents (e.g., bacteria, fungi, viruses, protozoa);
- Group 4—other living organisms (e.g., microscopic insects, plants and animals plus some organisms that have been genetically modified).

APVMA defines microbial agents as naturally occurring or genetically modified microorganisms, including bacteria, fungi, viruses, protozoa, microscopic nematodes, or other microbial organisms. The APVMA approves a list of microorganisms that have been reviewed by the US Food and Drug Administration, Center for Veterinary Medicine, as direct-fed microbials (Table 1.18). However, applicants must still provide up-to-date evidence that the direct-fed microbial has generally Recognized as safe (GRAS) status from the US Food and Drug Administration or the equivalent from the European Union.

1.8.2.8 New Zealand To determine whether a probiotic product is registrable, submission of class determination is necessary. Importing a probiotic product that contains microorganism would also require approval from MAF Biosecurity and ERMA. Usage of probiotic for animals in New Zealand is governed by the Agricultural Compounds and Veterinary Medicines Regulations (ACVM) 2001 (596). As an oral nutritional compound (ONC), probiotics can be exempted from registration if the Trade Name Product (TNP) are the following:

- It is not medicated;
- It contains no feed additive that is not listed in Schedule 7 Part A of the ACVM Regulations 2001;
- It makes no therapeutic of pharmacological claims attributable to a nutritional benefit;
- It contains no substance of uncertain status as either a nutrient or feed additive;
- It does not use any slow release mechanism containing high/concentrated levels of substance.

For a probiotic that requires registration, the information required by NZFSA includes information to support safety of the functional ingredient and where applicable the non-GRAS additives at the proposed feeding rate, and if appropriate, information to support the product's status as being fit for purpose and the Product Data Sheet (PDS). Details of the registration package can be found in ACVM Specified Requirements Products Standard and Guideline: Oral Nutritional Compounds Containing Nutrients with Known Therapeutic Uses (Functional Nutrients) non-GRAS ingredients (596). The GRAS status of the probiotic can be determined by the GRAS Register for ONC maintained by NZFSA (597). According to the current list, there are a total of 21 strains of microorganisms from nine genera (Table 1.18).

TABLE 1.18 Overview of Microorganisms Approved as Feed Additives in the Asia-Pacific Countries

Genus	Species	China	Japan	Korea	Thailand	Australia	New Zealand
Aspergillus	*niger*				✓	✓	✓
	oryzae				✓	✓	✓
Bacillus	*badius*		✓				
	cereus		✓	✓			
	coagulans		✓	✓	✓	✓	
	lentus				✓		
	licheniformis	✓			✓	✓	✓
	pumilus				✓	✓	
	subtilis	✓	✓	✓	✓ and strain BN, non-antibiotic producing strains only	✓	✓ no pathogenic strains
	toyoi				✓		
Bacteroides	*amylophilus*				✓	✓	
	capillosus				✓	✓	
	ruminocola				✓	✓	
	suis				✓	✓	
Bifidobacterium	*adolescentis*				✓	✓	
	animalis				✓	✓	
	bifidum	✓			✓	✓	
	infantis				✓	✓	
	longum				✓	✓	
	pseudolongum		✓	✓			
	thermophilum		✓	✓	✓	✓	
	sp						✓
Candida	*pinolepessi*				✓		✓

	utilis	✓						✓
Clostridium	*butyricum*			✓	✓			
Enterocuccus	*faecalis*	✓		✓	✓			
	faecium	✓		✓	✓			✓
	mundtii							
Lactobacillus	*acidophilus*	✓		✓	✓	✓	✓	✓
	bifidus							✓
	brevis					✓	✓	
	bucheri							
	bulgaricus	✓	only for pigs and poultry			✓	✓	✓
	casei	✓				✓	✓	✓
	cellobiosus					✓	✓	
	curvatus					✓	✓	
	delbruckii					✓	✓	✓
	fermentum					✓	✓	✓ subspecies *lactis*
	helveticus					✓	✓	
	lactis					✓	✓	
	pantarum				✓	✓		
	pentosus							
	plantarum	✓				✓	✓	✓
	reuteri				✓	✓	✓	
	rhamnosus							✓
	sakei							
	salivarius			✓	✓			✓
Lactococcus	*lactis*							✓ subspecies *cremoris*
Leuconostoc	*mesenteroides*					✓	✓	
	pseudomesenteroides							
Pediococcus	*acidilacticii*	✓			✓	✓	✓	✓

(*continued*)

TABLE 1.18 (*Continued*)

Genus	Species	China	Japan	Korea	Thailand	Australia	New Zealand
	cerevisiae (damnosus)				✓	✓	
	pentosaceus	✓			✓	✓	✓
Pediocuceus	sp				✓		
Propinibacterium	*freudenreichii*				✓	✓	
	globosum						
	shermanii				✓	✓	
Rhodopseudomonas	*palustris*	✓					
Scaccharomyces	*cerevisiae*	✓			✓	✓	✓
Streptococcus	*cremoris*				✓	✓	✓
	diacetylactis				✓	✓	
	faecium				✓ and *cernelle* 68	✓	
	intermedius				✓	✓	
	lactis				✓	✓	
	salivarius						✓ subspecies *thermophilus*
	thermophilus				✓	✓	
Yeast					✓		

1.8.2.9 Indonesia, Malaysia, Philippines, and Vietnam There is currently no positive list of microorganisms as feed additives for these Asian countries. Sale of probiotic products in these countries is subjected to the same registration requirements as other feed additives.

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