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Introduction to Prebiotics and Probiotics

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1.1 INTRODUCTION

1.1.1 History

During the second part of the 19th century, early scientific studies on microorganisms have also dealt with their interactions with the human host, albeit primarily from a negative perspective. However, as early as 1885, Escherich (1) described the microbiota and in 1886 early colonization (2) of the infant gastrointestinal tract (GIT) and suggested their benefit for

digestion, whereas Döderlein (3) was probably the first scientist to suggest the beneficial association of vaginal bacteria by production of lactic acid from sugars, thereby preventing or inhibiting the growth of pathogenic bacteria. These findings and other information on the early stages of development toward biotherapeutic concepts and the utilization of functional bacteria are summarized in Table 1.1. Bacteria producing lactic acid as the major metabolic product were generally grouped as "lactic acid bacteria" (LAB) even in those early days, and their association with fermented milk products was also recognized. Recent research has underlined the importance of a vital and "healthy" microbial population of the GIT. Particularly, the beneficial association of LAB with the human host, suggested more than 100 years ago on the basis of gut ecological and taxonomic studies by Moro in 1900 (4), Beijerinck (5), and Cahn (6), has been confirmed and extended by increasing research efforts during the last three decades. Metschnikoff (7, 8) in his bestseller *The Prolongation of Life* was probably the first to advocate, or rather postulate, the health benefits of LAB associated with fermented milk products. He suggested the longevity of the Caucasians to be related to the high intake of fermented milk products. Although Metschnikoff viewed gut microbes as detrimental rather than beneficial to human health, he considered substitution of gut microbes by yogurt bacteria to be beneficial. He considered that lactic acid production, resulting from sugar fermentation by LAB, to be particularly beneficial. The bifidobacteria, another group producing lactic acid, phylogenetically distant but commonly accepted to form part of the LAB, were discovered in 1889 and described in the early 1900s by Tissier (9, 10) to be typically associated with the feces especially of breast-fed infants. When compared to formula-fed infants, a lower incidence of intestinal upsets was observed for infants receiving mother's milk. Thereby the assumption was made about the benefical association of bifidobacteria with the human GIT.

1.1.2 Definitions

The expression "probiotic" was probably first defined by Kollath in 1953 (11), when he suggested the term to denote all organic and inorganic food complexes as "probiotics," in contrast to harmful antibiotics, for the purpose of upgrading such food complexes as supplements. In his publication "Anti- und Probiotika," Vergio (12) compared the detrimental effects of antibiotics and other antimicrobial substances with favorable factors ("Probiotika") on the gut microbiology. Lilly and Stillwell (13) proposed probiotics to be "microorganisms promoting the growth of other microorganisms." Although numerous definitions have been proposed since then (see Table 1.1), none has been completely satisfactory because of the need for additional explanations, e.g., with regard to statements such as "beneficial balance," "normal population," or "stabilization of the gut flora." A consensus and somewhat generalized definition as suggested by

TABLE 1.1

Chronology (Arbitrary) and Development of the Concept of Biotherapy and Probiotics

Period	Time	Concept/Approach/Definition	Literature
"Empiric"	<1850	Fermented foods (yogurt) consumed for therapy against diarrhea	
Early developments of microbiology as science	1850–1890	1857: LAB discovered and lactic acid fermentation described by Pasteur	
		1878: Lister isolates LAB ("*Bacterium lactis*") in pure culture from fermented milk	
		Particular micro-organisms beneficial for GI tract	
		The microbiota of the neonate and breast-fed infant	Escherich, 1885 (1)
		Early bacterial colonization of the infant GI tract (by *E. coli*) and relationship to digestion	Escherich, 1886 (2)
		1889: *Bifidobacterium* discovered in feces of breast-fed infants	Tissier, 1900; 1905 (9; 10)
		1890: First "commercial" starter cultures for sour milk and cheese in Copenhagen and Kiel	
Microbiology as basis for scientific approaches	1890–1930	Positive association of lactic acid bacteria in the stabilization of the vagina	Döderlein, 1892 (3)
		Discovery of *Lactobacillus acidophilus*	Moro, 1900 (4)
		"Industrial" lactic acid bacteria	Beijerinck, 1901 (5)
		Rod-shaped bacteria (lactobacilli) of the infant feces	Cahn, 1901 (6)
		Longevity of the Caucasians related to the high intake of fermented milk products. Gut microbes more detrimental but substitution of gut microbes by yogurt bacteria beneficial	Metchnikoff (1907; 1908) (7; 8)
		Prophylactic substitution by non-pathogenic, "physiological" *E. coli* directly after birth	Nissle, 1916 (14)
		"Antagonistic" treatment of chronic intestinal inflammation	Nissle, 1918 (15)
		"Mutaflor" treatment of diarrhea and dysentery	Nissle, 1919 (16)
Development of concepts toward probiotics and biotherapeutics, and their functions	1930–1990	1936: Isolation and early biotherapeutic application of "*Lb. casei*" Shirota	
		1953: First suggestion and definition of the term "probiotic," denoting all organic and inorganic food complexes as probiotics in contrast to harmful antibiotics – for the purpose of upgrading as supplements	Kollath, 1953 (11)
		"Probiotic" first defined: Promotion of body functions and beneficial microorganisms by microbes and their metabolites	Vergio, 1954 (12)
		Prophylactic treatment of acute infections with "physiological" bacteria	Kolb, 1955 (17)

TABLE 1.1

Chronology (Arbitrary) and Development of the Concept of Biotherapy and Probiotics (continued)

Period	Time	Concept/Approach/Definition	Literature
		Characterization of typical lactobacilli and bifidobacteria from different regions of the human GI tract	Lerche and Reuter, 1962 (18); Reuter, 1963 (19); 1965 (20); 1969 (21)
		Probiotic defined as microbiologically produced substances ("factors") which promote growth of other organisms	Lilly and Stillwell, 1965 (13)
		Oral administration of beneficial lactobacilli ("*Lb. acidophilus* Shirota") influences intestinal population of infants	Shirota et al., 1966 (22)
		Role of LAB and their fermentation products in antitumor activity and modification of biological responses	Reddy et al., 1973 (23); Kato et al., 1981 (24); Yokokura et al., 1981 (25)
		Feed supplements for animals – defined as "organisms and substances that have a beneficial effect on the host animal by contributing to its intestinal microbial balance"	Parker, 1974 (26)
		Intestinal population of breast-fed and infants established and similar to those receiving formula-milk	Hoogkamp-Korstanje et al.,1979 (27)
		Modulation of immune response	Schwab, 1977 (28); Conge et al., 1980 (29)
		Definition by Fuller: "live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance"	Fuller, 1989 (30)
Probiotics toward functional strains and understanding of mechanisms	1990– present-day	Improved definition: "mono- or mixed cultures of live micro-organisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora"	Havenaar et al., 1992 (31)
		Definition: "viable microorganisms (bacteria or yeasts) that exhibit a beneficial effect on the health of the host when they are ingested"	Salminen et al., 1998a (32)
		Definition: "living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition"	Guarner and Schaafsma, 1998 (33)
		Consensus definition: "Probiotics are defined, live microorganisms, which when reaching the intestines in sufficient numbers (e.g., administered via food), will exert positive effects"	BgVV, 1999 (34)

the Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (BgVV; now called BfR) states that probiotics are defined, live microorganisms, which when reaching the intestines in sufficient numbers (e.g., administered via food), will exert positive effects (34). The present-day concept refers to viable microorganisms that promote or support a beneficial balance of the autochthonous microbial population of the GIT. These microorganisms may not necessarily be constant inhabitants of the GIT, but their "…beneficial effect on the general and health status of man and animal" (26, 30) should be ascertained. This is also reflected in the suggestion of Havenaar et al. (31), defining probiotics as "…mono- or mixed cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora." Probiotics are best known by the average consumer with relation to food where they are defined by the EU Expert Group on Functional Foods in Europe (FUFOSE) as "viable preparations in foods or dietary supplements to improve the health of humans and animals" (35). Yet, particular pharmaceutical preparations containing viable microorganisms in capsules and which are being used for the restoration of the gastrointestinal population, e.g., after or during antibiotic treatment, have also been known as "biotherapeutics" for many years.

1.1.3 Administration and Consumption of Probiotics

Viable strains of especially the *Lactobacillus acidophilus* "group" and *Bifidobacterium bifidum* were introduced into dairy products in Germany during the late 1960s because of their expected adaptation to the intestine and the sensory benefits for producing mildly acidified yogurts. Such products first became known in Germany as mild yogurts or "bio-yogurts", while in the USA, acidophilus milk was better known (36, 37).

As is shown in Figure 1.1, probiotics are available and may be administered in different forms, comprising foods, mainly in a fermented state, and pharmaceutic products, mainly as capsules or in microencapsulated form. By definition, probiotic strains may even be undefined organisms from fermented foods, which survive the gut passage and may exert positive effects in the GIT. If probiotic microorganisms constitute a defined part of a food, they are defined by FUFOSE as "live consituents of a food which exert positive effects on health" (32, 38). Probiotic foods comprise between 60 and 70% of the total functional food market. A continued increase is observed among the dairy-type probiotic foods, but even in the range of nondairy probiotic food products such as fermented meats and vegetable and fruit juices. Taking into account the wide range of potential (fermentable) substrates and the different conditions under which LAB strains may be challenged for "functional performance," it can be expected that developments toward new food-based probiotics will proceed further in the future.

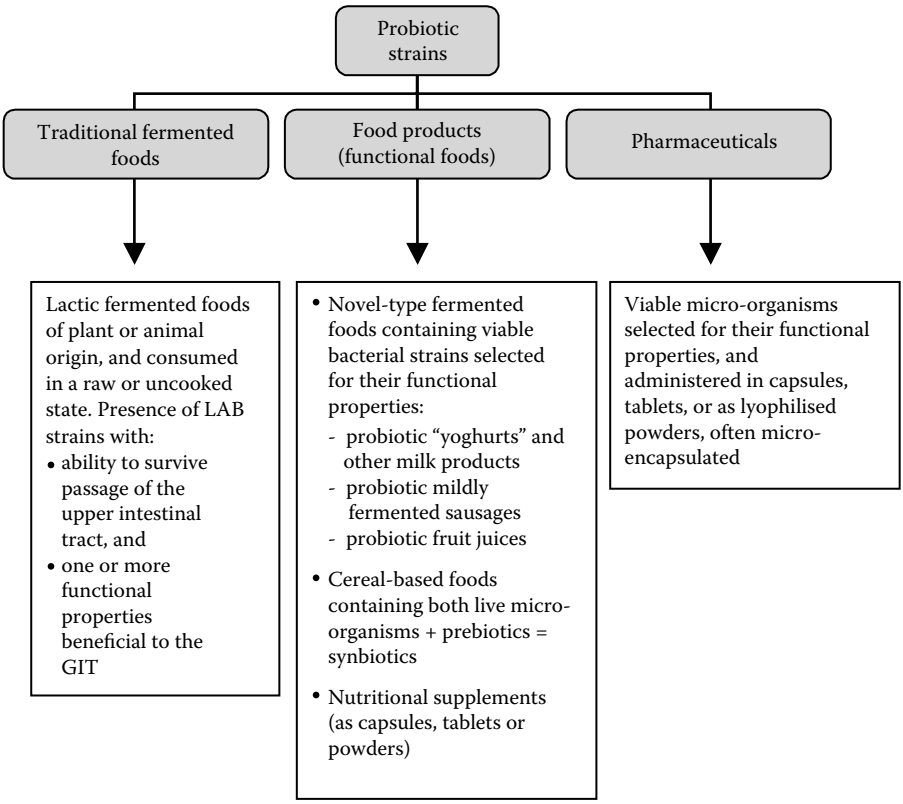

FIGURE 1.1 Administration of probiotics in different forms.

It is postulated that this positive effect is achieved when the proportion of lactobacilli and bifidobacteria in the intestinal population increases, either by increased intake of typical gut bacteria (e.g., as fermented foods or dehydrated preparations), or indirectly as a result of the stimulation of autochthonous gut bacteria belonging to these groups. The lactobacilli and bifidobacteria associated with the GIT are generally considered beneficial for such things as combating disturbances of the mucosa associated immune system and of the established gut population.

A particular feature of probiotic cultures is that they regulate the balance of the gut bacterial population, e.g., by competition for epithelium contact sites and nutrients and also by modulation of the pH value. Other features refer to the support of absorption of nutrients and the synthesis of vitamins such as riboflavin. Further stabilization of the gut microbiota is associated with the synthesis of nutritional physiologically important short-chain fatty acids (SCFAs) by which the gut mucosa is supported. In addition, probiotic cultures are also suggested to stimulate the immune system. These functional aspects are briefly discussed in Section 1.2.

1.2 GUT MICROBIAL ECOLOGY

1.2.1 The Gastrointestinal Tract as Ecosystem

The GIT with its diverse and concentrated microbial population is one of the key organs of the human body; it is in fact an ecosystem of highest complexity that mediates numerous interactions with the chemical (and nutritional) environment. The mucosal surface increase by circular folds, intestinal villi, and microvilli provides a large area for such interactions associated with digestion, adhesion to the mucosal wall, and colonization (39). Compared to about 2 m^2 of skin surface area of the average human body, the gastrointestinal system comprises an area of 150 to 200 m^2 (40), making available the necessary space for digestive interactions and for adhesion and colonization associated with the mucosal wall. The circular folds contribute to about a 3-fold increase, a 7- to 10-fold increase by the epithelium folding (intestinal villi), and a 15- to 40-fold increase by the microvilli in the enterocyte resorptive luminal membrane.

1.2.2 Microbiota of the Human Gastrointestinal Tract

Increasing microbial populations are found throughout the GIT (Table 1.2), ranging from varying numbers of food-associated bacteria in the esophagus, to 10^1 - to 10^3/ml (or g) in the stomach, 10^7/ml in the jejunum (comprising mainly lactobacilli, *Enterobacteriaceae* and streptococci), up to 10^9 CFU/g in the terminal ileum, and ca. 5 x 10^{11}/g in the distal colon. Many microbes isolated from the duodenum and jejunum are considered to be typical transients, especially considering rapid chymus flow; indigenous colonization is, however, more likely to occur in the lower parts of the ileum. The estimated total population of 10^{14} viable bacteria in the adult human GIT (41) represents about 10 times more than all body tissue cells. The microbial population therefore represents an immense metabolic potential that not only supports the digestion processes but is also interactive in detoxification and toxification processes and, most importantly, comprises the major part of the human immune system. *Bacteroides* and the Gram-positive, anaerobic genera *Eubacterium* and *Bifidobacterium* predominate in the densely populated large intestine. Other groups such as the clostridia, peptostreptococci, "streptococci," and lactobacilli also play an important role, e.g., in the maintenance of a stable gut mucosa and in the generation of SCFAs in a beneficial ratio. The role of the lactobacilli may be more important in the small intestines where they comprise a higher proportion of the total population. In healthy humans, lactobacilli are normally present in the oral cavity (10^3 to 10^4 CFU/g), the ileum (10^3 to 10^7 CFU/g), and colon (10^4 to 10^8 CFU/g) and are the dominant microorganism in the vagina (68-70).

TABLE 1.2

Estimated Numbers (Per ml or g of Intestinal Contents) and Suggested Role (Postulated Effects) of Major Microbial Population Groups in Different Segments of the Gastrointestinal Tract

Microbial Group	Stomach $10^1 - 10^3$ CFU/ml	Duodenum $10^1 - 10^4$ CFU/ml	Jejunum + Ileum $10^5 - 10^8$ CFU/g	Colon $10^9 - 5x10^{11}$ /g	Positive Effects	Negative Effects
Actinomyces spp.			10^4 - 10^6		?	
Bacteroides-Prevotella-Porphyromonas Group	Up to 10^2	ca .10^3	10^4 - 10^7	$10^9 - 10^{11}$		+
Bifidobacterium spp.				$10^9 - 10^{10}$	+	
Clostridium spp.			10^4 - 10^5	$10^8 - 10^9$	(+)	+
Coprococcus cutactus				$10^7 - 10^8$		
Enterobacteriaceae	Up to 10^2	$10^2 - 10^4$	$10^3 - 10^6$	$10^5 - 10^7$	(+)	(+)
Enterococcus spp.			$10^2 - 10^4$	$10^3 - 10^6$		
Eubacterium spp.				$10^9 - 10^{11}$	+	
Fusobacterium spp.			10^3 - 10^5	$10^5 - 10^7$		
Lactobacillus spp.	$10^1 - 10^3$	$10^2 - 10^4$	$10^4 - 10^6$	$10^5 - 10^8$	+	
Megamonas hypermegas				$10^7 - 10^8$		
Megasphaera elsdenii				$10^7 - 10^8$		
Methanobacteria				up to 10^9	(+)	(+)
Peptostreptococcus spp.			10^2 - 10^6	$10^8 - 10^9$	(+)	(+)
Proteus spp.				$10^3 - 10^6$		
Pseudomonas spp.				$>10^3$		
Staphylococcil				ca. 10^3		
Streptococcus spp.	$10^1 - 10^3$		$10^3 - 10^8$	up to 10^7		
Veillonella spp.			$10^3 - 10^7$	$10^5 - 10^8$		+
Yeasts				ca. 10^3		(+)

Source: Modified according to Holzapfel et al. (39); Tannock (42, 43); Sullivan et al. (44); Holzapfel (45).

Interestingly, *Streptococcus intermedius* and *Haemophilus parahaemolyticus* could not be detected in the jejunum of 18 patients with gastrointestinal diseases, as compared to healthy subjects, whereas lactobacilli were detected more commonly in diseased than healthy subjects (44).

In spite of increased research on gut microbial ecology, still only a relatively small number of the ca. 400 genera and species have been cultivated and studied with regard to their physiology, metabolic interactions, and taxonomy.

Considered as beneficial bacterial groups of major importance to the gut ecosystem, special attention was given to the Gram-positive genera *Lactobacillus*, *Bifidobacterium,* and, more recently, to *Eubacterium*. All Gram-positive bacteria cluster in two of the formerly recognized 17 eubacterial phyla that also coincide with their DNA base composition (71, 72). Practically all organisms used in probiotic foods or food supplements are representatives of the genera *Lactobacillus*, *Enterococcus* or *Bifidobacterium*. The genus *Bifidobacterium* shares some phenotypic features with the "typical" LAB, but traditionally and also for practical purposes they are still considered to form part of the LAB. The bifidobacteria exhibit a relatively high guanine plus cytosine (G+C) content of 55 to 67 mol% in the DNA and are phylogenetically distinct from the "true" LAB and form part of the so-called *Actinomycetes* branch. The "true" LAB form part of the so-called *Clostridium* branch which is characterized by a G+C content of <55 mol% in the DNA (70, 71). Based on the comparison of 16S rRNA sequences, *Carnobacterium*, *Enterococcus*, *Vagococcus*, *Aerococcus*, *Tetragenococcus*, and the newly described genus *Lactosphaera* are more closely related to each other than to any other LAB. *Lactococcus* and *Streptococcus* appear to be relatively closely related genera, whereas the genus *Lactobacillus* is phylogenetically diverse. Comparison of 16S rRNA sequencing data showed the genera *Lactobacillus* and *Pediococcus* to be phylogenetically intermixed as 5 species of *Pediococcus* cluster with 32 homo- and heterofermentative *Lactobacillus* species in the so-called *Lactobacillus casei-Pediococcus* group (73). The 16S rRNA sequence data of pediococci and lactobacilli clearly indicate that the taxa generated on the basis of phenotypic properties, such as cell morphology and fermentation type, do not correspond with the phylogenetic branching. Therefore, a number of species of LAB may have to be reclassified; this may have important consequences for commercial probiotic strains (37).

The early observations and hypotheses in the 20th century pointed toward the beneficial role of the LAB in food fermentations (46), the GIT (4, 6, 7), and the vagina (3) (*vide supra*; Table 1.1). Even so, studies on the types and numbers of LAB of the different regions of the human GIT were rare. Among the first comprehensive studies were those by Reuter and coworkers (18-21, 47). Thanks to their precise and well-documented observations, the three major groups of homofermentative lactobacilli, typical of the intestinal tract of the human host, were characterized in the 1960s and were confirmed by later taxonomic investigations, supported by improved sampling techniques and molecular biological methods comprising:

- The "*Lactobacillus acidophilus* group" involving strains that are presently recognized as *L. acidophilus*, *L. gasseri*, *L. crispatus*, and *L. johnsonii* (discussed in Section 1.3; see also Table 1.3)
- "*Lactobacillus salivarius*"
- The "*Lactobacillus casei* group," comprising strains of *L. paracasei* and *L. rhamnosus*

The heterofermentative lactobacilli were shown to comprise a major phenotypic group (later classified as *L. reuteri*) and, to some extent, also *L. fermentum* and *L. oris* (47).

Apart from the bifidobacteria, the LAB in the gut are mainly represented by the lactobacilli, but in contrast to their domination in the ileum, they only form a minor population in the colon. The lactobacilli, as major LAB representatives, in fact only make up about 1% of the total bacterial population of human feces but may be more numerous in the proximal colon (48). They do not appear to be detectable by conventional culture methods in the feces of all adults; yet, they seem to be consistently present in the colon, albeit in relatively low numbers. This may in part also result from the consumption of fermented food products (42, 49, 50). This has in fact been shown by Dal Bello et al. (51) using "alternative" incubation conditions (30°C, 2% O_2) and confirmed by polymerase chain reaction (PCR) – denaturing gradient gel electrophoresis (DGGE) analyses of resuspended bacterial biomass obtained from agar plates for revealing of the species composition. These workers in fact reported that food-associated LAB, such as *Lactobacillus sakei* and *Leuconostoc mesenteroides*, hitherto not described as intestinal inhabitants, were more easily detected with the alternative incubation condition (see also Table 1.4). Randomly picked colonies grown under the alternative condition showed *L. sakei* as one of the predominant food-associated LAB species, to reach counts of up to 10^6 CFU/g feces.

TABLE 1.3

Features of the Species of the So-Called "Acidophilus" Group of Lactobacilli

Species	Habitat[a]	mol% G+C in the DNA	"Biotypes" acc. to	DNA Homology groups acc. to	
			Lerche and Reuter (1962)	*Lauer et al. (1980)*	*Johnson et al. (1980)*
L. acidophilus	HSCP	32-37	I,/II	I a	A-1
L. amylovorus	S/C	40	IV (III)	I b	A-3
L. crispatus	H/P	35-38	III	I c	A-2
L. gallinarum	P	33-36	-	I d	A-4
L. gasseri	H/C	33-35	I	II a	B-1
L. johnsonii	H/S/P	32-38	I, II	II b	B-2

[a] H = humans; S = pigs; C = cattle; P = poultry.

Source: Modified according to Mitsuoka (70); Reuter (47); Holzapfel et al. (39).

TABLE 1.4

LAB Typically Associated with the Human Host

Lactobacilli	Other LAB
Intestinal Bacteria	
*Lactobacillus acidophilus** "group"	*Bifidobacterium adolescentis**
L. acidophilus senso strictu	*B. angulatum*
L. animalis	*B. bifidum*
*L. brevis**	*B. breve*
L. buchneri	*B. cantenulatum*
L. crispatus	*B. dentium**
L. curvatus	*B. infantis*
*L. deLrueckii **	*B. longum*
L. fermentum	*B. pseudocantenulatum*
*L. gasseri**	
L. johnsonii	*Enterococcus faecalis**
*L. paracasei**	*E. faecium**
*L. plantarum**	
*L. reuteri**	*Leuc. mesenteroides*
*L. rhamnosus**	
L. ruminis	*Pediococcus pentosaceus**
*L. salivarius**	
L. sakei	*Weissella confusa*
Vaginal Bacteria	
*Lactobacillus acidophilus**	*Bifidobacterium bifidum*
L. fermentum	*B. longum*
*L. casei**	*B. infantis*
*L. rhamnosus**	*B. breve*
L. cellobiosus	*B. catenulatum*
*L. plantarum**	*B. dentium*
*L. brevis**	
*L. delbrueckii **	
*L. salivarius**	
*L. jensenii**	
L. vaginalis	
*L. gasseri**	
L. crispatus	

[a] Also found in clinical samples.

Source: From References 42, 47, 50, 51.

Even so, detailed and scientifically well-founded studies on other "beneficial" groups of the human GIT were particularly rare until the last decade of the 20th century. This was mainly due to technical restrictions related to sampling techniques, and detection and cultivation methods. In contrast to the oxygen-tolerant lactobacilli, the study of anaerobic groups such as the bifidobacteria and eubacteria was made possible by the development of anaerobic techniques developed in the early 1970s (52, 53), which were

further improved in combination with improved cultivation media. Compared to their domination in infants, bifidobacteria comprise only up to 3% of the total fecal bacteria of humans and up to 10% of the total culturable population (54-56). With increasing age, however, their numbers in feces are reported to decline in adults (43, 57). They comprise up to 91% of the total population in breast-fed babies and up to 75% in formula-fed infants (58). A reduced environment and special media are applied for the selective cultivation of bifidobacteria; yet, such media do not equally support the growth of all *Bifidobacterium* species present in human feces (59). Moreover, the identification of *Bifidobacterium* species by phenotypic characteristics is difficult and unreliable (43, 60). These culture-related and other factors limit the research data and their quality with regard to bifidobacterial and other gastrointestinal populations. Also, it can be expected that the population detected in feces may probably more correspond to that of the distal colon than the proximal region. It is in the distal colon where fermentable but nondigestible carbohydrates (so-called "prebiotics") may play an important role in stimulating the *Bifidobacterium* and *Eubacterium* populations, or perhaps particular species only. With the aid of genetic fingerprinting techniques, it could be shown that particular *Bifidobacterium* and *Lactobacillus* strains are unique to each individual (43). In addition, it was suggested that the composition of these populations remains relatively constant for some individuals and to fluctuate considerably for others (43, 56). Using fluorescent *in situ* hybridization (FISH) with group-specific 16S rRNA-targeted oligonucleotide probes, it was possible to detect variations in bifidobacterial populations in the feces of different age groups. The percentage of bifidobacteria in the feces ranged from 0 to 78.9%, depending on the age group, with large variations within each group (58, 61, 62). Moreover, DGGE banding patterns of human gut bacteria have been found to differ significantly from those of other mammals. Furthermore, 16S rDNA sequences showed three bacterial species, *Ruminococcus obeum, Eubacterium halii,* and *Fusobacterium prausnitzii* to be most probably ubiquitous to humans; these groups were therefore suggested to play an important role in the human GIT (63-66).

Matsuki et al. (67) investigated the population structure of the human fecal microorganisms by applying 16S rRNA-gene-targeted group-specific oligonucleotide primers for the *B. fragilis* group, *Bifidobacterium* spp., the *C. coccoides* group, and *Prevotella*, and thereby detected and identified 74% of the predominant bacteria in the feces of six healthy volunteers. The other isolates were identified by 16S ribosomal DNA sequence analysis and consisted of *Collinsella*, the *Clostridium leptum* subgroup, and isolates of other clusters. As shown in Table 1.1, major microbial groups of the human GIT vary in numbers and their distribution among the different regions of the gut. Recent observations suggest the *Bacteroides-Prevotella-Porphyromonas* group, with numbers of up to 10^{11}/g, to dominate the colon population together with the Eubacterium group, and to reach 10- to 100-fold higher numbers than the bifidobacteria (43). As for the *Lactobacillus* species, the *Bacteroides-Prevo-*

tella-Porphyromonas group appears to be present in all regions of the GIT, indicating that not all representatives are strictly anaerobic (Table 1.2).

1.2.3 Role and Functions of the Microorganisms of the Gut

A healthy intestinal epithelium, in association with an established and stable intestinal microbial population, presents a vital barrier against the invasion or uptake of pathogenic microorganisms, antigens, and harmful compounds from the gut lumen, while the intestinal mucosa also efficiently assimilates antigens (36). Specific immune responses are evoked by the specialized antigen transport mechanisms in the villus epithelium and Peyer's patches (74). The positive role of gut microorganisms in human health was largely overlooked for a long time, and the main focus was placed on enteric pathogens and factors leading to gastrointestinal disorders or "dysbiosis" (36). A stable barrier, typical of healthy individuals, ensures host protection and serves as support for normal intestinal function and immunological resistance. The gut-associated lymphoid tissues (GALT) are considered to be the largest "immune organ" in the human body, and its "barriers" serve for intrinsic protection against infective agents. Around 80% (10^{10}) of all immunoglobulin-producing cells are found in the small bowel (75), while the gut microbial population is essential for mucosal immune stimulation and amplification of immunocompetent cells. Numerous physiological functions have been ascribed to the "normal" gut microbial population; some of the major functions are considered the following (36; 39; 76):

- Maintenance and restoration of barrier function
- Stimulation of the immune system
- Maintenance of mucosa nutrition and circulation
- Improvement of bioavailability of nutrtients
- Stimulation of bowel motility and reduction of constipation

1.3 Probiotic Microorganisms

1.3.1 Examples of Probiotic Microorganisms

Probably the longest history of proven health benefits and "safe-use" of probiotic bacteria in food is documented for *L. casei* strain "Shirota" (22) and some strains of the *L. acidophilus* group. Since at least 40 years in Japan and more than 30 years in Germany, LAB cultures of human origin are applied in the manufacture of fermented milk products. Viable strains of especially

"*Lactobacillus acidophilus*" and *Bifidobacterium bifidum* were introduced in Germany during the late 1960s into dairy products because of their expected adaptation to the intestine and the sensory benefits for producing mildly acidified yogurts (77). In Germany, such products first became known as mild yogurts or "bio-yogurts", whereas in the USA, acidophilus milk was developed. The functional properties and safety of particular strains of *L. casei/paracasei, L. rhamnosus, L. acidophilus,* and *L. johnsonii* have extensively been studied and are well documented (32, 78-80).

Viable probiotic strains with beneficial functional properties are at present found among a wide and diverse number of microbial species and genera. They are supplied in the market either as fermented (mainly "yogurt"-type) food commodities or in lyophilized form, both as food supplements and as pharmaceutical preparations. Most strains currently in use as probiotics in food, nutrition, and in pharmaceutical preparations are members of the LAB (Table 1.5). A number of "nonlactic" strains, e.g., *Bacillus cereus* ("*toyoi*"), *B. clausii, B. pumilis* (146), *Escherichia coli* (Nissle) (16), *Propionibacterium freudenreichii, P. jensenii, P. acidopropionici, P. thoenii* (147), and *Saccharomyces cerevisiae* ("*boulardii*"), are also available in the market mainly as pharmaceutic preparations and some also as animal feed supplements (39, 79) (Table 1.5).

With 65%, the probiotic milk products (mainly "yogurt"-like) represent the largest segment of the functional foods market in Europe, while in Japan they are estimated to comprise about 75% of the foods for specified health uses (FOSHU)) market. Initiated by a national project team under the auspices of the Japan Ministry of Education and Science, specific regulatory measures on functional foods were first initiated in Japan in 1984. This

TABLE 1.5

Microorganisms Reported to Find Application as Probiotics Mainly for Humans

Lactobacillus Species	Bifidobacterium Species	Other LAB	"Non-lactics"[c]
L. acidophilus	*B. adolescentis*	*Ent. faecalis* [a]	*Bacillus cereus* ("toyoi")[a,c]
L. amylovorus	*B. animalis*	*Ent. faecium*	*Escherichia coli* (Nissle 1917)[c]
(L. casei)	*B. bifidum*	*Sporolactobacillus inulinus* [a]	*Propionibacterium freudenreichii*[a,c]
L. crispatus	*B. breve*		*Saccharomyces cerevisiae* ("boulardii")[c]
L. delbrueckii subsp. bulgaricus[c]	*B. infantis*		
L. gallinarum[a]	*B. lactis*[b]		
L. gasseri	*B. longum*		
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			

[a] Mainly for animals.
[b] Synonym of *B. animalis.*
[c] Mainly in pharmaceutical preparations.

Source: Modified from Holzapfel et al. (39).

triggered the beginning of numerous academic and industrial studies on functional foods in relation to nutrition and evidence in support of functional claims. The Japan Ministry of Health and Welfare thereupon established a specific policy on FOSHU in 1993, by which health claims of some selected functional foods are legally permitted. The developments of functional food science in Japan focused, among others, on minimizing undesirable and maximizing desirable food factors. Three major requirements had to be met for FOSHU approval, viz.:

- Scientific evidence of the efficacy, including clinical testing
- Safety for consumption
- Analytical determination of the effective component

By the end of 1999, 167 items were approved as FOSHU, as compared to 293 by 2002. In April 2001, the Japanese government introduced a new regulatory system (foods with health claims), comprising FOSHU and foods with nutrient function claims (FNFC). Most of the descriptions of foods under the FOSHU system are similar to the category of enhanced function claims of Codex (81-83).

Functional food products primarily contain strains of the "acidophilus group" (mainly *L. acidophilus, L. crispatus,* and *L. johnsonii*), *L. casei/paracasei* and *Bifidobacterium* spp.; enterococci are rarely used in probiotic milk products (22, 32, 77-80). Information on the typical LAB species associated with probiotic milk products in the European market is given in Table 1.6. The problems still encountered with the correct identification of these strains are evident from these data (see also Temmerman et al., Reference 84) and may (among others) be related to the use of unreliable phenotypic methods (compare also Table 1.3 with regard to the acidophilus group). Although phenotypically difficult to assess, the heterogeneity of *L. acidophilus,* one of the most important "probiotic" species, was recognized in the 1960s by Reuter and coworkers (19), who suggested four different "biotypes". DNA-DNA hybridization studies reported in 1980 (20, 21) confirmed this heterogeneity, suggesting the existence of six different homology groups (see Table 1.3). Consequently, only strains belonging to the similarity group and showing a high degree of DNA relatedness with the type strain of *L. acidophilus* remained in this species, while members of the other homology groups were classified as separate species, i.e., *L. amylovorus, L. gallinarum, L. crispatus*, *L. gasseri*, and *L. johnsonii*. Although they are regarded as separate species, they are closely related and have been suggested to belong to one phylogenetic "group" or branch (37, 39, 47, 72). The exact identification of members of the "*L. acidophilus* group" is an important aid toward indication of the origin and typical host of a species (see Table 1.3).

Identification studies on various mild yogurts and novel-type probiotic yogurt-type dairy products showed the 26 isolated *Lactobacillus* strains to represent *L. acidophilus*, *L. johnsonii, L. crispatus, L. casei, L. paracasei,* and *L.*

TABLE 1.6

Lactic Acid Bacteria in Commercial Probiotic Dairy Products: Comparison between Claimed Identity and Identification Results

Product Name	Producer / Distributor/ (Country)	Strain Identity Claimed on Product	Confirmed Identity
ABC	Söbbecke (D)	*L. acidophilus, L. casei*	*L. acidophilus, L. paracasei*
Actimel	Danone (F)	*L. casei* Actimel ("Immunitas")	*L. paracasei*
Andechser Bioaktiv	Bioland (D)	BIOGARDE cultures	*L. johnsonii*
B'A Fruits	B'A France (F)	*Bifidobacterium* ("active bifidus")	*S. thermophilus*
BI'AC	TMA (D)	*L. acidophilus, L. casei*	*L. acidophilus; L. paracasei* ssp. *paracasei; S. thermophilus*
Biogarde plus (naturel)	Almhof (NL)	*L. acidophilus, L. casei, Bifidobacterium*	*L. acidophilus; S. thermophilus*
Bio Snac'	Danone (F)	*Bifidobacterium*, living yogurt cultures	*Lc. lactis* subsp. *lactis*
Biotic	Aldi (D)	*L. acidophilus* LA7	*L. acidophilus*
Do-filus	Arla (S)	*L. acidophilus*	*L. acidophilus*
Fitness Quark	Onken (D)	*L. acidophilus, Bifidobacterium*	*L. johnsonii, S. thermophilus*
Fysiq (Mona)	Campina (NL)	*L. acidophilus* Gilliland, *L. casei*	*L. crispatus, L. paracasei ssp. paracasei*
Gaio (Causido®)	MD Foods A/S (DK)	*Enterococus faecium, S. thermophilus*	*Enterococus faecium, S. thermophilus*
Gefilus	Valio (FIN)	*Lactobacillus* GG, living yogurt cultures	*L. rhamnosus*
Kinderjoghurt mild	J. Bauer KG (D)	*L. acidophilus, L. bifidus*	*L. acidophilus; L. johnsonii, S. thermophilus*
Lc1	Nestlé (D)	*L. acidophilus* LA-1	*L. johnsonii*
Probiotic LA7-Plus	Bauer (D)	*L. acidophilus* LA-7	*L. acidophilus*
Procult Drink	Müller (D)	*B. longum*, live yogurt cultures	*L. acidophilus, S. thermophilus*
Natreen Pro 3+	Milchwerke Köln (D)	*L. acidophilus* LA-H3, *L. casei* LC-H2	ND
Primo	Zott (D)	BactoLab cultures	*L. acidophilus*
Symbalance	Toni Lait (CH)	L. *acidophilus, L. casei, L. reuteri*	*L. acidophilus, L. paracasei, L. reuteri*
Vifit	Südmilch (D)	*L. casei* GG	*L. rhamnosus, L. acidophilus*
Vifit Drink	Mona (NL)	*L. casei* GG, *L. acidophilus, B. bifidum*	*L. acidophilus, L. rhamnosus*
Yakult	Yakult, Europe / (D)	*L. casei* Shirota	*L. paracasei*
Yogosan	Lidl (D)	*L. casei*	*L. paracasei (casei)*

Note: CH = Switzerland; D = Germany; DK = Denmark; F = France; FIN = Finland; NL = The Netherlands; S = Sweden

Source: Modified and extended data from References 36, 39, 84, 85, 89.

rhamnosus, revealing that some strains had been misclassified (85). Some strains designated as *L. acidophilus* were shown to belong either to *L. johnsonii* or *L. crispatus*. Most strains currently designated as *L. casei* may in fact be members of either *L. paracasei* or *L. rhamnosus* (85, 86); (compare also Collins et al. [87] and Dicks et al. [88]). Viable numbers of lactobacilli in mild and probiotic yogurts varied greatly, whereas a few products contained only low *Lactobacillus* numbers (85). This was followed up by a recent study which, once more, showed the identity given for strains in some products to differ from that found by DNA homology studies (36). An important but still controversial issue regarding the "minimal effective dose" of viable bacteria by which scientifically confirmed beneficial effects may be expected is still under discussion.

In addition to the wide range of probiotic "yogurt"-like dairy products in the market, increased attention is also given to other foods as carriers for probiotic lactobacilli and bifidobacteria (90). Particular interest is focused on different types of cheese with "added functional value" by addition of strains of, e.g., *L. acidophilus, L. paracasei,* and *Bifidobacterium* species to different cheese types, including Cheddar, Tallaga, and Ras, and soft cheeses (90, 91). Moreover, the use of *B. bifidum* , *L. acidophilus,* and *L. rhamnosus* GG has also been suggested for ice cream (92, 93), frozen dairy desserts (94), of *L. plantarum* 299v (95) or bifidobacteria (96) for a fermented oatmeal gruel, and of *Bifidobacterium* spp. for fermented sausages and ham (97, 98). Lee (99) suggests many traditional fermented foods to have functional properties, resulting both from the microbial strains involved (mostly LAB) and from other functional components, either originating from the ingredients or formed during fermentation. Several well-known traditional fermented foods may serve as examples, e.g., Korean kimchi (100), sauerkraut (101), and a number of African cereal gruels (e.g., ogi and uji), Nigerian gari, and Asian vegetable foods (99). This hypothesis is also supported by Molin (102), who focused on the role of *Lactobacillus plantarum*, an extremely heterogeneous species.

Numerous probiotic food supplements are available in the market, most commonly as capsules but also as powders and tablets. As for the probiotic milk products, controversies exist also for some products between label claims and sound scientific identifications (84). In addition to the *Lactobacillus* and *Bifidobacterium* species, typical of the probiotic milk products, nonlactics are also being applied, albeit only in a few products, e.g., *Bacillus* IP5832 (identified as *Bacillus cereus*) in "Bactisubtil" (Synthelabo Belgium), *Saccharomyces cerevisiae* in "Bifidus complex" (Biover, Belgium). Furthermore, *Enterococus faecium, L. reuteri,* and even *Pediocooccus acidilactici* have been used in some products (84).

Biotherapeutics for clinical applications are also based on selected probiotic strains, mainly LAB but may also include *Escherichia coli* strains (e.g., the "Nissle" strain), *Saccharomyces cerevisiae* (*boulardii*), and also a number of *Enterococcus faecium* and *E. fecalis* strains, the latter being marketed under the name of "Symbioflor 1".

1.3.2 Selection of Appropriate Strains

The prevalence of bifidobacteria in the feces of breast-fed infants may have been a major reason for selecting strains of this group for use as probiotics (13). Decisions on the use of *Lactobacillus* strains as probiotics have been determined by a number of favorable factors such as:

- Their association with traditional fermented foods earlier noted by Metchnikoff (7) when he postulated benefits from the consumption of yogurt by the Caucasians), together with the high acceptability of lactic fermented foods
- Their association with the human GIT, together with observations on their beneficial interactions in the gut ecosystem
- The adaptation of many lactobacilli to milk and other food substrates and the relatively long history of technical application of LAB with the use of the first industrial strains dating back to 1890 (see Table 1.1)

The selection of new strains presents a major challenge, both to science and industry. The primary objective is to select microbial strains with one or more proven functional properties. Even when probiotic microorganisms are suggested to promote health and well-being, the challenge remains to define particular end points or biomarkers by which such strains can be characterized and particular claims be sustained — either by *in vivo* or validated *in vitro* tests — even when all the mechanisms involved have not yet been fully elucidated (38). Approaches for selection of an "ideal" strain are therefore still difficult and indeed require considerable resources. Desirable technical features and factors related to health promotion or sustaining health serve as important criteria for strain selection. Five major aspects may be taken into account as key criteria for the selection of an appropriate functional strain (36, 38, 39, 86, 103, 104), viz.:

1. General aspects, e.g., origin, identity, and resistance to mutations
2. Technical aspects (growth properties *in vitro* and during processing, survival and viability during transport and storage)
3. General physiological aspects (resistance against environmental stress and to the antimicrobial factors prevailing in the upper GIT as encountered during the stomach-duodenum passage [pH 2.5, gastric juice, bile acid, pancreatic juice], adhesion potential to intestinal epithelium)
4. Functional aspects and beneficial features (adhesion, colonization potential of the mucosa, competitiveness, specific antimicrobial antagonism against pathogens, stimulation of immune response, selective stimulation of beneficial autochthonous bacteria, restoration of the "normal" population)

5. Safety aspects (no invasive potential, no transferable resistance against therapeutic antibiotics, no virulence factors)

Research during the past two decades focused mainly on functional features of strains selected for inclusion, e.g., in functional foods. Considering the worldwide increase in the consumption of dairy products containing probiotic strains of the bacterial genera *Bifidobacterium* and *Lactobacillus* during this period, relatively little attention has been given to technical and sensory properties of these strains and/or the resulting products (107). For the producer, technical properties related to growth, adaptation, and persistence of some probiotic strains, and also the sensory properties of the resulting products, are still major obstacles toward the large-scale production of functional foods containing probiotic strains. Information on particular production steps and modification of growth conditions are still well-protected industrial secrets for the technical production of some strains. Technical production of especially the bifidobacteria in milk substrate constituted a considerable technical challenge but was at least partly solved by some industries during the 1960s (77). Still, it is known that particularly strains of the "acidophilus" group and also bifidobacteria are not well adapted to the milk substrate and, in addition, do not influence the sensory properties of a product positively. Such strains therefore still constitute special technical challenges (108; Holzapfel et al., unpublished data).

From the viewpoint of regulatory authorities, the safety and nonpathogenicity of a new strain is considered of major importance. Ongoing and partly controversial discussions are particularly directed toward the assessment of new strains without a previous "history of safe use" and the definition of minimal requirements to be met before it can be classified as "safe" or "GRAS". According to Marteau (105), an extremely low potential of four types of side effects may exist for probiotic bacteria, viz., systemic infections, deleterious metabolic activities, excessive immune stimulation in susceptible individuals, and gene transfer. The following approaches for assessing the safety of probiotic and starter strains have been recommended by Salminen et al. (78):

- Characterization of the genus, species, and strain and its origin that will provide an initial indication of the presumed safety in relation to known probiotic and starter strains
- Studies on the intrinsic properties of each specific strain and its potential virulence factors
- Studies on adherence, invasion potential, and the pharmacokinetics of the strain
- Studies into interactions between the strain, intestinal and mucosal microflora, and the host

Lactobacilli and bifidobacteria are extremely rare causes of infection in humans. This, even more so, applies to probiotics based on these organisms; in fact, very few cases of adverse effects have been related to the consumption of probiotics. Even when in rare cases strains of some LAB are isolated from clinical specimens (see also Table 1.4), there is apparently no indication that the general public is at risk from the consumption of lactobacilli or bifidobacteria used as probiotics or starters (36, 78, 105, 106). In a paper published in 2003 by the Health & Consumer Protection Directorate-General of the European Commission (SANCO Secretariat) entitled "A generic approach to the safety assessment of micro-organisms used in feed/food production," a "qualified presumption of safety" (QPS) system was suggested as an approach for the safety assessment of microorganisms for use in food and feed. This suggests a key decision which takes into account, among other things, (a) experience and a history of safe use and (b) the detection of known strain-specific risk factors.

In addition to the LAB, strains of other microbial groups such as *Bacillus* spp. (146) and *Propionibacterium* spp. (147) have also been reported to show probiotic or functional properties. The safety, particularly of some "probiotic" strains of *Bacillus cereus*, may be questioned, some of which have been shown to produce Hbl and Nhe enterotoxins (146).

1.3.3 Functional Properties

In spite of research progress in recent years, our understanding of the gut ecosystem is still fragmentary and consequently limits our comprehension of a normal or balanced microbial population. Thus, the impact of a functional strain on the composition and function of the intestinal population is still difficult to ascertain (39, 109). Numerous beneficial functions have been suggested for probiotic bacteria (36, 109), e.g.:

- Nutritional benefits:
  - Vitamin production, availability of minerals and trace elements
  - Production of important digestive enzymes (e.g., β-galactosidase)
  - Production of β-galactosidase for alleviation of lactose intolerance
- Barrier, restoration, antagonistic effects against:
  - Infectious diarrhea (traveller's diarrhea, children's acute viral diarrhea)
  - Antibiotic-associated diarrhea, irradiation-associated diarrhea

- Cholesterol-lowering effects by:
  - Cholesterol assimilation
  - Modifcation of bile salt hydrolase activities
  - Antioxidative effect

- Stimulation and improvement of the immune system, e.g., by:
  - Strengthening of nonspecific defense against infection
  - Increasing phagocytic acitivity of white blood cells
  - Increasing IgA production
  - Regulating the Th1/Th2 balance; induction of cytokine synthesis

- Enhancement of bowel motility, relief from constipation
- Reduction of inflammatory or allergic reactions, by:
  - Restoration of the homeostasis of the immune system
  - Regulation of cytokine sysnthesis

- Adherence and colonization resistance
- Anticarcinogenic effects in the colon by:
  - Mutagen binding
  - Inactivation of carcinogens or procarcinogens, or prevention of their formation
  - Modulation of metabolic activities of colonic microbes
  - Immune response

- Maintenance of mucosal integrity
- Antioxidative activities (110)

Effects such as lowering of the serum cholesterol level are not fully substantiated yet by placebo-controlled, double-blind, randomized clinical trials. On the other hand, strain-specific effects of probiotic lactic cultures on the human immune system and on diarrhea are well documented, e.g., for counteracting rotavirus or antibiotic-associated diarrhea, by application of strains such as the LGG strain of *L. rhamnosus* and the Shirota strain of *L. casei* (*L. paracasei*) (48, 111, 112). Therapeutic use is also considered successful in cases of lactose intolerance, irritable bowel syndrome, colon cancer, and *Helicobacter pylori* infection (109). Complex underlying mechanisms, such as adhesive and immunomodulating properties of effective strains, are major challenges remaining to be solved by intensified research (36, 80).

Apparently, adhering probiotic strains may transiently colonize the GIT, and thereby cause an increase in IgA levels (113, 114), resulting in the enhancement of serum IgA response to pathogens such as attentuated *Sal-*

monella typhi Ty21a (115). Moreover, many probiotic effects are mediated through immune regulation and especially through balance control of proinflammatory and anti-inflammatory cytokines, thereby suggesting the use of probiotics as innovative tools to alleviate intestinal inflammation, normalize gut mucosal dysfunction, and down-regulate hypersensitivity (116). In the ideal situation, immune stimulation by probiotic strains would be based on transient or longer-term colonization through adhesion and aggregation without invasion (115).

1.4 Prebiotics

Prebiotics are defined as nondigestible but fermentable food ingredients that beneficially affect the host by selectively stimulating the growth and/or activitiy of one or a limited number of bacteria in the colon, and thus improve host health (116). The major prebiotics are resistant dietary carbohydrates, but noncarbohydrates are not excluded from this definition. In theory, Hartemink (118) states that "…any antibiotic that would reduce the number of potentially harmful bacteria and favour health-promoting bacteria or activities, can be considered as a prebiotic". Although these definitions do not highlight any specific bacterial group as such, prebiotics are considered to stimulate selectively bacterial groups such as bifidobacteria, lactobacilli, and eubacteria resident in the colon. These are considered particularly beneficial for the human host. Resistant short-chain carbohydrates (SCCs) are also referred to as nondigestible oligosaccharides (119) or low-digestible carbohydrates (LDCs) (119). These SCC or LDCs provide interesting possibilities for inclusion into conventional food products for their "bifidogenic" effects (36, 118). Several such "candidate prebiotics" are currently under consideration by the industry for human consumption (120). Inulin and fructo-oligosaccharides (FOSs) are considered as typical "bifidogenic factors" and are probably the most commonly used prebiotics in the market (121–123). In addition, Bouhnik et al. (124) have shown that ingestion of 10 g of lactulose per day increases fecal bifidobacterial counts. Other promising prebiotic oligosaccharides under consideration are galacto-oligosacccharides, isomalto-oligosaccharides, soybean oligosaccharides, lactosucrose, and xylo-oligosaccharides (125). A quantitative tool has been developed by Palframan et al. (126) for the comparison of the prebiotic effect of dietary oligosaccharides; the quantitatiove probiotic index (PI) equation may find application in quantifying prebiotic effects *in vitro*.

In some cases, prebiotics such as FOSs are added to probiotic yogurts, the combination of which would thereby result in a "synbiotic" (36). These substances should ideally be well tolerated in the GIT and also reach the cecum where they will be availabe to benefit bacterial groups such as the bifidobacteria and some lactobacilli and eubacteria for fermentation. In some

instances, however, dose-related undesirable effects, due to osmotic potential and/or excessive fermentation, may occur, e.g., excessive flatus, bloating, abdominal cramps, and even diarrhea. Although dose-related intolerance symptoms may occur after ingestion of LDCs, the dose of intolerance generally appears to be high, thereby allowing a relatively broad "therapeutic window," i.e., the dose above the minimal effective level (36, 125, 127). Although it is generally established that bifidobacterial numbers increase in the feces of humans upon ingestion of FOSs (123), the average increase is considered small, whereas the "biological significance" for the human population and in specific disease situations appears not to be fully clarified (43, 128, 129). When compared to the observations of Bouhnik et al. (124) with lactulose, it appears that also the type and quantity of probiotic ingested might be decisive. Another factor may, however, be the underestimation of the bifidobacterial population by selective plating techniques, with recovery rates ranging from 17 to 58% (depending on the species), as compared to 85% by culture-independent methods (130).

Still, general agreement seems to exist on a number of beneficial effects of prebiotics, which point to the favorable influence on the small bowel by improved sugar digestion and absorption, glucose and lipid metabolism, and protection against known risk factors of cardiovascular disease. In the actual "target region," the colon, the fermentative production of SCFAs is in fact considered a major beneficial feature related to the primary prevention of colorectal cancer (131). Other confirmed effects from prebiotics are related to the low energy value (<9 kJ/g) resulting from their nondigestibility, to an increase in stool volume, to the modulation of the colonic flora by stimulation of beneficial bacteria (*Bifidobacterium, Lactobacillus*, and *Eubacterium* spp.), inhibition of "undesirable" bacteria (*Clostridium* and *Bacteroides*), and colonization resistance against *Clostridium difficile* (132). Some of the postulated effects that have not been finally confirmed refer to the prevention of intestinal infections, the modulation of the immune response, the prevention of colorectal cancer, reduction of the serum cholesterol level, and to improved bioavailability (36). In spite of strong indications on the positive role of LDCs in the maintenance of the human GIT, this issue is not fully clarified and deserves further attention (131).

By definition, a synbiotic refers to a product in which a probiotic and a prebiotic are combined. The postulated synbiotic effect may involve two different "target regions" of the GIT, comprising both the small and the large intestines. Moreover, the growth of a probiotic strain that is able to utilize a prebiotic will be selectively stimulated in the gut. This combination of pre- and probiotics in a single product has been shown to confer benefits beyond those of either on its own. Convincing data showed, e.g., an enhanced reduction in the number of colonic aberrant crypt foci (ACF) (133) and for colon carcinogenesis in rats (134). Also, antibiotic-associated diarrhea could be prevented by the combined application of *Lactobacillus sporogenes* (syn: probably *Bacillus Coaguleus)* and fructo-oligosaccharides in children (135). On the

other hand, synbiotic therapy did not result in any improvement of gut barrier function in elective surgical patients (136).

1.5 Conclusions

The establishment of scientifically confirmed evidence of "functional" effects related to pre-, pro-, and synbiotics presents a tremendous challenge to interdisciplinary scientific research. More than a century has passed since the early scientific observations and careful reporting, with increased research efforts and continuous development of new and improved hypotheses in the last two decades. The important role of the intestinal flora in the maintenance of health and in the prevention of disease is well recognized and acknowledged. Disturbance of the delicate balance of the gut microbial ecosystem may lead to dysbiosis and other disorders and thus facilitate establishing a state of disease (39). Particular interest is increasingly focusing on the continuous interaction or "communication" of the gut microbiota with the environment, the central nervous system, the endocrine system, the immune system, and the complex underlying mechanisms (43, 137–140). Based on *in vivo* and *in vitro* studies, Freitas et al. (141) suggest that both the established intestinal bacteria and probiotic strains are able to modulate host–pathogen interactions in the gut. It appears that species-specific modulations of intestinal cell glycosylation may represent a simple, general, and efficient mechanism to adapt the host defense toward pathogens. The strong focus of recent research efforts and observations on the role of the gut microflora and probiotics (functional strains) in immunomodulation is extensively addressed by Fuller and Perdigón (142) and a number of experts in a book on this topic.

Even when Tannock (43) does not see much progress on the "understanding of how probiotics work," he admits that pre- and probiotics have stimulated and generated new interest by the medical profession in the gut microbiota. It is envisaged that probiotic and prebiotic products of the future may be targeted for use in the prevention or alleviation of symptoms of specific diseases, provided that "abnormal microfloras" can be recognized and the safety of probiotics be guaranteed also to immunologically dysfunctional persons (43, 143) and at the same time effect the modulation of the immune response of the immunodeficient host (144).

Another major challenge concerns the development and validation of *in vivo* and *in vitro* test models. Significant progress has been made in recent years in conducting placebo-controlled, double-blind clinical studies, for which important functional effects could be verified. Yet, both for pre- and probiotics, a number of postulated effects still need to be confirmed (36, 39, 43). For prebiotics, studies may particularly be directed toward their influence on blood serum cholesterol values, the role of some dominant but hardly studied

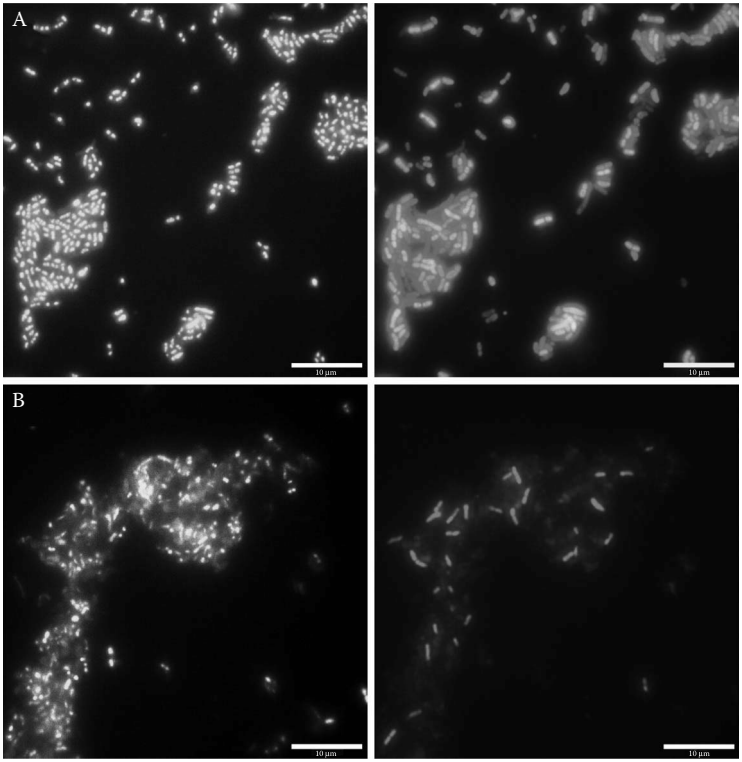

FIGURE 2.4
Microscopic photographs showing fluorescence conferred by DAPI-stained (left panel) and probe-labeled cells (right panel). In (A), cells of a pure culture of probiotic *Bifidobacterium lactis* 420 were hybridized with a specific 16S rRNA-targeting CY3-labeled probe using two helper oligonucleotides. Note that virtually all cells confer both DAPI and CY3 fluorescence. In (B), a fecal sample of a healthy subject was hybridized using the probe Bif164, being complementary to the primer Bif164 published previously (83). The scale bars represent 10 µm.

relationships among microorganisms or with gastrointestinal cell lines that are not accessible using conventional cultivation-dependent techniques. The high degrees of conservation of the ribosomal RNAs usually limit the taxonomic resolution of FISH to the species or subspecies level.

2.3.2 Animal Trials

Animal trials can be very helpful in identifying potential health benefits of pre- and probiotics in a safer, faster, and cheaper way than with human trials. In addition, they offer an opportunity to make investigations that would not be acceptable in human studies (e.g., *Salmonella* challenge studies). Apart from all the advantages of animal trials, the results cannot always be directly transferred to humans. The safety of new probiotic strains can be tested *in vitro* or in animal studies. BALB/c mice may be

used in order to study adverse effects, oral toxicity, and translocation potential of new probiotic strains (113–115). Challenge experiments with, for example, *Salmonella* have also been performed in BALB/c mice. In these studies, mice were fed with probiotic products or placebo and received a single oral dose of *Salmonella*. Differences in survival and immunological parameters between probiotic- and placebo-fed mice can be observed (116-118). In general, most health benefits of probiotics that can be observed in humans can also be investigated in animals, e.g., stimulation of the immune system in mice (119) and protection against rotavirus diarrhea in suckling rats (120). In some studies, germ-free animals, monoassociated or human flora-associated animals are used (121-123). Furthermore, there are animal models available for several human diseases that allow studying the effect of pre- and probiotics on these diseases. The transferability of the findings on human patients is a critical point also for these model systems. Ovariectomized rats serve as an osteoporosis model for investigations on calcium absorption and bone density (124). Interleukin-10 gene-deficient mice develop a Crohn's disease-like chronic colitis; hence, they are used as a model for IBD (125). Congenitally immunodeficient mice may help to assess the safety of probiotics for immunodeficient humans including neonates (126). In addition to several animal cancer models for different types of cancer exist (127–129), other examples are rat and mouse models for hypertension (130), allergies (131), and arthritis (132).

2.3.3 Human Trials

Human trials (clinical or dietary intervention studies) are essential for proving health benefits of probiotic strains. Different designs have been applied such as pre- and postintervention designs and placebo-controlled designs, parallel and crossover designs, and case-control studies. The best evidence is probably coming from double-blind, randomized, placebo-controlled trials. In addition to the study design, the methods applied for analysis of various parameters are also of great importance. Epidemiologic evidence relating probiotics or probiotic-containing foods and disease incidence would be valuable but is hardly available. However, these studies would be difficult to control as fundamental parameters such as specific strain and dose would be unknown for most probiotic-containing food products (133). In human studies certain markers are usually applied in order to observe the effect of pre- and probiotics or functional foods in general on the human body. Markers can be classified into three categories: [a] markers that relate to the exposure to the food component under study, [b] markers that relate to the target function or biological response, and [c] markers that relate to an appropriate intermediate endpoint (134). It was suggested that markers of type [b] might lead to enhanced function claims, whereas markers of type [c] might allow reduced risk of disease claims (134). In the case of probiotics, the detection of a certain probiotic strain

in feces could be classified as a type [a] marker, an increase in natural killer cell activity and phagocytosis as type [b] (enhanced functioning of the immune system), and a reduced incidence of respiratory symptoms (e.g., cough, sore throat, runny nose) as type [c] (reduced risk of respiratory tract infections).

2.4 Health Benefits of Probiotics

2.4.1 Health Benefits of Probiotics Established in Human Studies

Originally, probiotics were thought to balance disturbances of the gut microflora and thereby prevent or correct gastrointestinal-related dysfunctions. However, some health benefits, e.g., immune modulation, may be achieved even with dead bacteria (135). Many health benefits of probiotic bacteria have been shown in human studies. However, the mechanism of action behind most of these effects remains to be ascertained.

2.4.1.1 Diarrhea

The most common cause of acute diarrhea in childhood is rotavirus (5). Several probiotic strains — especially *Lactobacillus rhamnosus* GG — have been shown to prevent or alleviate infantile diarrhea (5, 136–138). It is also well-established that some probiotic strains can both prevent and shorten antibiotic-associated disorders (137–139). However, the evidence for the effects of probiotics on traveler's diarrhea remains low, because few studies have been conducted and they showed contradictory results (137, 138).

2.4.1.2 Stimulation of the Immune System

Many human studies have been performed to investigate the effects of probiotic cultures on the immune system. Some studies focused on the intestinal immune system, others on the systemic immunity. These studies reveal that probiotic bacteria are able to enhance both innate and acquired immunity by increasing natural killer cell activity and phagocytosis, changing cytokine profiles, and increasing levels of immunoglobulins (140–142). Two probiotic strains have been developed with a particular focus on their enhancing effects on immune responses: HOWARU™ Bifido (*Bifidobacterium lactis* HN019) and HOWARU™ Rhamnosus (*Lactobacillus rhamnosus* HN001) (113). Both strains have been demonstrated in several studies to enhance natural immune function in healthy people (143–149).

2.4.1.3 Inflammatory Bowel Disease

There is growing evidence that probiotics have a potential therapeutic benefit for patients suffering from IBD. Controlled clinical studies have shown that probiotics are efficacious in the maintenance of remission of pouchitis, prophylaxis of pouchitis after the formation of an ileoanal reservoir, maintenance of remission of ulcerative colitis, and treatment of Crohn's disease. The probiotics that have been used in these controlled clinical trials are two single strains (*Escherichia coli* Nissle 1917; *Saccharomyces boulardii*) and a product called VSL#3 that consists of a mixture of four strains of lactobacilli, three strains of bifidobacteria, and one strain of *Streptococcus salivarius* subsp. *thermophilus*, as has been reviewed lately by Hart et al. and Marteau et al. (11, 138).

2.4.1.4 Irritable Bowel Syndrome

The level of evidence that probiotics may alleviate the symptoms of subjects with IBS is low to date. Varying results have been obtained in the trials that have been conducted so far. Most trials have concentrated on the reduction or cure of symptoms. However, it is possible that the future role of probiotics may be in prevention rather than cure of IBS (16, 138).

2.4.1.5 Lactose Intolerance

Bacterial cultures — yogurt starter cultures as well as some probiotic cultures — are known to improve the lactose digestion in lactose maldigestors. The concentration of the lactose-cleaving enzyme β-galactosidase is too low in subjects suffering from lactose intolerance. Bacteria in fermented or unfermented food products release their β-galactosidase in the small intestine, where it supports lactose digestion. However, probiotic bacteria seem to promote lactose digestion in the small intestine less efficiently than do conventional yogurt cultures, but they may alleviate clinical symptoms arising from the undigested lactose (133, 138, 150).

2.4.1.6 Allergies

Pelto et al. (151) found that *Lactobacillus rhamnosus* GG confers an immunostimulatory effect in healthy adults, whereas the same strain downregulates the immunoinflammatory response in milk-hypersensitive subjects when challenged with milk. Moreover, probiotics have been applied successfully in the management of atopic eczema in infants (152). Furthermore, *Lactobacillus rhamnosus* GG was shown to be effective in the prevention of early atopic disease in children at high risk. The *Lactobacillus rhamnosus* GG product was given prenatally to mothers and postnatally for 6 months to the mothers or to their infants directly. The frequency of atopic eczema in the probiotic group was half that of the placebo group at the age of 2 years. The preventive effect was reconfirmed at the age of 4 years (153, 154).

2.4.1.7 Cancer

A few epidemiological studies indicate an association between a lower incidence of colorectal cancer and consumption of fermented dairy products containing lactobacilli or bifidobacteria. However, there is no direct experimental evidence that probiotics reduce the risk of colon cancer in man, but there is some indirect evidence based on several markers applied in human studies (e.g., fecal enzyme activities, fecal mutagenicity and genotoxicity, immunological markers) (5, 17, 140). The effect of *Lactobacillus casei* strain Shirota on recurrence of superficial bladder cancer was studied by Aso et al. (155, 156). The 50% recurrence-free intervals after tumor resection were significantly higher (1.8 times) for the probiotic group compared with the placebo group. A case-control study conducted in Japan with 180 cases and 445 controls revealed that habitual intake of lactic acid bacteria reduces the risk of bladder cancer (157).

2.4.1.8 Respiratory Tract Infections

The evidence for a potential positive effect of probiotic bacteria on respiratory tract infections has been hitherto very low (158). A probiotic yogurt drink containing *Lactobacillus rhamnosus* GG, *Bifidobacterium* species 420, and *Lactobacillus acidophilus* 145 was shown to reduce significantly the occurrence of potentially pathogenic bacteria in the nose compared with a control yogurt (159). Hatakka et al. (160) conducted a long-term study with 571 Finnish children attending day care centers. They found a slight reduction in the incidence of respiratory infections and antibiotic treatments after 7 weeks' consumption of a milk containing *Lactobacillus rhamnosus* GG compared with a control milk.

2.4.1.9 Constipation

Some studies have been carried out on the effects of lactic acid bacteria on constipation and intestinal motility (161). A reduced severity of constipation and an improved bowel movement frequency and stool consistency have been observed in constipated but otherwise healthy people after consumption of a fermented milk drink containing *Lactobacillus casei* strain Shirota (162). Administration of *Bifidobacterium longum* BB536 to constipated women resulted in a significantly increased defecation frequency and stool softness (163). A positive influence of *Bifidobacterium longum* BB536 on the "regularity" was also reported for elderly people (21).

2.4.1.10 Urogenital Tract Infections

Apart from the intestine, the urogenital tract is a promising field of application for probiotic bacteria. A case-control study with 139 females with acute urinary tract infection and 185 controls revealed that consumption of fermented milk products containing probiotic bacteria was associated with a decreased risk

of recurrence of urinary tract infection (164). To date there is just a small number of human studies showing positive effects of probiotics in urinary tract infections (158). Nevertheless, these studies suggest that probiotic preparations given orally or intravaginally may provide a therapeutic source of lactobacilli to help control urogenital infections in women (133, 165).

2.4.1.11 Helicobacter pylori *Infection*

Colonization of the stomach mucosa with *Helicobacter pylori* has been associated with gastritis, stomach carcinoma, gastric ulcer, and lymphomas. Several probiotic strains have been shown to inhibit *Helicobacter pylori in vitro*. Human studies confirmed this inhibitory effect on *Helicobacter pylori*, which seems to be independent of the viability of the bacteria (137, 138, 140, 158).

2.4.1.12 High Cholesterol

Many human studies have evaluated the effects of culture-containing dairy products or probiotic bacteria on cholesterol levels with equivocal results (140). Some examples are given below. A fermented milk containing *Enterococcus faecium* and *Streptococcus thermophilus* was reported to produce a small but significant decrease in total and LDL-cholesterol in patients with primary hypercholesterolemia. However, some subjects did not respond to the product and even showed a cholesterol increment (166). Richelsen et al. (167) investigated the effect of a long-term (6 months) consumption of the same fermented milk product. In normocholesterolemic subjects, the fermented milk resulted in a rapid reduction of LDL-cholesterol, but after 6 months the effect was similar to the placebo milk. In another long-term study (6 months), a yogurt containing *Lactobacillus acidophilus* 145, *Bifidobacterium longum* 913, and 1% oligofructose did not have a significant effect on total cholesterol and LDL-cholestrol in normo- and hypercholesterolemic women. But as the HDL-cholesterol concentration increased significantly, the ratio of LDL to HDL cholesterol decreased significantly (168).

2.5 Technology of Probiotics

2.5.1 Application of Probiotic Cultures in Food Products

Probiotic bacteria are applied in many different products worldwide. In addition to food products, probiotic cultures are also used in pharmaceuticals and animal feed. Most definitions of probiotics are based on live bacteria that confer a health benefit for the consumer. Thus, it is considered as important that probiotic products contain an effective dose of living cells during

their whole shelf life. However, for some health benefits, viability of the microorganisms does not seem to be essential. Nonviable bacteria are, for example, applied in some pharmaceuticals and food supplements. The selective enumeration of probiotic species in a fermented product is sometimes impossible due to the product's background flora. Also, the choice of media may have a major impact on the viable cell counts.

2.5.1.1 Dairy Products

Probiotic bacteria have been applied in fermented dairy products for many years. In some cases fermented milk products are monocultures of probiotic bacteria, but usually support cultures are applied to speed up the acidification process and provide the desired texture and flavor. Many lactobacilli and bifidobacteria survive in fermented milk products for 4 to 8 weeks. There are several parameters that may influence the growth and survival of the probiotics, e.g., the starter culture, fermentation temperature, pH, sugar content, presence of oxygen, packaging material, fruit preparations, and other ingredients. Therefore, the survival of a probiotic culture should be reconfirmed in the final product formulation. Probiotics may also be applied to unfermented milk products such as milk-based sweet or acidified drinks and ice cream.

2.5.1.2 Other Food Products

The applicability of probiotics in food products depends in general on factors like water activity, processing and storage temperature, shelf life, oxygen content, pH, mechanical stress, salt content, and content of other harmful or essential ingredients. For many products, excess water activity is a critical parameter that increases the death rate of bacteria. Products with an unfavorable water activity are, for example, cereals, chocolate, marmalade, honey, and toffees. These products are too "dry" for applying live bacteria and too "wet" for the application of freeze-dried bacteria. Freeze-dried bacteria could be applied in these products if the bacteria could be protected from moisture, as small amounts of moisture can be very detrimental to the dried culture. In addition to dairy products, fruit juices have been shown to be suitable carriers for probiotics. The limiting factor for many of the probiotic strains is the low pH of the juices. There is growing interest in applying probiotics to fermented meat products. Lactic acid bacteria have been used for the fermentation of meat products for many years, and today some strains are also utilized as protective cultures. Probiotics might be an instrument to change the perception of meat products toward a healthier image. This might, however, also be a hurdle in the marketing of probiotic sausages. Freeze-dried probiotic bacteria are applied to infant nutrition powders and powdered milk drinks. In these products, the water activity is very low, which is essential for the stability of freeze-dried bacteria.

2.5.1.3 Food Supplements and Over-the-Counter Products

Most probiotic food supplements and over-the-counter (OTC) products are available as powders, tablets, and capsules. As these products also contain dried bacteria, the water activity in the final product must be very low. Another critical parameter for tablets is the pressure applied in tableting and the heat that is produced. An enteric coating can be applied on tablets and capsules in order to protect the bacteria from the acidic environment in the stomach and improve their survival rate.

2.6 Conclusions

A very short conclusion to this chapter could be that probiotic food ingredients must be safe and have a well-documented functionality. When taken to the extreme, this statement describes the requirements set for all food ingredients. What makes probiotics different from most other ingredients is that the functionality — improving the health of the consumer — is not seen in the food product itself, but after consumption. The documentation of such functionality requires a substantial amount of science.

During the next few years we will see new probiotic products being developed through the use of genomics. The use of genomics on microorganisms has the potential to reveal the complete metabolic potential of each of the bacterial strains. Several probiotic strains have already been completely sequenced (169, 170). The genome of *Bifidobacterium longum* revealed a large number of genes potentially coding for enzymes in the metabolism of prebiotic carbohydrates (169). This opens the possibility to construct new combinations of pre- and probiotics. Also, the application of human genomics is likely to show a large impact on the innovation in this area. With the availability of the sequence of the entire human genome (171, 172), it is now possible to design DNA chips for the analysis of the regulation of particular genes or even to analyze simultaneously the regulation of all human genes. The combined use of genomics on the microorganisms as well as the human host is likely to result in the design of probiotics with increased health-improving effects.

In addition, the safety aspect of probiotics is somewhat different from most other food ingredients. The production on an industrial scale of highly concentrated live bacterial cultures without undesirable contamination by harmful microorganisms requires competencies possessed only by the dedicated food culture manufacturers and a few scientifically based food companies. Legislation on probiotics and microbial food cultures in general differs among major markets. In the European Union, the regulatory requirements are going to be harmonized and therefore subject to change. Hopefully, Europe in this area will avoid the usual tendency to overregulate. Future

innovations in probiotic food ingredients will be determined by a balance between the rapidly expanding scientific achievements and the regulatory framework imposed on the area. The scientific achievements open new possibilities, but they also open our eyes for previously unknown risks. Regulatory measures are justifiable if a real safety problem is being discovered. If, however, the problem is still hypothetical, regulation will probably be harmful, as innovation in the area will be delayed by costly approval procedures. This regulatory issue is also relevant for other food ingredients in the area of food safety.

Danisco is a company producing a large range of food ingredients including, among others, products for food safety, cultures, probiotics, and prebiotics. The company is actively conducting research to support the development of innovative products for food safety and healthy food.

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