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ORIGINAL CONTRIBUTION

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Progress in the science of probiotics: from cellular microbiology and applied immunology to clinical nutrition

Summary Probiotic research is progressing rapidly with strong scientific-based observations. New molecular biologic techniques for the more accurate identification of intestinal microflora and seminal studies that have helped define the function of commensal bacteria in the gut have been reported recently. In functional terms, new techniques are operational to study the affect of microbial–host "crosstalk" between both bacteria and the host. Probiotics have been shown to initiate the activation of specific genes localized to these cells. Both the bacterial and host aspects of microbiota–host crosstalk can now be studied, in particular thanks to simplified *in vivo* gnotobiotic mouse models. Their functional genomic studies enable the screening for probiotic potential and for investigating the modulated expression of genes involved in a broad range of intestinal functions including regulation of nutrient uptake and metabolism, mucosal barrier and epithelial cell function, xenobiotic metabolism, and strengthening of the innate immune system. An important function of probiotics is its effect on the gut immune system. The latter may work by enhancing mucosal barrier function, preventing apoptosis of epithelial cells and ultimately, decreasing antigen uptake, especially in the small bowel. Clinically, there is strong evidence that some probiotics improve the digestibility of lactose and others prevent the recurrence of pouchitis after inflammatory bowel disease (IBD) surgery. There is reasonably strong evidence for the efficacy of probiotics in childhood infectious gastroenteritis and antibiotic-associated diarrhea. Recent data suggest the potential efficacy of probiotic strains in atopic eczema, IBD, *Helicobacter pylori* gastritis, neonatal necrotizing enterocolitis and as a substitute for inadequate initial neonatal colonization.

Key words acquired immunity – cellular microbiology – microbial-epithelial crosstalk – gastrointestinal tract – glycosylhydrolases – gut-associated lymphoid tissue – host defense – innate immunity – intestinal epithelium – lactic acid bacteria – microbiota – molecular microbiology – mucins – mucosal barrier – probiotics – toll-like receptors

Abbreviations: *Btheta*: *Bacteroides thetaioitamicron*; FISH: fluorescence *in situ* hybridization; HMA: human microbiota-associated; IBD: inflammatory bowel disease; IBS: irritable bowel syndrome; NEC: necrotizing enterocolitis; RCT: randomized controlled trial; RIVET: recombinase-based *in vivo* expression technology; TLR: toll-like receptor

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Introduction

This is an important year for the study of probiotics in terms of research, clinical use, and cultural and regulator constraints imposed by various societies. Probiotic use is no longer based on antidotal experience but has evidenced-based scientific and clinical studies to support its value in health and disease [1]. The 2004 Danone International Probiotics Convention constituted a documented review of these striking advances. The Convention focused on the following topics: recent international probiotic guidelines and definition, new methods of evaluating the survival and function of gut flora, host–microbiota crosstalk, probiotic interactions with the immune system, the latest data generated by clinical studies on probiotic use in various age groups including infancy, and a cultural comparison of the manner in which probiotics are perceived in Europe and the United States.

From probiotic research to guidelines

As evidenced by the substantial increase in the number of papers published in both scientific journals and the lay press, interest in probiotics is booming not only in the scientific community but also in the market place among consumers. Given the extensive interest, guidelines were considered necessary. In response, FAO/WHO convened two comprehensive meetings: (1) a joint FAO/WHO expert consultants group to evaluate the health and (2) nutritional properties of probiotics in food including powdered milk with live lactic acid bacteria. Two documents were generated as a result [2, 3]. The first evaluated the latest information relating to probiotics based on working papers devoted to microbiology (L. Morelli, Piacenza, Italy), the regulatory and clinical aspects of dairy probiotics (G. Reid, London, Ontario, Canada) and technological and commercial aspects (S. Gilliland, Stillwater, Oklahoma, USA) [2]. The second document outlined general guidelines for the assessment of probiotics [3].

FAO/WHO define probiotics as "live microorganisms which, when consumed in adequate amounts as part of food, confer a health benefit on the host." It is noteworthy that no specific action of intestinal microflora is emphasized since the collective beneficial effects are comprehensive and long standing. In addition, both the FAO/WHO documents link probiotics to food and to food only, thus excluding any reference to the term "biotherapeutic agents." These reports underscore the need for a multidisciplinary approach: (a) In terms of taxonomy, the consultants recommended that probiotics be named in accordance with the International Code of Nomenclature and strongly urged that, for the sake of full disclosure, probiotic strains be filed with an internationally recognized culture registry. This is already required for patent applications and, in Europe, for probiotic use in animal feeds, but not human food. (b) With regard to health benefit, definition and measurement, the consultants recommended that each product should be labeled with the minimum daily amount required in order to confer specific health benefit(s). The methods of demonstrating health benefit(s) are to be via a randomized double-blind, well-controlled design and the studies are to be conducted with numbers of human subjects sufficient to enable statistical significance to be demonstrated. (c) With respect to probiotic evaluation for food use, the strain has to be identified and functionally characterized on the basis of *in vitro* and animal studies. Safety is to be assessed for new strains on the basis of *in vitro* and/or animal studies and phase 1 human studies and at least one double-blind placebo-controlled phase 2 clinical trial, preferably confirmed by a second trial. Phase 3 efficacy trials comparing probiotics with the standard treatment modality for a specific condition are to be designed to demonstrate their biotherapeutic effect. This approach is not necessary, however, to characterize a probiotic food. (d) With regard to labeling and regulatory issues, the consumer is to be provided with correct and relevant information including the genus, species and strain, the minimum number of bacteria to be used, the viable concentration of each probiotic present at the end of the shelf life, the storage conditions, etc.

The FAO/WHO expert panel agreed that the scientific evidence is sufficient to indicate that health benefits may be derived from consuming food probiotics, but more research is needed for many probiotics in order to confirm their actual health benefit in man. The research is to be conducted using a systematic approach and in accordance with recommended assessment guidelines.

Probiotics and functional food

Functional foods represent a new area of interest in the field of alternative/complementary medicine. The first randomized nutritional clinical trial ever reported in English was conducted in 1747. James Lind assessed the effects of lime (which at that time was neither a food nor a drug but a sailors' nemesis) on the crew of a Royal Navy ship, e.g. a large study population [4]. Lind randomized the crew located on the port side of the boat to receive lime with their diet

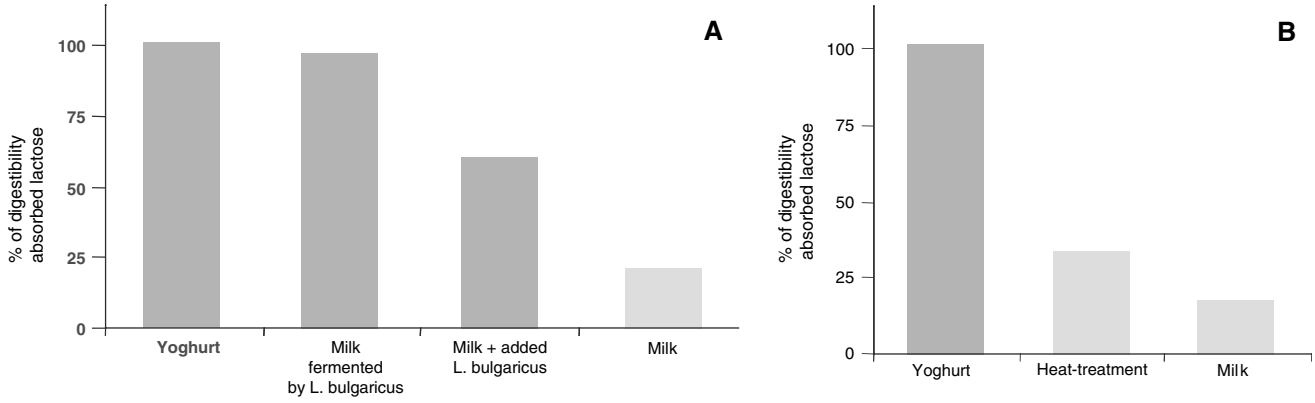

Fig. 1 Yogurt lactose digestibility. **A**. Comparison of yogurt and *L. bulgaricus* with or without fermentation. **B**. Deleterious effect of heat treatment on yogurt induced lactose digestibility. From Martini et al. [6]

and the crew on the starboard side to continue receiving the standard diet. As one would expect, the port crew fared better than the starboard crew, who developed scurvy that in some cases proved fatal. However, it took the Royal Navy a 100 years to establish the use of lime as mandatory on its ships. Of course, lime contains vitamin C, but it provides additional health active benefits other than vitamin C alone since it also contains bioactive phytochemicals whose effects still need to be investigated. Lime may have been the first functional food whose benefits were demonstrated by a nutritional clinical trial.

What is a functional food? In Europe, it is a food and not a dietary supplement and, as such, it needs to be palatable and balanced, e.g., in addition to its intrinsic nutritive value it should beneficially affect a target body function, improve health and well being and/or reduce risk(s) of disease. The criteria for definition and a means of assessing health benefits for functional foods were suggested by a report from a European commission in 2004 [5] The "Quality of life and management of living resources program" was coordinated by the European branch of the International Life Science Institute. On the basis of their criteria, there is strong evidence that foods containing certain probiotics, e.g. milk fermented with *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, which, while tasty and balanced, has been shown to significantly improve lactose digestibility (Fig. 1), and thus to fulfill the definition of a functional food.

Gut microbiota: new methods of evaluating flora survival and functions

The structural and functional diversity of bacteria is a key characteristic of gut microbiota. While the uterus provides a sterile environment for delivering a newborn, the neonate is rapidly and extensively colonized after birth in its passage through the birth canal. Human microbiota rapidly rises to 10^{11} bacteria per gram of large-intestinal content and, in adults, reaches 10^{14} microorganisms, ten times more organisms than the number of eukaryotic cells in the body [7]. The human digestive tract, in particular the colon, harbors a large portion of bacteria that are visible under the microscope and yet cannot be cultured. In recent publications [8–10] the fraction that can be cultured has been estimated to be in the order of 30% of total microorganisms present. The proportion of those identifiable bacteria rises sharply throughout life from 0% in preterm infants to 30% in very young children and 80% in adults and 87% in the elderly [8].

Identification of intestinal bacteria

We need to study microbial diversity and improve our understanding of the role of intestinal microbiota in gut function in order to elucidate the influence of exogenous factors, (e.g., diet including pre- and probiotics, lifestyle, environment) and endogenous (e.g., innate and acquired immunity, host physiology, genetic background) factors on the composition and activity of these microbiota. In turn, those factors strongly influence host functions, particularly stimulation of active substances and gene expression. These influences can be studied by elucidating the diversity of the gut microbiota by investigating the effects of their key functional activities on the host including *in situ* activation or inactivation of certain substances, by identifying functional groups of bacteria and by correlating bacterial activity with identity.

In order to enhance our understanding of bacterial diversity, we needed culture-independent methods of analysis, namely molecular tools [11, 12] that are based on identifying unique genome sequences

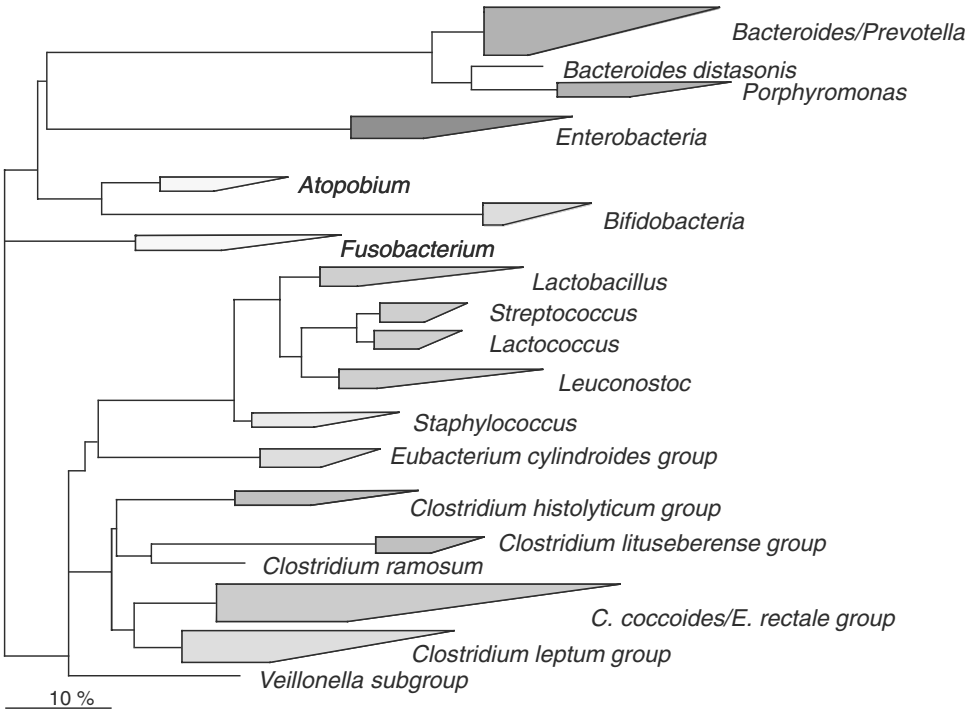

Fig. 2 Phylogenetic tree of the dominant fecal microflora of a healthy human adult derived from partial sequence data, using 16S rRNA targeted probes. Horizontal bar represents 10% sequence divergence

(Fig. 2). The identification of gut microbiota requires establishing a comprehensive database of 16S rRNA sequences for human gut microbiota and, when this has been done, designing 16S rRNA-targeted diagnostic oligonucleotide probes for accurate identification.

Over the last few years, whole cell fluorescence *in situ* hybridization (FISH) and dot-blot hybridization probing methods have been continuously improved and are now being used increasingly to identify human gut microbiota. *In-situ* hybridization coupled with flow cytometry shows that there are only a few, highly prevalent dominant phylogenetic groups in the gut microbiota [13–15] (Fig. 3). A pan-European study of 91 healthy adults from five countries has shown that three major groups are numerically significant, with no significant differences between the countries studied [16]. These include: the *Bacteroides* group, and among Gram+ organisms, *the Clostridium leptum* group [17] and the *Clostridium coccoides/Eubacterium rectale* group [12, 17, 18]. There are several other groups, including *Bifidobacterium*, *Atopobium*, *Lactobacillus* and relatives that are quantitatively less prevalent but found in many individuals. Thus, six dominant and prevalent phylogenetic groups have been identified with an increasingly large panel of probes. On average, however, 25% of the bacteria that are detected with universal probes are still not identified. These data do not apply to infants, in which *Bifidobacteria* predominate together with some *Bacteroides* if the baby is breast fed [7, 19], nor probably to older and very elderly people [20].

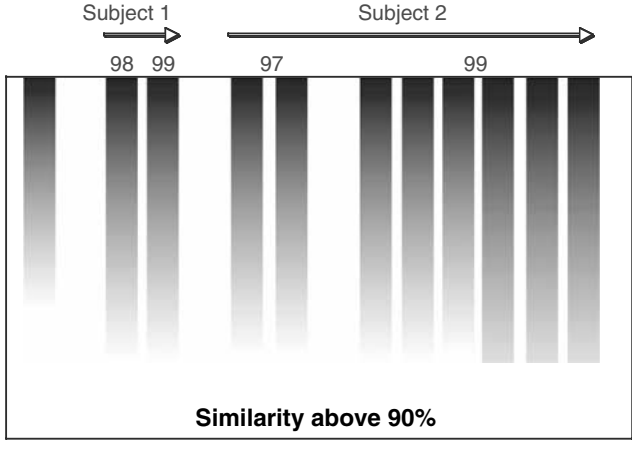

Fig. 3 Temporal temperature gradient gel electrophoresis of 16S rDNA amplicons of fecal samples from two subjects, studied at 1- and 2-year intervals. The dominant species diversity of human gut microbiota remains stable for years in a given adult subject

With regard to the dominant species, each human fecal microbiota appears unique, e.g., specific to the individual. Indeed, dominant species diversity is: (a) variable from one individual to the next as shown in a pioneer study in 1998 [21], but appears stable over time in a given subject on his/her usual diet [22] (Fig. 3); (b) resistant, in terms of classical ecology; (c) resilient, e.g. it is able to recover its original pattern within 30–60 days of exposure to stress such as

antibiotic treatment (de la Cochetière and Doré, unpublished observations, 2004); and (d) not markedly altered by probiotics [9, 23). The lack of disruption in the consortium of dominant species, most frequently observed in feeding trials, should be considered a positive outcome.

Functional studies of gut microbiota

Following ingestion, probiotics traverse the gut in a strain-specific, and probably a food matrix-dependent manner, as has been demonstrated by ileal recovery of *Lactobacillus casei* DN114 001 [24], *Bifidobacterium* sp12 [25], *B.* spp [26], *Bifidobacterium* animalis DN 173 010 [27], *L. plantarum* NCIMB 8826 [27], and, to a lesser extent, *Lactococcus lactis* MG [28], and by fecal recovery of *B.* sp [29] and *L. plantarum* NCIMB 8826 [28].

The passage of probiotics through the gut is not simply a passive act. As has been shown with human microbiota-associated (HMA) mice, a derivative of the *L. casei* DN114 001 strain survives transit at high population levels ($>10^9$ cfu/g content) and initiates transcriptional activity leading to protein synthesis for specific genes during transit. Transcriptional activity is initiated one and half hours post-ingestion [24].

Similar studies using intestinal fluid samples from intubated humans are in process using *L. casei* DN114 001 (J. Dore unpublished observations, 2004) and *L. plantarum* [30, 31]. In addition, a study initiated by Dutch investigators using fecal bacterial genes from healthy and diseased subjects have provided new insight into the metabolomics of human intestinal microbiota [32]. These investigators used DNA-dependent/culture-independent methods or large DNA fragments isolated and categorized from distal intestinal mucosa in conjunction with a bank of reference genes to make these observations.

The survival of ingested probiotics continues to be of importance in this context. However, since the functional contribution of probiotics to the ecosystem is related to its activity rather than simply its presence, assessment of *in vivo* adaptation of probiotics in the gut is an important focus. This should also extend to the overall influence of probiotics on the intestinal microbiota, in general, which may respond functionally to probiotics without actual changes in composition. The genome sequences of probiotic strains and gut commensals, together with recent developments in functional genomics of the intestinal ecosystem should provide new perspectives in this context.

Functional studies of gut microbiota have employed other methods, such as the activation or inactivation of bioactive compounds, as well as, *in situ* detection of microbial activity. One example of the activation/inactivation of bioactive compounds is equol, a bacterial induced metabolite (produced in about 30% of humans) of isoflavone daidzein, a soy constituent, with greater biological activity than the initial compound itself. Isoflavones have been suggested to have health-related effects in the prevention of hormone-induced malignant degeneration, atherosclerosis and osteoporosis, and in the alleviation of menopausal symptoms [33, 34]. Since microbial modification of food ingredients may influence their effectiveness, the organisms catalyzing alteration would be a good indicator of whether activity is present in a given subject. *In situ* detection of microbial activity includes detection of metabolic activities or that of mRNA. Examples are: (a) *In-situ* detection of catalytic activities using fluorogenic substrates is of considerable interest but still restricted to a relatively small number of enzymes (β-glucuronidase, β-galactosidase, β-glucosidase). Using flow cytometry technology with its cell sorting capabilities, Rigottier-Gois et al. have demonstrated the feasibility of enrichment of metabolically active labeled cells [15]. (b) *In situ* detection of mRNA, which is widely used in eukaryotic cells, requires that the cells be permeable to reverse transcriptase and RNA polymerase, that amplification products are not already existent in the cells and that mRNA be present in sufficient quantities, and its signal be detectable. This approach cannot be widely applied to bacteria simply because they are not sufficiently permeable to enzymes and because prokaryotes has a shortened half-life of mRNA and their mRNA's are much less stable than eukaryotes.

Of major concern with regard to any novel approach to the detection of relevant microbial activities is whether a differential expression of bacterial genes exists. Recently several seminal publications have reported that bacteria influence the expression of host genes in eukaryotic cells. In particular, bacteria influence genes involved in the regulation of nutrient uptake and metabolism, mucosal barrier function and epithelial cell activities [35–37]. However, despite these observations, very little is known about what actually happens to the gut when probiotics are ingested. Genomics and proteomics may be another helpful approach. In this context there are a number of important unanswered questions. For instance, does the consumption of certain food ingredients have an impact on the *in vivo* expression of bacterial genes? And how are the expressions of certain genes at various sites in the GI tract affected?

Two papers by Oozeer et al. have demonstrated the survival of and the capacity for protein synthesis initiation of a derivative of the *L. casei* DN114 001 strain during its transit through the GI tract of

gnotobiotic (HMA) mice [24, 38]. The physiologic adaptation of probiotics to the GI tract environment through modulation of promoter activities has also been demonstrated [39]. With a view to linking beneficial probiotic functions to new protein synthesis and obtaining specific information on probiotic adaptation, reverse transcriptase (RT-PCR) combined with other methods appears to be promising [39]. Recombinase-based *in vivo* expression technology (RIVET) [40, 41] is another technique which is now being applied to the study of gut microbiota. This very specific approach enables identification of unique, transiently expressed genes even after they have reverted to a basal activity state.

In addition to the development of new methods, two questions remain to be answered. (a) What is the relative contribution to the composition of gut microbiota of a specific host genotype compared to the influence of exogenous factors such as diet? The microbiota composition of twins show greater similarity than those of unrelated subjects or marital partners living together [42]. This would suggest that the composition of the microbiota has an important genetic component. However, the magnitude of that component has yet to be determined. (b) Is mucosa-associated flora more important to gut function than luminal flora, e.g., that usually obtained in fecal samples? This question is a central issue with respect to the crosstalk studied by immunologists and microbiologists. The importance of the luminal flora is illustrated by the striking spontaneous healing and protection from postoperative recurrence of Crohn's mucosal inflammation after diversion of the fecal stream. However, in addition to the need for a clear definition of what constitute mucosal bacteria (e.g. are they adherent bacteria?), it should be noted that fecal samples, while easily obtained, are probably not representative of the true composition of luminal microbiota at any site other than the rectal lumen and certainly not representative of mucosa-associated flora.

Host–microbiota crosstalk: application of probiotics

Communication, e.g., crosstalk, between microorganisms in the gut lumen and those attached to the mucosal surface and the host GI tract are diverse and complex. They include: competition/cooperation for nutrients; intra- and interspecies communication; direct contact between components of the bacterium, e.g., lipopolysaccharide, peptidoglygans, etc., and host cell surfaces, secretion of bacterial compounds that can interact with underlying intestinal epithelium and

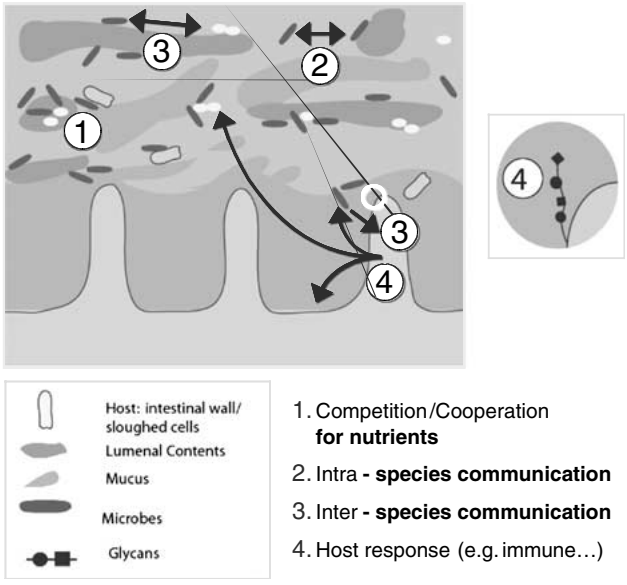

Fig. 4 Host–microbiota cross-talk: main subjects of the dialogue. Reprinted with permission from M. Lecuit and JL Sonnenburg

"modulins" which can directly effect host cell function and responses (e.g., immune response, glycosylation changes, etc.) (Fig. 4). To more completely understand this "crosstalk," studies that identify both the bacteria and host contribution require further investigation. For example, understanding the molecular basis for nutrient sharing among members of the normal gut microbiota is essential if we are to appreciate how the intestine's microbial community is established and maintained and how it may be modified by probiotics to the benefit of the host. In addressing this issue, an *in vivo* gnotobiotic mouse model, e.g. a microorganism-free state in the intestinal ecosystem has proven of particular value [43].

Using this animal model to define the specific role of gut flora in the development of gut functions, *Bacteroides thetaioitamicron* (*Btheta*) – strain VPI-5482, a dominant member of normal human distal intestinal microbiota originally isolated from the feces of a healthy adult, has been used as a model symbiont [37, 44–47] in the pioneering work of Midtvedt and Gordon et al. [47–49]. The organism is a readily cultured, Gram-negative, obligate anaerobe. It becomes a prominent member of human and murine microbiota during a critical phase (the suckling to weaning transitional period) of postnatal gut development. The bacterium can be genetically modified and its complete genome has been sequenced [50]. The 4779-member proteome of *Btheta* includes diverse molecules that function for example as an apparatus for

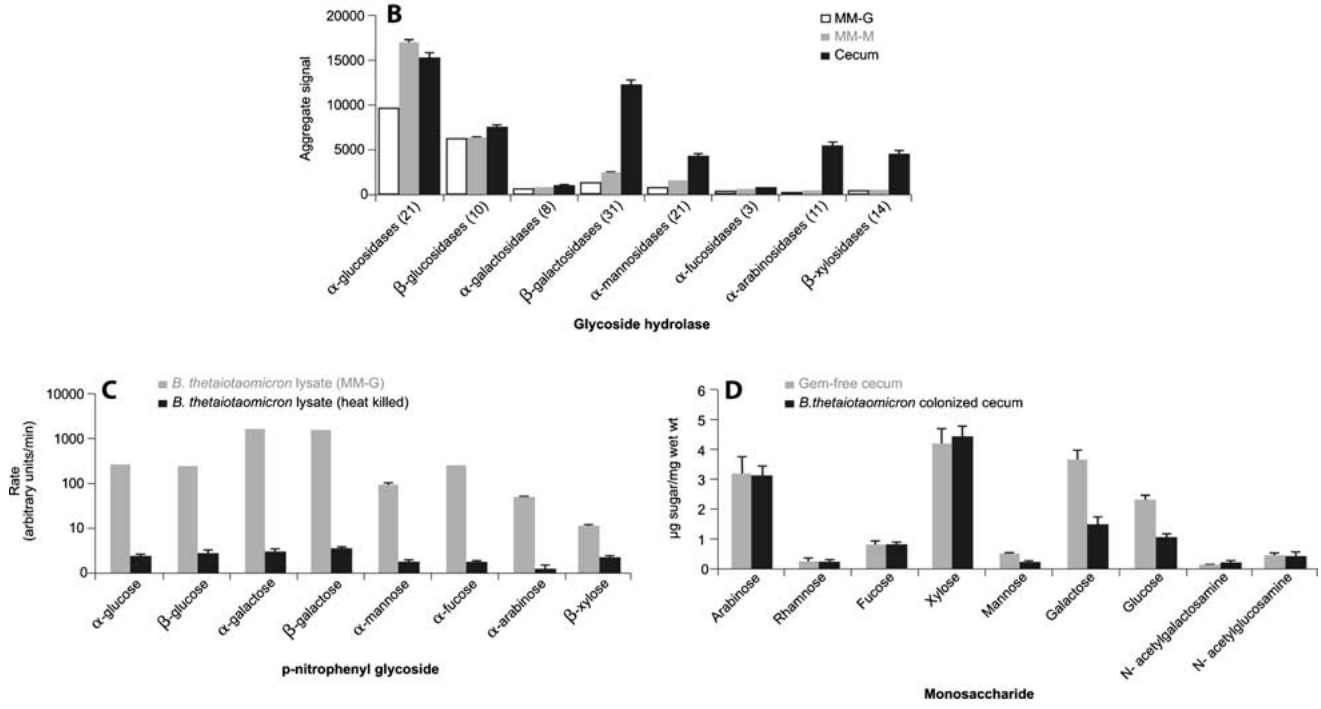

Fig. 5 Carbohydrate foraging by *Btheta* [50]. Reprinted partly, with permission from J.L Sonnenburg

acquiring and hydrolyzing dietary polysaccharides, an associated environment-sensing system consisting of a large repertoire of extra-cytoplasmic functions and one- or two-component signal transduction systems [50]. In a recent study Gordon et al. used DNA microarrays to identify whole genome *Btheta* transcription profiles from organisms harvested directly from the mouse cecum after a ten-day colonization period [44]. The authors showed that *Btheta* utilizes specific gut luminal polysaccharides via a specific uptake and degradation mechanism. GC-MS analysis of the standard mouse chow diet and of the total cecal contents harvested from microorganism-free and *Btheta*-associated animals has established the following: (a) The major responses *in vivo* occur in genes dedicated to carbohydrate transport and metabolism (glycobiome activation). (b) Expression of *Btheta* glycosylhydrolases mirrors the most abundant sugars in the environment. (c) *Btheta* preferentially consumes the subset of the monosaccharides available that can be metabolized with the greatest efficiency. (d) In control experiments, the repertoire of glycosylhydrolase genes induced in the cecum are specific to the glycan structures found in the host's mucus when mice are fed a custom chow diet containing glucose and sucrose as the only fermentable carbohydrates (JL Sonnenburg, J. Gordon et al. unpublished observations, 2004). And (e) finally, *Btheta* is not only able to degrade dietary plant polysaccharides [44, 50] but also host-derived polysaccharides (e.g. host mucus glycans) (JL Sonnenburg, J. Gordon et al. unpublished observations, 2004). Such flexible adaptation is known to strengthen complex food webs and is likely to promote stability in the microbial community. The *Btheta* genome encodes more than 200 glycosylhydrolases [50] (Fig. 5), many of which are secreted to allow for extracellular processing of those complex polysaccharides. Compared to *Btheta*, a gut commensal and probiotic, *Bifidobacterium longum*, has a more modest glycan-degradation capability but a more extensive repertoire of simple transporters [47]. These features suggest that *B. longum* may be a direct beneficiary of the products of *Btheta*'s polysaccharide degradation machinery. The approach used to examine *Btheta*'s adaptive behavior *in vivo* can also be extended to examine the effects of probiotics on members of the normal gut community such as *Btheta*.

This same technique has been used successfully to determine the role of microbial colonization on gut functions. Using gnotobiotic mice monocontaminated with *Btheta*, functional genomic studies have shown that this symbiont modulates the expression of genes involved in a broad range of important intestinal functions including nutrient absorption, angiogenesis, xenobiotic metabolism and strengthening of the

innate immune system [37, 45, 46]. In order to investigate the microbial determinants of beneficial versus pathogenic host–bacteria relationships, similar types of functional genomic studies were conducted on microorganism-free mice colonized by *Listeria monocytogenes*, or its non-pathogenic close relative, *L. innocua*. The results revealed that the host's response to *L. innocua* was very similar to that documented with *Btheta*. In contrast, *L. monocytogenes* elicited a complex sequence of host gene expressions that included NF-κB-dependent and IFN-responsive pathways. Of interest, a *L. monocytogenes* mutant for listeriolysin induced a host response that mimics that of *L. innocua* (M. Lecuit, J. Gordon et al. unpublished observations, 2004). These findings suggest that, in this case, the presence or absence of a single gene product which enables a microorganism to access the cytoplasmic compartments of host cells can profoundly influence the host–bacterial relationship.

Such studies underscore the value of the gnotobiotic animal model in identifying the genomic and cellular factors that regulate interactions between bacteria and their host within the intestinal ecosystem. Such a powerful approach enables a step-by-step identification of individual organisms contributing to intestinal host responses, including that of probiotics. This approach combines sophisticated tools such as laser capture micro-dissection to distinguish among individual cell types of those cells that are responsible for the host response and murine genetic techniques (e.g., knock-out and transgenic mice). Together with simplified *in vitro* (cell culture) systems enabling investigation of a highly controlled environment and screening for probiotic potential, the animal models hold the promise of providing a conceptual and experimental framework for exploring mechanisms of probiotic–host crosstalk in healthy and diseased humans. However, the transposition of these observations to humans and clinical relevance is yet a further step. Thus transposition will necessitate associating, within ethical limits, clinical trials and basic mechanistic studies including using organ cultures of human gut biopsies for microbiota exposure, repetition of studies already performed using animal model systems and the use of publicly available databanks in a "bedside to bench top" approach to elucidate the mechanisms of crosstalk.

Probiotic interactions with the immune system

Because the mucosal immune response of the gut is so important to host defense it is likely that probiotics will have an influence on this important gut function.

Immune function as a biological marker to assess health benefits of probiotics

Because recent evidence indicates that probiotics (e.g. *S. thermophilus* and *L. bulgaricus*) may influence both systemic and gut-associated immune responses [51], systemic and intestinal immune biomarkers have been suggested as the basis for assessing the nutrient requirements and/or health benefits of functional foods including probiotics. The number and function of circulating T-cells migrating from Peyer's patches to the intestinal mucosa (gut-homing T-cells) may constitute a surrogate immune marker for the effect of probiotics in healthy children and adults since those cells can be identified by their expression of integrin $\alpha4\beta7$. Similarly, mucosal IgA responses can be analyzed in blood and secretions, as T-cells also transit from organized lymphoid tissue to the mucosa via the circulation. *In vitro* intestinal mucosa organ cultures of intestinal biopsies have been used as a technique to determine the effects of exposure of normal and inflamed gut to probiotic bacteria. Measuring disease parameters directly or determining surrogate markers of immune modulation in healthy subjects will not only provide clues as to the mechanisms of probiotics on immune function but should also constitute a basis for making health claims.

Immunological effects of probiotics

Lactobacillus species markedly inhibit TNF-α production by normal and inflamed (e.g. Crohn's disease) mucosa. The mechanism has yet to be elucidated. Compelling *in vivo* data generated in children showing that feeding pregnant women and their newborns after birth with probiotic bacteria reduces by one half the incidence of allergic eczema [52, 53]. Unfortunately this clinical effect is not reflected by any of the laboratory markers associated with eczema (e.g., total IgE, specific IgE and skin prick reactivity) but there is evidence that probiotic bacteria can inhibit Th2 responses to house dust mites [54]. Further studies on atopy are ongoing in Finland. The public health value of using probiotics may be enhanced by the observed inverse relationship with the incidence, over the last 50 years, of prototypical infectious diseases and the incidence of autoimmune and allergic disorders including asthma, Crohn's disease, multiple sclerosis and type 1 diabetes mellitus [55]. This observation may help explain the positive effect of probiotics on atopic eczema prevention [53]. There are also studies to suggest the immunological effects of probiotics on the skin (e.g., *L. casei* has been shown to reduce experimental hapten-specific $CD8^+$ T-cell-mediated skin inflammation in the mouse) [56]. An association

between *L. casei* and the usual yogurt starter bacteria—*S. thermophilus* and *L. bulgaricus*—reduces the frequency of skin-homing CD4-CLA$^+$ (a skin-associated antigen) cells (T. T. MacDonald et al. unpublished observations, 2004).

Some probiotics enhance while others suppress immune responses. What mechanisms are involved? Recent preliminary data suggest that probiotics could act through stimulating regulatory T-cells which can activate both of these responses. This is a major area of investigation with respect to the immune enhancing function of probiotics.

Most of the immunobiological effects of probiotics are likely to take place in gut-associated lymphoid tissue, including Peyer's patches, in the small intestine. At that site, due to less difference between the number of probiotic and resident bacteria, probiotic bacteria may compete with luminal microbiota more easily than in the colon, which is already heavily populated with indigenous bacteria. Furthermore, the "crosstalk" between probiotics and the small intestine may be different from that in the colon. In addition, some of the "crosstalk" effects may be age-dependent. The maturing small intestine of the newborn is initially exposed to a large number of colonizing bacteria acquired while passing through the birth canal. In the absence of mature intestinal function (mucus production, peristalsis, etc.) large numbers of bacteria colonize the small intestine in contrast to the presence of large numbers of colonizing bacteria only in the distal ileum, cecum, and colon in the mature intestine. Thus this initial early exposure of the small intestine to colonizing flora may be an important step in the appropriate maturation of mucosal immune system.

It is extremely difficult to simulate the complex bacterial–mucosal immune interaction using *in vitro* models, although models of the human mucosal immune system have been developed using co-cultured colon cancer lines and blood mononuclear cells. In many studies, probiotics have been added to immune cells *in vitro*. However, there does appear to be a variable response among different probiotic bacteria, both live and dead, to induce cytokine production by macrophages and dendritic cells with large variations in the amounts of IL-12, IL-10 and TGF-β produced by individual strains. The mechanisms of these variable responses have not been elucidated and require further study. It is likely, however, that the probiotic changes in regulatory T-cell activity is due to their effect on antigen-presenting cells. *Lactobacillus* provides a good example of a specific probiotic-immunological effect. As a Gram-positive bacterium, *Lactobacillus* express ligands for toll-like receptors (TLRs) which initiate immune responses and enable gut epithelium and immune cells to recognize both pathogens and indigenous microbiota. With regard to probiotic–TLR interaction, a recent finding is of great interest. It has been reported that recognition of commensal bacteria by TLRs is necessary for intestinal homeostasis, protection of epithelial cell from injury and stimulation of repair [57]. Using *in vitro* epithelial cell line techniques, probiotics (e.g. VSL#3) have been shown to increase barrier function [58] via TLR2, the toll-like cell surface receptor that recognizes peptidoglycans of Gram-positive bacteria [59]. Thus, a probiotic signaling through the TLR2 can inhibit allergen uptake by maintaining mucosal barrier integrity and thereby preventing the expression of eczema. There is also evidence that different cells in the gut (e.g. epithelial vs. dendritic cells) express different TLRs at different stages of development. In addition, specific probiotics (e.g., *L. rhamnosus* [GG]) [60] can prevent cytokine-induced apoptosis and can inhibit NF-κB activation (e.g. *L. reuteri*) [61] when M-cells overlying Peyer's patches allow probiotic uptake and presentation to dendritic cells. These observations can be confirmed *in vivo* by demonstrating the presence of bacteria in ileal lymphoid follicles by endoscopic biopsy.

Thus with regard to mucosal immunology, probiotics may increase mucosal barrier function, prevent apoptosis of epithelial cells and ultimately decrease antigen uptake, especially in the small bowel.

Clinical use of probiotics

Important clinical data with regard to probiotic use in clinical disease have recently been generated in adults, children and infants. The FAO and WHO guidelines [2, 3], albeit several years away from implementation in United Nation countries, will undoubtedly contribute to ensuring that reliable, clinically proven probiotics are available in the future [62].

Adults

The evidence for the efficacy of various probiotics is already reasonably strong as a result of randomized controlled trials (RCTs) and meta-analyses for lactose malabsorption (significant improvement in lactose digestibility induced by *L. bulgaricus* and *S. thermophilus* present in yogurt) [63, 64], infectious gastroenteritis [65] and antibiotic-associated diarrhea (e.g. *Saccharomyces boulardii* and *Lactobacillus*) [66].

Other selected clinical conditions warrant further study because of mixed clinical results. These include inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), the effect on intestinal transit time, *Helicobacter pylori* gastritis and nosocomial infections

Inflammatory bowel disease

RCTs have been conducted with various probiotics (including VSL#3, *Escherichia coli* Nissle 1917, *L. rhamnosus* GG, *S. boulardii*, and other *Lactobacillus* or *Bifidobacterium* strains) on patients with refractory pouchitis after ileoanal anastomosis for ulcerative colitis, in ulcerative colitis (prevention of recurrence) and in Crohn's disease (prevention of spontaneous and postoperative recurrence) [67]. Currently, the most convincing evidence of clinical efficacy has been obtained with pouchitis (e.g., acute pouchitis prevention, chronic pouchitis prevention, maintenance of remission in recurrent and resistant pouchitis) which is a unique complication of IBD which transiently responds to antibiotics. VSL#3, a probiotic preparation containing eight bacterial strains has been reported to prolong the treatment remission [68–70]. In ulcerative colitis per se, *E. coli* Nissle 1917, a probiotic not given as a food but as a supplement, was shown to be as effective as low dose mesalamine treatment in preventing relapse [71–73]. However, no placebo-controlled trial is currently available. There are also studies in which probiotics are given as food (e.g. *Lactobacillus rhamnosus* GG and *Bifidobacterium*). The results, however, are not as convincing as with those with pouchitis. Several RCTs have been conducted in the treatment of Crohn's disease but the results are equivocal. While *E. coli* Nissle 1917 was thought to be superior to placebo in preventing a relapse, *Lactobacillus* GG, given for 1 year, was of no benefit in preventing postoperative recurrence [74]. In Crohn's disease, lessons from animal models may be particularly useful. For example, a novel approach to probiotic therapy has been suggested for the prevention and treatment of colitis in two murine models using *Lactococcus lactis* engineered to secrete recombinant human IL-10 [75] or trefoil factors [76]. This approach is now the subject of pilot trials in IBD patients [67].

Irritable bowel syndrome

In IBS, the usual therapeutic interventions are generally not much more effective than placebo. The placebo effect is marked but insufficient as a therapy in clinical practice. Therefore, large populations of patients are required to assess a legitimate probiotic effect. Most published RCTs included only a small number of patients for relatively short follow-up periods. Some probiotic strains (e.g., *L.* Shirota) and a cocktail of strains (VSL#3) appeared effective with IBS symptoms of bloating [77, 78], while others (e.g. *L. plantarum* 299V) proved ineffective [79, 80]. The therapeutic strategy in post-infectious IBS, an increasingly frequent entity within the IBS spectrum in certain countries [81], may benefit from the recent finding that *L. paracasei* normalizes muscle hypercontractility in a murine model of post-infectious gut motility dysfunction [82].

Intestinal transit time

In randomized studies, milk fermented with the *Bifidobacterium animalis* DN173 010 strain and lactic acid bacteria or yogurt bacteria significantly accelerated whole-gut and colonic transit time in healthy volunteers [83–85]. This effect with respect to acceleration of both total colonic and sigmoid transit times has been confirmed in a double blind cross-over RCT in subjects with prolonged basal transit duration [86]. These findings may prove of special interest in IBD patients with slowed colonic transit or with constipation.

Helicobacter pylori gastritis

Through 2004, 15 open-label or randomized clinical trials have addressed the effects of probiotics alone, or more commonly as adjuncts to the standard triple-agent therapy. The most frequent shortcomings of these RCTs were the small study populations and the short durations of probiotic administration and follow-up. Eradication of *H. pylori* was not observed with probiotics alone, except in two open-label studies for which 20%–40% eradication was reported. In studies combining probiotic and triple-agent therapy [(six trials and an additional three trials using *L. johnsonii* La1) [87], milk fermented by *L. acidophilis* La5, and *B. lactis* Bb12 with yogurt bacteria (*S. thermophilis* and *L. bulgaricus*)] [88] more positive outcomes have been noted. These included decreased *H. pylori*, urease activity, as determined by ^{13}C-urea breath tests, and decreased gastric inflammation and/or decreased bacterial mucosal density. In no study did addition of probiotics enhance the triple therapy-induced rate of *H. pylori* eradication but a significantly reduced incidence of side effects related to antibiotics was reported with *L.* GG [89].

Nosocomial and postoperative infections

While certain probiotic strains (*S. thermophilus*, *L. acidophilus, L. casei* DN-114 001) and a mixture of strains (VSL#3) may enhance intestinal barrier function in animal models and in *in vitro* human cell lines [58, 90, 91], *L. plantarum* 299V and synbiotics containing *L. acidophilus* La5, *B. Lactis* Bb12, *S. thermophilus* and *L. bulgaricus* do not modify the incidence of bacterial translocation or the postoperative septic complications in patients undergoing surgery [92] nor did they affect sepsis in critically ill patients in

intensive care units [93, 94]. However, *L. plantarum* 299V given with fiber supplements was shown to reduce sepsis in patients with acute pancreatitis [95] and in liver transplant recipients [96].

Infants and children

In 2001, the Committee on Nutrition of the European Society of Pediatric Gastroenterology, Hepatology and Nutrition extensively reviewed information available on the effects of probiotics given as dietary products to infants [97]. At the 2nd World Congress of Pediatric Gastroenterology, Hepatology and Nutrition held in Paris on July 3–7, 2004, numerous papers regarding probiotic use in children were presented. The topics included childhood diarrheal diseases, experimental models of intestinal inflammation and bacterial infection, *H. pylori* gastritis, chronic constipation, and intestinal bacterial overgrowth.

Acute diarrheal diseases

Three meta-analyses of RCTs clearly support the observation that probiotics significantly shorten the duration of acute infectious diarrhea by a mean of 18 h (8 RCTs, 773 patients) [98], 17 h (7 RCTs, 675 patients) [99], and 29 h (12 RCTs, 970 patients) [100], respectively. These findings were especially true using *Lactobacillus* [99], in particular *L.* GG [98]. Two of the meta-analyses also showed that certain probiotics significantly reduce the risk of diarrhea lasting three or more days [98, 100]. These data confirm the conclusions of a previous review which suggested a clinically significant benefit of probiotic use in the treatment of acute infectious diarrhea, particularly rotavirus gastroenteritis in infants and children [101]. Treatment of acute infectious diarrhea, especially in children, may prove to be a primary therapeutic application after probiotic therapy in pediatric practice [62]. However, additional large-scale RCTs are needed to definitively establish the clinical relevance of this finding.

With regard to the prevention of community-acquired and nosocomial diarrhea, two studies established, through home visits and daycare center evaluations respectively, that *L.* GG was associated with a reduced incidence of diarrhea in Peruvian babies [102] but not in Finnish children aged 1–6 years [103]. In contrast, studies in hospital-settings in Europe and USA demonstrated a decreased incidence of acute infectious diarrhea. The first study was conducted with *L.* GG [101], the second with *B. bifidum* + *S. thermophilus* [104] and the third, which showed no effect, with *L.* GG [105]. Effective probiotic prevention of antibiotic-associated diarrhea in children has been recently confirmed in a RCT using *S. boulardii*. This study included 269 subjects aged 6 months to 14 years [106].

The mechanism(s) of the probiotic effect in infectious diarrhea has (have) yet to be fully elucidated. Probiotics may protect the intestine by competing with pathogens for adhesion sites, strengthening the mucosal barrier and tight junctions between enterocytes and/or enhancing the mucosal immune response to pathogens [107].

Experimental models of intestinal inflammation and bacterial infection

The probiotic strain *E. coli* Nissle 1917 activates the human B defensin-2 promoter in Caco-2 cells. Activation does not occur following transfection of mutated NF-κB suggesting a possible new pathway of probiotic action mediated by the antimicrobial peptide human B defensin-2. *L. plantarum* 299V enemas can reduce the induction of dextran–sodium sulfate-induced colitis in rats and has been shown to upregulate Muc2 colonic mucin gene expression. These findings may prove to be clinically relevant. In a model of colonic bacterial (*Citrobacter rodentium*) infection in mice, *L. rhamnosus* and *L. acidophilus* prevented colonic hyperplasia, decreased bacterial internalization by the mucosa and reduced infection-related cell damage. A preliminary study of *L.* GG in a small number of children with active Crohn's disease showed therapeutic benefit and allowing for tapering of steroids [108].

Helicobacter pylori gastritis

The effects of probiotics on pediatric patients with this condition are more disappointing. One or three probiotic strains (*L. casei defensis*, *S. thermophilus* and *L. bulgaricus*) were administered in a prospective randomized clinical trial (conventional triple-agent treatment + 7 days of probiotic administration). An increased rate of *Helicobacter pylori* eradication was only observed when the three probiotic strains were combined with the conventional triple-agent anti-*H. pylori* treatment [109].

Childhood chronic constipation

In a double blind RCT, *L.* GG induced no beneficial effect to that of lactulose alone in 2- to 16-year-old children with chronic constipation [110].

Intestinal bacterial overgrowth

A striking finding, to be confirmed in extensive RCTs studies, is that killed *L. acidophilus* administration

Fig. 6 Comparison of probiotic markets: Europe vs. US (from the Frost and Sullivan Consultant Report: European and US Probiotic Markets, 2003)

was associated with normalization of expired ^{2}H breath-test results at one month in 14 out of 16 children with documented bacterial overgrowth [111].

Neonates

A recent (1999–2003) randomized trial of 208 very low or extremely low birth-weight infants given either *Bifidobacterium breve* or a placebo within the first 24 h after birth (Y. Yamashiro, et al. unpublished observations, 2004) suggests that probiotics may be useful in the prevention of necrotizing enterocolitis (NEC). No death from infection occurred in the *B. breve* group as opposed to a 13.5% rate in the control group. *B. breve* administration, which has been shown to promote colonization by *Bifidobacterium* [112], may also stimulate immunological development in very low birth-weight infants. One mechanism suggested is due to an increased production, of TGF-β, a cytokine that enhances IgA production, immune oral tolerance, intestinal mucosal wound healing and epithelial cell proliferation and differentiation. The clinical results obtained in this Japanese study warrant further randomized, multicenter RCTs to confirm this important observation.

Cultural views on probiotics: USA vs. EUROPE

In this section "cultural views" are comparatively considered regarding the commercial market for probiotics with respect to distribution and sales, sociocultural, considerations, regulatory constraints, probiotic research analysis and trends and forecasts.

Probiotic market and sociocultural considerations

The levels of and reasons for probiotic consumption differ considerably in Europe and the USA. Ninety-five percent of the consumption in Europe and less than 50% in the USA is accounted for by animal feeds and dairy foods, while dietary supplements account for 4% in Europe and up to 56% in the USA (Fig. 6) (Frost and Sullivan Consultant Report: European and United States Probiotics Markets, 2003). The global yogurt market is growing in both Europe and the USA, but consumption is far greater in Europe (Euromonitor 2004). The same difference exists for fermented dairy drinks (Fig. 7).

With regard to sociocultural conditions (Frost and Sullivan Consultant Report: European and United States Probiotics Markets, 2003), Europeans are more familiar with the health benefits of fermented dairy products, are not opposed to using such dairy products, have some rudimentary sense of the process of fermentation, need to be informed about and are sometimes skeptical about the harmlessness of bacteria. The situation is quite different in the USA. Americans do not have a tradition of consuming fermented dairy products, which they consider to have an unpleasant acid taste, do recognize the health benefits of fermented dairy products but to a lesser extent than Europeans are not in general aware of the live dimension of yogurts, associate bacteria with "germs causing disease," and have a poor understanding of the underlying value of probiotics. However, there are also some other societal issues which could contribute to an increase in the US probiotic market. These include (a) an expanding interest in alternative medical practices and in the prevention of disease; (b) a collective attempt to seek a healthier lifestyle; and (c) unlike Europeans, a view of probiotics as dietary supplements (e.g., nutraceuticals) and not as food. This is illustrated by their constantly increasing market as dietary supplements from 1990 to 2000. The collective market has been estimated to reach 14 billion dollars in 2000 [113] (Fig. 8). The market should continue to increase and is a major growth area of health-related products. Thus, Americans are very interested in improving the quality of their health through both diet and lifestyle changes. An increasing US probiotic market has been forecasted through 2010 (Frost and Sullivan Consultant Report: European and United States Probiotics Mar-

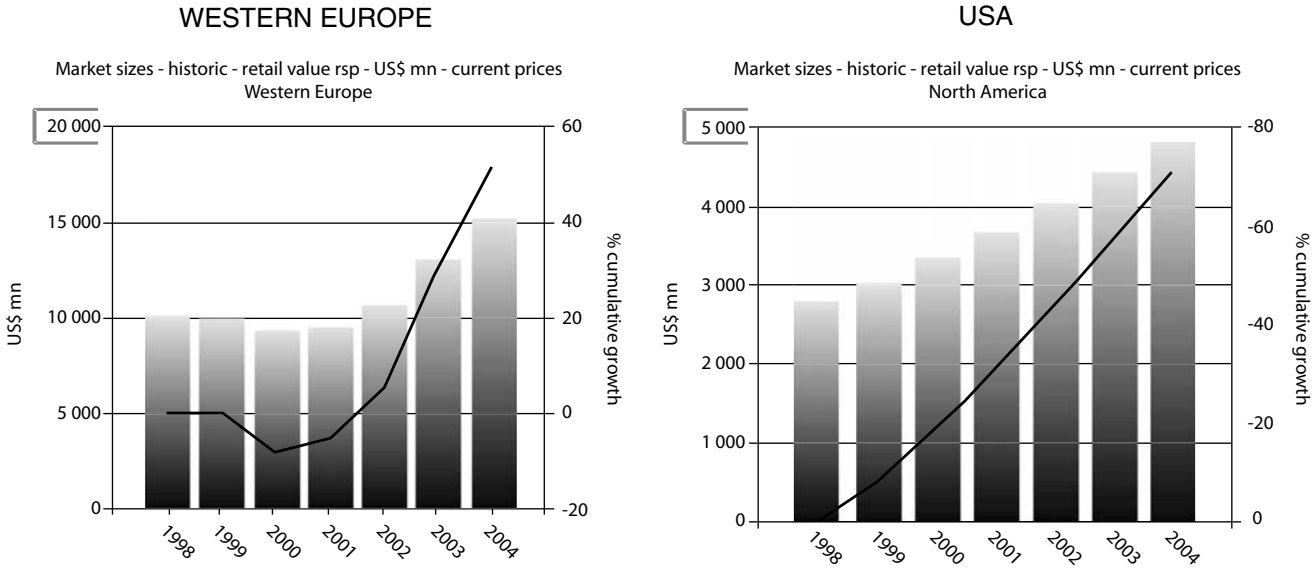

Fig. 7 Global fermented dairy drink market, Western Europe vs. North America: an overview up to 2004 (from Euromonitor 2004)

kets, 2003). In particular, specialized nutritional products for specific segments of the population (e.g., infancy, athletes, elderly, etc.) are potential major future growth areas. The US market for probiotic yogurt (solid and liquid) and fermented dairy drinks is also increasing.

With regard to probiotic consumption at present, there are two proteotypic countries: (a) the Scandinavian countries, Benelux, France, Germany, Austria and Switzerland, in which there is a good understanding of the probiotic concept due to a long tradition of consuming fermented dairy drinks and yogurts; and (b) the USA, Ireland, the United Kingdom, etc. where the concept is much less well developed.

Regulatory constraints

The regulatory constraints in Europe differ widely from those in the USA and Japan, probably due to the influence of sociocultural traditions. In Europe there is as yet no legal definition of "functional foods" and no legislation on their use in health claims. With regard to probiotic products, there has been no legislation on the use of microorganisms in human food and no prohibition of functional claims with regard to promoting health. In contrast, important health claims for probiotics are prohibited if they suggest disease treatment or disease prevention. Furthermore, the use of probiotics in animal feed is closely controlled. In the USA, all types of health claims (for health and disease prevention are authorized for functional products if the benefits have been demonstrated. Probiotics are

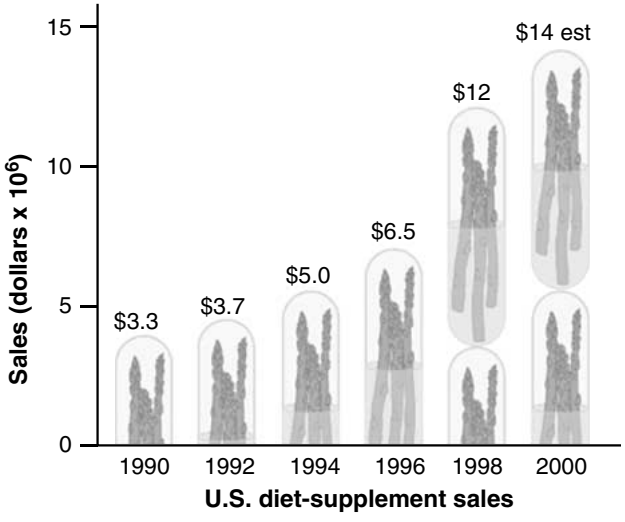

Fig. 8 Reprinted with permission from S.H. Zeisel [113]

generally considered dietary supplements and, as such, are subject to the "Dietary Supplement, Health and Education Act." This act was passed by Congress in 1994 and provides a new framework for the regulation of dietary supplements by the FDA. Although the US restrictions seem a bit stricter than those in Europe, the act is still very liberal, enabling manufacturers to sell supplements by listing label contents. Probiotic products require FDA approval or GRAS (Generally Recognized As Safe) status. The US regulations are thus not as stringent for functional foods as for the pharmaceutical products.

Probiotic research analysis

Over the past 10 years, there have been numerous publications on probiotics (2,632 papers) primarily addressing the digestive tract (1,440 papers), particularly *Lactobacillus* than *Bifidobacterium*. The principal studies addressed intestinal health and disease, but there was also considerable variety of emerging topics. These included: the immune system, eczema and other allergies, cholesterol levels, urinary tract infections, colonic polyps, cancer, etc. There were many more review articles than original publications. More publication emanated from Europe than the USA. Only a modest number of RCTs have been conducted. It is of interest that the largest consortium of investigators studying the lactic acid bacterial genome is led by the American Dairy Association and that a number of the *Lactobacilli* undergoing genome sequencing are owned by that consortium. Therefore, it is likely that a considerable quantity of follow-up results from the basic research conducted by US universities and corporations will emerge in the future.

Trends and forecasts

Several clinically pertinent trends are likely to become established soon. For example, probiotics can be used as surrogate flora for the initial colonization of the gut under circumstances in which adequate colonization does not occur in neonates. Based on seminal studies by Isolauri et al. [51], probiotics may become established as a preventative measure in allergic mothers giving birth to allergy-prone infants. However, before this measure can be established as a clinical recommendation confirmation as to its efficacy has to be established by large, multi-center RCTs. The adjuvant effects of probiotics used before standard vaccination, as was shown using *L.* GG with typhoid vaccination [114] may become established as standard clinical practice. A large multi-center RCTs of probiotics in day care centers published in 2001 has shown significant reduction in respiratory infection [103] and additional studies suggesting protection against nosocominal diarrhea in infants using *L. GG* [101] may result in new standard clinical care policies worldwide. Finally, the potential advantage of preparing genetically engineered "tailor made" probiotics, such as a study in which human IL-10, an anti-inflammatory cytokine, was shown to be locally secreted on the intestinal surface by a probiotic may be standard therapy for inflammatory intestinal conditions [67] in the future if shown through RCTs to be efficacious.

It is strongly suggested that probiotics taken as fermented foods has the added advantage of not only providing probiotic benefits but also providing the advantages of a healthy diet.

In summary, the potential future uses of probiotics are as follows: their use as functional foods (nutraceuticals) in dietary supplements may be as important as taking multi-vitamins. They could be routinely incorporated into infant formulas and weanling food (pediatric yogurt) to assure adequate development of mucosal immunity. With the reduction of host defenses in the elderly, they could be routinely used in nursing homes as a food source (e.g., yogurt, dairy drinks) or supplements to reduce nosocominal infection. However, before these uses become routine additional RCTs are required to confirm their clinical efficacy.

Conclusion

In 2001–2002, an expert panel from the FAO/WHO convened to assess the use of probiotics and agreed that adequate scientific evidence exists to indicate that there is potential for deriving health benefits from consuming food-containing probiotics. However more research is needed to confirm a number of those health benefits in humans by applying a systematic approach and complying with the assessment guidelines suggested by the FAO/WHO report.

A molecular approach to the identification of gut microbiota is now possible because genomic sequence of an increasing number of bacteria are available. In recent years, the genomic sequences of an increased number of probiotics or related lactic acid bacteria have been reported. The sequence information retrieved may be useful to establish a better understanding of the metabolic potential and function of those bacteria in the human intestinal tract. In conjunction with classic molecular biology and culture-independent techniques, these approaches may be used to define the mechanism by which probiotics may influence the health of the gut.

A major stumbling block exists to the clinical recommended use of probiotics in humans before they can be recommended for specific health benefits or to prevent/treat a specific illness. They include: [1] conclusive, statistically significant effects demonstrated by large, multi-center RCTs with a statistically reliable number of patients studied [2], furthermore, the specific clinical effect are probiotic strain and dose specific and their use can not be generalized to other probiotics or at other dose levels. The field of clinical research on probiotics is currently expanding to include studies of effects on the skin, joints, liver disease and obesity. In the latter condition, for example, very recent basic studies have shown that the conventional gut microbiota may induce a number of changes in

gene expression relating to body fat accumulation through interaction with the epithelial expression of a fasting-induced adipocyte factor, e.g. angiopoietin-like protein. This new identified factor inhibits lipoprotein lipase activity and triglyceride storage in adipocytes and interferes with insulin sensitivity [36].

Much work remains to be done. However, a scientific motivation to consolidate basic and applied research from molecular and cellular microbiology studies to immunologic and clinical nutritional application is underway.

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