Food Formats for Effective Delivery of Probiotics

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Key Words

functional foods, prebiotic, synbiotic, dairy, yogurt

Abstract

Probiotic bacteria are increasingly incorporated into food products intended to confer health benefits in the human gut and beyond. Little is known about how the food matrix and product formulation impacts probiotic functionality, even though such information is essential to scientific understanding and regulatory substantiation of health benefits. The food format has the potential to affect probiotic survival, physiology, and potentially efficacy, but few comparative studies in humans have been conducted. Human studies should account for the effects of the food base on human health and the bioactive components present in the foods that may augment or diminish interactions of the probiotic with the human host. Some studies show that food ingredients such as prebiotics and milk components can improve probiotic survival during the shelf life of foods, which may enhance probiotic efficacy through increased dose effects. Furthermore, there are indications that synbiotic products are more effective than either probiotics or prebiotics alone. Identification of probiotic adaptations to the food and gut environments holds promise for determining the specific cell components and potential bacterial-food interactions necessary for health benefits and determining how these factors are affected by changes in food formulation and host diet. These studies, combined with controlled human studies, are important future research activities for advancing this field.

INTRODUCTION

Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host (Food and Agricultural Organization 2001). Foods containing probiotics are typically active in the gut, although other sites of action have been described (Lenoir-Wijnkoop et al. 2007). Foods and beverages are increasingly popular carriers of probiotics and might drastically extend the option for regular consumption of probiotics in the human diet. Dairy products, mainly yogurts, are the most popular food carriers, and the probiotics used in these products typically are from the *Lactobacillus* and *Bifidobacterium* genera. Currently more than 80% of the yogurts available in the United States contain additional, putative probiotic cultures, although it is likely that not all these added cultures have established health effects (Sanders 2003). Because the inclusion of probiotics into foods and beverages is an attractive option for companies interested in new and healthy products, probiotic bacteria are also being added to other dairy and nondairy foods and beverages such as frozen yogurts, cheeses, chocolates, cereal bars, and juices (Champagne et al. 2005). This article will focus on foods as delivery vehicles for probiotics and specifically on what is known about the contributions of product formulations and food base to probiotic-conferred health effects.

HEALTH EFFECTS OF PROBIOTIC FOODS

Among foods studied clinically as carriers of probiotics, the most documentation exists for probiotics provided in a dairy matrix, either fermented or unfermented milk. Sanders & Merenstein (2009) summarized health effects attributed to yogurt. A few studies have documented changes in biomarkers or health effects of probiotics in nonyogurt foods, including fermented oat drinks (Pitkala et al. 2007, Klarin et al. 2008), cereal bars (Ouwehand et al. 2004), juice (Gotteland et al. 2008, Ouwehand et al. 2002), and milk-based fruit juice (Piirainen et al. 2008). In contrast, many other food products containing putative probiotics have not been subjected to controlled efficacy evaluation.

Numerous reviews have summarized the health effects of probiotics (Sanders et al. 2007). Probiotics are generally recognized for their immune and gut benefits, such as reduced side effects associated with antibiotic use, improved intestinal regularity, reduced symptoms associated with irritable bowel syndrome, and reduced gut inflammation. However, probiotics are also being recognized for an expanded range of effects, including reduced incidence of common infectious diseases (Weizman et al. 2005); antipathogenic effects on extraintestinal sites, such as skin (Peral et al. 2009), the vaginal tract (Martinez et al. 2009), and stomach (Park et al. 2007); and altered neuropsychological endpoints such as pain perception (Rousseaux et al. 2007), stress (Diop et al. 2008), and anxiety (Rao et al. 2009). Recent years have seen the publication of several meta-analyses on specific clinical endpoints for the most researched probiotic effects (**Table 1**). Although meta-analyses provide a cumulative view on probiotic efficacy, these reports are limited because the reviewed studies are not standardized and in most cases pooled studies used different strains or strain combinations in variable frequencies and doses. Only two of the meta-analyses shown in **Table 1** provide strain-specific analysis. Both of these papers reviewed evidence for effects on pediatric acute diarrhea, with one on *Lactobacillus rhamnosus* GG (Szajewska et al. 2007) and the other on *Saccharomyces boulardii* (Szajewska et al. 2007). Furthermore, there has been little differentiation in these publications between effects observed in products administered in food matrices compared with supplement forms.

A likely rationale for the lack of studies examining the contributions of the food format to probiotic function is that the probiotic is considered to be a functional ingredient, and the role of

Table 1 Summary of meta-analyses published evaluating probiotic efficacy in a variety of clinical endpoints

Clinical Endpoint	Conclusions from analysis	Reference
Treatment of infectious diarrhea	Probiotics are useful for rehydration therapy in treating acute, infectious diarrhea in adults and children.	Allen et al. 2004
Treatment of eczema	Probiotics are not an effective treatment for eczema, and probiotic treatment carries a small risk of adverse events.	Boyle et al. 2008
Antibiotic-associated diarrhea	Probiotics appear to benefit antibiotic-associated diarrhea, but available studies are flawed by the lack of a placebo design and by peculiar population features. Further studies are needed to confirm the benefit.	Cremonini et al. 2002
Prevention of necrotizing enterocolitis	Probiotics might reduce the risk of necrotizing enterocolitis in preterm neonates, but safety needs to be assessed in large trials.	Deshpande et al. 2007
Prevention of antibiotic-associated diarrhea	Probiotics can be used to prevent antibiotic-associated diarrhea but efficacy remains to be proved.	D'Souza et al. 2002
Management of pouchitis	The benefit of probiotics in the management of pouchitis was confirmed.	Elahi et al. 2008
Pediatric acute diarrhea	Probiotic therapy shortens the duration of acute diarrheal illness in children by approximately one day.	Huang et al. 2002
Pediatric antibiotic-associated diarrhea	Probiotics to prevent antibiotic-associated diarrhea in children do not withstand intention-to-treat analysis. Further studies are needed with most promising strains and doses.	Johnston et al. 2006
Prevention and treatment of pediatric atopic dermatitis	Current evidence is more convincing for efficacy of probiotics in prevention rather than treatment of pediatric atopic dermatitis.	Lee et al. 2008
Treatment of irritable bowel syndrome	Studies on irritable bowel syndrome suggest that probiotics may improve symptoms, but methodological limitations warrant further research to confirm therapeutic potential.	McFarland & Dublin 2008
Prevention of antibiotic-associated diarrhea and treatment of *Clostridium difficile* disease	Three types of probiotics (*Saccharomyces boulardii*, *Lactobacillus rhamnosus* GG, and probiotic mixtures) significantly reduced the development of antibiotic-associated diarrhea.	McFarland 2006
Prevention of traveler's diarrhea	Probiotics may offer a safe and effective method to prevent traveler's diarrhea.	McFarland 2007
Treatment of pediatric atopic dermatitis	Probiotics may modestly treat moderately severe pediatric atopic dermatitis.	Michail et al. 2008
Treatment of irritable bowel syndrome	Probiotics may improve symptoms of irritable bowel syndrome and can be used as supplement to standard therapy.	Nikfar et al. 2008
Prevention of pediatric allergic disease and food hypersensitivity	There is insufficient evidence to recommend the addition of probiotics to infant feeds for prevention of allergic disease or food hypersensitivity.	Osborn & Sinn 2007
Prevention of preterm labor	There is insufficient data to assess impact of probiotics on preterm birth and its complications.	Othman et al. 2007
Maintenance of remission and prevention of relapse of Crohn's disease	Probiotics have not been demonstrated to improve efficacy in maintaining remission and preventing clinical and endoscopic recurrence in Crohn's disease.	Rahimi et al. 2008

(*Continued*)

Table 1 (*Continued*)

Clinical Endpoint	Conclusions from analysis	Reference
Maintenance of remission of Crohn's disease	There is no evidence to suggest that probiotics are beneficial for the maintenance of remission in Crohn's disease.	Rolfe et al. 2006
Prevention of acute diarrhea	There is some suggestion that probiotics may be efficacious in preventing acute diarrhea. Data are lacking from community-based trials and from developing countries on acute diarrhea unrelated to antibiotic usage.	Sazawal et al. 2006
Prevention of pediatric antibiotic-associated diarrhea	Probiotics reduce the risk of antibiotic-associated diarrhea in children.	Szajewska et al. 2006
Treatment of pediatric acute diarrhea with *Saccharomyces boulardii*	A moderate effect of shorter duration of diarrhea was observed for *S. boulardii* in otherwise healthy infants and children with acute gastroenteritis. Results should be interpreted with caution owing to methodological limitations of existing studies.	Szajewska et al. 2007
Treatment of pediatric acute diarrhea with *Lactobacillus rhamnosus* GG	The use of *L. rhamnosus* GG is associated with moderate clinical benefits in the treatment of acute diarrhea in children.	Szajewska et al. 2007
Prevention of traveler's diarrhea	Probiotics were not shown to be effective in preventing traveler's diarrhea.	Takahashi et al. 2007
Eradication rates and adverse events during *Helicobacter pylori* eradication therapy	Supplementation with probiotics could be effective in increasing eradication rates when used as adjuncts of anti-*H. pylori* therapy, and could improve *H. pylori* therapy-related side effects.	Tong et al. 2007
Acute pediatric infectious diarrhea	*Lactobacillus* is safe and effective as a treatment for children with acute infectious diarrhea.	Van Niel et al. 2002
Use in adult intensive care settings	There is currently a lack of evidence to support the use of pre-, pro-, or synbiotics in patients admitted to adult intensive care units. Large well-designed trials are needed.	Watkinson et al. 2007

delivery matrix is not considered by some to be relevant in making functional claims. However, the delivery vehicle is likely to influence probiotic functionality in many ways including inducing changes in the cell composition and physiological status of the probiotic; providing other complementary physiologically active ingredients, such as fibers, known bioactive compounds, fermentation end-products such as organic acids, bacteriocins or bioactive peptides; or improving the likelihood of regular consumption through product palatability and incorporation of that product into the diet. These factors could also be expected to affect cell fitness throughout product shelf life. Ideally, cells in the product are consistently functional all throughout the shelf life of the product, although no published studies have specifically addressed this issue. **Figure 1** summarizes many of the factors that might conceivably impact probiotic physiology and functionality including the delivery vehicle.

Additional context for considering the importance of specific probiotic food formulations is that probiotic products are consumed as part of an overall diet containing a multitude of diverse foods. However, dietary patterns are typically not accounted for in clinical trials on probiotics, and differences in diet might explain some of the observed individual and study-to-study variation. The potential importance of host diet on probiotic functionality was recently demonstrated in a study that showed significantly different global response profiles and colonization levels of

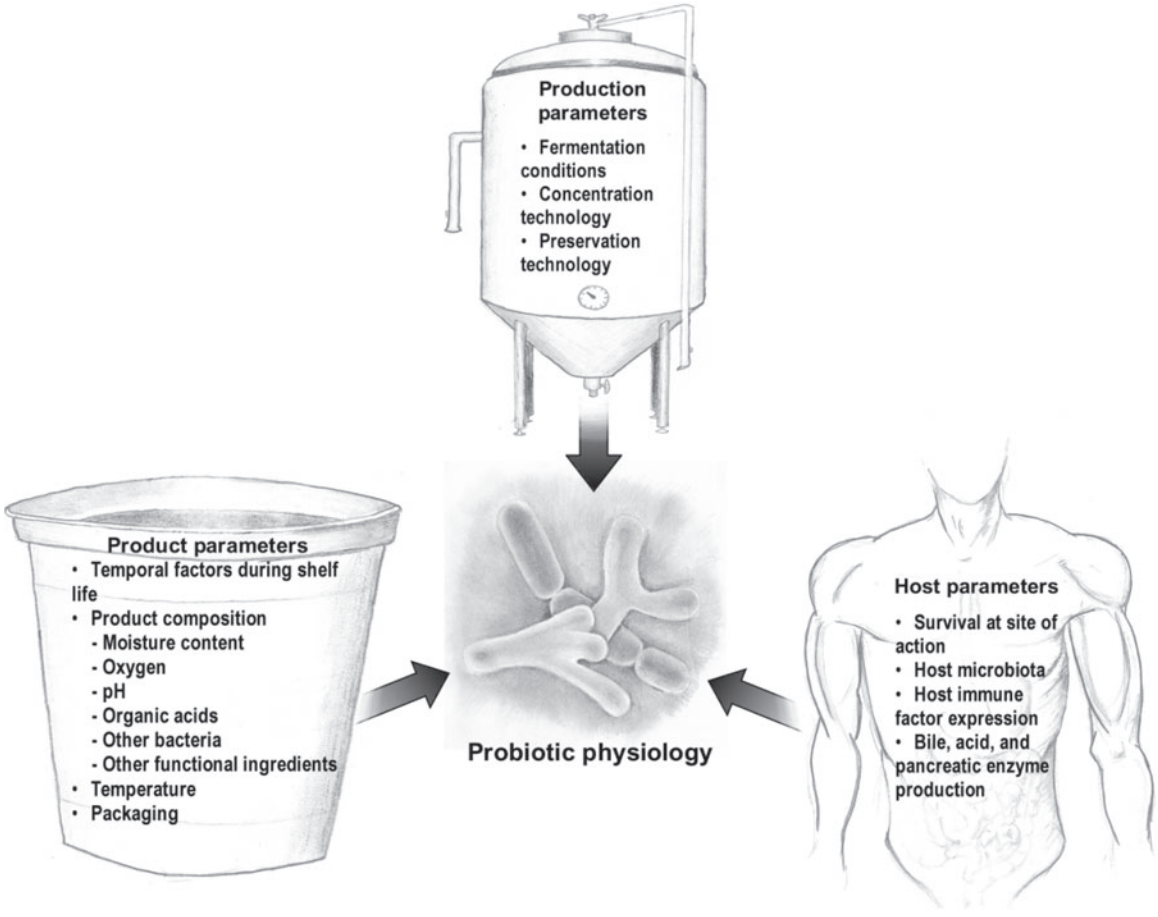

Figure 1

Factors that potentially impact probiotic physiology. Probiotic physiology will in turn impact in vivo functionality and stability. The extent to which these factors influence probiotic physiology should be expected to be strain-specific. Furthermore, the levels of probiotic recovered from a food will depend on methods used for enumeration.

L. plantarum WCFS1 in germ-free mice that were fed either standard polysaccharide-rich chow or a high-sugar and fat chow (resembling diets commonly consumed in the West) (Marco et al. 2009). Carbohydrate metabolism and cell surface composition encoding pathways differed in ways suggestive of different activity levels and interactions with the host (Marco et al. 2009). These results are complementary to the finding that the indigenous mouse gut microbiota cluster according to host diet (Turnbaugh et al. 2008). Therefore, to determine if dietary factors impact probiotic effects, human studies that provide detailed descriptions of the diet of subjects would be needed.

INFLUENCE OF FOOD FORMULATION ON PROBIOTIC-CONFERRED HEALTH BENEFITS

Individual human and animal studies that included comparisons between food formats are provided in **Table 2** with the outcomes described below. One of the few studies conducted in humans

Table 2 Studies that have examined the effects of food formulation on probiotic health effects

	Health Effect	Food format comparison	Strain	Reference
Clinical				
	Acute infectious diarrhea	Dairy versus freeze-dried powder	*Lactobacillus rhamnosus* GG	Isolauri et al. 1991
	Immunity	Dairy formulation; (lactose-hydrolyzed) low-fat milk	*Bifidobacterium lactis* HN019	Chiang et al. 2000
	Immunity	Dairy formulation; (lactose-hydrolyzed) low-fat milk	*Lactobacillus rhamnosus* HN001	Sheih et al. 2001
	Ulcerative colitis	Probiotic-prebiotic combination	*Bifidobacterium longum*	Fujimori et al. 2009
Animal				
	Arthritis	Dairy versus saline	*Lactobacillus rhamnosus* GG	Baharav et al. 2004
	Colon cancer	Dairy versus saline microencapsulated	*Lactobacillus acidophilus* ATCC 314	Urbanska et al. 2009
	Colon cancer	Probiotic-prebiotic combination	*Bifidobacterium longum* 25	Rowland et al. 1998
	Colon cancer	Probiotic-prebiotic combination	*Bifidobacterium longum*	Gallaher & Khil 1999
	Colon cancer	Probiotic-prebiotic combination	*Bifidobacterium lactis* Bb-12 and *Lactobacillus rhamnosus* GG	Femia et al. 2002
	Diabetes	Contribution of dairy product to probiotic function	*Lactobacillus acidophilus* NCDC14 and *Lactobacillus* NCDC19	Yadav et al. 2008
	Immunity	Milk versus fermented milk	*Lactobacillus rhamnosus* HN001	Gill & Rutherfurd 2001
	Inflammatory bowel disease	Probiotic-prebiotic combination	*B. infantis* DSM 15159	Osman et al. 2006
	Salmonella pathogenesis	Probiotic-prebiotic combination	*Bifidobacterium breve* strain Yakult	Asahara et al. 2001
	Whey allergy	Probiotic-prebiotic combination	*B. breve* M-16V	Schouten et al. 2009

tested *L. rhamnosus* GG in a fermented milk or as a freeze-dried powder in children recovering from acute infectious diarrhea (Isolauri et al. 1991). The duration of diarrhea was shorter in the groups that received either the fermented milk or freeze-dried powder compared with those receiving a pasteurized yogurt (Isolauri et al. 1991). Both delivery matrices worked equally well, suggesting that the delivery matrix was not critical for probiotic function. However, this study design did not meet inclusion criteria for a subsequent meta-analyses (Van Niel et al. 2002). Clearly, this single clinical trial is not sufficient for evaluating matrix-effects on probiotic performance. This consideration was recognized in a recent meta-analysis of randomized trials on *Helicobacter pylori* eradication by probiotics in fermented milk products (Sachdeva & Nagpal 2009). Preliminary comparisons showed that the efficacy of fermented milk-based products may be better than capsule/sachet-based bacteria-only preparations for eradicating intestinal *H. pylori* (Sachdeva & Nagpal 2009).

Similarly, studies done with animal models examining probiotic effects have typically not taken the delivery vehicle into account, and instead the probiotic microorganism is provided to the animals in saline or growth medium. The few studies that have been performed comparing

food matrices suggest that fermented milk might augment probiotic functionality. For example, Baharav et al. examined the potential of *L. rhamnosus* GG to ameliorate arthritis in Lewis rats when the probiotic was provided in water, milk, or yogurt (Baharav et al. 2004). *L. rhamnosus* GG in water or milk conferred minor preventative effects, whereas animals that received plain yogurt or *L. rhamnosus* GG-containing yogurt were more protected in the prevention of either adjuvant or tropomyosin arthritis (Baharav et al. 2004). Only the yogurt enriched with *L. rhamnosus* GG conferred significant effects in the treatment of preinduced arthritis (Baharav et al. 2004). Distinctions between yogurt and saline carriers were also found for microencapsulated *L. acidophilus* ATCC314 in a mouse colorectal cancer model (Urbanska et al. 2009). Significantly fewer ademonas were found in the small intestine of mice fed microcapsulated *L. acidophilus* in yogurt compared with mice fed the same strain in saline solution (Urbanska et al. 2009).

CONTRIBUTIONS OF FOOD COMPONENTS TO PROBIOTIC FUNCTIONALITY

When considering the contributions of food format to probiotic functionality, a key consideration is the presence of bioactive ingredients, which may provide independent benefits or augment or diminish probiotic efficacy. These ingredients may be added to, or are naturally present in, the food. Such ingredients might include prebiotics, other fibers, enzymes, vitamins, minerals, or food preservatives or flavors (for example, see Vinderola et al. 2002). Because clinical studies are rarely controlled in a manner conducive to deducing interactions among potential functional components in probiotic foods, little is known about additive or synergistic activities. This is true even in synbiotics, products that contain both probiotics and prebiotics.

Prebiotics

Studies have examined the effects of prebiotic compounds on probiotic function. Prebiotics are nondigestible (by the host) food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract (Gibson et al. 2004). Prebiotic compounds are typically oligosaccharides that are not degraded by mammalian cells but rather by certain groups of bacteria (commonly bifidobacteria and lactobacilli) inhabiting the gut. It should be noted that recently inulin was shown to stimulate the levels of fecal *Faecalibacterium prausnitzii* (Ramirez-Farias et al. 2009), an organism that when found in low levels is associated with an increased risk of development of Crohn's disease (Sokol et al. 2008), suggesting that *F. prausnitzii* may be a legitimate new target for prebiotic stimulation. However, the observation that some pathogenic *Escherichia coli* strains may metabolize fructooligosaccharides, thereby potentially improving intestinal colonization by the pathogen, emphasizes the importance of thorough documentation of effects on microbiota by candidate prebiotics (Schouler et al. 2009). As more information becomes available on the effects of different groups of commensal bacteria on health, the targets for prebiotic compounds will be better refined. For example, prebiotic human milk oligosaccharides are digested only by a limited number of gut-associated microbes, primarily *Bifidobacterium infantis* species, offering positive selective pressure for these bacteria in the guts of breastfed infants (Ward et al. 2007). The targets for prebiotic activity will likely expand beyond the traditional focus on lactobacilli and bifidobacteria to include newly identified health-promoting bacteria in the human gut microbiome.

Dietary prebiotic compounds are being investigated in their own right for beneficial effects on the treatment and prevention of numerous ailments, including cancer, allergy,

inflammatory bowel disease, constipation, and cholesterol metabolism (Macfarlane et al. 2008). Randomized, placebo-controlled human studies comparing the differential effects of probiotics, prebiotics, and synbiotics have yet to be performed. However, the synbiotic approach holds promise for delivering health benefits compared with probiotic-only preparations (Steed et al. 2008). This was shown in a recent open-label trial assessing the quality of life in ulcerative colitis patients receiving capsules containing prebiotic psyllium, *B. longum* or both (synbiotic) (Fujimori et al. 2009). In questionnaire-based examinations, the synbiotic group tended to score higher on social and systemic parameters compared with the other two groups (Fujimori et al. 2009).

Animal studies examining the effects of synbiotics suggest that some synbiotic combinations provide additional benefits compared with either prebiotics or probiotics alone. In a recent mouse study, administration of a prebiotic product with *B. breve* M-16V was found to confer better protection against the development of allergic symptoms in mice orally sensitized with whey (Schouten et al. 2009). The synbiotics were more effective than prebiotics or probiotics individually in reducing the acute allergic skin response, the anaphylaxis scores, and mast cell-derived mMCP-1 concentrations (Schouten et al. 2009). For protection against human pathogens, a synbiotic preparation of *B. breve* Yakult and transgalactosylated oligosaccharides was significantly more effective in reducing fecal excretion of *Salmonella enterica* serovar *typhimurium* in mice after pathogen challenge compared with either probiotic or prebiotic treatment (Asahara et al. 2001). However, synbiotics conferred no additional beneficial effects over prebiotic and probiotic treatments in a mouse model of inflammatory bowel disease (Osman et al. 2006). All prebiotic treatments (oligofructose and inulins) and those containing *B. infantis* DSM 15159 were equally effective in lessening the severity of disease and conferred decreased levels of bacterial translocation, myeloperoxidase activity, and colonic tissue IL-1β (Osman et al. 2006).

Cancer prevention by prebiotics and probiotics has also been investigated. The number of aberrant crypts in rats treated with the colon carcinogen azoxymethane (AOM) was significantly reduced when rats were fed a combination of inulin and *B. longum* strain 25 (Rowland et al. 1998). Effects on numbers of larger aberrant crypt foci suggested synergistic activity, compared with the individual prebiotic and probiotic treatments alone (Rowland 1998). Additive effects were found in another study showing that administration of *B. longum* and oligofructose resulted in reduced numbers of aberrant crypts in rats given the carcinogen 1,2-dimethylhydrazine (Gallaher & Khil 1999). In another study, tumor incidence was lower in AOM-treated rats fed the prebiotic Raftilose-Synergy1® and *L. rhamnosus* GG and *B. lactis* Bb-12 compared with untreated rats, and the prebiotic and probiotic organisms appeared to function in an additive manner (Femia et al. 2002). These effects appear to be mediated by modulating gut-associated lymphoid tissue, particularly through the Peyer's patches (Roller 2004). This formulation was also recently shown to reduce cancer risk factors in colon cancer patients (Rafter et al. 2007). Unfortunately, a truly synergistic effect of probiotics and prebiotics has not yet been investigated in randomized, placebo-controlled clinical studies.

Dairy Matrix

Milk is an excellent example of a functional food that may contribute to the health benefits described for probiotic bacteria. Bovine milk offers complete nutrition, being a source of macromolecular proteins, fatty acids, and carbohydrates as well as vitamins and minerals. Bovine milk is also a source of bioactive compounds, including but not limited to calcium, oligosaccharides, glycosphingolipids, lactoferrin, and immunoglobulins, although the molecular interactions of these compounds with human epithelial and immune cells are not well understood

(Ward & German 2004, Baldi et al. 2005, Severin & Xia 2005). Bioactive components of milk have been shown to confer antimicrobial, immunomodulatory, anticarcinogenic, and prebiotic properties (Severin & Xia 2005, Argov et al. 2008, Dewettinck et al. 2008, Florisa et al. 2003) that could be expected to complement probiotic functions in the gut. For example, the calcium in milk was shown to improve resistance to enterotoxigenic *E. coli* infection (Bovee-Oudenhoven et al. 2003). Calcium ions also significantly increased the binding capabilities of probiotic strains to a piglet jejunal epithelial cell line IPEC-J2 (Larsen et al. 2007), suggesting the advantage of calcium-rich, milk-based matrices for the delivery of probiotic bacteria. Milk also interacts with and can be modified by probiotics and starter cultures during fermentation. Proteolysis of milk proteins by some *Lactobacillus* strains results in some peptides that demonstrate bioactive properties conferring immunostimulatory, opioid, or angiotensin I-converting enzyme inhibitory activity (Haque & Chand 2008, Vasijevic & Shah 2008). Recently, the production of conjugated linoleic acid was demonstrated in dahi (fermented buffalo milk commonly consumed in India) containing *L. acidophilus* NCDC 14 and *L. casei* NCDC 19 (Yadav et al. 2007).

Although fermented milks and yogurts are common carriers of probiotics in clinical trials, the rationale behind the delivery of probiotics in these foods is largely driven by the high consumer acceptance of these foods as being healthy and natural carriers of living bacteria rather than because of milk's scientifically verified beneficial value as an appropriate carrier of probiotics for improving human health. Clinical trials and animal studies examining preventive and therapeutic measures typically compare the probiotic preparation against a control product that is similar in composition but lacking the probiotic organisms. This design does not permit assessments of the possible contributions of the food (e.g dairy) product on the health end-points. In a few animal studies, this possibility was taken into account, and dairy was found to confer beneficial effects, although probiotic-containing dairy products were more effective than dairy alone. For example, skim milk and a control dahi conferred protection against strepozotocin (STZ)-induced diabetes in male Wistar rats (Yadav et al. 2008). These dairy products were less effective than dahi with *L. acidophilus* NCDC14 and *L. casei* NCDC19 in delaying insulin resistance (Yadav et al. 2008). Moreover, feeding a dahi containing *L. casei* NCDC19 but not control dahi or a dahi-free diet enhanced the expression of Th1 type cytokines (IFN-γ and IL-2) in the Peyer's patches in mice (Jain et al. 2009). Ultimately, additional treatment groups should be included in animal and clinical studies to evaluate the potential separate contributions of the probiotic and food matrix on the observed health effects.

In addition to food or beverage matrix effects, the specific dairy product formulation might affect probiotic functionality, although this has yet to be validated in vivo. In a double-blind, three-stage, before-and-after intervention trial, *L. rhamnosus* HN001 contained in a low-fat milk or lactose-hydrolyzed low-fat milk was equally effective in increasing the in vitro phagocytic capacity of peripheral blood polymorphonuclear (PMN) leukocytes and the activity of natural killer leukocytes (Sheih et al. 2001). A similar result was found for *B. lactis* HN019, although the natural killer cell activity was significantly higher in human subjects consuming lactose-hydrolyzed milk containing *B. lactis* HN019 compared with the other products (Chiang et al. 2000). In a mouse study, direct oral feeding of mice with *L. rhamnosus* HN001 in fresh whole milk, fermented-milk, or HN001-fermented milk equally enhanced phagocytic activity of blood and peritoneal cells compared with feeding whole milk alone (Gill & Rutherfurd 2001). These limited human and animal studies suggest that product formulation might not be critical for probiotic function. However, more substantiation for different product formulations is needed. Because biologically active components in milk are vulnerable to treatment, processing, and storage conditions, these

factors should also be taken into account when evaluating the health modulatory properties of the final product.

CONTRIBUTIONS OF FOOD FORMAT TO PROBIOTIC SURVIVAL IN THE PRODUCT AND AT THE ACTIVE SITE IN THE HOST

The impact of food format on survival of a probiotic in food products and through the mammalian gut has been examined. Probiotic cell viability generally declines during processing and product storage, limiting the shelf life of food products (Champagne et al. 2005). To meet the definition of a probiotic, the microbe must be administered at an efficacious level. Commonly, efficacious levels of probiotics are in the range of 100 million-10 billion cfu/day, which leads to cell levels of ~1–100 million cfu/g of food, depending on serving size. Preserving this level of viability can be a technological challenge, and often is achieved by targeting significant overages during initial formulation to account for a drop in viability over the course of shelf life. It has long been recognized that the survival of probiotic bacteria in final products depends on various factors including strains used, interactions between species, solids content, sugar concentrations, dissolved oxygen levels, incubation, storage temperature, and storage time (**Figure 1**) (Lourens-Hattingh & Viljoen 2001). Probiotics intended to function in the gut are also generally expected to survive transit through the human digestive tract. Frequently, such strains are evaluated for resistance to gastric pH and bile (Morelli 2007). Note that this trait is not a prerequisite for probiotic applications that target regions of the body proximal to the gut such as the oral cavity or throat, or for non-orally administered probiotics, such as intravaginal or topical probiotics. Recent reports have highlighted the importance of physiological state of the probiotic cell with regard to numbers that are recovered during enumeration. A cell that is in a viable but nondividing state might remain active in the human gut (Bunthof & Abee 2002, Lahtinen et al. 2006).

Studies examining probiotic cell survival during strain production, food processing, and gut delivery have been the subject of numerous reviews and therefore will not be examined here (see Kosin & Rakshit 2006, Lacroix & Yidirim 2007, Champagne & Gardner 2008). Methods under investigation to achieve improvements in probiotic cell survival include encapsulation (Anal & Singh 2007) and induction of cellular stress-tolerance pathways (Corcoran et al. 2008). Some food matrices might also be better than others for ensuring high amounts of viable probiotic cells. Numerous probiotic strains were shown to survive storage and simulated gut transit conditions in olive (Lavermicocca et al. 2005) and artichoke preparations (Valerio et al. 2006), fruit juices (Saarela et al. 2006, Sheehan et al. 2007), oat-based cereal bars (Ouwehand et al. 2004), and chocolate bars (Nebesny et al. 2007). Some studies have shown that probiotic bacteria survive better in milk, cheese, and yogurt compared with either saline or buffer solutions during exposure to gastric conditions (Conway et al. 1987, Gardiner et al. 1999, Sharp et al. 2008). Milk was also the superior storage and carrier vehicle for probiotic *B. lactis* Bb-12 compared with fruit juice (Saarela et al. 2006). However, this strain was more susceptible to gastric pH and bile salts after prolonged storage in both matrices compared with freshly grown cultures (Saarela et al. 2006). Additionally, prebiotic ingredients were shown to improve probiotic survival in food products. Additions of inulin, high amylase corn starch powder, or fructooligosaccharides improved the survival of probiotic cells in dairy products (Desai et al. 2004, Capela et al. 2006, Varga et al. 2006, Donkor et al. 2007). The addition of dextran, polydextrose, and oat-flour fibers to chocolate-coated breakfast cereal and apple juice improved the long-term survival of *L. rhamnosus* GG in those products (Saarela et al. 2006). Synbiotic preparations containing mixtures of probiotic bacteria and prebiotics have yet to be shown to improve the survival of probiotics in the human gut (Alander et al. 2001), although there is evidence that coadministration of prebiotics improves the

survival of at least certain probiotic *Lactobacillus* and *Bifidobacterium* strains in the guts of mice (Su et al. 2007).

Human studies comparing probiotic survival after gastrointestinal transit in different food matrices and product formulations are important for validating in vitro digestive tract simulation studies and providing a baseline of probiotic strain survival in the human gut. Rochet et al. (2008) measured the survival of *B. animalis* strain DN-173 010 administered to 12 healthy subjects in a fermented milk or in a lyophilized form. The composition of the fecal microbiota was monitored using colony immunoblotting, fluorescent in situ hybridization with group-specific DNA probes, and temporal temperature gradient gel electrophoresis using group-specific primers. Enzyme activities and fecal metabolites were also measured. No difference was observed for average survival between lyophilized probiotics (22%) and probiotics delivered in fermented milk (20%). Furthermore, no significant differences were observed in the composition of the dominant members of the fecal microbiota or their activities. The authors concluded that *B. animalis* DN-173 010 survived equivalently whether delivered in lyophilized or fermented milk formats. Likewise, Varcoe et al. (2002) showed no difference in fecal survival as measured by colony counting of *L. acidophilus* NCFM when administered in skim milk versus water. Collins et al. (2002) showed equivalent fecal recovery of *L. salivarius* UCC118 when consumed in milk versus fermented milk. Goldin et al. (1992) compared fecal survival of *L. rhamnosus* GG when fed to 76 volunteers in three different formats (frozen concentrate at 4×10^{10}/d, fermented milk at 3.6×10^{11}/d, and a fermented whey drink at 1.6×10^{11}/d). Unfortunately, the design of this study did not result in direct comparisons of the same dose but different format. However, the frozen concentrate and fermented milk preparations were tested after seven days of daily feeding, with equivalent results. Comparisons in nondairy products include an indirect comparison of *B. lactis* Bb-12 survival in the human gut administered in an oat-based cereal bar and to that found for dairy products. *B. lactis* Bb-12 survived equally as well in cereal bars and dairy products containing galactooligosaccharides (GOS) and better than in dairy products without GOS additions (Ouwehand et al. 2004). Finally, *L. plantarum* MV12198 in fermented sausages was recovered from human feces in similar amounts compared with amounts of freeze-dried preparations of the same strain (Klingberg & Budde 2006). Although these studies are limited, there is no convincing evidence to date that the food format affects probiotic survival in the human gut. However, survival is but one measurement for evidence of probiotic function; it does not substitute for the critical need to confirm that efficacy is not altered in different food formats.

MOLECULAR MECHANISMS BEHIND PROBIOTIC FUNCTION IN FOODS

Probiotics are living organisms with the ability to dramatically change their cellular composition in order to adapt to new environments. Cell physiology can be substantively altered in response to culture conditions and phase of growth. The cell surface composition is a likely target for host-microbe interactions (Lebeer et al. 2008) and is also modified under different environmental conditions (for examples, see Kelly et al. 2005, Cohen et al. 2006). As a result, the physiological status of the microbe is dictated to a large extent by growth conditions, harvesting, and concentration technologies (as applicable for specific foods), as well as by how it is delivered in the final product. These parameters could also affect the fitness of the probiotic. The probiotic fitness could in turn impact the viable number and functional capacity of the probiotic as it reaches the active sites in the body, which will affect the net health effect conferred. This possibility would be bolstered by

controlled human studies comparing probiotic functionality in different food formats, studies that are not currently part of the published scientific literature database.

Recent studies using global gene, protein, and metabolite expression techniques are starting to provide evidence of probiotic adaptations in food products and survival and host-microbe interactions in the mammalian gut (Marco et al. 2006, Lebeer et al. 2008, Klaenhammer et al. 2007). Transcriptome profiling was performed for *B. longum* during growth in human milk (Gonzalez et al. 2008). This study showed that *B. longum* redirects sugar metabolism toward oligosaccharide consumption during growth in the milk matrix and that this activity might be relevant for the high abundance of this organism in the guts of breastfed infants (Gonzalez et al. 2008). Similarly, metabolomic and proteomic analyses of *B. longum* subsp. *infantis* ATCC 15697 during growth on human milk oligosaccharides resulted in the identification of oligosaccharide degradation pathways of this organism (Sela et al. 2008). Recently, transcript profiles of actively-dividing (4h and 8h) and stationary phase (12h) *L. acidophilus* NCFM cultures in bovine milk were determined. Results showed that this organism undergoes dynamic changes in carbohydrate and amino acid metabolism over time during growth in milk and expresses genes with putative probiotic function, some of which are required for survival in milk (Azcarate-Peril et al. 2009).

To identify traits important to conferring gut survival and host-microbe interactions, both in vitro and in vivo studies have been performed. In vitro studies have identified the global responses of probiotics in bile (Bron et al. 2004, Bron et al. 2006, Sanchez et al. 2007, Whitehead et al. 2008) and gastric conditions (Azcarate-Peril et al. 2004, Azcarate-Peril et al. 2005). Genes induced in the digestive tracts of mice models were identified for *L. plantarum* and *L. reuteri* (Walter et al. 2003, Bron et al. 2004) and confirmed by mutant analysis (Walter et al. 2005, Bron et al. 2007) and transcript quantification in the stomach to colon of the mouse gut (Marco et al. 2007). Global gene expression profiles of probiotics and host cells in mice and humans have shown the intimate host-probiotic relationships in the gut (Sonnenburg et al. 2006, Denou et al. 2007, Martin et al. 2007, Marco et al. 2009, Troost et al. 2008, Yuan et al. 2008, van Baarlen et al. 2009). These studies demonstrated the key relevance of probiotic energy metabolism and cell surface composition in vivo as well as reported significant changes in host gene expression and global metabolome profiles upon ingestion of probiotic cells. Remarkably, modern genomic approaches to study the molecular host-probiotic interactions have largely not taken into account the relevance of the food matrix in modulating these effects. Considering that probiotics will respond to the environments encountered within the food matrix, the inclusion of the food carrier is essential to determining underlying activities in the gut. This is illustrated by the recent finding that human transcriptome response profiles to probiotics depend on the physiological status of the probiotic cells at the time of ingestion (van Baarlen et al. 2009). Human duodenal epithelial cells were activated in pathways for division and growth subsequent to ingestion of exponential-phase *L. plantarum* cells, whereas NF-κB immune response pathways were induced in response to stationary-phase and heat-killed *L. plantarum* WCFS1 (van Baarlen et al. 2009). Therefore, it is likely that the physiological status of probiotics in common food matrices is important when aiming to understand and improve probiotic efficacy in foods.

SUBSTANTIATION OF HEALTH EFFECTS FOR PROBIOTIC FOODS

The importance of the food matrix to product functionality is currently of great relevance to those involved in the functional food industry. Regulatory authorities including EFSA, Health Canada, and the FDA are grappling with how different food matrices might influence the type of evidence required to substantiate claims of health benefits for foods. For example, although a probiotic may have been evaluated extensively as a component in yogurt, the question remains

if this evidence is sufficient support for claims made for this same probiotic being delivered, for example, in juice or dried format. The answer to this question is predicated on the extent that the matrix specifically contributes to probiotic physiology, gene expression, stability, and efficacy. Ideally, this issue would be addressed directly, with human studies establishing efficacy in different food formulations. Because cost constraints limit the extent of possible studies, a rationale for bioequivalency must be developed. In other words, use of data from one product matrix to substantiate efficacy in another product matrix would require a scientific rationale for why specific formulation changes would not be expected to change efficacy. This is extremely relevant when considering minor formulation changes, addition of other active ingredients, or product line extensions.

In some cases, it might be advisable to identify a biomarker that is tracked in all human studies dealing with a specific probiotic strain that can provide a benchmark for bioequivalency. For example, although not relevant for all probiotic applications, tracking recovery of the probiotic strain, alive, from feces of study subjects, might serve as a biomarker for equivalency of success of delivery of the probiotic in different forumlations. Although in itself such recovery is not a health endpoint, it could serve to show probiotic availability through different formats. Alternatively, the specific probiotic cell constituents responsible for conferring health effects could be monitored for amounts and stability in different consumer products and for maintenance of these levels in cells recovered from fecal samples. These probiotic effector molecules are starting to be identified and include primarily surface associated compounds including proteins (Konstantinov et al. 2008), lipoteichoic acids (Grangette et al. 2005), enzymes (Ivanov et al. 2006), and exopolysaccharides (Yasuda et al. 2008). The molecular monitoring of these cell constituents offers an exciting opportunity to select the most appropriate strains expressing optimal levels of these effector molecules and follow their levels in the food product and human gut. These measurements are especially important because it is not economically feasible to conduct controlled human studies on every conceivable product formulation. Evidence for plasticity of efficacy across different formulations could perhaps be obtained by testing clinical endpoints at the formulation extremes (for example, in yogurt and orange juice). If equivalent results are observed for the two disparate delivery vehicles, then a rationale can be provided for extrapolation to the middle ground. Lastly, understanding mechanism of action will certainly help in developing a rationale that effects should be expected in food formats different from what was specifically tested in studies. This could be further bolstered by employing in vitro or animal testing of different matrices and formulations.

DEVELOPMENT OF A DOSSIER TO SUBSTANTIATE HEALTH EFFECTS OF A PROBIOTIC FOOD

The path to developing a research dossier that provides substantiation for a probiotic food was outlined in a report issued by the Food and Agriculture Organization (2002). These guidelines describe a general approach for evaluating the efficacy and safety of probiotics, even in the absence of a legal definition of the term probiotic, as is the case in many countries. This document, along with information that has become available since its publication, indicates steps that are central to establishing a validated probiotic strain (**Table 3**). Note that particular local regulatory constraints may impact this approach. For example, a dossier submitted to the European Food Safety Authority requires clear identification and characterization of the food or ingredient, a clear statement of the nature of the claimed health benefit, a clear statement of evidence supporting the claimed benefit, and finally a rationale for why the benefit is substantiated by the evidence.

Table 3 Guidelines for development of a probiotic food. The guidelines developed in 2002 (Food and Agriculture Organization 2002) serve as a foundation for this list, but unlike the initial document, the impact of delivery vehicle is included in the criteria below

Efficacy substantiation for a probiotic food must include:
1. Thorough identification of the test product.
• Genus and species determination based on most current genetic methods
• Generation of strain-specific identification, with patterns from appropriate molecular techniques. For example, reproducible genetic methods such as pulsed field gel electrophoresis or randomly amplified polymorphic DNA analysis are useful.
• Description of the food product delivering the probiotic. Understand the extent to which the food format impacts probiotic function including, if applicable, the impact on functionality of any food transformation that results from probiotic growth.
2. Deposit of the probiotic strain in an international culture collection. This assures that a historical reference substance can be available and enables research to be repeated by independent investigators.
3. Thorough safety assessment, considering the following factors as they relate to safety for the intended use of the final product:
• Description of use (dose, format, stability)
• Validation that the product is manufactured under good manufacturing practices specific for the product category
• Survey of literature to determine to what extent the strain, species, or genus was involved in adverse events
• Description of physiological and genetic capacity for toxic activity
• Description of physiological and genetic capacity for pathogenic/opportunistic pathogenic activity
• Genetic stability of the probiotic
• Presence of transferable antibiotic resistance markers
• Review of the above information by regulatory authority or a panel of experts qualified in the field to evaluate the safety of the substance for its intended use, depending on the product category and requirements
4. Efficacy assessment. This will be specific for the particular effects being targeted.[1] Only in vitro characteristics that are confirmed to be related to in vivo functionality are useful.
• Valid in vitro assessments, as dictated by specific probiotic strain and intent of use
• Valid animal studies using test product, which might be useful for safety assessment, mechanistic studies, and suggestions of endpoints to confirm in human studies
• Human efficacy studies using test product, which provide the basis for substantiation of health benefits and the rationale for the dose being delivered in the product.
5. Product labeling (on product label or in accompanying information on product website)
• Genus, species, and strain of each strain included in the product
• Level of live microbes present in the product, through the end of shelf life
• Expiration date
• Dose to consume for labeled effects
• Description of health benefits
• Proper storage conditions
• Company contact number for answering questions and reporting any problems with the product
• Product website URL
6. Post-market surveillance

[1]The traditional assertion that probiotics possess certain traits such as specific source of isolation (human, animal, environmental, food), acid resistance, bile resistance, and other such traits may not be relevant for specific applications, and therefore cannot be considered essential.

CONCLUSIONS

Little is known about the role of delivery vehicle in probiotic functionality. Few controlled human studies have been conducted, although animal studies have provided some insight suggesting that different food formats may alter genetic expression of important functional probiotic traits. This is clearly an area where additional research is needed. Such research would contribute to

optimal product development efforts. Relevant to this issue is the fact that probiotic foods are components of varied diets both within a person and among populations, and as such are expected to function with a degree of plasticity with regard to dietary components. The current emphasis in the marketplace seems to be on probiotics as ingredients, without much knowledge of how effectiveness may vary depending on food format or the role of probiotic-induced transformations of food products that may result in improved efficacy of the food compared with the probiotic ingredient alone.

DISCLOSURE STATEMENT

M.E.S. is a paid consultant to numerous companies involved in the commercialization of probiotics.

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