Symposium: Probiotic Bacteria: Implications for Human Health

Aspects of In Vitro and In Vivo Research Approaches Directed Toward Identifying Probiotics and Prebiotics for Human Use¹

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ABSTRACT The microbiota of the human gastrointestinal tract plays a key role in nutrition and health. Through the process of fermentation, gut bacteria metabolize various substrates (principally dietary components) to end products such as short-chain fatty acids and gases. This anaerobic metabolism is thought to contribute positively toward host daily energy requirements. However, under certain circumstances, the fermentative process may produce undesirable metabolites. This may cause the onset of gut disorders that can be manifest through both acute and chronic conditions. Moreover, the gut flora may become contaminated by transient pathogens that serve further to upset the normal community structure. There has been a recent increase in the use of dietary components that help to maintain, or even improve, the gut microflora “balance.” Probiotics are live microbial feed supplements added to appropriate food vehicles (usually fermented milks), whereas prebiotics are dietary carbohydrates that have a selective metabolism in the colon and serve to increase numbers of bacteria seen as desirable. Because of their purported health-promoting properties, lactic acid–producing bacteria, including bifidobacteria, are the usual target organisms. The market value and biological potential of both approaches are enormous. This article will summarize how efficacious types can be identified. J. Nutr. 130: 391S–395S, 2000.

KEY WORDS: • gut microbiology • probiotic • prebiotic

A delicate balance exists between the human intestinal microflora and its host. Upset of this community structure may lead toward the symptoms of acute gastroenteritis, and there is also the possibility of more chronic disorders (inflammatory bowel disease, colonic cancer). It is therefore important that gut microflora interactions be controlled and sustained in an optimal manner. Many different environmental factors may affect the gut microbial ecology; these include diet, medication, stress, age and general living conditions. Knowledge of the gut microflora and its interactions has led to the development of dietary strategies that serve to sustain, or even improve normal gastrointestinal microbiology. Both probiotics and prebiotics are popular concepts that have been developed to target the gastrointestinal microflora.

Definitions and development

The Greek meaning of the word probiotic is “for life.” One early formal definition was that of Parker (1974), i.e., “Organisms and substances which contribute to intestinal microbial balance.” However, this was subsequently refined by Fuller (1989) whose revised definition is “A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.” This version emphasizes the need for the supplement to be composed of viable microorganisms and is the most widely used and accepted definition. Probiotics, therefore, aim to produce a beneficial effect on the host by administration of viable microorganisms such as those found in traditional yogurts and other fermented foods, as well as powders, tablets, liquid suspensions and lyophilized forms in capsules. Monocultures as well as mixed species (up to nine) can be used in individual products. Primarily, these are lactic acid–excreting bacteria such as lactobacilli, streptococci, lactococci or bifidobacteria, although some yeasts and other fungi are also used.

Metchnikoff (1907) first developed the concept of what we now know as probiotics at the beginning of this century. His hypothesis was that the complex microbiota of the colon was having an adverse effect on the host through what he termed the “autointoxication effect.” As such, he believed that modification of the activity of the colon microflora could occur through the ingestion of soured milks. The theory was developed after he observed that Bulgarian peasants consumed large quantities of such milks and exhibited longevity. Metchnikoff isolated the bacteria responsible and used them in human feeding trials. After Metchnikoff’s death, Rettger and colleagues became interested in the mechanism of the probiotic
effects and researched the use of intestine-derived species (Rettger et al. 1935). The field then took a number of scientific progressions to reach today's situation in which live microbial feed additions are ubiquitous. Three landmark observations were as follows: 1) germ-free animals are more susceptible to infection than are their conventional counterparts (Collins and Carter 1978); 2) oral antibiotics increase susceptibility of animals to infection (Freter 1955); and 3) administration of fecal enemas may control antibiotic-associated diarrhea (Schwass et al. 1984).

For a probiotic, the view is that the human large intestine contains indigenous bacteria that are beneficial, benign and detrimental for host health; it has been defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson and Roberfroid 1995). In this context, a probiotic is a dietary ingredient that reaches the large intestine in an intact form and has a specific metabolism therein, one directed toward beneficial rather than harmful bacteria. This would ultimately lead to a marked change in the gut microflora composition. Preferred target organisms for probiotics are species belonging to the Lactobacillus and Bifidobacterium genera. The most efficient probiotics may also reduce or suppress numbers and activities of organisms seen as pathogenic. For the substrate to be classified as a probiotic, the following three criteria should be met: 1) the substrate must not be hydrolyzed or absorbed in the stomach or small intestine; 2) it must be selective for beneficial commensal bacteria in the colon by encouraging the specific metabolism therein, one directed toward beneficial rather than harmful bacteria. This would ultimately lead to a marked change in the gut microflora composition. Preferred target organisms for probiotics are species belonging to the Lactobacillus and Bifidobacterium genera. The most efficient probiotics may also reduce or suppress numbers and activities of organisms seen as pathogenic. For the substrate to be classified as a probiotic, the following three criteria should be met: 1) the substrate must not be hydrolyzed or absorbed in the stomach or small intestine; 2) it must be selective for beneficial commensal bacteria in the colon by encouraging the growth/metabolism of the organisms; and 3) it will alter the microflora to a healthy composition by inducing beneficial luminal/systemic effects within the host.

Any food substrate that enters the colon is a potential probiotic; however, selectivity of the fermentation is a required determinant. Nondigestible oligosaccharides (NDO) are dietary substrates that seem to possess good prebiotic potential.

Much of the early and ongoing work on probiotics has been carried out in Japan. The search for bifidobacteria-promoting substances began by screening a range of carbon sources for their ability to increase these organisms in pure culture. For example, Yazawa et al. (1978) screened a range of dietary carbohydrates for their ability to promote bifidobacteria in comparison to other intestinal isolates. Further studies used mixed culture, animal models and human trials to determine the efficacy of oligosaccharides to modulate the gut flora composition (for reviews, see Crittenden 1999, Gibson et al. 1999). The term prebiotic was first coined in the mid-1990s (Gibson and Roberfroid 1995).

Methods for testing probiotics and prebiotics

Various different in vitro and in vivo approaches have been used to measure the efficacy of probiotics and prebiotics. For probiotics, "challenge tests" may be undertaken to determine survivability. Here, the test strain would be added to a fermenter and/or administered to a laboratory animal or humans. Subsequent effects, including those on immune function, lipids and pathogens, would then be determined. One important requirement is that the probiotic strain be detected in the study. Here, specific metabolic traits may be of some value, as are selected antibiotic resistance markers. However, neither is likely to be as efficacious as the use of certain molecular markers, which allow high discrimination and are genetically conserved. There have been some studies in which the green fluorescent protein is expressed by target strains and thus allows the probiotic to be "tracked" (Collins and Gibson 1999).

For prebiotics, it is important that their nondigestibility as well as selective fermentation be determined. Substrate integrity in the upper gastrointestinal tract is a requirement and can be measured using either in vitro conditions that simulate this environment or an ileostomy model (Gibson et al. 1999). A popular approach for determining bacterial fermentability is to use agar thought to be selective for gut microorganisms and measuring the response of predominant colonic genera during prebiotic fermentation. However, such methods are not wholly reliable; they do not recover the full gut diversity, and the techniques are both laborious and susceptible to operator subjectivity. There has been a large shift toward the use of molecular procedures in microbiology generally and gut bacteriology specifically. For a review of applicable techniques see O'Sullivan (1999).

The simplest in vitro fermenters are static batch cultures; the substrate is added at a known concentration to a vessel containing a fecal suspension, or defined cultures, incubated anaerobically at 37°C for a short period (usually 24–48 h) and sampled at regular intervals (Wang and Gibson 1993). This method takes a relatively short time; thus, it can act as a quick screening procedure when substrates are being compared. Moreover, only small volumes are required, which is important if the test material is in short supply. However, batch fermenters are closed systems in which the substrate is limited and the culture follows a typical bacterial growth curve; therefore, they may be used only for short time course experiments.

Continuous culture systems (chemostats) can be used to simulate the intestinal conditions more closely. By varying dilution rates and other parameters, optimum conditions for growth can be determined under steady-state conditions (Gibson and Wang 1994a). Semicon tinuous cultures in which the nutrient medium is added and spent culture removed at specific intervals have also been used. There are other variations on the single-stage chemostat approach. For example, Gibson and Wang (1994b) used a two-vessel system, with a membrane between the two chambers, called a diffusion chemostat. This allowed diffusion of growth factors and metabolites between the vessels, but not of the cells themselves. Multistage chemostats have also been developed and are used as efficient "gut models" in that each vessel represents a different physico-chemical region of the intestine. The model validated (against gut contents from human sudden death victims) by Macfarlane et al. (1998) consists of three vessels aligned in series. The first has nutrient-rich, fast transit and acidic conditions; the third has much less substrate, slow and neutral conditions (see Fig. 1 for a diagrammatic representation of the system). The first vessel is set to resemble the proximal colon, the second the transverse colon and the third the distal colon.

The ultimate test for probiotic or prebiotic functionality is the in vivo situation, in particular, well-controlled human studies. Animals, usually rats or mice, have been used to determine the effect of substrate on the fecal microflora. Animals associated with their normal flora may be used for certain effects such as gas production, weight changes and toxicology. However, differences exist between animal and human microflora; thus comparative results are likely to be compromised. Gnotobiotic rats may be used to investigate interactions between the host and the organisms, but these situations do not resemble the usual situation in the gut. Human flora-associated rats, or mice, give one representation of the situation in the human colon, although the intestinal physiology is not the same. Obviously, the best test of efficiency is a human volunteer trial with placebo control and blind coded samples. How-
IDENTIFYING PROBIOTICS AND PREBIOTICS

**Mechanisms**

Little is known about the way in which probiotics can influence the gut microflora; therefore, mechanisms for beneficial effects on the host are difficult to determine. However, it may be assumed that resistance to low (stomach) pH, bile acids and ability to compete effectively in the gut ecosystem are likely to enhance probiotic effects. For prebiotics, strain survival is not required, but the nonviable food component should enter the large gut intact and have a selective fermentation therein.

One facet that would improve survival of a probiotic agent would be an ability to attach successfully to the gut epithelium. McCartney et al. (1996) carried out a study in which ribotyping was used to identify population dynamics of lactic acid bacteria in two individuals over a 1-y period. In one volunteer, the flora existed in a relatively transient state with varying ribopatterns frequently being detected. However, in the other volunteer, some indigenous strains of lactobacilli and bifidobacteria were detectable over the entire 12-mo period. Although it is unclear whether these strains were attach-

![Diagrammatic representation of the three-stage multiple continuous culture chemostat as used to mimic the human colonic microbial environment.](image)

**FIGURE 1**

TABLE 1

<table>
<thead>
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<th>Examples of criteria that aid the selection of efficacious probiotics</th>
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<tr>
<td>● Strain origin—those isolated from the same species as the intended use ought to have an enhanced chance of survival</td>
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<td>● Safety—probiotics should be generally recognized as safe (GRAS) with minimal possibilities for the transfer of antibiotic resistance</td>
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<td>● Survivability—both in the product and after ingestion. Strains that have improved resistance to acid, bile secretions and able to attach to the gut epithelium would have better survival characteristics</td>
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<td>● Production characteristics—able to be grown in bulk culture, without genetic variation</td>
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<td>● Processing—robust enough to withstand the rigors associated with incorporation into oral delivery systems</td>
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<td>● Sensory properties—when added to foods, the quality should not be diminished</td>
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<tr>
<td>● Microbiological properties—required to survive in the gastrointestinal microbial ecosystem</td>
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<td>● Effects on the consumer—no adverse side effects such as bloating and effects on gut transit should occur</td>
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<td>● Adherence—to enhance survival in the gut</td>
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<td>● Effects on pathogens—many probiotics are able to inhibit adverse microorganisms by the production of acid, bacteriocins or competitive exclusion</td>
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<td>● Modulation of metabolic activities—such as the inactivation of procarcinogens</td>
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<tr>
<td>● Immunomodulation—probiotics may affect the immune system such that improved pathogen resistance occurs, as well as positive aspects with respect to food allergy</td>
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the application of molecular biological techniques to human gut bacteriology are of enormous value in this respect in that they offer the degree of fidelity, automation and discrimination that is required.

Fructooligosaccharides are the most extensively studied NDO in terms of their prebiotic properties. These carbohydrates contain both G₄,F₉ (α-D-glucopyranosyl-[β-D-fructofuranosyl]₃,1-D-fructofuranoside) and F₆,F₉ (β-D-fructofuranosyl-[β-D-fructofuranosyl]₃,1-D-fructofuranoside) molecules, with the number of fructose units varying from 2 to >70. They are available either as inulin, which is the storage carbohydrate in many thousands of plants, or can be synthesized enzymatically from sucrose (Van Loo et al. 1995). Bifidobacteria possess a cell-bound β-fructofuranosidase enzyme that allows preferred utilization of fructooligosaccharides over sucrose (Muramatsu et al. 1994) and clearly offers this genus a competitive advantage in the human gut. The fructose moiety is then metabolized in the specific “bifidus” pathway. Similarly, bifidobacterial α-galactosidase activity likely allows a prebiotic effect for soybean oligosaccharides (Desjardins et al. 1990). Galactooligosaccharides are manufactured from lactose by transglycosylation reactions and consist of galactosyl derivatives of lactose with β1–3 and β1–6 linkages. The purported prebiotic nature of galactooligosaccharides may be due to the linkage-specificity of the Bifidobacterium β-galactosidase (Dumortier et al. 1994). Isomaltooligosaccharides (α1–6 linked) and glucooligosaccharides (β1–6 linked) are candidate prebiotics, as are xylooligosaccharides. However, specific enzymes for the degradation of these molecules have not yet been evaluated; thus the explanatory mechanism for any purported prebiotic effect is not yet evident.

**SUMMARY**

This article has introduced the rationale for probiotic and prebiotic use in humans. Both approaches serve to improve the gut microflora composition because the importance of intestinal microbiology in health and disease is now clear. Other reviews in this series will identify the possible health outcomes. However, it is clear that microflora management through diet is achievable. Importantly, the scientific tools for determining probiotic and prebiotic effects now exist and should be exploited. In particular, this involves a molecular approach to the fermentation process in conjunction with well-controlled human trials.

**LITERATURE CITED**


