A REPORT FROM THE AMERICAN ACADEMY OF MICROBIOLOGY

PROBIOTIC MICROBES:
THE SCIENTIFIC BASIS

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MICROBIOLOGY

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PROBIOTIC MICROBES: THE SCIENTIFIC BASIS

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John Cebra was the inspiration and driving force behind this AAM colloquium to critically examine the potential that probiotics offer humankind. Unfortunately, John died shortly before the colloquium was held. Yet, thanks to the outstanding efforts of the Organizing Committee under John's direction, the colloquium participants, and Carol Colgan and her staff of the American Academy of Microbiology, the meeting proceeded flawlessly and was a huge success. Had John been there, he not only would have experienced an enjoyable and stimulating meeting, but I believe that he would have been satisfied that his vision was realized. My personal thanks to Carol Wells and John Bienenstock, who stepped in at the last minute when my own illness prevented my full participation in the meeting, and everyone else who worked on this meeting and enabled the production of this report.

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EXECUTIVE SUMMARY

The American Academy for Microbiology convened a colloquium November 5-7, 2005, in Baltimore, Maryland, to deliberate the current state of knowledge regarding probiotics. Participants with expertise in microbiology, medicine, periodontics, animal science, immunology, nutrition, and other relevant fields conferred on potential applications of probiotic therapies, regulatory issues, the human acquisition of normal microbiota, interactions between commensal microorganisms and their hosts, interactions among commensal microorganisms, and the identification of prospective probiotic organisms. Recommendations for ensuring the safety and efficacy of probiotic therapies were made.

The definition of "probiotic" set forth by the United Nations Food and Agricultural Organization and the World Health Organization in a joint report on the topic identifies probiotics as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). This definition was adopted by the colloquium participants and is the one used in this report.

In theory, microorganisms could be used to combat pathogenic microorganisms and the diseases they cause. They could also conceivably be used to prevent infectious diseases and immune dysfunction. Some probiotic formulations have been subjected to more scientific scrutiny than others, and many of these applications show considerable promise for alleviating certain illnesses. Many combinations of therapies are being evaluated for the treatment and prevention of various diseases and conditions.

Humans are colonized by bacteria, viruses, and other microbes from birth; we live with a suite of commensal microorganisms from that moment on. Neonatal gut colonization is strongly affected by the mode of birth. In vaginally-born infants, colonization follows a more or less predictable course, starting with certain groups of facultative anaerobic bacteria, then obligate anaerobes. The distribution of microorganisms within the adult gastrointestinal tract is difficult to determine given the current technical limitations on sampling and analysis. Studies indicate that the gut microbiota is relatively stable in an individual over time and that great variability in the composition of these microbial communities exists between individuals.

Interactions between a host and its commensal microorganisms, and the interplay between the commensal microorganisms themselves, can be critical to the health of a host. Proper development of the human immune system is reliant on microbial stimulation. Probiotic organisms may have the potential to interact with both the innate and acquired immune systems with possible benefits to the host.

In identifying prospective probiotic organisms for use in humans, particular attention must be placed on the safety of the organism for a wide variety of individuals, including the immunocompromised, the very young, and the aged. A number of *in vitro* test systems and *in vivo* models are now available for studying probiotics prior to their use in human subjects, although relevance of the resulting data may not be predictable.

INTRODUCTION

Are probiotics the future of medicine? Theoretically, beneficial microorganisms could be used to treat a range of clinical conditions that have been linked to pathogens, including gastrointestinal problems like irritable bowel syndrome and inflammatory bowel disease (e.g., ulcerative colitis and Crohn's disease), oral diseases like tooth decay and periodontal disease, and various other infections, including vaginal infections and possibly skin infections. Probiotics could also conceivably be put to use in preventing disease or thwarting autoimmune disorders. A number of these possibilities are being explored in research laboratories and hospitals around the world.

Probiotics are not only making an impact in research; they are also turning heads in the global marketplace. Today, hundreds of probiotic foods and dietary supplements that offer a variety of health benefits are available to the consumer. Vendors of powdered probiotic dietary supplements declare their products aid in constipation and fatigue. Bottles of pills with tailored mixtures of bacteria are touted as cancer preventatives and treatments for high cholesterol. Traditional foods with microbial components, such as *kombucha*, a fermented tea spiked with strains of yeast and bacteria, and *kefir*, a fermented milk drink, are widely believed to ameliorate conditions ranging from indigestion to migraines. Probiotics are even used in companion and farm animals. The global market for commercial probiotic products is now estimated at billions of dollars per year (Stanton, et al., 2001).

The buzz about probiotics has become a roar. But what can beneficial microorganisms really accomplish? Can these products benefit human or animal health? When it comes to probiotics, what is real and what is fiction?

The science surrounding probiotics is maturing, and it is now possible to investigate probiotics with more rigor and detail than ever before. Great leaps in technology have given rise to new methods in molecular biology, genomics, and clinical science that can be used to investigate probiotic functions and impacts. Medicine has provided important new insights into the human body, immunity, and disease. Finally, as interest in probiotic therapies increases, more and more scientists and institutions are becoming involved in researching the possibilities behind these treatments.

In light of the current public and scientific interest in probiotics and the newly revealed possibilities for scientific exploration and discovery, an evaluation of the current state of knowledge about probiotics is required. The American Academy of Microbiology convened a colloquium in November 2005 to discuss these issues, and this report represents a unified effort by the 38 professionals attending the colloquium to summarize the lessons probiotics have offered about the relationships between microbes, immunity, and disease, evidence behind probiotic therapies that are in use today, and the possibilities these therapies might offer in the future.

The precise definition of the term "probiotic" has been the subject of considerable debate. There is disagreement about whether dead or deactivated microorganisms or microbial products should be included in the term. Another contentious issue is whether the definition of a probiotic treatment should include a stipulation about the effective dose. For purposes of this report, colloquium participants adopted the definition of probiotics developed by the Food and Agriculture Organization of the United Nations and the World Health Organization in their joint report on probiotics published in 2001 (FAO/WHO). According to this definition, probiotics are:

...live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host.

Probiotic treatments are used not only for the benefit of human health; they are also routinely applied in livestock production. Unless otherwise specified, references to "probiotics" in this document include treatments for both humans and animals. Hence, references to "hosts" include both human and animal subjects.

"UNTIL NOW, THE DESIGNATED USAGE OF MOST PROB
SUPPORTIVE TREATMENT OF DISEASES, NOT CURATIVE

WHY ADMINISTER MICROORGANISMS TO A HOST?

Probiotic therapies have been developed to address the health concerns of both sick and healthy individuals. The case for developing probiotic therapies to treat ailing individuals is clear. In theory, altering a human's microbiota, and thereby immunologic defenses, could be useful in managing certain diseases and infections (Dugas, 1999; Wills-Karp, 2001; Yazdanbakhsh, 2002; Teitlebaum and Walker, 2002). Hence, treatments for disease are an understandable application for probiotics, but why would science and medicine aspire to develop probiotic products for healthy people? To date, there is no conclusive evidence that altering the microbiota of a healthy human adult is beneficial, but probiotics that alter the normal commensal microbiota of healthy farm animals are widely and routinely used to prevent certain conditions, including gastrointestinal distress resulting from switching feed types, for example. Similarly, there are a number of conceivable scenarios in which adjusting the microbiota of healthy human subjects could have a beneficial prophylactic effect. For example, displacing the oral bacterium *Streptococcus mutans* with a probiotic strain of bacterium that does not produce enamel-eroding acid could reduce the risk for tooth decay in otherwise healthy people.

Modulation of the host immune system in sick or healthy patients is another important potential effect of probiotic therapies. Recognition of microbes by the host is based upon recognition of conserved signature molecules in microorganisms, called Microbe- (or Pathogen-) Associated Molecular Patterns (MAMPs). Pattern Recognition Receptor (PRR) families mediate the response of host immune cells to MAMPs that include the Toll-Like Receptor (TLR) family. Microbes recognized by PRRs expressed by host cells may have the ability to influence the host immune system, physiology, and metabolism. The "hygiene hypothesis" suggests that allergies and autoimmune diseases are caused or aggravated by depriving the immune system of microbial stimulation during its development (Shanahan, 2000; Willis-Karp, 2001; Yazdanbakhsh, 2002; Liu and Murphy, 2003; Sartor, 2004; Strachan, 1989; Feillet and Bach, 2004; Warner, 2003; Sheikh and Strachan, 2004; Liu and Murphy, 2003; Smit, Folkerts and Nijkamp, 2004; Rautava, et al., 2004). If the hygiene hypothesis is correct, and the human immune system requires the stimulation of microorganisms in order to fully develop, an argument can be made that humans living in developed nations (where hygiene standards are high and bacterial exposure is low) ought to be exposed to supplemental bacteria at an early age. It is unclear whether exposure to any particular strains is helpful or necessary, however. Also, there is some evidence from animal models that shows autoimmune diseases may be influenced by the administration of bacteria (Oliviera, 2005; Souza, et al., 2004).

In practical terms, the ability of a probiotic to alter the host microbiota or host MAMP-recognizing molecules could have several beneficial outcomes for the host, including:

- Anti-infectious properties,
- Immune modulatory effects,
- Enhanced barrier functions,
- Metabolic effects, and
- Alterations of intestinal mobility or function.

Careful experimentation and clinical research suggest that probiotics can elicit many of these outcomes, but the efficacy and reproducibility of probiotic treatments has yet to be demonstrated.

It should be noted that certain probiotic formulations have had detrimental effects on hosts. Like any immune reaction, immune responses to probiotic organisms can have both positive and negative effects. For example, administering *Lactobacillus rhamnosus GG* (LGG) to Crohn's patients actually worsened the condition of some individuals. Also, in at least one study in which LGG was administered to babies prenatally, the bacterium produced significant reductions in the rate of atopic eczema by the time the children reached two and four years of age, but it also apparently brought about a slight (but not statistically significant) increase in the rate of respiratory allergy by four years of age (Kalliomaki, et al., 2003).

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EXAMPLES OF PROBIOTIC USE THAT BENEFIT HUMAN AND ANIMAL HOSTS

Until now, the designated usage of most probiotics in humans has been prophylaxis or the supportive treatment of diseases, not curative treatment. Of the curative treatments, there are many examples that have demonstrated health benefits, but the efficacy of most formulations is low, and the effects that have been measured have been difficult to differentiate from placebo effects. The list below includes some of the disorders for which probiotic therapies have been tested in people. The outcome of testing is briefly described.

TARGET DISORDERS

Diarrhea

The clearest example of an effective probiotic therapy may be the use of beneficial bacterial strains to treat diarrhea resulting from rotavirus infection. Several probiotics have been shown to shorten the duration of acute watery diarrhea caused by rotavirus in children (Szajewska, et al., 2001; Van Niel, et al., 2002). Other causes of diarrhea may also be addressed through probiotics, as demonstrated by the effective use of the yeast *Saccharomyces boulardii* to reduce the recurrence of *Clostridium difficile*-induced diarrhea in elderly patients (Kotowska, et al., 2005). There is also some success with other causes of diarrhea in infants (Correa, et al., 2005; Figueiredo, et al., 2001).

Pouchitis

Pouchitis is a recurrent inflammatory condition in the ileal pouch, a cavity constructed after surgery to remove the colon. Pouchitis is most common in patients whose colons have been removed because of ulcerative colitis and is rare in individuals whose colons have been removed because of cancer or trauma. Hence, the condition must be linked in some way to ulcerative colitis. Probiotics have proven effective as therapy for pouchitis and as prophylaxis to prevent the condition (Gionchetti, et al., 2000; Gionchetti, et al., 2004).

Irritable Bowel Syndrome

Certain probiotic preparations appear to be effective in the treatment of irritable bowel syndrome and are accompanied by changes in cytokine profiles that suggest anti-inflammatory mechanisms (O'Mahony, et al., 2005). This report indicated for the first time in an extensive blinded randomized controlled study that one bacterium was effective in regulating both inflammatory cytokines and also symptoms, whereas the other probiotic was not.

Bladder Cancer

Administration of probiotic doses of *Lactobacillus casei* may be effective in reducing the recurrence of bladder tumors (Hoesl and Altwein, 2005).

Urogenital Infections

Clinical trials have now been performed which substantiate the beneficial effects of probiotic strains of some lactobacilli against urogenital infections, such as urinary tract infections and bacterial vaginosis (Hoesl and Altwein, 2005).

Clostridium difficile Infection

Treatment of patients with *C. difficile* infections with *Saccharomyces boulardii* may be effective in shortening the duration of infection (Castagluiuolo, et al., 1999).

Atopic Eczema

Oral administration of *Lactobacillus rhamnosus* and *Lactobacillus reuteri* is beneficial in the management of atopic dermatitis (Rosenfeldt, et al., 2003). The preventive effect of probiotic flora on this condition may extend beyond infancy (Kalliomaki, et al., 2003)

Many probiotic formulas have also been used in farm animals – some with great success. *Lactobacillus reuteri* is used in chicken and turkeys to prevent infections and support growth and development. Other strains, including *Propionibacterium* species, *Lactobacillus* species, *Bacillus cereus*, *Bacillus licheniformis*, and *Saccharomyces cerevisiae* have been put to use in production animals to reduce adverse effects resulting from dietary changes. In the European Union, mandated withdrawal of the use of antibiotics as livestock growth stimulators has led the farming industry to apply probiotic therapies more routinely than farmers in the United States and other countries.

"GIVEN THE POTENTIAL HAZARDS INHERENT IN ADMIN
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POTENTIAL FUTURE APPLICATIONS OF PROBIOTICS

Given the possible benefits of using probiotics, many potential applications for these therapies can be identified. They include, but are not limited to:

- *Biotherapy using antibiotic-sensitive bacteria to displace resistant strains.* Colonization of the gut by antibiotic-resistant bacteria, including vancomycin-resistant enterococci, is a growing problem. It may be possible to replace a resistant enterococcus in the gut with a sensitive one.

- *Preventing translocation of bacteria* (the occurrence of bacteria beyond the skin or mucosal barriers). "Mucosa" is a term that describes the membrane lining of all body passages. Patients suffering from burn, shock, trauma, and immunosuppression are at high risk of infection resulting from bacterial translocation. This could possibly be prevented using benign probiotic organisms to counter pathogens on the skin and mucosa.

- *Probiotic supplementation to encourage weight gain.* The administration of *Bifidobacterium* to newborns and lactobacilli to children with AIDS may have modest benefits in encouraging weight gain.

- *Microbiota removal.* There are a number of scenarios in which it could be advisable to use probiotic organisms to rid the body of certain bacterial species, including *Helicobacter pylori* in patients with gastric ulcers and *S. aureus* in patients with Wegener's vasculitis. The approach may also be advisable in the future for patients with conditions for which the causative organism has yet to be identified, including necrotizing enterocolitis and inflammatory bowel disease. However, high quality studies documenting the effectiveness of microbial elimination are lacking in most cases.

- *Restoring microbiota disturbed by antibiotic treatment.* Children who suffer from recurrent ear infections frequently experience antibiotic-associated diarrhea. These patients could benefit from probiotic therapy to re-establish a normal suite of gut microorganisms following antibiotic treatments.

- *Custom-molding the degradative capacity of the microbiota to the nature of the diet in order to encourage adequate nutrient absorption without unwanted weight gain.*

- *Using probiotic organisms to improve oxalate metabolism in order to reduce the incidence of kidney or bladder stones.*

- *Degradation of chemicals of potential harm, particularly in cases of chronic exposure, e.g., workplace exposure.*

- *Suppression of the pathogen S. aureus and Clostridium difficile in hospital patients.*

- *Prevention of bladder infections.*

CAN MICROBIAL END PRODUCTS REPLACE PROBIOTICS?

Given the potential hazards inherent in administering a dose of live organisms to patients, including the risk of infection, it may be desirable in some cases to replace live microbes with isolated microbial components or end products. Further, this approach may be more efficient and effective than adding live cells. In the future, once the bioactive molecules at work in effective probiotic organisms are identified, these materials will likely be used in their pure forms. End products do not fall within the limits of the designation "probiotic," but, rather, might be identified as "derived from probiotic organisms." Some candidate products include Short Chain Fatty Acids (SCFA), cell wall peptidoglycan, and Deoxyribo-Nucleic Acid (DNA).

SCFAs are catabolic and anabolic products of anaerobic intestinal microbial metabolism of compounds deriving from exogenous and endogenous sources. The term SCFA includes acetic acid, propionic, butyric, isobutyric, valeric, iso-valeric, caproic, and iso-caproic acids. They are postulated to provide 10% of the caloric requirement in humans and much more in ruminants. Most of them are absorbed and are used as substrates for fat synthesis (acetate), gluconeogenesis (propionic acid), or conversion into various amino acids for subsequent use as carbon skeletons in nitrogen metabolism (valeric and

ISTERING A DOSE OF LIVE ORGANISMS TO PATIENTS, ABLE IN SOME CASES TO REPLACE LIVE MICROBES ODUCTS."

caproic acids). Butyrate is the preferred energy source of colonocytes and is a major controlling factor for their growth and differentiation (Litvak, et al., 1998). It was shown to inhibit the genotoxic activity of nitrosamides and hydrogen peroxide in human colon cells (Wollowski, et al., 2001) and to induce apotosis in human colonic cell lines in a p53-independent pathway (Hague, et al., 1993). SCFA may help maintain the integrity of the intestinal mucosa during periods of parenteral nutrition (Koruda, et al., 1990).

Short DNA fragments containing CpG sequences can have beneficial probiotic effects. CpG is a site in DNA where a cytosine (C) is adjacent to a guanine (G), and the "p" indicates a phosphodiester bond between the two bases. Methylation, which affects gene activity and gene expression, can occur at CpG sites, and it may be responsible for the observed immune effects on the host. Administering bacterial-derived CpG to newborn pigs has been shown to awaken the acquired immune system, allowing the pigs to respond to T-dependent antigens, Type 2 T-independent antigens, or to lipopolysaccharides (Butler, et al., 2005), abilities they do not otherwise possess until they have been colonized by commensal bacteria.

Strong evidence is available that bacterially-derived DNA can induce the production of defensins (antimicrobial proteins) in special defensive cells in the small intestine, called Paneth cells (Eckmann, 2004).

REGULATION OF PROBIOTICS

PLACEBO EFFECTS

There is a need for properly designed prospective clinical trials to evaluate probiotics for therapeutic uses. In general, characteristics desirable in such clinical studies include a high quality product and placebo, enrollment of a well-defined population (or population subset) of consenting subjects, broad sampling of host microbiota, use of validated testing instruments in determination of clear, clinically meaningful, endpoints, and adequate statistical power. It should be noted that placebo effects can be very strong in trials of probiotics, particularly with respect to products in the self care market, a category in which most current probiotic formulations fit (Beecher, 1959).

REGULATORY CONSIDERATIONS

In the U.S., probiotics are commercially available as foods, a category which includes dietary supplements. At present, no probiotic product is licensed in the U.S. as a biological drug product for use in the treatment, prevention, cure, mitigation, or diagnosis of a specific human disease condition. Commercially available probiotic products are manufactured according to food regulations, not the more stringent biological drug regulations.

A quality clinical study begins with a quality product. At present, the quality of probiotics available to consumers in food products around the world is unreliable, though some manufacturers have been better able to deliver high quality than others. Probiotic products that make false claims or fail to deliver promised doses of active organisms harm the reputation of valid probiotic research and stymie future work. Testing to ensure the identity of the organisms within probiotic products, the potency of those organisms, and the purity of those products needs to be put in place. Data indicate that the organisms cited on the labels of certain probiotic products are not actually contained within the product (Huff, 2004; Elliot and Teversham, 2004). With respect to the potency of these formulations, the numbers of viable bacteria within a product should be stated on the packaging and verified by regulators. The shelf life of these products also needs to be advertised to the consumer. Finally, the number of other organisms present in a product needs to be evaluated and kept within the limits allowed by food standards when intended for use as food.

REGULATORY CONSIDERATIONS

The application of probiotics to prevention or treatment of specific diseases will require attention to regulatory considerations not necessarily encountered in the health food market. These considerations were reviewed in a presentation to the colloquium participants by Jennifer Ross of the U. S. FDA and are summarized below:

- Intended use of a product determines how a substance is regulated by the U.S. FDA. Probiotic-type products are mainly regulated as foods or as biological products at FDA.

- The Center for Food Safety and Applied Nutrition (CFSAN) at FDA regulates probiotic products under the broad category of food, including dietary supplements. Briefly, a dietary supplement is an orally administered product intended to supplement the diet (1). Limited labeling claims may be made for products regulated as foods, including dietary supplements (Dietary Supplement Health and Education Act) (2).

- A long history of use of a microorganism in food may not in itself be supportive of administration to humans for clinical uses. A drug is an article intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease (Food Drug &Cosmetic Act United States Code (U.S.C.) Title 21, Chapter 9) (2). Traditional drugs are small, chemically synthesized molecules. A biological product is a type of drug that contains any virus, therapeutic serum, toxin, anti-toxin, or analogous product (Public Health Service Act of 1944, 42, U.S.C. 262) (2). Historically, the term "virus" conceptually referred to products containing minute living causes of infectious disease. In modern times the definition of a virus in this context encompasses products containing microorganisms such as bacteria, fungi, and protozoa. The Center for Biologics Evaluation and Research (CBER) at FDA regulates biological products, including probiotic-type products, for human clinical use (3).

- Within CBER, the Office of Vaccines Research and Review (OVRR) has had regulatory jurisdiction over most probiotic-type products to date. To avoid confusion with, and differentiate from, the many definitions of "probiotic" the term Live Biotherapeutic Product (LBP) is used to refer to products containing whole, live microorganisms (i.e., bacteria, yeast) with an intended therapeutic effect (i.e., cure, treat, prevent, mitigate, diagnose a disease or condition) in humans regardless of the route of administration. LBPs may contain commensal microorganisms isolated from a healthy human host and/or recombinant microorganisms.

- A Biologics License Application (BLA) (4) is submitted when a license to market a biological product, such as a LBP, in the U.S. for a specific disease claim is desired. Typically, a BLA includes data from a series of clinical trials conducted under an Investigational New Drug Application (IND) (5). Generally, the clinical trials were prospectively designed to evaluate safety and efficacy of a product for a specific clinical indication. A clinical indication is usually defined by a disease or condition as well as by a population (i.e., children or adults). An IND includes (i) cover sheet, (ii) table of contents, (iii) introductory statement and general investigational plan, (iv) description of the composition, manufacturing process, and control testing of the drug substance and drug product, (v) pharmacological and toxicological studies of the drug *in vitro* or in animal models to support the proposed clinical investigation, (vi) previous human experience, if any, (vii) proposed clinical study protocol, and (viii) any other information deemed relevant for review. Of note, current Good Manufacturing Practices (cGMPs) for biological products differ from those for foods and dietary supplements (5). A biological product must be shown to be safe, pure, potent, and manufactured with lot-to-lot consistency. In preparation of an IND for a LBP, including manufacturing information, guidance documents for vaccines are useful because vaccines can be live bacterial products (6).

- Plans for submission of an IND can be discussed with FDA prior to formal filing of an application. Those contemplating clinical research are advised to consult with FDA early to determine whether IND regulations apply to a particular situation (7). Many clinical studies conducted strictly for research purposes, as opposed to licensure, i.e. marketing approval, also require an IND. When an IND is required, a pre-IND meeting may be requested to facilitate compliance with regulations and identifying regulatory challenges prior to investing in the time and resources to submit an IND (6).

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HUMAN ACQUISITION OF GASTROINTESTINAL, VAGINAL, AND ORAL MICROBIOTA

We live in a microbial world. Over the millennia, humans have evolved in the presence of microorganisms, and we have adapted to their activities within and around our bodies. Indeed, it is neither advisable nor possible to separate an adult from the microbiota that populates his or her skin, gut, and other orifices, for eradication of these commensal microorganisms could compromise many critical bodily functions and possibly lead to chronic diarrhea, vitamin deficiencies, inadequate immune tolerance of foods, and opportunistic infections. Colonization by commensal organisms is crucial for the proper development of both the innate and adaptive immune systems (Duarte, et al., 2004; Neumann, et al., 1998; Souza, et al., 2004; Oliviera, et al., 2005) ***(see section Host-Microbe and Microbe-Microbe Interactions)****. Moreover, the presence of commensal organisms in the host can act as a barrier that prevents colonization by potential pathogens. Although a diverse microbiota provides some ability to counteract colonization, it has been found that bacterial members of the microbiota generally compete with and specifically occlude invading strains of the same genus or family (Freter, et al., 1983; Wells, et al., 1988; van der Waaij, et al., 1971). This is probably due to the fact that closely related bacteria share many of the same requirements for substrates and physical niches and can produce bacteriocins.*

Despite the importance of microorganisms to our well being, the general public, particularly in the United States, is possessed of a fear of these unseen legions. Microbes are viewed by many as "germs" that need to be cleaned from the body and its surrounds. One way to inform the public about "good and bad" microbes would be through public education campaigns.

HOW DO MICROORGANISMS COLONIZE THE HOST?

Any contact between a human and microorganisms may lead to colonization, including the consumption of microbe-laden food and interactions with animals and other people. Microbial colonization in humans takes place from the moment an individual is born, possibly even during birth.

Human infants and livestock young are sterile prior to birth, and in mammals, which give birth to live young, primary colonization occurs directly afterwards and largely depends on the mother. Upon exposure to the mother's body and to various other environmental sources, the gut, skin, and upper respiratory tract are colonized by an array of microorganisms. In the gut, this colonization takes place in a more or less orderly fashion that is probably dependent on the availability of anaerobic spaces to colonize. Some studies indicate that bacteria, including *L. reuteri* and *L. gasseri*, can be passed from mother to infant in breast milk.

Anaerobic bacteria, which thrive in the absence of oxygen, are probably transferred to an infant by the mother during vaginal birth. However, obligate anaerobes cannot proliferate freely in the gut of a newborn until oxygen has been consumed by facultative anaerobes—microbes that can grow either in the presence or absence of oxygen. Once the oxygen has been consumed, the earliest obligate anaerobic colonizers of the gastrointestinal system, including bacteroides, bifidobacteria, and clostridia, establish themselves.

A complex antagonistic relationship between obligate and facultative anaerobes exists in the gut, and a large number of obligately anaerobic species may be required to overcome the facultative strains (Freter, 1983; Freter, et al., 1983). Obligate anaerobes produce compounds that appear to interfere with the metabolic activities of facultative organisms. Over time, as more and more obligate anaerobic species are acquired and establish themselves, the number of facultative

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anaerobes dwindles, and a complex microbiota develops. In infants, this process of transforming a completely sterile gut habitat into a stable community of microbes takes approximately two years (Midtvedt and Midtvedt, 1992). In the end, obligate anaerobes outnumber facultative anaerobes by 1,000:1.

Infants in developing countries are more rapidly colonized by many types of commensal bacteria than their counterparts in industrialized nations, apparently because of differences in hygienic standards. In industrialized countries, there is evidence that intestinal colonizers, like *Escherichia coli*, which formerly colonized all human infants within a few days of birth, are less abundant than they once were. Consequently, it takes approximately six months for the average infant to acquire *E. coli* in western countries (Nowrouzian, et al., 2003; Adlerberth, et al., 2006), and bacteria that were formerly most often associated with skin colonization, like *Staphylococcus* species, are now among the first gut colonizers in these infants (Lindberg, 2004).

Birth by cesarean section also appears to affect the sequence of bacterial colonization. Infants born by cesarean section exhibit delayed acquisition of bacteria, most notably *Bacteroides* species and *E. coli*. Other bacterial groups, including *Clostridium* species which form spores and are abundant in all environments, are found at higher frequencies in the guts of cesarean section-delivered infants than in vaginally-delivered babies.

There are pronounced differences in the ease by which different types of bacteria are acquired by the host, some of which could be explained by differences in bacterial abundance in the environment or by the mode of colonization. Most microorganisms in the intestines are exchanged via the fecal-oral route. Some bacteria, including *Bacillus* and *Clostridium* species, are known to circulate in the air and can achieve colonization by settling on the skin, eyes, and mouth, or in the lungs.

Studies of twins show that the final composition of the gut microbiota is determined, at least in part, by host genetics. Microbial communities in the guts of homozygotic (identical) twins are more similar to one another than are those in the guts of dizygotic (fraternal) twins (Zoetendal, et al., 2001).

CHARACTERIZING THE INTESTINAL MICROBIOTA

The commensal microbiota of the human intestine is an extremely complex community of organisms. Although these communities vary greatly between individuals, in general they consist of hundreds of different species, most of which are obligate anaerobes. A number of different approaches are available for characterizing these communities, but researchers have yet to exhaustively catalog the diversity of the human gut microbiota.

Regrettably, complete descriptions of the intestinal microbiota of humans and animals are not available. Hence, an inventory of all the microorganisms that can influence a host cannot yet be compiled. Microbes of all varieties, including bacteria, viruses, archaea, fungi, and protozoans, can be part of the commensal microbiota of humans and could, in theory, be used as probiotics. Worms and worm eggs, including the intestinal helminth *Trichuris suis*, may align with many of the concepts of probiotic therapies, but because they are not microorganisms they are not currently classified as "probiotic" organisms.

Classical investigations that require the cultivation of hundreds of microorganisms (Finegold, et al., 1974; Holdeman, et al., 1976; Moore and Holdeman, 1974) can be time consuming and are not considered affordable today. Moreover, cultivation captures only 40-50% of the bacteria in the human microbiota as identified by microscopy and can, therefore, fail to account for a great deal of the diversity in these communities.

The best descriptions of the intestinal microbiota available today were obtained through the use of techniques that exploit the 16S ribosomal RNA gene, a sequence that can be used to identify a bacterium and determine its relatedness to the rest of the bacterial domain (Eckburg, et al., 2005; Suau, et al., 1999). A great deal of untapped diversity has been uncovered through these studies. Unfortunately, these methods have been applied in examining the microbiota of only a limited number of individuals, so a wide sampling of the diversity of this ecosystem is still lacking.

Many of the techniques for fingerprinting microbial communities, including Temporal Temperature Gradient Gel (TTGG) and Terminal

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Restriction Fragment Length Polymorphism (T-RFLP) analyses, use variations in 16S rRNA gene sequences to discern between different bacteria, but these methods can only detect populations that exceed 108 colony-forming units per gram of sample (CFU/g) (Seksik, et al., 2003; Zoetendal, Akkermans and de Vos, 1998). Many of the species that establish smaller populations in the intestine, including *E. coli* and *S. aureus*, are known to play significant roles in health and, in order to detect these minority groups by fingerprinting methods, specific probes and primers must be used to specifically target these groups. Hence, the current fingerprinting methods are only capable of detecting numerically dominant populations and groups, many of which have been discovered and characterized previously (Zaborovsky, et al., 2003). It should be kept in mind that 50% of the dominant gut microbes have yet to be described and are only accessible using molecular tools.

Metagenomic approaches, in which the DNA of a microbial community is extracted and analyzed as a whole, have also offered insights into the intestinal microbiota. Attempts have been made, for example, to use metagenomic techniques to characterize the entire microbiota of an individual. Metagenomics has inherent limitations, however. For example, only the numerically dominant groups of bacteria present in a sample are detected in most metagenomic analyses. In the future, this weakness could be overcome by using techniques to specifically isolate DNA from rare microbial groups in samples prior to analysis, but this capability has yet to be developed.

It is often contended that the physiology of commensal bacteria is more germane to the issue of human health than is the phylogeny of those organisms. By extension, it can be argued that, in researching the functions these organisms carry out in their ecosystem, analysis methods that characterize the physiology of the bacteria in the gut are, therefore, more useful than methods that classify them phylogenetically. On the other hand, probiotic organisms often have immune effects, and closely related organisms may share similar impacts on the immune system. For example, pattern recognition molecules in the host recognize structural details in the peptidoglycan backbone in the cell membranes of bacteria. These details may be shared among related classes of bacteria. Hence, both phylogeny and physiological capabilities can be important to understanding probiotic functions, and both require further research.

DISTRIBUTION OF THE MICROBIOTA IN THE HUMAN GUT

In addressing the question of spatial distribution within the human digestive system, the ability to sample accurately is of utmost importance, but technical difficulties in sampling continue to stymie efforts to determine the niche preferences of our microbial symbionts. Nevertheless, some deductions and observations about microbial distributions in the human gastrointestinal tract have been made.

One study has shown that the community of commensal bacteria within the lumen (the cavity or open space of the gut) does not differ in composition from the community associated with the mucous that lines the gut and the epithelium (the surface membranes). It has yet to be conclusively demonstrated, however, whether commensal bacteria in a healthy individual adhere to the epithelium of the gut or whether they are exclusively found in the lumen and in the mucous layer. Moreover, some studies have shown that, within the mucous layer, bacteria prefer sites close to the epithelium, while other studies have been unable to demonstrate such a preference. Technical difficulties associated with sampling and *in situ* analyses within the gut currently limit thorough exploration of spatial organization within the gut community.

Although microbial adhesion to the healthy epithelium has not been conclusively proven, it is probable that adhesins (which are proteins on the surface of bacteria that bind to receptor molecules on the host and allow the bacterium to adhere) are useful to commensal bacteria. Studies have shown that *E. coli* strains that are capable of colonizing a host for long periods of time are more likely to carry genes for adhesins than are their more transient relatives, suggesting a role for adhesion in the ability of a bacterium to colonize the gut. It is possible that, in the gut, adhesins are used not only for binding to surfaces but also for adhering to mucus or to dislodged epithelial cells that have been shed from the mucosa. In order to persist in the gut, a microbe must divide and produce progeny faster than the rate at which those progeny are moved out of the system (Lee, et al., 2004). Hence, it may not be necessary to adhere to the epithelium in order to colonize the gut, but adhesion to gut contents may help a strain to persist in spite of slow rates of replication (Freter, et al., 1983).

While it is not clear whether commensal bacteria normally adhere to the gut epithelium in healthy individuals, adherence to the gut epithelium has been associated with certain disease states. For example, there is some evidence that bacteria adhere to the epithelium in individuals with Inflammatory Bowel Disease (IBD), and that these epithelial populations differ from the non-epithelial community.

STABILITY OF THE HUMAN MICROBIOTA

Microbial colonization is the persistence and replication of a microorganism in an ecological niche. A given strain of bacteria may colonize the human gastrointestinal tract for any length of time—anywhere from a few days up to several years.

In examining the gut microbiota in a healthy individual, the apparent stability of the community probably depends on the level of phylogenetic resolution at which community stability is assessed. At coarse levels of phylogenetic resolution (such as at the group or phylum level as determined by 16S rRNA gene-dependent techniques), some studies have shown that individuals have a relatively stable gut community while at the strain level gut communities are relatively stable in some individuals but highly variable in others (McCartney, et al., 1996; Kimura, et al., 1997). It is suspected that stability at the group level masks a high turnover of individual strains within a group or phylum. Members of the genera *Bacteroides* and *Clostridium*, for example, are present for many years in the microbiota of an individual, but there may be constant renewal of individual strains that belong to these groups. The turnover rates of some specific strains, including *E. coli*, *S. aureus*, and lactobacillus strains, have been studied, but it is unknown how these rates relate to the rest of the microbial life in the gut. Also, there is some indication that gut microbial communities are constantly evolving at the genomic level. Hence, changes in the gut community may result from colonization and loss or from evolution.

Factors that impact stability of the gut microbiota include the transit time of food in the gut (which varies between individuals), diet, and the stability of the various niches of the gut. In terms of niche stability, the mouth appears to be a relatively stable niche since the microbiota there is generally stable over time. Also, the microbiota of the colon tends to be more stable than that of the ileum (the terminal portion of the small intestine), probably because of stronger competition in the ileum for the niches available. The niches of the gut are also influenced by the host's diet. For example, supplying a high fiber diet to pigs changes the populations in the animals' ceca and also apparently inspires changes in the gut wall.

Although populations of the human gut show a dynamic and adaptive response to modification of available niches, it is difficult to induce a long-term alteration of an established population. In experiments in which new strains of bacteria were transplanted into an individual, for example, it has been shown that the profile of the community changes over the short term but rebounds after a period of time. A longitudinal study in which the gut microbiota of individuals were tracked for 12 years using 16S rRNA gene-dependent methods also shows the gut community to be stable. Other studies show that a patient's original populations of gut microorganisms return after antibiotic treatment (De La Cochetiere, et al., 2005). Laboratory mice are more resistant to a second treatment to induce colitis than they are to a first treatment, suggesting that it is more difficult to bring about changes in the gut microbiota once those populations have once adapted to a stressor (Rachmilewitz, et al., 2002, 2004).

It is apparently easier to alter the gut microbiota of infants, who are undergoing neonatal colonization and whose gut communities are relatively simple, than to alter the established, complex microbiota of adults. Also, it has been noted, using strain typing methods, that the bacterial strains present in the gut of infants in developed countries are more stable than those of infants in the developing world (Adlerberth, et al., 1998).

Prebiotic treatments (carbohydrates that nurture the growth of specific fermentative probiotic organisms in the colon) often have greater impacts on the indigenous microbiota of individuals than do probiotic treatments. Prebiotics are consumed, for the most part, by strict anaerobes, and supplementation with prebiotics encourages the growth of these populations. The widely used prebiotic inulin, for example, has been shown to increase the population size of bifidobacteria, which are indigenous anaerobes (Gibson, et al., 1995). While changes in diet can influence the abundance of particular populations within the gut, these changes have not been shown to significantly alter the composition of the gut microbiota at the group level.

HOST-MICROBE AND MICROBE-MICROBE INTERACTIONS

The interplay between host, probiotic organisms, commensals, and pathogens is highly complex. Science is only beginning to uncover the details of these interactions, but answers about the activities of probiotics in the body and how those organisms interact with other microbes are beginning to emerge.

INTERACTIONS BETWEEN HOSTS AND MICROBES

Humans and animals mount two lines of defense against pathogens: innate and acquired immunity. Innate immunity works to prevent microorganisms from entering tissues, or, once microbes have gained entry, uses non-specific receptors to recognize and destroy suspected pathogens. Acquired immunity, on the other hand, involves a specific response to a particular infection and uses specifically tailored components, such as antibodies, to eliminate the offending microorganisms.

Exposure to commensal organisms is necessary for the appropriate development of both the innate and acquired immune systems. Once established, probiotic organisms interact with these immune defenses (Gill, et al., 2001a, b), possibly changing the nature of the immune response to other antigens, including commensal and pathogenic organisms.

THE ROLE OF MICROORGANISMS IN THE DEVELOPMENT OF THE IMMUNE SYSTEM

Numerous clinical and experimental studies have clearly demonstrated the pivotal role commensal bacteria play in the development of the immune system (Duarte, et al., 2004; Neumann, et al., 1998; Souza, et al., 2004; Oliviera, et al., 2005). For example, the life spans of lymphocytes, which are a type of white blood cell (also called T and B cells), depend on stimulation by commensal bacteria. The complete activation of inert regulatory T cells in newborns takes up to two years and depends on stimulation by bacteria (Sartor, 2004; Shanahan, 2000). Stimulation of germfree animals with commensal bacteria induces the development and activation of the immune system associated with the mucous membranes that line the gastrointestinal, respiratory, and urogenital tracts (Neumann, et al., 1998; Crabbe, et al., 1970; Sartor, 2004; Shanahan, 2000).

Immunoglobulin A (IgA) (antibodies that counter pathogens on the mucosal surface) is strongly induced by microorganisms. Different "natural" IgA antibodies are induced by particular organisms but they do not appear to provoke an immune response against the stimulating organism. The relative proportions of natural IgA and pathogen-specific IgA that are made by the body depends on the microorganisms involved (Bos, Jiang, and Cebra, 2001). The role of natural IgA in controlling the gut microbiota is still debated, but high affinity IgA antibodies are very important in clearing infections and in preventing re-infection. Hence, it is possible that low affinity natural IgA antibodies maintain a balance with the commensal bacteria and high affinity antibodies are responsible for the exclusion of pathogens (Wijburg, et al., 2006).

THE ROLE OF INNATE IMMUNITY IN PROBIOTIC ACTION

Innate immunity serves to recognize and react to microorganisms and to either destroy them if possible or contain them if they cannot be eliminated. The machinery of innate immunity includes soluble factors like defensins and lysozyme as well as cells like macrophages, Poly-Morphonuclear Neutrophils (PMNs), mast cells, and dendritic cells. Intestinal epithelial cells which can produce a variety of immunomodulatory proteins and cytokines in response to microbes can also be considered part of the innate immune system in the gut. One goal of probiotic treatment could be to either stimulate or prevent the various innate immune functions of these components (Gill, et al., 2001a). Immune homeostasis is the goal; the body must be able to respond to commensal microbes and potential pathogens alike without causing excessive inflammation. In theory, probiotics can also induce production of soluble innate defense factors, such as IgA and cytokines and microbicidal defensin proteins by Paneth cells in the crypts of the small intestine (Hooper, et al., 2003). It has recently been reported that secretory IgA induced in an antigen-unspecific manner by commensal flora protects mice against infection with *Salmonella*

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enterica var Typhimurium (Wijburg, et al., 2006). In addition, the presence of the microbiota seems to render the macrophages more active (Neumann, et al., 1998; Oliviera, et al., 2005) and to promote a general pro-inflammatory state, favoring the production of the pro-inflammatory cytokines, such as TNF and the chemokine MCP-1 (Souza, 2004). Innate immune responses can be rapidly called into action in defense of the host and then deactivated just as rapidly when a threat is removed, a fact that could have consequences in the long-term usage of probiotics.

TOLL-LIKE RECEPTORS AND PROBIOTICS

Probiotics may regulate immune responses by stimulating toll-like receptors (TLRs). TLRs are transmembrane proteins that are part of the innate immune system, but in vertebrates they can also serve to activate the adaptive immune system. Studies have found that mice that are missing the myeloid differentiation primary response gene 88 (Myd88) (which serves in TLR signaling) get a much worse form of colitis than their normal counterparts when exposed to dextran sodium sulfate, indicating that signaling via TLRs may be necessary to maintain intestinal homeostasis (Sartor, 2004). Probiotic strains may interfere with the interactions between other bacteria and TLRs on epithelial cells (Lan, et al., 2005). This capability to modulate some TLR responses, like increased TLR4 production during inflammation, may be one of the benefits that commensal organisms provide to the host. There is some indication that adhesin molecules can be regulated by TLRs (Sartor, 2004; Shanahan, 2004). There is also evidence that TLR-mediated responses to commensal bacteria are modulated by activation of other PRRs present within the cytosol of innate immune cells and epithelial cells. The NOD2 (nucleotide oligerization domain 2) PRR can in the presence of its ligand muramyl dipeptide, up or down regulate TLR2 signaling which can alter the production of pro- versus anti-inflammatory cytokines (Strober, et al. 2006). Mutations of the NOD2 gene are found in 10-15% of Crohn's disease patients (Ogura, et al., 2001; Hugot, et al., 2001; Lesage, et al., 2002).

THE ROLE OF ACQUIRED IMMUNITY IN PROBIOTIC ACTION

There are many claims that probiotics can enhance acquired immunity, but this has yet to be demonstrated conclusively. It has been shown that microbes can have an adjuvant effect; they enhance and orchestrate the immune response to other antigens.

The innate immune system instructs the acquired immune system; hence, probiotics may modulate the acquired immune response to other antigens in the environment through interactions with the innate immune system. Macrophages and dendritic cells, components of the innate immune system, interact with microbes in the body and process and present antigens to T helper cells. T helper cells determine the immune response to an antigen. Depending on the microbe with which they interact, macrophages and dendritic cells are induced to produce either T helper cell stimulatory cytokines (like IL-1 and IL-12) or T helper cell inhibitory cytokines (like IL-10). Therefore, different probiotics may, in theory, be used to modulate the action of the antigen presenting cell, and, hence, the function of the acquired immune system.

In one example of the interplay of the innate and acquired immune systems, neonatal pigs must first encounter certain microbial components, such as a section of DNA called a CpG site, in order for the piglet to mount an acquired immune response. It is possible that microbial products like CpG sites prime the innate immune system so that it may properly present antigens to the cells of the acquired immune system (Payette, et al., 2006; Ivory, et al., 2006; Babiuk, et al., 2004; Kennedy, et al., 2006; Iborra, et al., 2005; El Malky, et al., 2005).

ROLE OF THE HOST EPITHELIUM IN PROBIOTIC ACTION

The first point of contact between microorganisms and the host is at the epithelial layer, the tissue that lines the internal and external surfaces of the body. With respect to probiotics, attention usually focuses on the interactions between microorganisms and the epithelium of the small and large intestines, but microbes may also interact with the epithelium of the mouth, tonsils, upper digestive tract, urogenital tract, and skin.

Epithelial cells recognize microorganisms and communicate with and orchestrate both the innate and acquired immune systems. They may recruit dendritic cells (which trigger the immune response) into the epithelium within minutes of exposure to a pathogen, but they may also suppress T cell activation, thereby stifling an immune response.

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They also appear to be discriminatory, producing different cytokines in response to different commensal bacteria (Lan, et al., 2005).

Epithelial cells take up and load antigens onto major histocompatibility complex class II proteins (Telega, 2000). This process may be an alternate route of antigen presentation or a way of inducing specific immune tolerance to antigens that are not associated with pathogens, including antigens in food.

The epithelium is not a complete barrier to microorganisms, and translocation, in which microbes cross the mucosal layer into the tissues of the body, is common. Uptake of bacteria by intact epithelial cells has been observed using electron microscopy, but the cells in the intestine that are responsible for this activity have yet to be identified. There is also some evidence that translocation may occur at higher rates in the epithelium of the mouth than in the large and small intestines.

The lower portion of the small intestine, called the ileum, is lined with Peyer's patches, lymphoid tissue that helps to mediate immune responses to the contents of the gut. Peyer's patches are covered with microfold cells (M cells) that absorb and transport proteins and antigens to the cells beneath. The relative importance of M cells and absorptive epithelial cells to the immune response to probiotics and commensal microorganisms is not well studied, but absorptive epithelial cells cover a great deal more surface area than M cells and may have a proportionally larger effect on the immune response.

In humans, antigen uptake can vary according to the age and clinical condition of the individual. Uptake differences and differences in the gastric epithelium have also been detected between different animals. These differences could have significant consequences for the effectiveness of probiotics.

Several tight junction proteins (materials that bind cells closely together and prevent the movement of materials through a cell layer) in the epithelium are targets for pathogens. Hence, probiotics may be recruited to improve the barrier function of the epithelium by altering the expression and phosphorylation status of tight junction proteins.

WEBSITE LIBRARY OF MONOGRAPHS CONCERNING PROBIOTICS AND RELATED ISSUES

The biology of the host-microbial interactions at the mucosal surface, the medical consequences of this interaction, and ways to manipulate the interaction of the host with its microbes has been a topic studied in depth through the Old Herborn University Seminar (OHUS) Series. Currently nineteen volumes of monographs containing articles relevant to this subject are available and a new volume of articles by experts in the field is added to the series each year. This website library can be accessed through that of the International Study Group on New Antimicrobial Strategies (www.isgnas.org).

INTERACTIONS BETWEEN MICROBES

Bacteria on and in the human body impact each other in a variety of ways that may benefit or harm the interacting parties. Bacteria can communicate with one another through quorum sensing, a phenomenon in which a single bacterium can perceive population density by detecting the accumulation of signaling molecules. Interactions also take place within multiorganism assemblages on surfaces known as biofilms. Whether biofilms exist in the human gut is still a topic of debate, but they are the norm in the oral cavity and the vagina.

Specific ways microorganisms in the body can benefit one another include the production of extracellular beta-lactamase by certain bacteria. By breaking down penicillin, beta-lactamase can help penicillin-sensitive bacteria survive antibiotic treatment (Hackman and Wilkins, 1975; Hackman and Wilkins, 1976). Transfer of antibiotic resistance is also known to take place within the gut (Huycke, et al., 1992). Finally, some bacteria may have closely interwoven metabolic pathways (Schell, et al., 2002) that could influence the functions of probiotic treatments. For example, bacteria capable of breaking down complex carbohydrates provide substrates to bacteria in the community not being able to do so. Similarly, bacterial populations forming lactate produce substrates that may be utilized by butyrate-producing bacteria (Bourriaud, et al., 2005). Hence, by virtue of their ability to produce lactate, probiotic bacteria may provide additional substrate to other organisms.

Competitive exclusion (including competition for nutrients) and physical niche exclusion are probably important factors in microbial colonization of the human body. Growth inhibition has also been noted among commensal organisms. For example, one study has shown that *S. mutans* cells kill competitor cells in the mouth.

Many different organisms produce bacteriocins, but whether these compounds combat commensals or pathogens in the human body is highly controversial. Also, nitrate in saliva may be converted to nitrite by indigenous bacteria or by probiotic strains. This conversion may play a role in the ecology of the oral cavity (Hillestad, et al., 2005).

CONSEQUENCES OF MICROBE-MICROBE INTERACTIONS FOR THE HOST

In most cases, probiotics do not induce gross changes in the gut microbiota of an individual, but examples in which administration of a probiotic bacterial strain led to an alteration of the population sizes of certain other bacterial groups have been noted. For example, consumption of a probiotic *Lactobacillus rhamnosus* strain transiently altered the *Lactobacillus* and enterococcal contents of the feces without markedly affecting other bacterial groups (Tannock, et al., 2000).

The transfer of antibiotic resistance capabilities among pathogenic and commensal bacteria could pose a serious threat to the well being of an individual. Also, interactions between bacteria in the body and the potential for probiotics to intervene in the cross-talk and cross-feeding of the intestinal microbiota highlights the need to study diverse host types (including the very young, the aged, and the immunosuppressed) in order to verify the safety of these formulations.

It should be noted that fecal samples may not be able to reveal the effects that a probiotic organism has on the microbiota of the small intestine. For example, probiotic strains *Lactobacillus plantarum* 299/299v and *L. rhamnosus* GG have been detected in gastrointestinal tract biopsies of volunteers long after the probiotics were administered and the original cells would have died off. In theory, the newly established probiotic strains could have replaced commensal organisms that previously colonized these sites in the intestine, but their presence in the body would have been overlooked if the researchers relied solely on fecal samples.

IDENTIFYING PROBIOTICS CANDIDATES

Selecting and testing microorganisms for use as probiotic treatments can be a long, arduous process. First, careful consideration must be taken when selecting a strain for testing. Then, researchers must select in vivo *and* in vitro *testing protocols from among a range of options. Any organisms selected for trial should be relatively resistant to acid and bile to ensure effective passage to potential sites of colonization.*

SELECTING ORGANISMS

In selecting a new microbe for testing as an effective probiotic, a number of criteria need to be met. The formulation should, first and foremost, be safe to use. Safety validation includes making sure the strain in use is not pathogenic, and particular attention must be focused on the potential for the organism to infect immunocompromised individuals. Probiotic organisms must also be free of plasmid-encoded antibiotic resistance genes which could potentially be passed to pathogenic organisms in the patient.

A probiotic formulation must have a proven function and be shown to be effective in accomplishing that function. Manufacturing and regulatory considerations also play roles in probiotic selection.

Many professionals assert that, in the name of safety and effectiveness, a probiotic organism should only be used in the species of host from which it was originally derived. This appears to be an unnecessary precaution, as a number of probiotic organisms (including *Bifidobacterium lactis*, *Lactobacillus ruminis*, *L. rhamnosus*, and *L. reuteri*) have been transferred from one type of host to another with no apparent detrimental effects on the recipient.

IN VITRO TEST SYSTEMS

A number of *in vitro* test systems are available for studying the effects of probiotics in the human body. They include tests for:

- *The barrier functions of the intestinal epithelium (surface membranes).* Primary cultures of intestinal epithelium cells are useful for examining the interactions between bacteria and the epithelium.
- *Immune function in the presence of probiotic organisms.* The induction of cytokines (mediators of the immune response) by epithelial cells, peripheral blood mononuclear cells, and spleen cells in response to probiotic organisms can be evaluated outside the body.
- *The production of beta-galactosidase (the enzyme responsible for lactose degradation).*
- *The survival of probiotic strains (in vivo and on the shelf) and for resistance to acid, enzymes, and intestinal contents.*
- *The production of desired metabolites.*
- *The performance of certain functions, e.g. bile salts deconjugation.*
- *The presence of immune biomarkers.*
- *The ability of a probiotic strain to bind a toxin.*
- *Genetic sequencing of microbial genes relevant to probiotic function, e.g., ectopolysaccharide production.*
- *The production of nitric oxide and carbon monoxide by probiotic strains.*

IN VITRO VS. CLINICAL EFFICACY

At least one example of a correlation between *in vitro* effects of a probiotic organism and clinical efficacy has been demonstrated. The induction of anti-inflammatory cytokines IL-10 (Hart, et al., 2004; O'Mahony, et al., 2005) and TGF-beta (which ameliorate inflammatory bowel disease) by probiotic strains of bifidobacteria and lactobacilli has been shown to occur both *in vitro* and under clinical circumstances. However, it is not clear that this concordance between *in vitro* and clinical studies can be expected for many potential probiotics. Adherence to bodily surfaces is often crucial to the activities of commensal microorganisms and cannot be adequately mimicked with *in vitro* systems in the lab. Also, the mucus binding properties and mucolytic properties of commensals, which could mediate certain effects these organisms have on the body, cannot be effectively evaluated *in vitro*.

IN VIVO MODELS

In vivo models for testing potential probiotic therapies are available for several diseases. They include:

- *Models of* IBD.
- Mice with the interleukin-10 (an anti-inflammatory cytokine) gene knockout.
- Mice with the T-cell receptor alpha and beta chain (which recognize antigens) genes knocked out.
- Severe combined immune deficiency spontaneous mutation (SCID) mice, which can muster limited immune responses, can be used to test the relationship between immunity and disease.
- Mice with colitis induced by chemicals (including dextran sodium sulfate, trinitrobenzene sulphonic acid, and acetic acid).
- Mice with the E-cadherin (which mediates cell-to-cell adhesion) gene knockout.
- SAMP1/Yit mice, which exhibit spontaneous intestinal inflammation much like human Crohn's disease (Matsumotto, 2004).
- $CD45RB^{high}$ CD4+ T cell transfer model.
- Others
  1. An asthma model in mice in which allergic asthma is induced using allergens.
  2. A model of rotavirus infection in mice.
  3. A model of atopy (hypersensitivity reactions) in mice.
  4. A model of vaginal colonization in *Macaca nemistrina*.
  5. Mice with human-derived microbiota.
  6. A model for studying enterocyte kinetics.

For the purposes of modeling the effects of prospective probiotics, primate models of human disease would probably be very useful, but they are impractical for a number of reasons. Healthy volunteers offer another possible route for modeling.

RECOMMENDATIONS

- There is a pronounced need for large, carefully designed (randomized, placebo controlled) clinical trials of probiotics that undertake broad sampling of host microbiota, have clear end points, and have well informed participants who consent to treatment. Investigations like these are needed to overcome the placebo effect and other barriers to the thorough investigation of probiotic products.

- At present, the quality of probiotics available to consumers is unreliable. Testing to ensure the identity of the organisms within probiotic products, the potency of those organisms, the purity of the products, and their shelf life needs to be put in place. Moreover, these parameters should be verified by the appropriate regulatory bodies. The number of non-probiotic organisms present in probiotic products needs to be evaluated and kept within the limits allowed by food standards when intended for use as food.

- Each claim made for a given effect ascribed to a probiotic needs to be substantiated for each probiotic strain. Effects observed for one strain of a species should not be extrapolated to another strain of this species. Research into mechanisms of action of individual probiotics is needed as is testing to determine whether common mechanisms of action can usefully explain activity in various physiologic and disease states.

- Despite the importance of microorganisms to our well-being, the general public, particularly in the United States, fears microbes and views them strictly as "germs" that need to be cleaned from the body and the household. This misconception must be addressed through public education campaigns.

- Bacteria in the body inevitably interact with one another, and these interactions may be crucial to an individual's well-being. The potential for probiotic organisms to intervene in the cross-talk and cross-feeding of the intestinal microbiota highlights the need to study diverse host types (including the very young, the aged, and the immunosuppressed) in order to verify the safety of these formulations. A basic understanding of microbial ecology is needed.

- New probiotic formulations should, first and foremost, be safe to use.

- Validating the safety of prospective probiotics must include testing to ensure the strain in use is not a pathogenic strain.

- Particular attention must be focused on the potential for the organism to infect immunocompromised individuals.

- Probiotic organisms must also be free of plasmid-encoded antibiotic resistance genes, which could be potentially passed to pathogenic organisms in the patient.

- *In vitro* and *in vivo* models are needed to clarify the mechanisms of probiotic action.

- The molecular mechanisms underlying probiotic activity need to be unraveled to increase the credibility of the probiotic concept. Questions to be answered include:

  - Which microbial component(s) mediate the observed effects?

  - Which host processes/components are influenced?

  - Is viability a prerequisite for a given probiotic activity?

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