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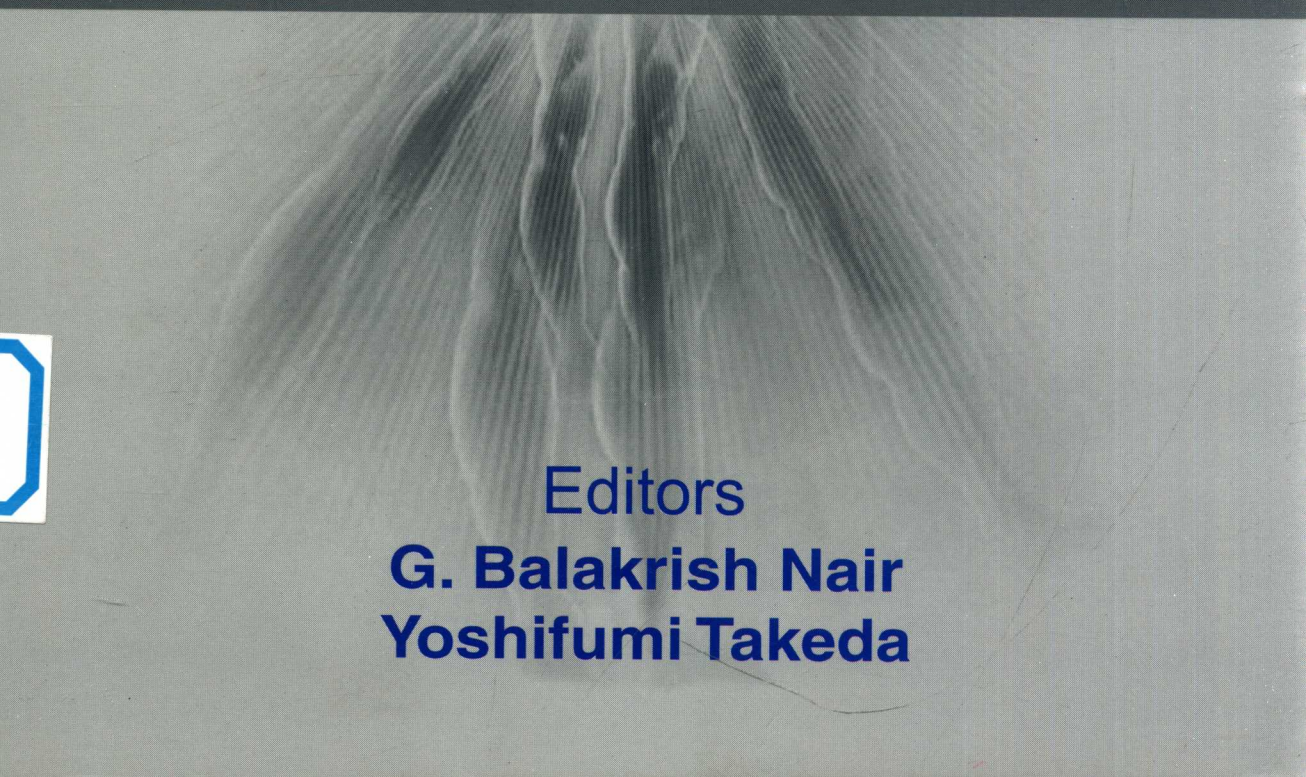
Probiotic Foods in Health and Disease



Editors
G. Balakrish Nair
Yoshifumi Takeda

PROBIOTIC FOODS IN HEALTH AND DISEASE

Editors

G. Balakrish Nair
Director, National Institute of Cholera and Enteric Diseases, Kolkata, India

Yoshifumi Takeda
Director, Collaborating Research Center of Okayama University for Infectious Diseases, National Institute of Cholera and Enteric Diseases, Kolkata, India

National Institute of Cholera and Enteric Diseases, Kolkata, India
Indian Council of Medical Research, New Delhi

Yakult Danone India (P) Ltd., New Delhi

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Foreword

It is sometimes easy to forget that scientific knowledge about probiotics and its role in health has its own history. Probiotics have been around since time immemorial with the realization of the impact that they were exercising but without understanding the science behind the impact. However, the situation changed markedly during the past decade or so with the advent of high throughput molecular tools and sequencing technologies providing greater insights into the complex assemblage of the gut microbial ecology unfolding its significant role in maintaining optimum health. With better understanding of the host-gut microbe interactions also came the recognition that interventions such as Probiotics that help in favorable modification of the gut flora may offer remarkable potential for improved health. This led to a rapid expansion of the probiotic arena through an escalation in probiotic research heralded by the large number of publications in the area highlighting the importance of this unique science. Scientific evidence soon pointed to the fact that besides playing a role in digestive health, probiotics could also modulate the immune system and have a significant effect on the alleviation of infectious diseases in children, adults and high risk groups. The past decade has validated its utility as an important therapeutic and preventive modality for gastrointestinal diseases, treatment and prevention of allergic disorders, chronic inflammatory diseases, prevention of cancers, immune stimulation and reduction of respiratory diseases.

This prompted the Indian Council of Medical Research (ICMR) to provide a thrust to the entire gamut of probiotic science in the country by initiating a series of annual probiotic symposia. The initiative aimed at providing a common scientific platform for basic scientists and clinicians to share and exchange knowledge and views and hence delve into newer areas of probiotic research. The 3rd India Probiotics Symposium in keeping with its theme "Probiotic Foods in Health and Disease" was an endeavor to bring together a panel of experts to review and present the more recent and relevant findings in the field that could finally contribute to integrating the advances for the development of scientifically substantiated products. This symposium organized for the third consecutive year by the National Institute of Cholera and Enteric Diseases, Kolkata also aimed at the dissemination of mechanisms involved in the translation of basic scientific findings into clinical studies and potentially new probiotic applications in the prevention and possible treatment of various diseases. Recognizing the need for establishing standards for the probiotic industry the symposium also helped address the Indian regulatory status of probiotics in food and reviewed the global regulatory milieu and its impact on scientific research. Policy recommendations that would advance the field of probiotics were made by experts present at the meeting.

The sponsors of the symposium, "Yakult Danone India" have more than 70 years of research to back the scientific efficacy of their strain, *Lactobacillus casei* strain Shirota in their product Yakult which has been accredited by the Ministry of Health, Labor and Welfare, Japan (FOSHU). Being pioneers in the area both Yakult and Danone engage in collaborative research through well conducted human trials across the globe to validate the health benefits of their probiotic products. Their endeavor remains to promote the science behind this niche category.

The 3rd India Probiotic Symposium, a continuation of a series, will be critical in establishing scientific credibility for this new evolving science in the country. I congratulate all those who have come along to create this difference and help develop a road map for the future……..

Jaimal Kumar Ganguly

Prof. N.K. Ganguly
Former Director General Indian Council of Medical Research,
President, Jawaharlal Institute of Post Medical Education and Research, Pondicherry
Distinguished Biotechnology Fellow & Advisor, Translational Health Sciences &
Technology Institute, National Institute of Immunology, New Delhi

Preface

Microbiology is witnessing change of unprecedented dimension. Microbiologists have worked for eons on how microbial pathogens overwhelm the host to cause disease. Extensive work has been done on a whole range of microbial armaments that pathogens use to evade host defences and cause disease. The fact that microbes can be used to treat and prevent disease is an emerging concept permeating into the way of thinking of pathogen hunters. There is, therefore, a paradigm shift in the mindset of how microbes are conceptualized. Such a change has also been catalysed by the knowledge that only about 5% (if not lower) microorganisms have been cultured and the vast majority remain anonymous, uncultured and unrecognized. The other central citadel in microbiology, the concept of culturability, is crumbling. And the concept that is gaining a foothold is assessing a niche by its community rather than individual culture. The intestinal microbiota of humans and animals is an ecological niche of great importance and is a formidable organ within an organ and its function in immune stimulation, competitive inhibition and a host of other beneficial roles is beginning to be recognized in the past decade or so. Commensurate with these changes, microbiologists are now homing on how the intestinal microbiota protects through a range of mechanisms and how the microbiota can be manipulated with probiotics is which are just being revealed. These changes have also ushered the science of Therapeutic Microbiology.

While there have been rapid advances in the science and practice of probiotics in the west, the pace has been much slower in countries like India. The grand plan of the symposium and this book is to disseminate this new found enthusiasm in probiotics both among clinicians and basic scientists. The intention is to spread the message of the need for more research and the need for application based on solid science. This proceeding captures the essence of the talks delivered at the third Probiotic symposium conducted in Delhi.

We would like to thank the authors for their valuable contribution and for cooperating with us to meet deadlines. The invaluable help extended by Dr. Neerja Hajela, Head, Science, Yakult Danone India, Delhi in skilfully coordinating, editing the first draft and collating the chapters of this book is gratefully acknowledged. We are certain that this book would not have come to this stage without her assistance. We also wish to thank Mr. Sunil Bernard and Mrs. Saheli Samanta, both from National Institute of Cholera and Enteric Diseases, for help rendered in formatting and compiling the references.

G. Balakrish Nair, Ph.D.
Yoshifumi Takeda, M.D., Ph.D.

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Gregor Reid, Jean Maclaim, Ruben Hummelen, Joke Dols and Wayne L. Miller
Canadian Research and Development Centre for Probiotics
Lawson Health Research Institute
London
Canada
E-mail: gregor@uwo.ca

PROBIOTICS TODAY

The field of probiotics has never been stronger, as measured by numbers of publications on the topic and the growing number of products entering the marketplace. Still, nine years after the United Nations and World Health Organization landmark definition of probiotics "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (22), this is a good time to reflect on where the field stands and the challenges and opportunities that lies ahead.

The recognition of the importance of microbiota in human and animal health is long overdue, given the volume of organisms inhabiting the body. The exogenous application of selected organisms to restore and maintain health has biblical roots, yet has taken nearly a century since Metchnikoff's observations, to be delivered to the masses. Modern methods of bulk fermentation, drying and delivery of probiotic organisms in various food types have made it possible to widely distribute products. However, many challenges remain.

All too often, companies ignore the FAO/WHO guidelines (23) as to what it takes to be appropriately called a probiotic. The majority of products in the market claiming to be 'probiotic' are in fact foods with bacteria added. We need to reiterate that the companies that sell a product are responsible for proving that it confers a health benefit and the only way to do this is through placebo controlled clinical studies. Studies that report probiotic use when infact the product had never been shown to be probiotic can lead to adverse publicity and confusion amongst consumers (9). Examples abound for this point, with three main categories being the most problematic:

1. Using say two strains shown to be probiotic in capsules, and adding them to another two strains in milk and expecting the same outcomes.

2. Composing a formulation of multiple strains and claiming that more the merrier and there must somehow be highly functional in the host.
3. Making claims about one product that has not been appropriately tested in humans, and using the work of another product to support it being efficacious.

(1) In the first example, distributors often go to fermentation companies and ask, or are recommended to purchase, 'probiotic' strains so that they can be added to a product. But, what if the same strain in dried encapsulated form shown to increase natural killer cells in elderly people, is now to be added to milk, fruit juice, a capsule with other strains, a yogurt with two starter cultures, a dried yogurt, frozen produce, or to a capsule that will not be appropriately handled in terms of exposure to temperature and moisture? What if the end users are children or people with diarrhoea? Even though regulatory agencies will not normally permit disease claims for foods, the company is still claiming that it is selling a 'probiotic'. But, while the product is unlikely to cause harm, unless studies are performed there is no way of knowing if it will actually confer measurable health benefits: thus, it does not meet the criteria for 'probiotic'. This might seem pedantic or asking for standards more associated with pharmaceutical agents, but it speaks to the core of the field. If probiotics are to gain wide acceptance and the respect of scientists, healthcare professionals and others, there must be an expectation that they meet minimum standards and not just the normal absence of contaminants and suitable viable count at expiry.

If two strains administered in milk daily to elderly subjects increases tumouricidal effects of natural killer cells (27), could we expect the same effect with these strains in a different formulation and concentration or in younger healthy adult subjects? These issues are at the core of the current probiotic field and there are no clear answers. In the elderly subjects there can be an age-related decline in lymphoid cell activity (immunosenescence) and therefore a benefit to administering probiotics. But, if no such impact (benefit) occurs with a different formulation or younger target group, then the product would not be probiotic. Thus, if the new product is marketed to children and adults, there should be at least one randomized controlled study showing it to be beneficial—probiotic.

(2) The issue here is not to make foods adhere to pharmaceutical evidence-based standards, but rather to ensure that a standard for 'probiotic' is upheld. If companies want to add Lactobacilli or Bifidobacteria or other 'probiotic' strains to their foods, without doing human studies, they should be permitted to do so but be made to call the product something other than probiotic. For example, Theralac capsules contain "Five Probiotic Species": *Lactobacillus acidophilus* LA-1–10 billion CFU; *Lactobacillus paracasei* F-19–5 billion CFU; *Lactobacillus rhamnosus LR-44*–2 billion CFU; *Bifidobacterium lactis* BL-34–10 billion CFU; and *Bifidobacterium lactis Bi-07*–3 billion CFU with a guarantee of 30 billion CFUs altogether (http://www.theralac.com/). It also contains 2 prebiotics that stimulate probiotics (patent pending) and natural acid-proof gel formulation (patented). The company claims that Theralac promotes a healthy soft-lining (wall) in the intestinal tract which results in improved digestion, regularity and nutrient absorption. A search of PubMed will be futile if you want to find any studies on this formulation. Strain LA-1 has been used in combination with *B. lactis* Bb12 as a potential immunomodulator (53), F-19 has been used orally in a pilot study which used vaginally applied *L. acidophilus* to treat bacterial vaginosis (BV) (20), while *Bifidobacterium animalis* subsp *lactis* Bi-07 has been used with *L. acidophilus* NCFM daily for 6 months to reduce fever, rhinorrhea and cough incidence and duration and antibiotic prescription incidence and the number of missed school

days attributable to illness, for children 3 to 5 years of age (39). None of these studies examined the softness of the gut wall, nor would it be likely that such a thing could be assessed easily. None of the studies examined improved digestion, regularity or nutrient absorption. Furthermore, different strains induce different immune responses in the host (30, 32, 40), meaning that some strains could counter the activity of another.

Another example is iFlora which contains *Bifidobacterium lactis, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus rhamnosus, Streptococcus thermophilus* with a total 15 billions cells per capsule, apparently designed to help maintain normal levels of yeast in the body and support healthy vaginal flora and urinary tract balance, as well as healthy digestion and normal bowel function (http://sedonalabs.com/shop/pc/viewPrd.asp?idcategory=2&idproduct=2). This is a lot of very different health effects for a product whose strains are not designated and therefore impossible to track through PubMed. Not all probiotic strains enhance vaginal or urinary health (6, 37) and there is no evidence of what a 'normal' level of yeast in the body represents.

(3) Three examples will be used to illustrate the third point. In 1973, Bruce and others showed that women who did not have recurrent urinary tract infections (UTI) had a vaginal microbiota dominated by lactobacilli (13). This led to an extensive research program that culminated in two strains, *L. rhamnosus* GR-1 and *L. reuteri* RC-14 being successfully shown to populate the vagina and out-compete a range of pathogens. The selection of these strains was a laborious process that included *in vitro* assays, some animal studies, extensive development of drying and encapsulation, and a number of clinical trials followed by acquisition of FDA approval in the USA and regulatory approvals elsewhere. Assessments were performed on the strains alone and in combination, in milk and dried formulations and studies were undertaken to provide insight into mechanisms of action. The latter includes displacement of pathogens (15, 52) and modulation of immunity (35, 36, 61). Thus, although the process could be shortened, it is far from simple.

It is gratifying when others cite your work and indeed model theirs after yours, however, this is different from implying that a completely different product in content, strain properties and formulation is somehow the same or could give the same responses *in vitro* or *in vivo*. A product sold by Natural Factors as Women's Multi Probiotic comprises the following:

Proprietary Synergistic Blend:	
Total Active Cell Count 12 Billion:	
Lactobacillus acidophilus	2.4 Billion
Lactobacillus casei	2.4 Billion
Lactobacillus rhamnosus	2.4 Billion
Bifidobacterium longum	1.2 Billion
Lactobacillus plantarum	1.2 Billion
Lactobacillus fermentum	0.6 Billion
Lactobacillus lactis	0.6 Billion
Streptococcus thermophilus	0.6 Billion
Bifidobacterium breve	0.4 Billion
Bifidobacterium bifidum	0.2 Billion
Cranrich Cranberry Concentrate	250mg
Other ingredients: Vegetable capsule (cellulose, purified water), FOS (Fructooligosacchardies) inulin, Magnesium stearate (vegetable grade), Ascorbic acid	

The product does not contain either *L. rhamnosus* GR-1 or *L. reuteri* RC-14, yet on its web site ostensibly set up to explain their product "The ideal probiotic blend for every woman", it is the successful studies on strains GR-1 and RC-14 that are prominently cited and no studies on the actual product being sold. The site also does not explain how the 250mg of cranberry affects bacterial viability, or why fructooligosaccharide and inulin are added and what they do (http://www.naturalfactors.com/ca/en/products/137/womens-multi-probiotic-with cranrich). A disclaimer is included presumably to appease the FDA by stating that the information is not intended to self-treat conditions that should be treated by a healthcare provider, when in fact the whole document covers infections, cancer and other diseases for which foods cannot be used to prevent, at least not without a major portfolio of studies.

In a somewhat related case, a press report on a Winclove Bio Industries six-strain probiotic supplement that received a vaginal infection-related health claim certification, included not only a description of the six strains: *Bifidobacterium bifidum, Lactobacillus plantarum, L. acidophilus, L. rhamnosus, L. salivarius and L. casei,* but also "a Winclove version of one (*L. rhamnosus*) of the two strains highlighted as being most efficacious by the leading researcher and developer in the area – Professor Gregor Reid." (http://www.nutraingredients.com/ Industry/Winclove-targets-female-niche-with-probiotic-health-claim). To the reader, it may appear that the Winclove product somehow contains a strain as good as or better than *L. rhamnosus* GR-1 in terms of functionality, or that the product has an extra five strains that could make it even better than GR-1 and RC-14. The report then stated that the claim certification was based on a pilot study, several *in vitro* studies and clinical trials conducted with similar products, and that a spokesperson for the company said "Further studies have been commissioned but it was not necessary to have any clinical trials for this claim."

Two issues need to be raised here. The first is to emphasize that no two strains or products are alike and the onus is on the manufacturers to perform studies proving that a product confers health benefits. The second is to show that even regulatory agencies do not enforce the use of the term 'probiotic' or the requirement for clinical efficacy before allowing an infection-related health claim, thus making it very confusing for consumers and healthcare professionals to know which products have been clinically proven.

The final example is so-called probiotics that survive heat treatment. This has become popular for production of chocolates, bread, cookies and potentially other 'probiotic' foods. With 523 new stock keeping units globally in the probiotic foods and beverages sector in 2007, the question is how many truly meet the minimum requirements with designation of strains, suitable end-of-shelf viability, and proven benefits? (http://www.nutraingredients-usa.com/Industry/Probiotics-grow-on-innovation-Datamonitor). In the case of chocolate, one technique combines three strains in microencapsulation, while another uses *Bacillus coagulans*, a spore forming organism that was long marketed as *Lactobacillus sporogenes*, and which has limited human data on its potential benefits (21). While there are merits to technical advances that allow different organisms to be added to food, the consumer needs to know how many pieces of chocolate or bread should be eaten per day and to what physiological benefit? In the case of chocolate, such studies need also to assess potential negative outcomes such as on cholesterol. If consumers are to decide between taking only one daily probiotic, how can they do this with limited information permitted on the label, few or no studies for certain products on Pub Med, and web pages that cite studies on other products?

In the developing world, the diversity of probiotic products is small at present, but as distribution channels open up and more people become financially able to purchase products, the same issues listed above will come to the forefront. Hopefully, by then the standards will have risen in the developed world.

PROBIOTICS IN THE DEVELOPING WORLD

Fermented foods with specific *Lactobacillus* strains play a major role in the diet of several regions in the developing world. Fermentation improves the digestibility of the fermented product and degrades anti-nutritive factors and toxins. Fermented products are consumed in the developing world because of an inherent belief that they promote health. An intriguing example is found in Kenya, where recent typing of *Lactobacillus plantarum* strains used by the Massai was found to have probiotic potential. The strains attached well to epithelial cell walls, were bile resistant and able to survive intestinal passage (43), albeit such characteristics are insufficient to be certain of what the organism will do in the host.

Besides the traditional use of *Lactobacillus* strains, the addition of probiotic *L. rhamnosus* GR-1 to a locally made yogurt has been proven to be successful in Mwanza, Tanzania. At this site, local women produce GR-1 supplemented yogurt for approximately 230 people each day of which at least 120 are living with HIV (59). Early results of this administration are presented below. It is important to note that probiotic supplementation is not merely dependent on a milk supply. Fermentation of cassava porridge with *L. plantarum* was shown to be feasible and improve the pace of recovery of malnourished children (Mbugua, M.K. personal communication). The use of probiotic strains in fermented products in a controlled way could have substantial benefits in the developing world in at least the following areas:

1. Gastrointestinal infections and diarrhoea.
2. HIV and immunity.
3. Women's health.
4. *Helicobacter pylori* infections in the stomach.

(1) Especially in developing countries, diarrhoea is a major killer. In 1998, diarrhoea was estimated to have killed 2.2 million people, most of them less than five years of age. In Tanzania, 17% of mortality in children under 5 years of age can be attributed to diarrhoeal diseases compared to 9.3% due to HIV. Besides efforts to increase sanitation and the availability of clean water, a simple food-based intervention could make a significant difference. In Peru, where 12% of mortality in children under 5 years of age are due to diarrhoeal diseases, a randomized controlled trial (RCT) of 200 children showed that *L. rhamnosus* GG reduced the number of episodes from 6.02 episodes / year to 5.21 episodes / year ($p = 0.03$) (45). This is still only making a small dent in the overall incidence, but other benefits are provided once diarrhoea occurs. A Randomized, Controlled Trial (RCT) of 287 children showed that the mean duration of a diarrhoeal episodes was reduced from 72 hours to 58 hours with the addition of *L. rhamnosus* GG to Oral Rehydration Solution (ORS) ($p = 0.003$) (29). Another RCT among 559 children showed that not only the duration of diarrhoea was reduced with this approach, but also the length of stay in hospital

and the need for intravenous fluid (7). Sadly, however, the *L. rhamnosus* GG strain or other anti-diarrhoeal probiotics are not available in countries like Peru or India.

In addition to the treatment and prevention of diarrhoea, various trials indicate that probiotics could also play a role in treating and preventing stunting (low length for age) and malnutrition. These are both problems that pose an enormous threat to the future potential of developing countries. In India, 48% of all children under 5 are stunted, while in Tanzania the problem is similarly high at 44%. An RCT among 100 Indian children with stunting showed that a simple *Lactobacillus* fermented product could significantly increase weight gain and height, with fewer episodes of fever (51). Note that this represents weight gain not a gain in fat or obesity. Even more promising is a recent RCT among 795 Malawian children with malnutrition which used a combination of four different lactic acid bacteria (10^{11} colony-forming units of bacteria total; *Pediococcus pentosaceus* 16:1 LMG P-20608, *Leuconostoc mesenteroides* 23-77:1 LMG P-20607, *Lactobacillus paracasei ssp paracasei* F-19 LMG P-17806, and *Lactobacillus plantarum* 2362 LMG P-20606) and four fermentable bioactive fibres (2·5 g of each per 10^{11} bacteria; oat bran [rich in β-glucans], inulin, pectin, and resistant starch). Probiotics and Prebiotics did not enhance nutritional status but did reduce mortality (p=0.06) (34). It is worth noting that 52% of these children were infected with HIV.

(2) Among people living with HIV, the gut is one of the most severely affected sites. Given that 80% of the entire T-cell population resides in the gut, it is easy to understand how the gut of HIV patients is the centre of many problems. Their intestinal barrier is severely damaged, causing an influx of bacterial products into the bloodstream, poor uptake of nutrients, and often chronic diarrhoea. The resulting systemic inflammation may fuel HIV replication and cause faster progression towards AIDS (11). Quantitative PCR shows that the intestinal microbiota of HIV patients contains fewer Lactobacilli and Bifidobacteria and more potential pathogens such as *Candida albicans* and *Pseudomonas aeroginosa* (28). An RCT among 57 HIV patients who were given prebiotics showed significantly reduced intestinal pathogenic load and significant immune system activation after 12 weeks supplementation (8, 58). Whether probiotics can reduce the pathogenic intestinal load among people living with HIV has not yet been studied. However, an RCT among 77 children living with HIV showed an increase in the CD4 count among the group receiving *B. bifidum* and *S. thermophilus* of +118 cells/ml after two months of follow-up compared to a decrease of -42 cells/ml among the placebo group (p= 0.049) (57). These results were confirmed in a pilot study among 24 Nigerian women living with HIV where those supplemented with *L. rhamnosus* GR-1 experienced an increase of +6.7 cells/ml compared to a decrease of -2.2 cells/ml among the placebo group after 30 days (p=0.04) (4). Whether modifying the intestinal microflora may result in a reduced immune activation and an increased CD4 count is a link that warrants further investigation.

(3) Globally, an estimated 7,000 women become infected with HIV each day. Over 67% of all new HIV infections are in women and the rapid spread is facilitated by the high prevalence of sexually transmitted infections (STI) and a disturbed vaginal flora, consisting of depletion in Lactobacilli and over-colonization of BV pathogens. In a large cohort in Tanzania it has been estimated that 17% of all new HIV infections can be attributed to this condition. The supplementation of indigenous *Lactobacillus* in a food-based product or a capsule provides a potential way to improve the vaginal flora. *Lactobacillus* strains have

been shown *in vitro* to directly inhibit the growth of various vaginal pathogens such as *Gardnerella vaginalis*, *Neisseria gonorrhoea* and HIV (54). They also compete for adherence sites, stimulate the local antimicrobial immune response and lower the pH to one less hospitable for many pathogens. *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 have been shown to populate the vagina after administration (12, 17, 26), reduce the colonization by pathogens and restore and maintain a normal vaginal flora (48, 49). Therefore, exogenously applied *Lactobacillus* may prove a cost-effective, female-initiated method to prevent HIV infection and STI in the developing world.

(4) More than half the global population is infected with *Helicobacter pylori*, a Gram-negative bacterium known to cause peptic ulcers, cancer and other diseases (60). Most cases are found in Asia, and the preferred treatment consists of antibiotics and compounds that affect the acidity of the stomach such as proton pump inhibitors although these often cause side effects. The option to use probiotic organisms to treat *H. pylori* came from *in vitro* studies showing an ability to inhibit or kill the pathogens. However, given the ability of *H. pylori* to create a niche in the stomach, it has not been possible to eradicate them in humans using probiotics. Nonetheless, there has been some use of probiotics in reducing drug side effects and in potentially helping dislodge and kill the pathogens. A review of ten eligible randomized controlled trials on 498 patients who received fermented milk plus triple drug therapy, and 465 in the control group who received the drugs, showed the pooled odds ratio for eradication by intention-to-treat analysis in the treatment versus control group was 1.91 (1.38-2.67; $P<0.0001$) (Cochran's $Q=5.44$; $P=0.488$) (50). The pooled odds ratio for the number of patients with adverse effects was 0.51 (95% CI 0.10-2.57; $P=0.41$; random effects model; heterogeneity by Cochran's $Q=68.5$; $P<0.0001$), suggesting that fermented milk-based probiotic preparations improve *H. pylori* eradication rates by approximately 5–15%.

WHAT DO WE KNOW ABOUT MECHANISMS, AND POTENTIAL MECHANISMS, OF ACTION?

In recent times, much of the research in probiotics has been dedicated to understanding the underlying mechanisms of their beneficial effects. Probiotic action by microorganisms can be divided into two broad categories: effects on other microbes, and effects on the host. A number of modes of action have been attributed to probiotics, such as pathogen exclusion, modulation of host gene expression and immunity and production of antimicrobials. Through controlled experimentation many of the biochemical mechanisms have been uncovered, albeit not proven in humans. With the advancement of 'next-generation' sequencing technology, it is becoming easier to sequence the entire genome of an organism and investigate what armamentarium it possesses that could contribute to probiotic action.

ANTIMICROBIALS

One of the earliest probiotic effects to be exploited for human benefit is the production of lactic acid as a normal end product of fermentation by various "lactic acid bacteria", including *Lactobacillus*. The antimicrobial action of lactic acid has long been used for food preservation, but with

respect to the human body, it is in the vagina where lactic acid appears to play a more important role in lowering pH and making the environment less hospitable for pathogens. In several cases lactic acid production has been directly attributed to the maintenance of a healthy vagina (10, 33).

Many probiotic organisms are able to produce specific antimicrobial compounds that have been attributed, at least in part, to the organism's probiotic effect. There are many bacteriocins and antimicrobials reported from lactobacilli strains, and some have been employed in food preservation (19, 55, 56). In the case of *L. reuteri*, several strains are able to produce a broad-range antimicrobial called reuterin through glycerol fermentation (5). It was nearly two decades after the description of reuterin and its anti-microbial action that the genomic island for the production of reuterin was identified through comparative genomics with a closely related, non-reuterin producing species (44). Although reuterin has in several cases been attributed to the probiotic action of the species not all strains express it. For example probiotic *L. reuteri* RC-14 is unable to produce reuterin yet still exhibits probiotic effects (16). Examination of the genome sequence of *L. reuteri* RC-14 has confirmed that several genes required for reuterin production are missing meaning that other factors make this strain beneficial to humans. This highlights the significance of strain-specific probiotic action. A mechanism of action defined in one strain cannot necessarily be applied to other strains of the same species without experimental validation.

ADHERENCE OF STRAINS TO SURFACES AND PATHOGEN EXCLUSION

One selection criteria used to identify potential probiotic organisms is the ability to adhere to epithelial cells or mucosal surfaces. The concept is that good adherence allows for effective habitation at a site, interaction with host cells, and exclusion of pathogens. Compared to pathogens such as *E. coli*, relatively few adhesions have been described for lactobacilli. These include fibronectin binding proteins (31), protein-lipoteichoic complex (2), fimbriae (47), and mucin binding proteins (14). One group of "sortase-dependent" proteins are so named because they contain a conserved amino acid sequence which is cleaved by the enzyme sortase which then links the protein to the cell surface. Sequencing the genome of an organism and identifying protein-coding genes in the sequence provides the ability to identify these sortase-dependent adherence proteins and the sortase enzyme through the conserved sequence motif. In one particular case of *L. plantarum* WCFS1 (46), two sortase-dependent proteins were identified for their role in mannose-specific binding. Previously it had been demonstrated that *L. plantarum* strains use mannose to bind to the surface of intestinal epithelial cells (1). Using the genome sequence available for the strain, the authors were able to knock out the gene for sortase or the sortase-dependent proteins. In both cases, the authors observed that WCFS1 could no longer agglutinate yeast strains displaying mannose on their cell surface. The experiment showed a direct relation between a set of genes and the important colonization mechanism of mannose-binding.

MODULATION OF HOST GENE EXPRESSION

Through various host cell interactions, probiotic organisms have been shown to elicit beneficial host responses through changes in host gene expression. In a study by Mack *et al.* (41), *L. plantarum* 299v incubated with intestinal cells in vitro increased the mRNA levels of the mucin-

producing genes, MUC2 and MUC3, which then inhibited enteropathogenic *E. coli* adherence. The mucosal layer of the intestine is an important barrier between the epithelial cells and the outside environment (24). The mucins produced by the epithelial cells are able to bind pathogens to prevent pathogen adherence to the epithelial layer of the intestine (18, 24).

The above examples demonstrate some of the varied mechanisms by which probiotic organisms can beneficially affect their host. With the advancement of the "genomic age" and the release of several whole genome sequences of probiotic organisms, it has become easier to uncover these mechanisms and find the genes controlling their action. The challenge for the future will be to show that mechanistic effects can be proven in humans. While mutant strains can prove this in animals, they are not generally permitted for human applications, making the task of proving the cause and effect more difficult.

CLINICAL CHALLENGES FOR THE FUTURE

The most important step in evaluating whether a product is actually probiotic, in food or other formulations, is an appropriately designed and adequate sample sized human study (23). Double-Blinded, Randomized, Placebo-Controlled design is one of the best among such designs.

Before an appropriate sample size can be selected, the primary endpoint must be chosen, whether this is an improvement in some physiological state such as nutritional status, reduction in obesity, reduced duration of colds or infection, lowering of cholesterol, or reduction in disease-associated symptoms such as diarrhoea, rhinorrhea, or fever. The sample size must be large enough to detect significant differences yet of a size that is manageable in terms of recruitment and follow-up of subjects.

The use of a placebo to assess the ability of probiotics to maintain health is essential to rule out any 'placebo effects'. However, when the goal is to assess the efficacy of probiotics to alleviate disease symptoms, the use of a placebo may not always be ethical, particularly if a standard treatment exists. In such cases, the probiotic must be evaluated in combination with or compared to, the standard therapy (i.e., an *active control*). Two studies have shown that *L. rhamnosus* GR-1 and *L. reuteri* RC-14 can improve the cure of bacterial vaginosis (BV) when used with antibiotic therapy (3, 42). The mechanisms are not known but may involve helping restore the vaginal lactobacilli post eradication of the pathogens. In another study of 120 children, the use of *L. acidophilus* b.i.d was as effective at preventing UTI as trimethoprim/sulfamethoxazole during a second year of follow-up (incidence 18.3% (11/60) in the probiotics group, versus 21.6% (13/60) in the antibiotic group; P = 0.926) (38).

Evidence is growing for the ability of probiotics to improve gut health (25), alleviate inflammatory conditions, and reduce the incidence and severity of various infections. Several challenges wait, including as stated above uncovering mechanisms of action of probiotics. The ability to sample relevant body sites in real time may be aided by advances in robotics and imaging technologies such as Single Photon Emission Computed Tomography (SPECT), Positron Emission Tomography (PET), Nuclear Magnetic Resonance (NMR) and capsule endoscopy (CE). One of the most common claims of probiotics is that they "restore the normal flora", but until results emerge from the Human Microbiome Project (http://nihroadmap.nih.gov/hmp/), the true nature of the microbiota will not be known, nor will the ability of it to be balanced by use of probiotics. Given the important role that microbes play in health and disease and the complexity of the interaction between microbes and the human body an understanding of the microbiota

will be a key to the optimal implementation of probiotics. For now on an everyday basis far too many people are dying or suffering in regions of the world where no properly documented probiotics are available. This challenge, a commercial one at its core, may be the biggest to overcome in the near future.

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REFERENCES

1. **Adlerberth, I., S. Ahrne, M. Johansson, G. Molin, L. Hanson, and A. Wold**. 1996. A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. Appl. Environ. Microbiol. **62:**2244–2251.
2. **Anokhina, I. V., E. G. Kravtsov, N. V. Yashina, A. V. Ermolaev, V. L. Chesnokova, and M. V. Dalin**. 2006. Characterization of surface adhesions of lactobacilli used in production of probiotic preparations. Bull. Exp. Biol. Med. **141:**716–719.
3. **Anukam, K., E. Osazuwa, I. Ahonkhai, M. Ngwu, G. Osemene, A. W. Bruce, and G. Reid.** 2006. Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: randomized, double-blind, placebo controlled trial. Microbes Infect. **8:**1450–1454.
4. **Anukam, K. C., E. O. Osazuwa, H. B. Osadolor, A. W. Bruce, and G. Reid**. 2008. Yogurt containing probiotic *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 helps resolve moderate diarrhoea and increases CD4 count in HIV/AIDS patients. J. Clin. Gastroenterol. **42:**239–243.
5. **Axelson, L. T., T. C. Chung, W. J. Dobrogosz, and S. E. Lindgren**. 1989. Production of a broad spectrum antimicrobial substance by *Lactobacillus reuteri*. Microbial Ecol. Health Dis. **2:**131–136.
6. **Baerheim, A., E. Larsen, and A. Digranes**. 1994. Vaginal application of lactobacilli in the prophylaxis of recurrent lower urinary tract infection in women. Scand. J. Prim. Health Care **12:**239–243.
7. **Basu, S., D. K. Paul, S. Ganguly, M. Chatterjee, and P. K. Chandra**. 2009. Efficacy of high-dose *Lactobacillus rhamnosus* GG in controlling acute watery diarrhoea in Indian children: a randomized controlled trial. J. Clin. Gastroenterol. **43:**208–213.
8. **Ben Amor, K., G. Rizzardini, C. Torti, T. Quirino, M. Moroni, M. Knol, D. Bray, A. Vriesema, M. Clerici, and A. Gori**. 2008. Disturbed gut microbiota in HAART-naive HIV-1 positive adults: Effect of intervention with a specific prebiotic oligosaccharide mixture. Kroi Conference, Poster abstract.
9. **Besselink, M. G., H. C. van Santvoort, E. Buskens, M. A. Boermeester, H. van Goor, H. M. Timmerman, V. B. Nieuwenhuijs, T. L. Bollen, B. van Ramshorst, B. J. Witteman, C. Rosman, R. J. Ploeg, M. A. Brink, A. F. Schaapherder, C. H. Dejong, P. J. Wahab, C. J. van Laarhoven, E. van der Harst, C. H. van Eijck, M. A. Cuesta, L. M. Akkermans, and H. G. Gooszen; Dutch Acute Pancreatitis Study Group**. 2008. Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. Lancet. **371:**651–659.
10. **Boskey, E. R., K. M. Telsch, K. J. Whaley, T. R. Moench, and R. A. Cone.** 1999. Acid production by vaginal flora in vitro is consistent with the rate and extent of vaginal acidification. Infect. Immun. **67:**5170–5175.
11. **Brenchley, J. M., D. A. Price, T. W. Schacker, T. E. Asher, G. Silvestri, S. Rao, Z. Kazzaz, E. Bornstein, O. Lambotte, D. Altmann, B. R. Blazar, B. Rodriguez, L. Teixeira-Johnson, A. Landay, J. N. Martin, F. M. Hecht, L. J. Picker, M. M. Lederman, S. G. Deeks, and D. C. Douek**. 2006. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat. Med.**12:**1365–1371.