Selected Topics in Probiotics and Prebiotics: Meeting Report for the 2004 International Scientific Association for Probiotics and Prebiotics

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Abstract
On August 29–31, 2004, 84 academic and industry scientists from 16 countries gathered in Copper Mountain, Colorado USA to discuss certain issues at the forefront of the science of probiotics and prebiotics. The format for this invitation only meeting included six featured lectures: engineering human vaginal lactobacilli to express HIV-inhibitory molecules (Peter Lee, Stanford University), programming the gut for health (Thaddeus Stappenbeck, Washington University School of Medicine), immune modulation by intestinal helminthes (Joel Weinstock, University of Iowa Hospitals and Clinics), hygiene as a cause of autoimmune disorders (G. A. Rook, University College London), prebiotics and bone health (Connie Weaver, Purdue University) and prebiotics and colorectal cancer risk (Ian Rowland, Northern Ireland Centre for Food and Health). In addition, all participants were included in one of eight discussion groups on the topics of engineered probiotics, host-commensal bacteria communication, 'omics' technologies, hygiene and immune regulation, biomarkers for healthy people, prebiotic and probiotic applications to companion animals, development of a probiotic dossier, and physiological relevance of prebiotic activity. Brief conclusions from these discussion groups are summarized in this paper.

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Introduction

The International Scientific Association for Probiotics and Prebiotics (www.isapp.net) was founded in 2001 as an international non-profit collaboration of scientists dedicated to advancing the science of probiotics and prebiotics. Its mission is to engender and disseminate information on high quality, multidisciplinary, scientific investigation in the fields of probiotics and prebiotics, and to advance the development of scientifically substantiated, health-promoting probiotic and prebiotic products worldwide.

Convening meetings with targeted discussion topics and key specialists in the field have led to progress toward these goals.

Numerous reviews on the topics of probiotic and prebiotic functionality have been advanced during recent years (Isolauri *et al.*, 2004; Marteau *et al.*, 2004). The focus of this report will be conclusions from the eight specific topic areas addressed by the discussion groups (Table 1) at the meeting. This report presents current thinking for eight areas of research in the field as addressed by selected discussion groups at the 2004 meeting.

Biomarkers for healthy people

Certain safe and effective probiotics and prebiotics appear to be used by, and apparently beneficial to, individuals who are healthy as well as those who have an illness. The concept of using probiotics and prebiotics to maintain or enhance health is of interest to many people. However, documenting such an effect, especially when dealing with healthy subjects, is a scientific challenge.

What is health? According to the WHO "Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" (WHO, 1946). From a practical perspective, it is likely that health as so defined is not usually achieved in real life. It would be hard to find individuals with complete well-being considering that many individuals who generally considered themselves healthy might often have minor ailments and many individuals without identified illness/ disease will consider themselves not healthy. Rather than being able to compartmentalize people into one of two groups, healthy or not healthy, more descriptive is a depiction of health as a continuum (Fig. 1). A more practical (less restrictive) definition for health might be a state with no recognizable disease (normal functions), no symptoms that require treatment (according to well-defined guidelines), and no nutritional deficiencies. In this case, there might be a window of opportunity for the use of probiotics and prebiotics for healthy people.

Table 1. Composition and topics for discussion groups.	
Engineered probiotics as therapeutics: formats and challenges	Chair: David Mills, Univ. of California – Davis, USA Co-chair: Todd Klaenhammer, North Carolina State Univ., USA Peter Lee, Stanford Univ., USA Gianni Pozzi, Univ. of Sienna, Italy Philippe Langella, National Institute of Agronomical Research, France Lothar Steidler, Alimentary Pharmabiotic Centre, Ireland Jerry Wells, Institute of Food Research, Colney, Norwich, UK Michiel Kleerebezem, NIZO Food Research, The Netherlands Jos Seegers, Lactrys Biopharmaceuticals BV, The Netherlands Lennart Hammarstrom, Karolinska Univ. Hospital, Sweden Jim Heimbach, JJHeimbach LLC, USA
Host commensal interactions - who talks to whom and how?	Chair: Gregor Reid, Univ. of Western Ontario, Canada Co-chair: Rex Gaskins, Univ. of Illinois, USA Thaddeus Stappenbeck, Washington Univ. Sch. Med, USA Corinne Grangette, Pasteur Institute of LILLE, France John McCormick, Univ. of Western Ontario, Canada David Mack, Univ. of Ottawa, Canada Maeve Murphy, General Mills, USA Yoshimi Benno, Japan Collection of Microorganisms, Japan Miguel Freitas, Danone, France Jane Leedle, Chr. Hansen, USA Ben Dias, Kraft, USA
Omics technologies - exploration of the interaction of pro and prebiotics with the host	Chair: Joe O'Donnell, CA Dairy Research Found, USA Co-chair: Ian Rowland, Univ of Ulster, UK Bruce German, Univ. of California, Davis, USA Anne Kurilich, National Dairy Council, USA Mike Gasson, Institute of Food Research, UK Marion Leclerc, INRA, France Nina Rautonen, Danisco Innovation, Finland Ralph N. Landau, Bradley Pharmaceuticals, Inc., USA Yvonne Clune, Univ. College Cork, Ireland Jay Tiesman, Procter and Gamble, USA Karen Scott, Rowett Research Institute, Scotland
Hygiene and immune regulation	Chair: Francisco Guarner, Hospital General Vall d'Hebron, Spain Co-chair: Graham Rook, Univ. College London, UK Willem van Eden, Utrecht Univ., The Netherlands Harsharn Gill, Department of Primary Industries, Australia Joshua Korzenik, Massachusetts General Hospital, USA Peter McGuirk, Immune Regulation Research Group, Ireland James Versalovic, Baylor College of Medicine, USA Joel Weinstock, Univ. of Iowa, USA Per Brandtzaeg, LIIPAT, Inst. of Pathology, Norway Alice Heth, General Mills, USA Raphaëlle Bourdet-Sicard, Danone, France
Biomarkers for healthy people	Chair: Maria Saarela, VTT Biotechnology, Finland Co-chair: Joseph Rafter, Karolinska Institutet, Sweden Yehuda Ringel, Univ. of North Carolina at Chapel Hill, USA Daniel Bunout, INTA Univ. of Chile, Chile Martin Kullen, Wyeth, USA Greg Leyer, Rhodia, USA Robert Martindale, Medical College of Georgia, USA Jean-Michel Antoine, ILSI/Danone, France
Prebiotic and probiotic applications to companion animals	Chair: George Fahey, Univ. of Illinois, USA Co-chair: Bob Rastall, Univ. of Reading, UK Christina Khoo, Hills Pet Food, USA Marie Louise Baillon, Waltham Centre for Pet Nutrition, UK Arland Hotchkiss, USDA-ARS, USA Glenn Gibson, Univ. of Reading, UK Anne McCartney, Univ. of Reading, UK Ram Nimmagudda, Chr. Hansens, USA Mike Ceddia, Mead Johnson, USA Marko Stojanovic, Procter and Gamble, USA
Development of a probiotic dossier using science-based criteria	Chair: Bruno Pot, Institut Pasteur de Lille, France Co-chair: Mary Ellen Sanders, Dairy and Food Culture Technologies, USA Dominique Brassart, BDN Bio sarl, Switzerland Luc Devuyst, Vrije Universiteit Brussel, Belgium Thomas Tompkins, Institut Rosell, Canada Irene Lenoir-Wijnkoop, Danone, France Yuan Kun Lee, National Univ. of Singapore, Singapore Kevin Collins, Univ. College Cork, Ireland Trish Conway, VRI, Australia Peggy Martini, Kraft, USA Fabrizio Arigoni, Nestle, Switzerland Norio Ishibashi, Morinaga Milk Industry Co., Japan

Table 1. Continued	
Physiological relevance of prebiotic activity	Chair: Jan Van Loo, Orafti, Belgium Co-chair: Kieran Tuohy, Univ. of Reading, UK Bryon Petschow, Mead Johnson, USA Ian Griffin, Baylor College of Medicine, USA Cyril Kendall, Univ. of Toronto, Canada Bob Hutkins, Univ. of Nebraska, USA Doug Willrett, Genencor, USA Connie Weaver, Purdue Univ., USA Sandra Macfarlane, Univ. of Dundee, Scotland Robert Ward, Univ. of California - Davis, USA

Key intervention targets include immunology and defense systems, gastrointestinal functions, gut and oral microbiota, mucosal systems and integrative functions (e.g. quality of life). Some promising or developing targets with only preliminary data include probiotic and prebiotic effects on bone and cardiovascular health, mental state and performance.

Healthy target groups, which could potentially benefit from probiotic and prebiotic dietary intervention, include:

- Infants (e.g. in families with atopic disease; prevention of diarrhea)
- Toddlers (e.g. prevention of upper respiratory tract infections)
- Teenaged girls (bone mass)
- Pregnant women (atopic disease, bone mass)
- Adults with acute symptoms (e.g. with functional gastrointestinal symptoms)
- Elderly (e.g. with impaired immune functions)
- Athletes (e.g. restoration of natural killer cell functions)
- Travelers (e.g. prevention of diarrhea).

Most difficult is the specification of biomarkers for health (or preferably markers for health, since the term biomarker is usually connected to disease) for health. Numerous biomarkers related to disease but also to health

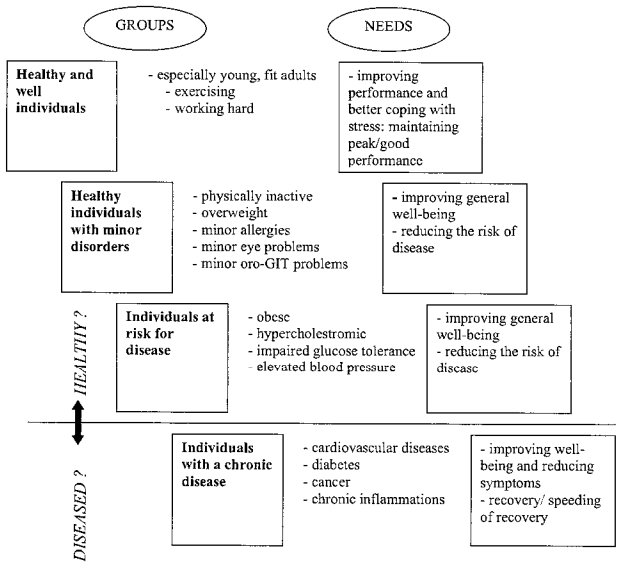

Fig. 1. Consumer groups with different health status and needs regarding functional foods.

have been exhaustively listed in previous publications (Crews *et al.*, 2001; Saris *et al.*, 2002; Asp *et al.*, 2003, 2004). In general terms, three levels of generic markers for health are available. The aim of these is to assess changes (increasing or decreasing) in health status over time with specific interventions. They are (i) health status and quality of life measures, (ii) physiological/biological markers, and (iii) psychosocial markers/factors.

Validated tools for measuring health status (Nottingham Health Profile, Hunt *et al.*, 1985), activities of daily living [Physical Self Perception Profile (Fox and Corbin, 1989; Fox, 1990)], and quality of life [MOS General Health Status Questionnaire 36-SF (Ware and Sherbourne, 1992)] are available. In addition, it is possible to measure prevention of disease, utilization of health care and absenteeism from school or work.

Guidelines to use markers are available (Howlett and Shortt, 2004). These include the following important specifications:

- Markers should be validated methodologically (to include their precision and accuracy, specificity and sensitivity, reproducibility and repeatability) and biologically (so that they reflect closely the process leading to the claimed health effect and respond appropriately with changing events).
- Within a study, the marker should change in a biologically relevant way and be statistically significant for the target group.

Examples for different categories for physiological/ biological markers include:

- Integrative, such as infection incidence, skin test, growth rate
- Specific system function, such as natural killer cell function, gut transit
- Response to a challenge test, such as vaccination or hydrogen breath test
- Mechanism, such as DNA repair
- Regulatory system, such as insulin secretion
- Metabolites, such as short chain fatty acid (SCFA) production.

When planning probiotic and prebiotic dietary intervention studies the researcher should choose relevant physiological/biological markers belonging to the above listed categories. However, within a single study, markers representing one/some categories can be selected – not all categories have to be represented.

Validated tools to measure psychosocial well-being [e.g. Symptom Checklist-90 (Derogatis *et al.*, 1973) or Mental Health Index SF-36 (Strand *et al.*, 2003)] should be used. The individual subjective sense of well being is influenced not only by presence and severity of a disease or physical symptoms, but also by the individual's physical, emotional, and psychosocial status. Therefore, clinical research, particularly in healthy people, must use sensitive and responsive measures to assess these subjective domains of health. Quality of life (QOL) incorporates the individual's perceptions of health and functional status and is reflective of the perception of his or her health. Its measurement incorporates psychosocial (e.g., daily function, recreation, sexual function) as well as physiological (e.g., blood pressure, weight) factors. It is important to notice that the validation of the measure rests with the individual. Several standardized instruments to measure QOL are available including single-item global measures or multi-item health measures; generic measures or condition-specific measures. Other clinical measures might also be useful although they do not directly measure individual's perception of own health. These might include daily functioning measures, health care utilization or absenteeism from work or school. The specific measure should be chosen according to the study population, design, and the specific question to be answered.

It is possible to conduct human intervention studies looking at the effect of probiotics or prebiotics on healthy people. It must be remembered, however, that "healthy people" is not a homogeneous group. When conducting studies on healthy people, consider and specify the target population and carefully choose markers and biomarkers to measure and demonstrate the effect

Hygiene and immune regulation

Industrialized countries have experienced a steady rise in the incidence of diseases due to immune-dysregulation, particularly during the past four decades. These diseases include both Th2-mediated disorders, like allergies, and Th1-mediated disorders, such as Crohn's disease, multiple sclerosis or type I diabetes (Bach, 2002). Defects in natural immunoregulatory mechanisms have been recognized to have a causative role in some of these disorders (Karlsson *et al.*, 2004; Kriegel *et al.*, 2004; Viglietta *et al.*, 2004).

The so-called 'Hygiene Hypothesis' is based on epidemiological observations that suggest a link between increased incidence of allergies and reduced incidence of infectious diseases in Western industrialized countries (Bach, 2002). This correlation prompted the assumption that modern hygiene reduced contact with pathogens that prime Th1 responses. The reciprocal down-regulation of Th2 cells by Th1 cytokines and of Th1 cells by Th2 cytokines suggests that frequent infections would help in adequate balancing of the immune system, and explain why infectious diseases protect against allergy or autoimmunity.

However, important advances in recent years in the identification of regulatory mechanism of innate and adaptive immunity suggests that the 'Hygiene Hypothesis' must be re-interpreted. While there are some inhibitory interactions between Th1 and Th2 lymphocytes, growing evidence shows that both effector T cell types are mainly regulated by specialized subsets of regulatory T lymphocytes (Treg). This specialized regulatory T cells are formed by clonal expansion from 'naïve' T cells primed by antigen-presenting cells under particular conditions (Rook *et al.*, 2004). The condition or conditions that result in induction of regulatory subsets of lymphocytes instead of effector T cells are currently being investigated.

The gut microbiota is essential for development of the immune system including regulatory pathways. It has been shown that a few pathogens are able to induce immunoregulatory pathways in the host to evade the immune system (Mills and McGuirk, 2004). In this particular case, the pathogen uses this strategy to survive within the host. However, induction of immune regulation depends primarily on the exposure to harmless bacteria within the gut. By this regulatory mechanism, the host can resist the antigenic burden of foreign structures within the gut or on the contact surfaces (skin, eyes, respiratory tract), without responding with immuno-inflammatory reactions that would damage the host's own tissues.

Experimental data suggest that balanced exposure to a range of microbes is critical for immune homeostasis. Hypothetically, an appropriate combination of microbes will develop an optimally functioning immune system. However, the specific effects of any given microorganism (including probiotics, as well as helminths) need to be investigated. Intake of probiotics has been shown to correct dysregulated immune responses both *in vitro* and *in vivo* (animal models of inflammatory bowel disease, allergy, multiple sclerosis, arthritis). There is also evidence of efficacy in humans (Gill and Guarner, 2004). Ability of helminths to restore immune homeostasis in inflammatory bowel disease has also been demonstrated (Weinstock *et al.*, 2004). It is thought that a balanced gut microbiota with adequate presence of commensals or 'in transit' bacteria able to stimulate regulatory pathways would be critical for the development of such pathways, and subsequently for the prevention of allergies, inflammatory bowel disease and autoimmunity.

A major drawback is the fact that scientific knowledge on gut bacteria is too scarce to determine what is a 'balanced' microbiota or even a 'normal' microbiota. The gut microbiota include microbial communities assembled on the mucosal surfaces and in the lumen of the gut. These communities include both native species, that permanently colonize the tract, and a variable set of living microorganisms, that transit temporarily through the tract (Guarner and Malagelada, 2003). However, most bacteria in the gut are not culturable and their phenotypic characteristics are unknown. They are acquired after birth and initial colonization largely depends on environmental factors. In addition, transient bacteria are continuously acquired from the environment. Recent evidence suggests that host genotype may influence final composition, which is host specific and relatively stable over time in a given individual (Zoetendal *et al.*, 2004).

Differences in the gut microbiota composition have been observed between individuals in developed versus underdeveloped countries, babies born by caesarean section versus birth canal delivery, breast-fed versus

formula fed infants and hospitalized versus home-living children (Guarner and Malagelada, 2003; Schwiertz *et al.*, 2003; Zoetendal *et al.*, 2004). Interestingly, studies have shown composition differences in the gut microbiota of children that are likely to develop allergy later in life (Gill and Guarner, 2004).

The group concluded that lack or limited exposure to relatively harmless microbes and helminths rather than reduced rate of infectious diseases during childhood would be responsible for the increase in chronic immunodysregulatory diseases in industrialized countries. Theoretically, appropriate use of probiotics and prebiotics would favour the exposure of the developing immune system to harmless microbes able to induce immunoregulatory mechanisms. Thus, strategies based on probiotics and prebiotics may prove useful in the prevention of such disorders.

Many open questions remain to be answered, including the specific role of Treg lymphocytes in any of the disease conditions mentioned above (allergies, Crohn's disease, multiple sclerosis), and the identification of specific mechanisms to properly stimulate Treg cells. A better knowledge of the gut flora (particularly, mucosa-associated flora) is essential. Finally, the new strategies, for instance feeding of specific probiotics or prebiotics early in life, need to be tested in controlled studies to ultimately prove efficacy in the prevention and treatment of immunoregulatory disorders. The feasibility of such an approach has already been demonstrated (Kalliomaki *et al.* 2003).

Host commensal interactions – who talks to whom and how?

The ability of bacteria to communicate with each other, and with the host, has been known for over a quarter of a century (Koshland, 1977). However, genetic and molecular tools have made it possible to isolate the signals, study their mechanisms and potentially manipulate their effects. The ability of probiotic organisms to affect host gene expression has been demonstrated in different systems. For example, the turning on of mucin expression by *Lactobacillus* sp. (Mack *et al.*, 1999) and by activating transcription factors involved in cytokine signaling directly, leading to NF-kappa B activation, and indirectly via cytokines, leading to signal transducer and activator of transcription (STAT) activation (Miettinen *et al.*, 2000). The studies showing that *Bacteroides thetaiotamicron* induces angiogenesis and development of a healthy intestine further illustrates the importance of the host-commensal interaction (Stappenbeck *et al.*, 2002). Further evidence suggestive of communication comes from studies showing that immune regulation can occur in newborns fed *L. rhamnosus* GG resulting in a reduced incidence of atopy (Kalliomaki *et al.*, 2003). Human genome arrays now provide a means to study the effect of introduction of a probiotic organism on host gene expression. Such systems can document changes in differential gene expression (Cox *et al.*, 2001). In addition, quantitative morphometrix (numbers of T cells, B cells, macrophages, and sub populations of CD4, CD8 for example) can provide a means to study the key gene events changed with probiotic use, thereby leading to identification of site of action and molecules involved. However, these methods simply provide evidence of activity, but not the identity of the factors involved.

Communication between bacteria has been studied. One method involves molecules called autoinducers that are secreted by organisms to regulate gene expression and control behavior on a community-wide scale (Henke and Bassler, 2004). A study showed that the determinants for pheromone binding and specificity are contained within the transmembrane domain of *Lactobacillus plantarum* (Johnsborg *et al.*, 2003). In many lactic acid bacterial strains, bacteriocins function as quorum sensing molecules, in that they are produced, and are controlled in a cell-density dependent manner, using a secreted peptide-pheromone that can enable the organism to switch on bacteriocin production at times when competition for nutrients is likely to become more severe (Eijsink *et al.*, 2002). While the primary focus has been on communication related to virulence and disease, there is evidence now emerging that probiotic *Lactobacillus*, such as *L. reuteri* RC-14, produce signaling molecules that interfere with gene expression in other organisms (McCormick, personal communication). Specific signaling molecules will soon be identified, but it will only represent the cusp of our understanding. Experiments need to be designed, preferably in the human, to determine the point at which microbes enter the system (in the newborn), what causes them to release signaling compounds, how they are detected and processed by existing bacteria, and what physiological outcome this has for the host. While methods are in place to monitor more obvious outcomes, such as changes in stool, electrolytes and immunity, it is not a simple task to show cause and effect *in vivo* of small, mostly peptide, molecules in a dynamic peptide rich gut. Indeed, many of these reactions are occurring over a 1 micron distance within natural biofilms (Egland *et al.*, 2004). Studies in the vaginal mucosa may be more practical as the microbiota is simpler and sampling as well as microbial and human gene studies more accessible. Conventional and germ free mice may also provide a means to understand these complex processes.

As signaling functions become better known, recombinant or mutant probiotic strains can be designed to insert or up-regulate signaling molecules to enhance probiotic benefits in the host. Alternatively, signaling molecules could become a new generation of drugs designed to interfere with virulence and pathogenesis (Raffa *et al.*, 2004).

Omics technologies – exploration of the interaction of pro- and prebiotics with the host

The application of the new omics technologies to the study of the human gastrointestinal tract and the influence of pro- and prebiotics on the system represents perhaps the greatest opportunity and challenge to date for microbiologists and nutritionists to elucidate the complex interactions between gut microbiota and host (van Ommen and Stierum, 2002; Davis and Milner, 2004; Ebert, 2004; German and Young, 2004). The importance of indigenous microbes in a whole range of acute and chronic diseases and syndromes has been established (Rowland, 1999; Hughes and Rowland, 2003; Guarner and Malagelada,

2003) yet the mechanisms involved remain obscure in most cases, hindering development of more effective, targeted nutritional and microbiological interventions.

Essential to this discussion is the fact that the gastrointestinal tract is a multidimensional system ideal for an integrated, non-biased, systems biology approach, which could be combined with clinical and health information to enable debilitating human health issues to be addressed. The technologies associated with systems biology, namely genomics, transcriptomics, proteomics and metabolomics are now widely available (although some are more highly developed than others) and are being applied to the gastrointestinal tract in studies of the effects of diet and specific disease states on gene expression in the intestinal mucosa [for example in the recently completed SYNCAN European study on synbiotics and cancer risk (www.syncan.be)]. A true systems biology approach needs to integrate both the host and microbial components of the gastrointestinal tract, but as yet these techniques have seen little application in terms of the microbial component. Challenges with this approach include the difficulties of accessing and sampling many regions of the gastrointestinal tract without altering gene expression of microorganisms and issues of variability between individuals.

One ambitious result from this approach is a comprehensive, unbiased database documenting gastrointestinal commensal bacteria – the 'human microbiome'. A crucial component would be the development of a metagenomic database of human microbes, preferably all regions of the gut and all stages of microbe development. This would constitute an invaluable public database for investigations of the role of microbial communities in disease (hypothesis-driven candidates include allergy, inflammatory bowel disease, irritable bowel syndrome and cancer). It was also proposed that a metabolomic database of human gastrointestinal microbes should be established. In practice this may be easier to achieve than the above mentioned metagenomics database, since the technologies required, including magic angle spinning, nuclear magnetic resonance and high pressure liquid chromatography and mass spectroscopy systems, techniques which are well developed and are indeed being used to establish a metabolomics database of the rat intestinal tract. Furthermore, a human microbiota metabolome would complement the initiative being funded by the U.S. National Institutes of Health to define the human blood metabolome.

Clearly, such a systems biology approach would require a major funding initiative to develop the technologies and databases needed to define the gut microbiome. One approach to acquiring funds for developing the microbiome would be to identify a diseased state for which compelling evidence exists for an aetiological role of the gut microbiota. A potential candidate could be allergy, since it appears that changes in microbiota precede allergic development in children and it is unlikely that the disease itself has a major impact on the microbiota as irritable bowel syndrome or colon cancer undoubtedly do. The experimental approach would then be to compare the disease state phenotype with that of a healthy phenotype on the basis of genetic variation in host genotypes, gene expression differences at site of the phenotypic target, genetic variation and gene expression differences in the microbiota, and molecular differences across all classes of biomolecules, proteins, signals and metabolites.

The systems biology approach outlined above could result in a tremendous increase of knowledge of the physiology and genetics of disease and result in a wide range of applications:

- Defining molecular mechanisms in health and disease associated with gastrointestinal tract
- Development of assessment tools for health or disease status
- Dedicated human dietary intervention studies with pro/prebiotics
- Development of novel functional biomarkers, based on transcriptomics, for critical events in gut mucosal health (for example, cell proliferation or apoptosis) to facilitate human interventional studies with prebiotics and probiotics
- Identification of diverse functional biomarkers for assessing disease risk and for use in dietary trials.

Engineered probiotics as therapeutics: formats and challenges

Probiotic bacteria, very often members of the genera *Lactobacillus*, *Bifidobacterium*, *Lactococcus* and *Streptococcus*, have been linked to an assortment of health benefits (Sanders and Huis in't Veld, 1999). Recently, engineered strains of some of these lactic acid bacteria have been employed as oral vectors for delivery of therapeutic or prophylatic proteins ("engineered probiotics", Wells *et al.*, 1996; Steidler, 2003). Researchers have examined engineered probiotics as a means to abate enzyme deficiencies (Drouault *et al.*, 2002), to deliver neutralizing antibodies (Kruger *et al.*, 2002), cytokines (Steidler *et al.*, 1998), or antimicrobials (Hillman *et al.*, 1987), as well as to present numerous bacterial and viral antigens (Zegers *et al.*, 1999; Maggi *et al.*, 2000; Enouf *et al.*, 2001; Lee *et al.*, 2001; Bermudez-Humaran *et al.*, 2002; Hughes and Rowland, 2002; Ribeiro *et al.*, 2002; Chang *et al.*, 2003; Grangette *et al.*, 2004) with varying results. Using lactic acid bacteria as delivery vehicles is an attractive approach for a number of reasons. Lactic acid bacteria are consumed frequently in fermented products and are thus generally considered safe for human consumption. Some lactic strains are also members of the animal gastrointestinal tract microbiota, and are able to survive passage through, and persist within, the intestinal tract. Finally, due to their commercial prevalence, there is a wealth of knowledge on growth, metabolic end products and preservation characteristics. These latter properties position engineered probiotics as an ideal route for creation of stable oral therapeutics that can be delivered without injection.

Any therapy or prophylactic use presents its own inherent challenges, format restrictions and containment considerations. Since there is a wide variation in possible target applications, no single delivery format is suitable for all approaches. To consider engineered probiotics for a particular therapy or prophylactic goal one must consider various issues including but not limited to choice of probiotic host, *in situ* expression versus delivery of preloaded cells, and intra- versus extracellular expression

formats. Moreover, use-specific issues of biocontainment and potential regulatory aspects influence the chosen formats as well. To make matters more complex each format choice can impact another (for example, dead cells won't express proteins *in situ*) thereby restricting options for certain choices.

Numerous lactic acid bacterial species have been used as hosts for delivery of recombinant proteins. In general, two strategies have emerged, use of commensal lactic acid bacteria or use of industrial starter cultures. Commensal lactic acid bacteria have included *Lactobacillus casei*, *L. plantarum*, *L. jensenii*, and *Streptococcus gordonii* (among others), strains which are known to persist in or on animals (Bryan *et al.*, 2000; Mercenier *et al.*, 2000). Commensal strains are attractive in that individual strains themselves can differentially stimulate the immune system thus providing some adjuvant capacity when desired (Pouwels, 1998). Perhaps more importantly, an extended persistence in the host may result in a prolonged therapeutic or prophylactic treatment. *Lactococcus lactis* is considered the prototypical industrial starter culture that has also been employed as a therapeutic delivery vehicle (Wells, 1996; Steidler *et al.*,1998). *L. lactis* can be a desirable host because it can survive passage through, yet not persist in, the gastrointestinal tract (Klijn *et al.*, 1995). This latter aspect enables transient delivery of therapeutic proteins and thereby has the potential for better dosage control in comparison to commensal lactic acid bacterial strains which might differentially persist in the gastrointestinal tract.

When considering preloading cells with recombinant protein versus *in situ* expression, perhaps the most straightforward and desirable format is to preload cells. This format is desirable for several reasons. First and foremost, preloaded cells that do not persist *in situ* can be advantageous in terms of the ability to carefully control the timing and extent of the delivered product. Moreover, preloading cells also permits the option of delivering killed cells, a format that decreases biocontainment concerns. However, not all therapeutics can be delivered in this way. Many target proteins may require *in situ* expression (Steidler, 2003) due to problems with protein formation or a niche specific expression requirement (such as antiviral agent expression in the vaginal tract). Expression in three different bacterial cell locations has been examined; cytoplasmic, expression onto the cell surface and direct secretion of proteins into the medium (Mercenier *et al.*, 2001). There does not appear to be a single optimal mode of expression as each offers different advantages and/or surmounts specific hurdles for specific target proteins. Perhaps the most studied model examined via oral delivery within lactic acid bacteria is the subunit C of the tetanus toxin (Mercenier *et al.*, 2001; Grangette *et al.*, 2004). This antigen has been presented in all three formats (intracellular, cell surface and secreted) in different lactococci, lactobacilli and streptococci and tested for immunogenicity after subcutaneous or oral delivery (Seegers, 2002). While the various expression formats behaved differently, all appeared capable of eliciting an immune response. Most often, the chosen target protein and/or target disease addressed dictates the expression format in the probiotic host. Many target proteins that require additional folding modifications and/or contain disulfide bonds are better served by extracellular expression *in situ* (Steidler, 2003). In response, much work has focused on probiotic secretion mechanisms for heterologous proteins (Pouwels *et al.*, 2001; Miyoshi *et al.*, 2002; Grangette *et al.*, 2004). Not all targets are best served by this format either, as extracellular transport of some proteins has been challenging and/or the half-life of extracellular proteins in the gastrointestinal tract may be short.

A variety of plasmid-based expression schemes have been developed for expression of proteins in lactic acid bacteria (Kok,1991; Bryan *et al.*, 2000). Plasmid-based systems are ideal for expression work as they allow rapid analysis of various format options. Plasmid-based expression presents some technical challenges, however. Engineered plasmids typically house antibiotic resistance genes that would be delivered as part of a therapeutic. This can be overcome by use of "food grade" markers for plasmid maintenance (MacCormick *et al.*, 1995; Platteeuw *et al.*, 1996; Fu and Xu, 2000; Sorensen *et al*, 2000; Bron *et al.*, 2002). Another problem is that autonomously replicating plasmids may inadvertently transfer to other commensal microbes in the gastrointestinal tract, leading to uncontrolled dosage of the target therapeutic. To surmount this problem, most groups have chosen to integrate heterologous genes within the probiotic genome (Steidler, 2003). This is typically accomplished via homologous recombination of a gene cassette into specific chromosomal loci (Mills, 2001; Russell and Klaenhammer, 2001). A drawback to this approach is lower level of expression, most likely due to the lower copy number. Another problem is that performing chromosomal manipulations in some probiotic hosts is difficult.

Containment considerations revolve around three primary issues, (1) spread of antibiotic resistance genes, (2) gene transfer into or out of the engineered probiotic, and (3) shedding of the engineered probiotic into the environment. The first concern is eliminated by performing chromosomal manipulations in a fashion that removes any antibiotic resistance markers and results in a strain that contains only the heterologous gene(s) at a select chromosomal site. Gene transfer of the target therapeutic genes to other commensal microbes is problematic in that it may lead to uncontrolled dosage of the delivered therapeutic. Others have shown transfer of conjugative elements between gastrointestinal tract based microorganisms (Netherwood *et al.*, 1999). Incorporation of the target gene into the chromosome lessens (Steidler *et al.*, 2003), but does not eliminate, the opportunity for such transfer (Torres *et al.*, 1991). In this situation availability of genomic sequence becomes critical by allowing strategic design of integration sites unlinked to mobile elements such as phage sequences or insertion sequences. Moreover, prior knowledge of host genome sequence enables potential transfer elements to be removed and/or inactivated prior to integration of the heterologous gene(s).

Containment of engineered probiotics is an obvious public concern. Of particular importance is the potential to shed a therapeutic to a non-target population. Since some

engineered probiotics are designed to persist *in situ* (by virtue of the host selection) prior planning for containment becomes significant and several mechanisms exist to limit shedding into the environment. Perhaps the most obvious containment device is to deliver killed engineered probiotics, however this format is not feasible for those targets that need to be expressed extracellularly or *in situ*. The use of lactic acid bacteria strains that are sensitive to antibiotics allows rapid elimination of the engineered probiotic from the human/animal host following antibiotic treatment. Several groups have designed attenuated strains that do not persist outside the production environment. This is accomplished by engineering mutant strains that require specific medium additives to complement the mutation during the initial production of the engineered probiotic, prior to delivery. Upon ingestion of the engineered probiotic, the complementing factor is no longer present and further growth of the strain becomes halted. Various food grade systems have been developed to ensure plasmid maintenance by complementing a specific mutation. Unfortunately, as described above, plasmid-borne systems are less desirable for engineered probitoics. Several groups have exploited this approach for containment of the engineered lactic acid bacteria through alanine racemase or thymidylate synthase (thyA) mutations (Steidler *et al.*, 2003; Palumbo *et al.*, 2004), genes essential for cell growth. Alanine racemase is required for synthesis of D-alanine, an essential cell wall component (Grangette *et al.*, 2004). Additions of D-alanine to the media can complement this mutation. Similarly, the thyA mutation is complemented by addition of thymidine or thymine to the medium. Importantly the viability of thyA mutant strains diminishes rapidly in the absence of complementing thymidine, an attractive feature for containment of an engineered probiotic.

With the advance of engineered probiotics to clinical reality, governmental regulatory concerns, as well as public perception of these therapeutic options, become critical. While the regulatory aspects of engineered probiotics as they relate to human health remain to be determined, the panel considered genetic engineering itself not the critical factor in this calculation. [A workshop convened by the FAO (2001) considered the safety assessment of foods derived from genetically modified microorganisms].

Other issues such as product traceability, public access to scientific information on engineered probiotics, and the nature of the disease addressed will shape the public and regulatory environment surrounding commercialization of engineered probiotics. This environment is also likely to shift over time as encouraging results from early clinical trials, such as abatement of inflammatory bowel disease by oral delivery of IL-10-secreting *Lactococcus lactis* (L. Steidler, personal communication), attracts public interest and engenders further government support. Indeed, the panel thought that as the field matures opportunities to use engineered probiotics in other realms, such as to enhance human nutrition, will expand.

Development of a probiotic dossier using science-based criteria

The science of probiotic characterization, safety and efficacy has become more sophisticated over recent years. Companies marketing these products, and the government agencies responsible for regulating them, have begun to develop and insist on quite extensive dossiers documenting product attributes. It is not always clear, however, what information these dossiers should contain. A multitude of *in vitro* tests and animal models have been used to characterize probiotics, and many different biomarkers and study designs have been used to establish efficacy and safety. In some cases, results from studies have questionable significance in providing support for probiotic function in humans. The time is right for scientists to recommend based on current scientific understanding what criteria should be provided for commercial probiotic strains.

The aim of this discussion group was to use the concise guidelines published by the FAO (2002) as a starting point for determining the key criteria important for assessing probiotic function. It should be noted that subsequent to this ISAPP meeting, the International Dairy Federation convened a panel of experts in Brussels (October 2004) with the aim of compiling the methods needed for measuring these essential traits.

The scope of this discussion was limited to a non-genetically manipulated, single strain probiotic ingredient for human consumption. Consumption of the probiotic in the form of a food or dietary supplement was considered. The general attributes considered important to include in a probiotic dossier were:

1. Description of strain isolation.
2. Establishment of strain identity. Genus, species and differentiating strain-specific fingerprint is the minimum. In general, DNA-based approaches are considered essential for this. Full genomic sequencing is desirable.
3. Description of physiological and metabolic characteristics. These characteristics contribute to the overall description of the strain, and may or may not provide insight into expected human physiological activity of a strain. Examples of useful traits might be Gram reaction, morphology description, sugar fermentation patterns, enzyme activities, acid tolerance, bile tolerance, production of antimicrobial compounds, and antibiotic resistance patterns. Generally, these attributes are determined through *in vitro* analysis.
4. Substantiation of functionality (Sanders *et al.*, 2004). The focus of this characterization is generally animal and human studies. *In vitro* analysis can be important in justifying endpoints used in *in vivo* studies. Often, markers rather than clinical or disease endpoints are useful. A special challenge is in finding relevant, valid markers of structure and function of the human body. Physiological relevance of some chosen markers is not always apparent (e.g., fecal levels of groups of microorganisms or blood concentrations of different immune markers). Other considerations in conducting animal and human studies include dose,

delivery format, and how studies might be used for fashioning claims for communication of health effects to consumers.

5. Proposed mechanism of action and definition of active principle. Although not essential, it is very useful, for scientific and regulatory reasons, to establish a mechanism for any observed physiological effect. Development of a hypothesis for effect provides a basis for meaningful interpretation of clinical results.
6. Technological characteristics. As delivery of consistent, viable probiotics is essential, the most pressing technological characteristics revolve around stability. Genetic stability during growth of the strain and ability to remain viable during preservation, shipping and storage over time are important to document for the probiotic dossier.
7. Safety. The probiotic dossier should comment on the following if known and deemed relevant:

    - Recommended consumption level if an upper limit is known
    - Contraindications in specific groups
    - Virulence factors known in strain
    - Listing of species on any generally recognized as safe list (FDA 2001; Mogensen *et al.*, 2002) or specifically approved for similar use by governmental agency
    - History of consumption
    - Mutagenicity
    - Acute and chronic toxicity
    - Post marketing surveillance.

It is worth noting that the key points raised here are functionally equivalent to those required by the International Committee for Harmonization for any drug master file and by most governments who are currently accepting registration of microbial products, including probiotics.

Physiological relevance of prebiotic activity

Prebiotics, by definition, are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. There is growing evidence that prebiotics confer physiological changes in the host, but their clinical relevance is only now becoming understood. Thus, as with probiotics, it is timely to study the relevance and function of prebiotics. Aspects of particular interest in this definition are (1) the requirement for, mechanism of action of, and scientific verification of selective fermentation of prebiotics within the colon, and (2) what evidence exists for improved host health upon the selective fermentation within the colon. A range of non-digestible oligosaccharides are available and to varying degrees have been proven to reach the colon intact (Tomio and Tomohiro, 1993). For example, in feeding studies with ileostomy patients, over 90% of ingested inulin was shown to pass through the small intestine intact (Ellegard *et al.*, 1997). There is also strong evidence that once in the colon, a range of oligosaccharides are selectively fermented, in that their fermentation results in changes in population levels of specific bacteria. There exists strong evidence from human feeding studies that inulin (of varying degree of polymerization), fructooligosaccharides (both derived from inulin and synthesized from sucrose) and lactulose elicit a particular change within the gut microbiota (De Preter *et al.*, 2004), whereby relative numbers of *Bifidobacterium* spp. and sometimes *Lactobacillus* spp. increase in number. In some studies, this increase in what are presumed to be beneficial members of the gut microbiota, coincided with concomitant decreases in potentially harmful bacteria (e.g. species of *Bacteroides* and clostridia, Gibson *et al.*, 1995; Kruse, *et al.*, 1999). The recent sequencing of the *Bifidobacterium longum* genome has indicated that within this organism, the function of a large proportion of the genome is carbohydrate metabolism, both degradative enzymes and transport mechanisms. Such activities are likely to play an important role in governing metabolism of prebiotics within the gut microbiota. Studies on the genetic determinants of prebiotic metabolism within the bifidobacteria and lactobacilli have shown that such activities are carried out by enzymes with high substrate affinity and that specific transport systems are available for capture and import of prebiotic breakdown products into these bacteria.

However, not all prebiotic oligosaccharides share the same scientific support for their beneficial impact on bacterial numbers within the gut. There exists a need to confirm *in vivo* the ability of emerging prebiotics such as galactooligosaccharides, soyoligosaccharides, isomaltosaccharides, and xylooligosaccharides to modulate the gut microbiota in a selective manner. Conversely, quite a substantial amount of scientific information exists about the prebiotic nature of inulin, fructooligosaccharides and lactulose, with a minimum dose of 3 to 5 g lactulose or fructans respectively shown to elicit a prebiotic effect *in vivo*.

Intrinsic in the definition of a prebiotic is the need for a positive impact on host health. Specifically, microbiota modulation by prebiotics should result in improved host health. Evidence describing prebiotic benefits in colon cancer, lipid metabolism, mineral absorption/bone health, inflammatory bowel disease (ulcerative colitis and immuno-modulation) and colonization resistance has been published (Van Loo *et al.* 1999; Van Loo, 2004). For colon cancer, mineral absorption, lipid metabolism and inflammatory bowel disease strong evidence from *in vitro* studies and animal models are corroborated with results of recent human nutritional intervention studies (Jackson *et al.*, 1999; Griffin *et al.*, 2002; Klinder *et al.*, 2004; Furrie *et al.*, 2004; Langlands *et al.*, 2004). However, not all studies have proven to be positive. In these no-effect studies experimental design often proved to be inadequate to capture hypothesized effects. Parameters which should be addressed in future human studies with human volunteers on the efficacy of prebiotics in particular disease states include study cohorts inclusion criteria (e.g. genotype or disease diagnosis of patients), standardization of clinical measurements, identification of efficacious biomarkers and use of an interdisciplinary approach. Of particular importance is the need to determine microbiological effects using state of the art molecular techniques to link the changes in gut microbiota

due to prebiotic ingestion with specific health outcomes or biomarkers of disease. The advent of omics technologies will have a direct impact on this field of study. The systems biology approach (genomics, transcriptomics, proteomics and metabolomics) will enable direct measurement of the physiological relevance of changes within the gut microbiota and provide essential mechanistic data on how, through microbiota modulation, prebiotics affect human health. Positive results have been obtained with the use of substrates containing stable isotopes to show differences in gastrointestinal metabolism induced by the intake of pre- and probiotics (De Preter *et al.*, 2004).

Prebiotic and probiotic applications to companion animals

To assess the role of probiotics and prebiotics in the health of companion animals it is essential to understand the bacterial communities in their guts. Only limited literature exists on the normal gut microbiota of pets (Greetham *et al.*, 2002; Rastall, 2004). Most studies have used culture-based techniques and have revealed much greater variation in the canine and feline gut microbiota than in humans. Current culture methods need to be modified for companion animal applications. A polyphasic strategy based on selective plate culture for screening and colony isolation, with molecular techniques for speciation, would go far towards addressing this knowledge gap. Fluorescent *in situ* hybridization provides reliable quantitative data for physiologically related groups of bacteria and detects non-culturable organisms. Gut bacterial profiling can also be achieved using denaturing gradient gel electrophoresis.

Outcomes tested with feeding studies in companion animals are frequently based on targets used in human research. However, the range of targets for companion animals can be expanded and include fecal and oral odor, bacterial and viral infections, atopic dermatitis, inflammatory bowel disease, urinary tract health, cancers, inflammation, immune modulation, oral health, skin and coat health in addition to gastrointestinal health. As a result, the effectiveness of probiotics and prebiotics are likely to differ for different species, based in part on varying nutritional needs. Human probiotics have been used successfully in companion animals (Rastall, 2004), but a more effective approach would likely be use of strains found naturally in the target animal (Fuller, 1989). New bacterial genera may be discovered to be effective probiotics in companion animals.

One economical source of novel prebiotics is waste biomass from food processing operations. Such material typically contains cellulose, xylans and pectins. Xylans are also a good source of prebiotics and xylooligosaccharides are produced commercially in Japan. Biomass polysaccharides may also be a source of beneficial carbohydrates other than prebiotics. Oligosaccharides derived from pectins are protective against *E. coli* O157: H7 verocytotoxin, stimulate apoptosis in a human colon cancer cell line and are prebiotic (Olano-Martin *et al.*, 2002; Olano-Martin *et al.*, 2003a; Olano-Martin *et al.*, 2003b). Probiotic bacteria isolated from companion animals may be a source of enzymes that can be used to produce effective prebiotic structures. Such prebiotics can be combined with the probiotics to produce targeted synbiotics.

Some additional challenges to probiotic and prebiotic applications to companion animal feed are:

- The retort and extrusion processes currently used to produce petfoods are designed to kill bacteria. Novel post-extrusion methods must be developed to deliver viable probiotics in petfood.
- It is likely that prebiotics would need to be effective at ≤ 1% w/w due to animal feed cost sensitivity.
- A sizable barrier to commercialization of new prebiotics for companion animals is the regulatory process. The process is considerably expensive, differs throughout the world, and is laden with tighter regulations than required for human applications.

Prebiotics and probiotics may be most beneficial for animals with an abnormal gut microbiota and/or immune status. This may include senior dogs and cats, weanling puppies and kittens and animals undergoing prolonged antibiotic treatment. Further research is needed that should answer the following specific questions:

- Which prebiotic, probiotic or synbiotic is most efficacious for different targets in companion animals?
- What are the effects on different companion animal life stages?
- What is the lowest effective prebiotic dose in companion animals?
- What are the mechanisms of action for prebiotics and probiotics in companion animals?

Conclusions

The diverse topics covered in this symposium highlight areas of significant advance in the field of probiotics and prebiotics. Understanding at the cellular level the interactions of specific probiotics with other intestinal bacteria and with different types of host cells is fundamental to understanding how probiotics function, the basis for observed effects on human and animal physiology, and what types and levels of effects might be anticipated by probiotic or prebiotic intervention. Genetic technologies will be essential to providing necessary precision for revealing the secrets of probiotic and prebiotic actions as well as driving invention of strains with targeted function. Developing approaches to studies that will provide insight into the ability of exogenously applied bacteria to impact healthy consumers is essential to leveraging probiotics broadly as healthful dietary components. Pulling all this information together to enable companies to assess potential, evaluate safety and communicate benefits is necessary for this field to progress in the marketplace. ISAPP's goal is to provide opportunities for scientists to meet, read ISAPP reports, to collaborate, to cross-fertilize other related disciplines and thereby contribute to the advancement of the science of probiotics and prebiotics. Future ISAPP-sponsored meetings, symposia, written papers and other activities will hopefully help scientists make progress toward these goals.

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