



NUTRITION AND HEALTH COLLECTION

Immunity and Probiotics



AN INITIATIVE OF THE DANONE RESEARCH CENTERS



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Immunity
and
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CONTENTS

Introduction	9
1. Probiotics: myth or reality	13
2. Selection and implementation of probiotics	16
3. The immune system	18
Innate and adaptive immunity.....	18
Humoral and cellular immunity.....	20
Mucosal immunity.....	21
Cellular regulation of the mucosal immune system.....	22
Tolerance and immunity: a dual function of the intestinal immune system.....	23
4. Mucosal immune system and age	25
Aging population.....	25
Babies and young children.....	27
5. The mucosal barrier: a physical protection	29
6. Probiotics and immunomodulation: animal model studies	31
7. Probiotics and immunomodulation: in vitro studies	34
8. Probiotics and intestinal disorders	37
Treatment and prevention of children’s diarrhea.....	37
Inflammatory bowel disease.....	38
Colorectal cancer.....	40
9. Public and legal implications	45

INTRODUCTION

“It is a great honour for me today to open the second Danone Symposium on ‘Fermented Food, Fermentation and the Intestinal Flora’. This symposium will be focused on a very important, complex and scientifically challenging field: that is, the relationship between probiotics and the immune system.

Today, a number of presentations will provide us with some insight on how the effects of probiotics are measured and on how they function. A growing body of evidence suggests that a direct relationship exists between probiotics and the innate and adaptive immune systems... Some lectures will focus primarily on demonstrating that, in order that the defence mechanism be better coordinated, the interplay between the innate and adaptive immune systems has to be working at the same pace.

It is now known that probiotics have a notable impact in creating the optimal conditions for the coordination of both arms of the general immune system. But all these mechanisms, as will be discussed today, are obviously influenced by a number of factors. One of the reported factors is diet, food and the different components of our dietary intake. The effects of these factors, their measurement and evaluation have also been reported. This leads us to investigate the following questions: what is the role of food, and where do we want to go in terms of the evolution pattern of food consumption? And then we shall proceed to relate that to probiotics, which are a growing segment in terms of food consumption.

If we have a look on what was happening only 30 years ago, and compare this to what is happening now in the food area, we can see that there is a tremendous evolution in terms of what we expect from food. Thirty years ago, we all expected food to be a source of energy to sustain us in our daily activities. But we have moved beyond that. Obviously, the role of energy remains important, but I do not think the lack of it is such a big problem in Western countries any more. Obesity is becoming one of the main problems that we are facing today, and is related to over-accumulation of energy. So we have moved

from the concept of energy to: 'What is a balanced diet?' A balanced diet was described in terms of the percentage of energy derived from carbohydrates, fat, and proteins which was necessary according to the physiological needs and age of a given person.

Today, food is not just an accumulation of a source of energy.

A striking feature of the 90's is that food is even more in demand. Food is considered as a source of prolonged well-being. Pleasure is always an important aspect of food, but, in addition, well-being is becoming an increasingly accepted consumer requirement. Moreover, we want food to reduce some of the risks that we might encounter in life. We are moving into a common acceptance of food as playing a major role in terms of health prevention. Nowadays, health prevention and well-being are two determining factors in the consumer's choice of a food product.

Obviously, this cannot be achieved without science. Science must be the basic, architectural, building blocks of today's food and of the next generation. And one of the growing fields in this regard is the role of probiotics. Probiotics represent a growing market, of which consumers are increasingly aware; consumers want to know how they work. And this is where science comes in.

Thus, it is important that food consumption and the architectural basis of food should be founded on scientific facts, if we want to be able to generate credibility and confidence.

If we go back to our first symposium at the Pasteur Institute in February at the beginning of this year, it focused more on fermented food and on its impact on the digestive tract, and led to two main conclusions.

The first one was that food and the diet will have a dramatic impact on the composition of the microflora, in terms of numbers and nature of the microorganisms.

The second one was that, in addition to the fact that the microflora could be modified by the food we eat, there is a close relationship between the microflora itself and our defence mechanisms.

These defence mechanisms could consist in direct competition with the incoming pathogen; either by bacterial competition, or by production of other components such as bacteriocines which could interfere with the growth of pathogenic microorganisms. A relationship might also exist between the microflora itself and the interplay between the innate and the adaptive immune systems.

This is the reason why we were eager to organise this meeting today and answer some of the questions that we all have been asked.

One important question that often comes to mind is: 'Have we overused our immune system to the point that it is fatigued?' This is a question that needs to be debated and answered.

The second important question that I think should receive an answer pertains to the mediation between the immune system and the microflora.

- How does it work?
- Which are the immune cells involved?
- Are they the M-cells or the T-cells?
- What are the factors that really modify those specific cells so that they become the interface or the mediators?

Other domains that will be discussed are, for example, the genetic and immune determinants affected by age, and particularly the mucosal immune system. What happens? Are they programmed or is it a combination of genetic programming and different environmental factors having an increasing impact as we grow older?

So, with some scientific support, we should be able to communicate that information either via food labels or directly to the consumers, in order to have a positive impact on the general population. Communication of science by itself becomes an important factor upon which we must rely. And this is one of the reasons why we organise symposia.

I would like to emphasise again the importance of understanding and identifying the mechanisms of the relationship between probiotics and immunity, in order to communicate this information to the consumers either via food labels or through direct communication.”

Akram Fazel
Director of Research
Danone Dairy Division

CHAPTER I

PROBIOTICS: MYTH OR REALITY

The beneficial effect of lactic acid bacteria (LAB) in human or animal health has interested scientists since the early observations of Ilya Metchnikoff. Almost a century ago, this Nobel Prize winner clearly stated that bacteria are not necessarily detrimental to man, but may on the contrary play an important role in our well-being. He was actually the first to recommend ingestion of live cultures of microorganisms, the LAB, whose natural role was to prevent "putrefaction". In his opinion, this effect was an indirect cause of prolonged life expectancy of Caucasian populations consuming large quantities of fermented milk products. Since then, considerable research has been carried out in the field of what has come to be known as "probiotics". The definition of "probiotics" is constantly evolving, but essentially designates "living microorganisms which favorably influence the health of the host by improving the indigenous microflora" (Fuller, 1989). This may be differentiated from non-probiotic health effects of microorganisms which do not survive the mouth-caecum transit and therefore do not alter the intestinal microbial balance. A typical example of the latter case is the improvement of lactose maldigestion by the ingestion of yogurt. In certain countries, a high proportion of the population suffers from lactase insufficiency, a deficiency which can best be alleviated by the β -galactosidase activity of yogurt cultures. Also, it is possible that some probiotic effects result from biological activity present in the supernatant of cultures that do not impact on the composition of the intestinal microflora. Nevertheless, as the complexity of the latter remains only partially understood, it may be difficult to differentiate probiotic from non-probiotic health effects.

During the symposium, probiotics were defined as "preparations to be consumed orally containing viable microorganisms that affect the intestinal flora and show health effects that go beyond those of a normal diet". Whether fermented dairy products prepared with probiotic starters should be called nutraceuticals was discussed.

Positive health effects may also be obtained by "prebiotics" which are essentially non-digestible substances (mainly fructo- and galacto-oligosaccharides) that increase

the growth or the activity of specific microorganisms in the gastro-intestinal tract. Promotion of desirable bacteria, such as bifidobacteria, can further lead to displacement of undesirable microorganisms (e.g. Clostridia, coliforms) as was observed in healthy persons fed a controlled diet containing oligofructose. Nowadays, the term “synbiotic” designates the synergistic combination of pre- and probiotics.

Although the concept of synbiotics looks very promising, most research has thus far focused on probiotics. The list of potential health-promoting traits attributed to LAB in particular is quite impressive (table I). Nevertheless, most of the suggested effects remain to be proven or substantiated by human randomized double-blind and placebo-controlled clinical studies. Among these effects, the following observations must still be consolidated:

- reduction of *Helicobacter pylori*-induced ulcers and malignancies (evidence mostly obtained in vitro);
- relief from constipation or irritable bowel syndrome;
- reduction of allergic symptoms (with the exception of atopic eczema, see below);
- beneficial effects on mineral metabolism, particularly on bone density and stability;
- cancer prevention.

Table I. Probiotics: suggested effects

-
- Increased nutritional value (better digestibility, increased absorption of minerals and vitamins)
 - Promotion of intestinal lactose digestion
 - Positive influence on intestinal flora (antibiotics or radiation-induced colitis)
 - Prevention of intestinal tract infections (bacteria or virus induced, *Candida enteritis*, *Helicobacter pylori* ulcer, neoplasia)
 - Regulation of gut motility (constipation, irritable bowel syndrome)
 - Improvement of immune system
 - Prevention of cancer
 - Reduction of catabolic products eliminated by kidney and liver
 - Prevention of atherosclerosis (reduction of serum cholesterol)
 - Prevention of osteoporosis
 - Better development (growth)
 - Well-being
-

Source: J. Schrezenmeier, this symposium.

While it is generally accepted that the health claims mentioned above are supported by progressively increasing evidence, the potential reduction of cholesterol and triglycerides levels remains a controversial issue. It should also be mentioned that the growth-promoting effect has been observed only in young rats. In contrast, it has been proven, or documented by solid clinical data, that specific probiotic LAB strains may:

- lower the frequency and duration of travelers’ diarrhea and of diarrhea associated with *Clostridium difficile*, rotavirus infection (especially in hospitalized children) and chemotherapy;

- decrease unfavorable metabolites (heterocyclic amines, NH₃) and cancerogenic enzymes (β -glucuronidase, nitroreductase, azoreductase) in the colon;
- induce beneficial immunological effects, an aspect which will be further discussed below.

Strikingly, while an important effort is presently being made to confirm or prove the claimed health benefits, virtually no information is available on the mechanisms underlying these different actions. This constitutes an open avenue of research, covering numerous questions that still remain open:

- What are the mechanisms accounting for quite different biological effects?
- Is there a small bowel terrain of probiotics which must be differentiated from a colonic site of action?
- Is there a luminal and/or mucosal site of action?
- Which and how many studies are needed to provide sound evidence of health effects?
- Is there a critical number of viable bacteria in the product to be defined?
- What is the dose-response relationship of probiotics?
- What is the duration of effects during or after cessation of intake?
- Can the effectiveness be increased by gene technology, e.g. adhesion enhancement?
- What is the potential of using probiotics as vehicles for other active principles?

These questions have been partly addressed in the case of protection against bacteria-induced enteritis, where knowledge of the physiology of LAB and data generated mostly *in vitro* have led to the hypothesis that the mode of action results from a combination of factors. These factors include the inhibition of the growth of pathogens by competition for the nutrients, production of antimicrobial substances (H₂S, H₂O₂, bacteriocins) and local lowering of pH and redox potential linked to an increase in mucosa-cell regeneration and gut motility as well as competition for mucosal adhesion sites and stimulation of the immune system.

It is important to bear in mind that not all LAB strains exhibit probiotic effects. In fact, a strong variation between species and strains belonging to the same species is expected and has indeed been observed in certain cases. Furthermore, strains presenting the desired characteristics required for a specific health effect may not present appropriate technological properties. Alternatively, the strain may be unstable, generating variants with altered levels of the beneficial traits. It thus becomes evident that progress in the area of probiotics will depend on the capacity to define and apply adequate strain selection criteria, based on a better understanding of the mechanism underlying the targeted health benefit.

SELECTION AND IMPLEMENTATION OF PROBIOTICS

In the development of a probiotic food, the selection of a functionally active strain is a crucial step. The implementation of the strain in the preparation of the final product should nevertheless not be overlooked, as it will determine whether or not the strain is utilized at its full potential. Therefore expertise is required from both human physiologists and food microbiologists. Also, from a legal point of view, it will be necessary to establish a consensus definition of probiotics and probiotic foods that is agreed upon by international experts from different disciplines.

With regard to probiotic strain selection from microbial ecosystems (figure 1), there are two mandatory filters: safety (only non-pathogenic non-toxic strains can be used) and survival through gastrointestinal (GI) transit. The latter corresponds to only one of several functional screenings which have been proposed. Unfortunately, most of these are still based on working hypotheses which remain to be demonstrated. There is thus a pressing need to set up validated functional models, preferably *in vitro*. Their development will rely on unraveling the mechanisms of action of probiotics with already proven effects and verification of the working hypothesis with the help of isogenic strains lacking the proposed active factors. This latter target is now attainable thanks to progress in LAB molecular genetics. The most difficult part will probably involve linkage of desired phenomenology to a specific strain characteristic. For example, in the case of lactose maldigestion improvement, will the β -galactosidase level of the candidate probiotic strain be the main criteria? For GI protection and immunomodulation, should factors other than the capacity of inducing immunoglobulin and interleukin production, adhesion and hydrophobicity, or bacteriocin synthesis be considered? In order to improve the efficiency of the selection process, it would be ideal to have access to a series of cross-validated *in vitro* screening tests, to be further completed by *in vivo* studies in animal models. The strain selected in this way will correspond to a “potential human probiotic” whose functionality can only be proven definitively

by clinical studies. The selection phase will generally include a microbiological-biochemical characterization of the selected strain as well as its unambiguous identification.

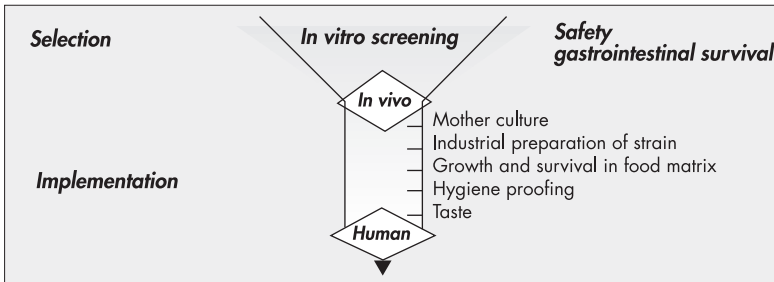


Figure 1. Probiotic strain selection criteria.

Implementation of the probiotic strain in the food substrate necessitates to verify a number of technological properties and to perform successive steps. A mother culture must first be established to ensure durable stocks. It should be noted that the process utilized for long-term storage may change certain properties of the strain, such as its acidification capacity, for example. Industrial inocula are prepared from the mother culture and this large-scale fermentation must be fully controlled. Adequate cultivation and storage conditions (substrate, temperature, pH, oxygen content, water activity) must be defined to ensure the highest multiplication rate, i.e. the highest population in the final product, with the best survival during the shelf life of the food. While association of strains from various species may help to meet these goals (e.g. the yogurt symbiosis), it may be difficult to maintain all individual strains at high viable counts. Also, it is known that the viability of different strains is affected by external stress conditions such as those encountered in industrial processes or passage through the GI tract in various ways. It is thus quite possible that even when starting with a product containing high counts of viable cells, not all active factors reach the gut.

Another critical point in manufacturing a probiotic food is to run hygiene proofing of the overall production process. Indeed, probiotic bacteria are often slow acid producers or slow growers in milk, and risks of contamination by non-desirable microbes are higher than in the case of classical dairy starters. The large scale fermentation of probiotic strains thus requires special treatment that is adjusted with the help of simulation models and contamination challenge tests. Lastly, the final probiotic food should taste good and be pleasant to consume.

Optimization of the different parameters mentioned above leads to fine-tuned fermentation achieved under particular conditions and often with specially-designed strain associations. Obviously, the property required for functional activity must be maintained, as proven by adequate controls, throughout the overall process. At this stage, the probiotic product is ready to be tested in humans to determine the real health benefit of the final mix.

THE IMMUNE SYSTEM

INNATE AND ADAPTIVE IMMUNITY

The immune system evolved with a very important function to carry out, namely to protect the host from pathogenic and lethal infections. An intriguing aspect of the immune system is that it can manifest itself in the defence against an unlimited number of pathogenic microorganisms or viruses. The immune system has been arbitrarily divided into two compartments, namely the innate immunity and the adaptive immunity. Both compartments consist of cellular and humoral factors that mediate the immune defence response. The innate immune system (macrophages, neutrophils, NK cells, serum complement, etc.) provides a first line of defence against many common microorganisms and is essential to control infections. However, it cannot always eliminate infectious organisms, and there are many pathogens that it is unable to recognize. The lymphocytes of the adaptive immune response (T cells and B cells) have evolved to provide a more versatile means of defence, that, in addition, provides an increased level of protection from a subsequent re-infection with the same pathogen. The cells of the innate immune system play a crucial part in the initiation and subsequent direction of the adaptive immune responses.

A first exposure to a foreign pathogen is encountered by the innate immune response. This response is non-specific to any particular pathogen but features many recognition systems. For example, macrophages and neutrophils (phagocytes) have surface receptors that evolved to recognize and bind many common constituents of many bacterial surfaces. Bacterial molecules binding to these receptors trigger the cells to engulf the bacteria and also induce the secretion of mediators such as cytokines that cause inflammation. Also, many bacterial surfaces trigger the complement system which results in both bacterial phagocytosis and bacterial destruction. Viral infection results in infected tissues

and such infected tissues are recognized by the natural killer cells and are destroyed to control the viral replication.

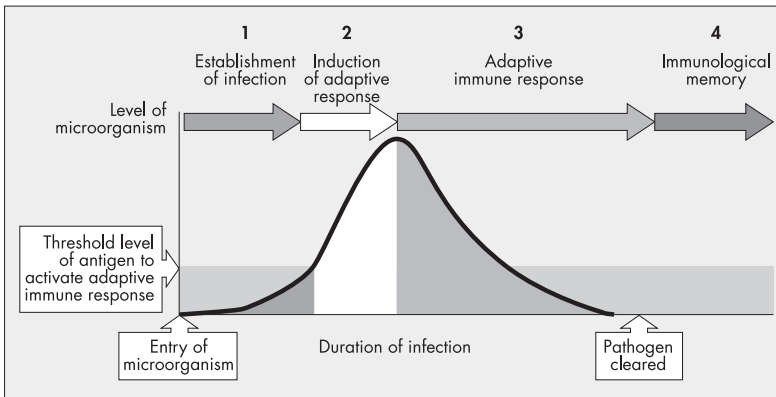


Figure 2. Evolution of the immune response to a foreign pathogen.

Escape from innate immunity and/or failure of the innate system to recognize the pathogen results in the pathogen mediated induction of the adaptive immune system (figure 2). This exquisite system consists of lymphocytes each bearing a receptor which is specific for a particular unique antigen of a pathogen. The specificity of these receptors is determined by a unique genetic mechanism that operates during the development of lymphocytes in the bone marrow (B lymphocytes) and in the thymus (T lymphocytes). Although the individual lymphocytes carry receptors of only one specificity, the specificity of each lymphocyte is different and thus the millions of lymphocytes in the body give rise to millions of various specificities. Thus, for an adaptive immune response to take place, antigens from the pathogens are processed and presented in the context of the major histocompatibility antigens (MHC class I and II) by antigen-presenting cells to corresponding specific T cells bearing the receptors for the MHC-peptide complex. These T cells are activated, become effector cells and secrete cytokines. Certain cytokines secreted by one subset of helper T cells (Th2) activate the specific B cells for the antigen to differentiate and secrete antibody. The other T helper cells subset (Th1) is primarily involved in inflammatory responses and also secretes cytokines to activate CD8 cytotoxic T cells. Both the humoral antibody response and the cellular response participate in the elimination of the pathogen or virally infected cells. These clonally expanded antigen-specific T and B cells, after containment of the infection, differentiate into memory cells. Upon a second encounter with the same pathogen, these memory lymphocytes respond rapidly and effectively to fight the infection. This memory response is the principle of vaccines utilized nowadays in infants and adults.

HUMORAL AND CELLULAR IMMUNITY

Antibodies are made by plasma cells derived from B-lymphocytes, each of which is programmed to make only one antibody which is placed on the cell surface as a receptor. Antigen binds to the cell carrying the complementary antibody, activates it, and causes clonal proliferation and finally maturation to antibody-forming cells and memory cells. Like B cells, T cells have their individual antigen receptor and recognize antigen in association with molecules of the major histocompatibility complex (MHC) (figure 3).

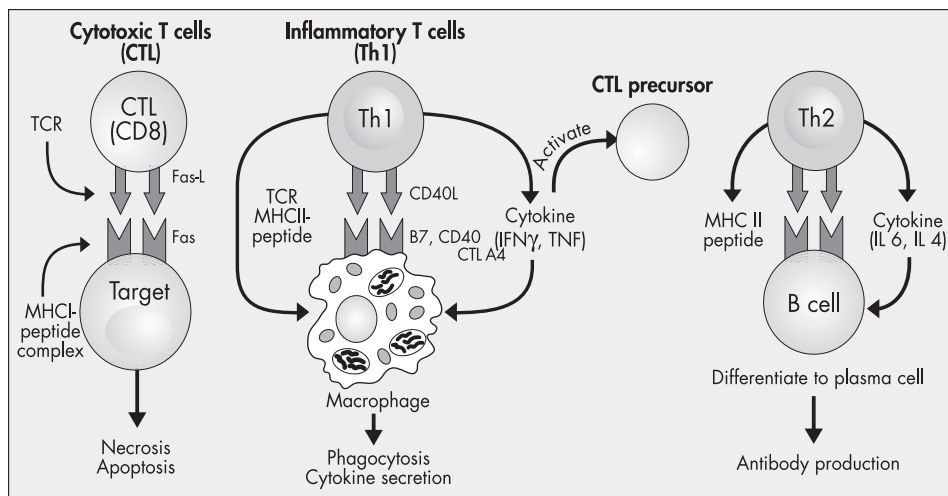


Figure 3. The different arms of the immune system and their major cellular components.

Cell-mediated immunity is under the control of two different T cell populations. A subset of cytotoxic T cells (CD8+) recognizes the antigen in association with class I MHC molecules. By intimate contact with corresponding targets, they kill the targets and are particularly implicated in the elimination of viral and also microbial infections. They also release γ -interferon (IFN γ) which can make surrounding cells resistant to viral spread. T helper cells (CD4+) recognize antigens with class II MHC on the surface of antigen-presenting cells (APC) and produce upon activation a variety of soluble factors called lymphokines. These subsets of T cells are derived from a common precursor and differ from each other by their function, their cytokine production profile, their migration pattern and the expression of selected surface molecules. The Th1 subpopulation produces certain types of cytokines (IFN γ , IL2, TNF β) and confers protection against intracellular pathogens (e.g.: virus and certain bacteria) by recruiting and activating macrophage phagocytosis (upregulation of class II MHC and accessory molecules). It also promotes the production of opsonizing antibodies (IgG1 and IgG3) by B cells. Th2 are more implicated in humoral immunity by mediating B cell activation and differentiation, especially IgE and IgA antibody production (by IL4, IL5 and IL6 secretion). They also participate in the recruitment of

eosinophils, mast cells and basophils, conferring protection against certain parasitic infections (figure 4).

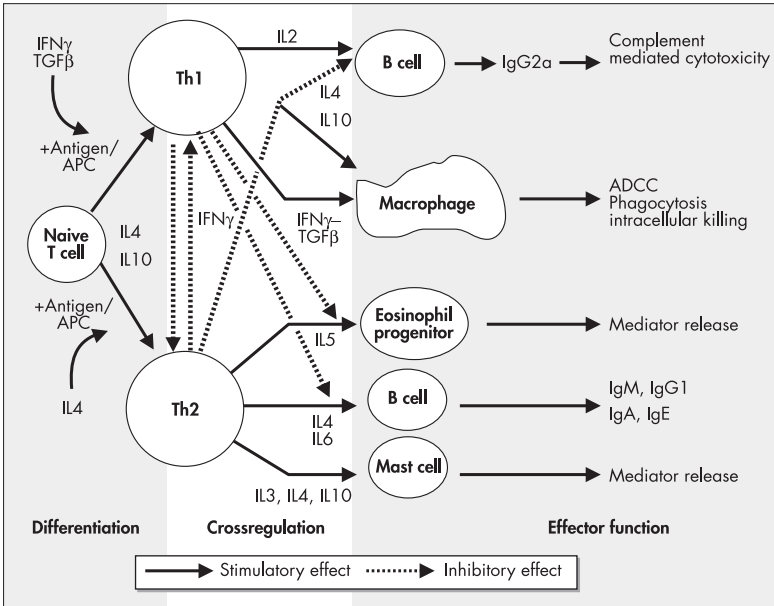


Figure 4. Regulatory interactions between CD4+ T cell subsets.

Th1 and Th2 subsets cross-inhibit each other's function and development. Th0 cells, which can be considered as an intermediate stage of differentiation, produce a large spectrum of cytokines, generally at low level, and are not associated with chronic inflammatory conditions. The relative proportion of all these T cell subpopulations depends upon the nature of the pathogen and the duration of the infection and will lead to a protective or inefficient response according to the nature of the CD4+ T cell response. Inappropriate polarization of these Th1/Th2 responses can eventually lead to disease, for example allergy (Th2) or auto-immunity (Th1). Another subset of regulatory T cells, named Th3, generated in the gut-associated lymphoid tissue (GALT), suppresses the function of effector cells by releasing the inhibitory cytokine TGF β . It is also implicated in the IgA production and in the regulation of oral tolerance by blocking Th1 or Th2 response.

MUCOSAL IMMUNITY

The balance of the different Th subsets is particularly important in mucosal immunity. The mucosal immune system consists of specialized local inductive sites (Organized Mucosa-Associated-Lymphoid Tissue or O-MALT) and widespread effector sites (Diffuse Mucosa-Associated-Lymphoid Tissue or D-MALT) which are separated from the mucosal surface by epithelial barriers. The O-MALT, characterized by mucosal lymphoid

follicles, occurs in the tonsils, bronchi and intestine. Induction sites in intestinal mucosa are constituted by the Peyer's patches. They have the anatomical appearance of classic secondary lymphoid organs, with clearly defined T- and B-cell-dependent areas separated from the intestinal lumen by epithelial cells. In the intestine mucosa, antigen uptake (especially particular antigens) occurs either through the specialized sampling system represented by the M cells overlying the Peyer's patches or across normal epithelium overlying the lamina propria. Historically, Peyer's patches have been thought of as the major inductive site for immune response in the intestine. The presence of M cells that overlay organized lymphoid tissue and are in close contact with APC makes this an attractive possibility. However, on a quantitative basis, the majority of T cells found in the intestine are by far present in the diffuse lymphoid system of the lamina propria. After priming, memory B and T cells migrate to effector sites, followed by active proliferation, local induction of certain cytokines and production of secretory antibodies (IgA).

CELLULAR REGULATION OF THE MUCOSAL IMMUNE SYSTEM

Local as well as peripheral immune mechanisms contribute to the regulation of the mucosal response in the intestine. Intra-epithelial lymphocytes (IEL) have been grouped into a category of non-professional antigen-presenting cells that do not constitutively express class II MHC molecules and do not activate T-cells in a conventional manner (lack of costimulatory molecules such as B7-1 and B7-2). In contrast, primary immune responses require the presence not only of appropriately processed peptide-MHC complexes but also the expression of a number of costimulatory molecules on the surface of the APC to amplify T-cell activation. Binding to their appropriate ligands (CD28 and CTLA-4), APC potentiate T-cell responses and rescue cells from anergy or apoptosis. Classical or professional APC include bone-marrow derived B-cells, macrophages and dendritic cells (DC). DC are the most potent cells to initiate a primary T-cell dependent immune response. They are also present within non-lymphoid tissues (skin, respiratory and digestive tracts). Two different subsets of DC are defined according to their lymphoid (lymphoid derived DC: LDC) or monocyte (monocyte derived DC: MDC) origin. According to the nature of the antigen presentation (i.e. nature of the antigen itself), different types of signals will be delivered to the T-cell precursor and lead to different costimulation (B7-CD28 or B7-CTLA4) and different Th1/Th2 polarization. Two subtypes of MDC can be defined: MDC1 responds to LPS, lipoteichoic acid or CpG DNA by the means of Toll mannose receptor (which leads to NF κ B activation) and potentiates Th1 response; the MDC2 subtype responds to immune complexes and cellular factors (cytokines, PGE2, MCP1) and leads to a Th2 response mediated by CD40/CD40L recognition. Today, it is not known whether there is a DC3 subtype involved in Th3 induction.

Many pathogenic microorganisms exploit M-cells to cross the digestive epithelial barrier. M-cells are derived from the stem cell compartment of the epithelial layer and their development appears to depend on the presence of lymphoid cells in model systems. Thus,

passage of antigens or microorganisms through M-cells is an essential step in the development of mucosal immune responses on the one hand, and in the pathology of many infectious diseases on the other. Because M-cells are a minor population in the follicular associated epithelium (FAE), they are difficult to characterize, and little is known about their biology and especially about the molecular mechanisms of microorganism M-cells interaction that lead to translocation. A culture system that reproduces the main characteristics of FAE and M-cells has been established by co-cultivation of Peyer's patch lymphocytes (PPLC) with the differentiated human intestinal cell line CaCO₂. In these experimental conditions, phenotypic conversion of enterocytes into M-cells was observed: reorganization of the brush border, accumulation of lymphocytes in epithelial pockets, and loss of expression of digestive enzymes. The ability of Peyer's patch lymphocytes to convert enterocytes into M-cells was also assessed in vivo. Such in vitro models are used to study the ontogeny of M-cells but also to establish the nature of the specific interaction between various pathogens and the apical membrane of M-cells, as well as the translocation of molecules and microorganisms. For example, *Vibrio cholerae*, which is a non invasive pathogen, can be transported by CaCO₂ differentiated in M-cells in the presence of PPLC. Electron microscopy reveals that they are located in the cytoplasmic vacuole of M-cells. Translocation of other bacteria was also observed, some with efficacy (*Shigella flexneri*), other showing less transport (*Listeria monocytogenes*). Very tight adhesion of *Yersinia enterocolitica* was also measured in this model. Information on the interactions of microorganisms with these transepithelial transport systems could lead to more effective targeting of non-living vaccines and live microbial vectors producing recombinant proteins to the mucosal immune system.

Another promising route for inducing specific immunity with mucosal vaccines is buccal immunization. In contrast to the intestine, oral tolerance does not occur at the buccal mucosa, which corresponds to an efficient site of immunization even with soluble antigens. In the buccal mucosa, a network of antigen-presenting DC, similar to Langerhans cells, migrate after antigen uptake to distant draining cervico-mandibular lymph nodes where they can prime T-cells.

TOLERANCE AND IMMUNITY: A DUAL FUNCTION OF THE INTESTINAL IMMUNE SYSTEM

Mucosal surfaces are specialized in two opposite functions: tolerance to environmental antigens (dietary antigens, probiotics) and immunity to mucosal pathogenic microorganisms. The intestinal tract represents an attractive system to study this functional duality of tolerance versus immunity inasmuch as it comprises various lympho-epithelial tissues which differ with the type and structure of the epithelium and the proximity of lymphoid nodules. Whereas infection with a mucosal pathogen leads to an active primary immune response followed by memory upon a secondary infection, oral administration of soluble antigen results in a suppressed response to subsequent exposure (oral tolerance). Mainly early studies demonstrated the presence of active suppression in vivo (following

oral administration of antigens) which could be transferred to naive recipients. This inhibitory mechanism could be mediated by class II restricted CD4+ T-cells and cytokines with immunosuppressive function (IL10, TGF β). This hypothesis is strengthened by the observation that knock-out mice deficient in either class II MHC molecules, TCR α/β , IL2R or IL10 develop an inflammatory bowel disease similar to that seen in human. The microflora also seems to play a crucial role in tolerance induction, as suggested by the fact that germ-free animals are defective in oral tolerance. In experimental models, after tolerization by hapten feeding, a down-regulation of delayed type hypersensitivity (DTH) is observed. In contrast, in MHC class II deficient or IL10-/- knock-out mice, no modification occurred. The same lack of tolerance is obtained when animals are treated with anti-CD4 monoclonal antibodies. Epithelial cells seem to contribute to oral tolerance by providing IL10 and TGF β , which cause bystander inhibition of T-cell responsiveness to haptens by anergyizing the T-cells. Mucosal DC could be critical APC for inducing both active immunity and tolerance, with the eventual outcome depending on the level of inflammatory and costimulatory activity induced by the antigen. According to this hypothesis, inert antigens such as soluble proteins may induce systemic T-cell tolerance because they are delivered to the immune system by antigen-loaden DC which exit from the gut without acquisition of significant costimulatory activity. The issue of how antigen is taken up, processed and presented by APC at mucosal surfaces is fundamental for understanding both the induction of mucosal and systemic immune response and oral tolerance.

Another cell population within the intestinal epithelium, the $\gamma\delta$ T lymphocytes, may play an important role in mucosal immunity. There is increasing evidence that $\gamma\delta$ T-cells are important in orally induced tolerance, and they have also recently been implicated in providing help for mucosal IgA production. Cross-talk between IEL and epithelial cells has been reported. Such interaction, mediated by the secretion of growth factors for stem cells (SCF, GF) and cytokines such as IL7 by epithelial cells, could influence the proliferation of $\gamma\delta$ IEL and regulate the mucosal IgA response. Moreover, $\gamma\delta$ lineage represents a logical evolutionary link between the non-specific, non-lymphocytic cells of the classic innate immune system and the very specific $\alpha\beta$ T-cells primarily responsible for acquired immunity.

In the same way, probiotics have been reported to favorably modulate both innate and acquired immunity. These safe microorganisms may interact with endogenous bacteria and/or host mucosal cells to induce or modulate the immune response. The immune system needs to discriminate between infection and non-infection. Innate immunity controls infection until the adaptive immune response can take over. As such, it must discriminate between self and non-self perfectly. This process could be mediated by triggering cell receptors that lead to intrasignaling and induction of cytokines. A new class of receptors called Toll-like receptors (TLR), could be a key initiator of innate immunity via activation of the NF κ B and AP1 pathway. The identification of bacterial components which are able to trigger "receptors" on cells that lead to intracellular signaling and induction of cytokines could be very useful in understanding how the immune response is induced and how it can be modulated.

CHAPTER IV

MUCOSAL IMMUNE SYSTEM AND AGE

The different aspects and components of the immune system presented above are valid for healthy adult individuals. However, on the frontiers of life, this situation is naturally altered: while the immune system of babies or young children must be “educated” after birth, it progressively loses efficiency with aging.

AGING POPULATION

The old (> 65 years) and the oldest old (> 85 years) age groups are the fastest growing subpopulations in industrialized societies. They correspond to an increased-risk population with high rates of morbidity and mortality due to infectious diseases. Most pathogens actually penetrate the body through the mucosal surfaces, which constitute a huge area (300-400 m²/adult) directly exposed to viruses and bacteria. The immune compartment involved in this first line of defence is largely independent from the systemic compartment. In mammals, the intestinal tract represents the largest single immunological organ. It contains > 70% of the immunoglobulin-producing cells and produces more IgA, the major immunoglobulin isotype in this location, than the organism’s total production of IgG. Despite the fact that mucosal surfaces correspond to the port of entry of most pathogens, the effects of aging on mucosal immunity have not been studied extensively.

There is, however, sufficient epidemiological and research evidence to suggest that intestinal mucosal immunity diminishes with increasing age, and, as a result, the intestinal tract of elderly subjects is especially susceptible to infectious diseases. WHO statistics indicate a 400-fold increase in mortality attributed to intestinal infections in elderly versus young adult populations and patients over 60 years account for 85% of the deaths due to diarrhea. Several studies, including a few in humans, did not detect age-related changes in intestinal IgA titers and concluded that mucosal immunity is unaffected by age. However,

these studies measured non-specific IgA, a poor index of mucosal immune responsiveness, rather than specific IgA antibody titers, a direct measure of immune vigor.

Several mechanisms direct the transition of immunological surveillance to active protection. One or more of them may become less efficient with age, including initiation of immune response, maturation and/or migration of IgA lymphoblasts from Peyer's patches to the intestinal lamina propria, and local antibody production or epithelial transport of antibodies to the mucosal surface. Even though limited data are available in humans, studies conducted with rats or rhesus macaques have shown that the intestinal IgA antibody response to intraduodenal immunization with cholera toxin (CT) diminishes with increasing age.

This does not seem to be linked to an age-related decline in antigen uptake by intestinal M-cells. No observations have documented thus far that this critical event is affected in the elderly. In rodents, the number and distribution of Peyer's patches, a major inductive site of the gut-associated lymphoid tissue (GALT), are not affected by age. In fact, the number of IgA positive cells in Peyer's patches in rats immunized with CT seems higher in old animals than in young or naive ones. Therefore, any suggestion that immunological surveillance or antigen uptake in the intestine is compromised in the elderly is speculative.

The second step which may lead to a decreased immune response is impaired maturation/migration of the IgA plasma cells. Both appear to be compromised in old animals. Immunohistochemical and flow cytometric studies have shown that aging leads to a reduction or a different B and T lymphocyte subpopulation distribution in the GALT. For example, in Fischer rats of 24-26 months, T-cells are equivalent in relative amounts but not in their distribution at the level of Peyer's patches, when compared to young (4 months) animals. Their migration from Peyer's patches to intestinal mucosal sites may be evaluated by tracking the number of antibody-containing or -secreting lymphocytes in peripheral blood using flow cytometry, for example. Intraduodenal immunization of old rhesus macaques with CT leads to three- to four-fold lower numbers of specific antibody-containing cells in the peripheral blood in comparison to values in young animals. This suggests that fewer antibody-secreting cells are migrating to mucosal sites in old animals, an observation which correlates well with the concomitant decline in the number of antibody-containing cells in the intestinal lamina propria of old animals.

The lower intestinal IgA antibody titers observed in older individuals may also reflect a reduced local antibody response. Experiments conducted in rats, i.e. intraduodenal immunization with CT, demonstrate that the immune response mounted by senescent rats is delayed but finally reaches levels similar to that of young animals. Nevertheless, differences between the two groups of rats were observed in the quantities of IgA, IgG and IgM antibodies produced by mesenteric lymph nodes or Peyer's patch lymphocytes of toxin-primed animals.

Furthermore, local antibody production in the intestinal lamina propria is down-regulated by T suppressor lymphocytes, a population which was observed to be three times

greater in old rats. This suggests that aging is accompanied by a dysregulation of local antibody production at mucosal sites due to increased T-cell suppression.

Finally, IgA antibodies produced in the intestinal lamina propria must be transported across the enterocytes via receptor-mediated endocytosis to their effector site, the mucosal surface. This transport is dependent on the expression of the polymeric immunoglobulin receptor, pIgR, expressed on the basolateral surfaces of most mucosal epithelial cells. Basolateral membranes from rat intestinal crypt cells exhibit a threefold greater IgA binding capacity than do membranes isolated from villus tip enterocytes. This crypt-to-villus tip gradient of IgA binding is identical in young and old rats and similar spatial and age patterns are seen in rhesus macaques. The fact that pIgR expression in the rat's small intestine correlates with cell rather than organism age suggests that diminished antibody titers in the intestinal lavage of old animals reflect a deficit upstream of IgA transport and secretion.

In summary, present knowledge indicates that decreased IgA antibody titers in the intestinal lumina of old animals and elderly humans may be linked to more than one factor. Impaired maturation of IgA immunoblasts, delayed IgA plasma cell migration from Peyer's patches to lamina propria and reduced local antibody production may all explain the senescence of the mucosal immune system.

Given that the constantly growing population of the elderly is making heavy demands on the health care system, it is evident that any treatment able to prevent or reduce the intestinal mucosal immunosenescence, such as the use of probiotics, would have major health benefits and economic impacts. This area thus deserves more extensive research support and activity, especially in humans.

BABIES AND YOUNG CHILDREN

In contrast with the elderly, whose immune system is well-established but weakens with aging, the situation is completely different in babies or young children. The intestinal tract, which is sterile at birth, becomes progressively populated with different bacterial species. The role of the gut microflora in the maturation of the mucosal immune barrier is currently well-recognized and can best be seen in newborns. The latter are rapidly exposed to foreign antigens after birth when delivered by the vaginal route (maternal bacterial flora) and breast fed (food antigens). After about one year, the immune system begins to establish tolerance. In some cases, an atopic status characterized by excessive IgE responses may occur, and this normally reverses spontaneously to tolerance. However, one-fourth of young children develop multiple food allergies, a so-called "atopic disease".

It should be mentioned that the incidence and severity of allergic diseases has doubled in the last decades in industrialized countries. This phenomenon is linked more to environmental than to genetic factors, as indicated by the fact that the frequency of allergies has increased over a relatively short period of time.

In babies, atopic eczema or dermatitis accompanies intestinal disorders, leading to a reduced growth rate. The classic treatment of this disease consists in eliminating the incriminated antigen in the food source (elimination diets) which may be very inconvenient from a practical point of view. In addition, immunotherapy and stabilization of the gut microflora and mucosa may be attempted. Clinical studies have been conducted on infants developing dermatitis or cow milk allergy to evaluate if LAB probiotic strains could prevent or reduce allergic symptoms. In one such trial, *Lactobacillus rhamnosus* GG or a placebo was given to babies with atopic eczema. The “GG group” showed a clear amelioration of symptoms and after one month of treatment, the *in vivo* (fecal) level of TNF α , a pro-inflammatory cytokine, was considerably lower. Additional immunological parameters which reflect the Th1/Th2 balance were measured, testifying to reduced intestinal inflammation. To observe a positive effect, 10^8 to 10^{10} viable cells of *L. rhamnosus* GG were necessary depending whether the bacteria were supplied in milk or as freeze-dried powder respectively. This study is an example of a well-conducted clinical trial proving that selected LAB strains are able to exert a healthful effect in the context of allergy in infants.

Currently, maturation of the mucosal immune system is poorly understood in humans for obvious ethical reasons. This is primarily due to the fact that mucosal tissues are difficult to access and are obtained mainly from adults during certain surgical procedures that are seldom performed in infants. Therefore a study was undertaken to analyze duodenal and bronchial tissues sampled in the course of autopsies performed in infants who died from Sudden Infant Death Syndrome. Gut and bronchus samples from 90 infants, aged between a few minutes and 9 months, were collected. The maturation of the gut and bronchi was examined by histology and immunofluorescence in a group of children between 0 and 23 weeks of age. The pattern derived from quantitative analysis of the samples showed that, at birth, only small IgM-bearing B-cell foci are detectable, first in the gut and later in bronchi. After a few days, organized germinal centers develop and IgM plasma cells appear. IgA plasma cells are detected at 12 days and IgA become predominant after three weeks in the gut and after six weeks in bronchi. Taken together, the results confirm that, in man, MALT organization at birth is still in its fetal form and that maturation depends on exposure to environmental challenges occurring first in the intestine and evolving over several weeks before IgA become the predominant isotype. The lower respiratory tract matures more slowly, even though the plasma cell populations ultimately reach similar numbers, indicating the importance of both GALT and BALT in the protection of mucosal surfaces.

CHAPTER V

THE MUCOSAL BARRIER: A PHYSICAL PROTECTION

The concept of “mucosal barrier” was first restricted to the physico-chemical structure that covers the gastric lining and protects the stomach against autodigestion by acid and pepsin. The concept has now expanded to the entire gastrointestinal tract, as this barrier separates the internal “milieu” from an aggressive intraluminal environment, which contains a number of chemical and bioactive pollutants (bile salts, enzymatic activities, bacterial products and toxins, phenols, indols and amines derived from anaerobic proteolysis, etc). This physical barrier includes the epithelial cell lining and a mucous gel formed by the interaction of mucin glycoproteins and trefoil peptides. A surfactant mono-layer of phospholipids on top of the mucus provides hydrophobic properties to the surface. This hydrophobic layer constitutes a line of defence against some pathogens. It has been shown that in gastric ulcers, *H. pylori* considerably decreases this hydrophobicity, an effect which can be reversed with bismuth.

In several mammalian species including humans, surface hydrophobicity on top of the gastric mucosa and in the colon is very high, whereas it is much lower throughout the small intestine which is the absorptive surface. In the stomach, this hydrophobic layer prevents the flux of acid and proteolytic enzymes into the epithelium, while in the colon it is thought to prevent the influx of water-soluble bacterial products and toxins. The large variety of bacterial populations and the high concentration of chemical and bacterial toxins present within the colonic lumen are constantly challenging the structure and function of the mucosal barrier.

Morphological or functional disturbance of the mucosal barrier may lead to changes in permeability. The role of bacterial colonization on intestinal permeability has been investigated in a rat model. A colonic segment was surgically excluded from fecal transit and brought out of the abdominal wall through two colostomies. This segment

provides a suitable *in vivo* environment to study the effect of colonization with preselected strains after elimination of the native flora with antibiotics (“germ-free” animals) (figure 5). In this model, mucosal hydrophobicity was found to be higher in rats kept germ-free than in those recolonized with a mixed rat microflora. The latter showed higher lumen-to-blood passage of toxins instilled into the segment lumen, and were more susceptible to mucosal injury and inflammation than rats kept “germ-free” with antibiotics. Further studies using the same model tested lumen-to-blood clearance of dextran (mw 70.000) and D-mannitol (mw 182) in rats recolonized with a single bacterial strain from rat colonic origin (*Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus viridans*, *Bacteroides fragilis*, *Lactobacillus* spp), and in control rats of which the colonic segment was kept “germ-free”. Colonization by a single species did not change lumen-to-blood clearance of dextran, suggesting that colonizing bacteria did not cause any injury to the epithelial cell layer. However, some commensal bacteria increased colonic mucosal permeability to mannitol, a small molecular weight probe cleared through transcellular and paracellular pathways (tight junctions). In contrast, the *Lactobacillus* strain tested improved the barrier function and decreased mucosal permeability to mannitol. This suggests that given colonizing bacteria may impair the structure and function of the colonic barrier and may increase mucosal permeability to luminal toxins, whereas other species seem to improve the barrier function.

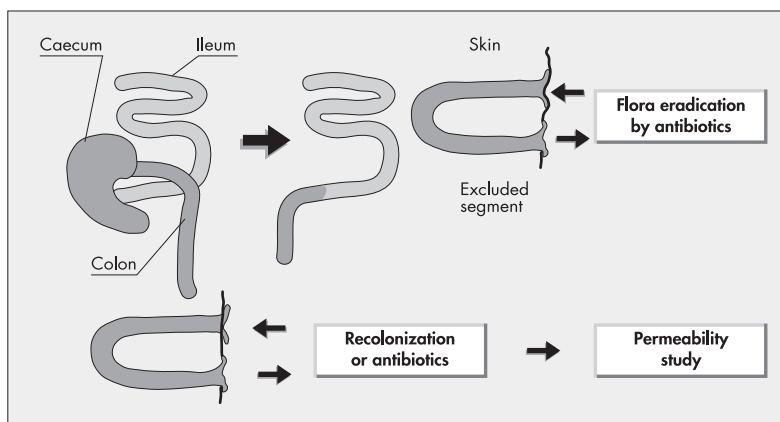


Figure 5. Model to study the role of bacterial colonization on intestinal permeability: experimental design.

This kind of studies may be relevant to some human situations. Indeed, altered intestinal permeability and increased passage of probes from intestinal lumen to peripheral blood have been shown in a number of disorders of growing incidence in Western societies, including inflammatory bowel disease (IBD), atopic eczema, food allergies, liver cirrhosis, etc. Improving the mucosal barrier with the help of probiotic bacteria, for example, may prove beneficial for the prevention of such diseases.

CHAPTER VI

PROBIOTICS AND IMMUNOMODULATION: ANIMAL MODEL STUDIES

It is generally accepted that given strains of LAB may act as immunomodulators. However, the majority of the data generated in this field derive from *in vitro* studies or experiments conducted in animal models. Most of them aim at proving the adjuvant or immunostimulation capacity of LAB strains primarily belonging to the *Lactobacillus* genus, although strains of *Streptococcus thermophilus* and bifidobacteria were examined as well. Because of the wide variety of bacterial strains and test systems used, it is hard to come to a general conclusion. What seems to hold true in any case is that the observed effects may differ in nature and in intensity in a species- or even strain-dependent manner. Therefore, it is of utmost importance to properly identify the strains under study, with the aid of today's molecular taxonomy tools, and to verify whether the results demonstrated with a certain strain may be extrapolated to the entire genus.

Several reviews cover the progress achieved in the analysis of the adjuvant or immunostimulatory properties of probiotic strains, of which only a few examples are given below.

From the existing literature, it would appear that LAB administered orally to rodents are able to stimulate both the non-specific and specific immune responses. This effect is mediated by activation of different cell subpopulations, typically macrophages or dendritic cells for example, or by regulation of the numbers of T-cell subsets or immunoglobulin-secreting cells, especially in the intestine mucosa. It was also shown that germ-free animals are characterized by delayed cellular immune and lower humoral responses than their conventional counterparts (animals harboring a conventional intestinal microflora).

During the meeting, different experiments conducted in mice or rats were presented in which lactic acid bacterial strains such as *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* spp. *bulgaricus* and *Streptococcus thermophilus* were compared. Yogurt (*S. thermophilus* + *L. bulgaricus*) was also tested as were, in some cases, strains of *Lactobacillus rhamnosus* and *Lactococcus lactis*. Targeted biological effects included protection against *Salmonella typhimurium*, tumor growth inhibition and reversion of defects caused by malnutrition or immunodepression. From these studies, *L. casei* appears to be an interesting candidate as an oral adjuvant that does not induce inflammatory immune responses. Additional experiments were designed to clarify the interaction of LAB with the intestinal epithelial cells. It appears that different strains penetrate the gut mucosa by different routes, not all of them being able to bind to M-cells, as seems to be the case for *L. casei* and *L. plantarum*. As a consequence, LAB strains differing by their site of action in the small intestine would induce variable immune responses.

Another example described experiments conducted on rats in which enterocolitis had been induced chemically. The animals received different types of diet or nutritional supplements. When sacrificed, the two groups of rats fed with either *L. reuteri* R2LC or *L. plantarum* DSM9843 showed less severe symptoms than the controls. Even though the normal situation was not restored, increased levels of ileal and colonic secretory IgA as well as higher counts of CD4+ and CD8+ cells were observed.

As for other health promoting traits, while immunostimulation by certain probiotic strains has been documented, there is virtually no understanding of the mechanisms accounting for the observed effects, and this will necessarily become an important area of investigation.

It has often been suggested that bacteria must adhere to susceptible cells in order to evoke an immune response. In addition, occupation of common binding sites on the mucosal surface by probiotic bacteria such as lactobacilli may competitively exclude pathogens from these sites. Adhesion can result from specific or non-specific types of interaction such as receptor-ligand association, hydrophobic adhesion or charge interaction, which all involve bacterial surface components. Bacteria may adhere to the epithelial cells themselves or rather to the mucus overlaying the cells. While several *in vitro* studies have demonstrated that given LAB strains, mostly belonging to the *Lactobacillus* genus, are indeed able to adhere to human epithelial cell lines, the nature of the interaction has generally not been characterized. Potential adhesion factors have been identified, but their definitive role in the cell-bacteria interaction has not yet been established.

Some *L. reuteri* and *L. crispatus* strains able to bind collagen via a lectin interaction have been described. Collagen is not normally part of the intestinal mucus, but it is sometimes exposed during shedding of the gut epithelium. An aggregation promoting factor (Apf) has also been reported for *Lactobacillus crispatus* and, in this case, isogenic strains which are agg+ or agg- are available. The comparative *in vivo* study of the colonization capacity and immunogenicity of this pair of strains will help to clarify the biological function

of Apf. Moreover, it has been proposed that the S-layers produced by some lactobacilli may mediate adhesion to epithelial cells through their hydrophobic domains.

One of the most advanced analysis of LAB adhesion factors was conducted on the mucus adhesion promoting (Map) protein of *L. fermentum* 104R. This probiotic strain, isolated from pigs, adheres (in vitro) to porcine gastric epithelium and small intestine mucus. The gene encoding for the 29 kDa surface-bound MapA has been isolated and sequenced. Computer-assisted analysis of the mapA gene showed that MapA is related to the cluster III bacterial solute-binding proteins. MapA contains three short amino acid sequences which are similar to those which compose the sialic acid-binding motif of adhesins from *E. coli*, *Vibrio cholerae* and *H. pylori*. Quite remarkably, MapA shows structural similarities to the major cell binding factor of *Campylobacter jejuni*. Furthermore, the addition of MapA to human mucus or HeLa cells in an in vitro assay inhibited the adhesion of a human (but not a bovine) *C. jejuni* strain, supporting the model of competitive exclusion. The construction of a mutant defective in MapA is presently attempted.

In conclusion, it should be emphasized that adhesion is expected to be host- and tissue-specific. Thus, while in vitro tests and animal models are of great assistance in screening for adherent strains, the results of these studies may not be systematically extrapolated to man and will have to be ultimately validated in humans.

PROBIOTICS AND IMMUNOMODULATION: *IN VITRO* STUDIES

The animal studies are likely to have a predictive value in humans, in terms of probiotic action, only limited to the study of the biochemical mechanisms at the cellular level. An alternative approach for pre-clinical screening of probiotics consists in the development of tailored *in vitro* systems that allow a rapid and clear-cut definition of biological activities. This would greatly facilitate the study of the mechanisms of action and help define new immunological markers to be followed in clinical trials. Furthermore, dose-response curves, which remain poorly documented in probiotic studies, could be established as well.

In this respect, *in vitro* models based on the use of eucaryotic cell lines or specific cells subsets of the immune system are particularly attractive. They may be used to measure the induction of non-specific and specific immune responses by following indices such as production of cytokines and mediators by antigen presenting cells (APC) and cellular markers on different types of cell subpopulations.

Human peripheral blood mononuclear cells (PBMC) or intestinal epithelial cell lines (CaCO₂, HT29) were used, for example, to study the protective effect of the strain *L. casei* DN-114-001 (designated LC hereafter) or LC-derived products (supernatants of ACTIMEL® and of LC grown in milk or in MRS). These *in vitro* studies have shown that dead LC or LC culture supernatants were able to modulate the production of IgG, IgA and IgE immunoglobulins. For example, preincubation of human PBMC with LC-derived products suppressed the IL4 induced IgE production as did exogenously added superoxyde dismutase (SOD). This observation points to a possible role for LC in the protection against oxydative stress, a hypothesis which is corroborated by the fact that HT29 or CaCO₂ cells pre-incubated with LC products did not produce increased levels of TNF α , a

pro-inflammatory cytokine, when exposed to TSST1. This effect was not accompanied by a phenotypic alteration (cellular markers) of immune cells but rather resulted from a reduced NO^* production.

In a healthy person, the balance between inflammation and immunity is maintained through fine-tuned regulation of several factors. When exposed to an antigenic stimulus, the immune system responds first by initiating an inflammatory response, which leads to recruitment and activation of APC and finally to the induction of a protective immune response. This cascade of events is accompanied by the release of several mediators that regulate the unrolling of the process. Notably, the generation of oxydants, i.e. free radicals and NO^* , plays an important role in upregulating inflammation. In normal cells, oxydative molecules (O_2^* , OH^* , H_2O_2 , NO^*) are counteracted by antioxydants which include enzymes (SOD, catalase, peroxydase, glutathion transferase) and quenchers (thiols, glutathion, vitamins A, C, E...). This equilibrium may be disrupted when the individual is invaded by a pathogen, and when the oxydative balance is not restored naturally, inflammatory responses are set up and may even persist, as in the case of chronic inflammations where an immuno-redox vicious circle is established. Exacerbated NO^* levels favor Th2 type immune responses as a result of clonal deletion of the Th1 associated cells. B-cells are also involved in the oxydative balance, as they may synthesize nitric oxyde synthase (NOS), an enzyme that is important for controlling cell apoptosis.

As non specific immune reactions are known to help the control of the infectious processes of various classes of viruses, the effect of LC and ACTIMEL[®] supernatants on the infection of human PBMC by HIV (Human Immunodeficiency Virus) and EBV (Epstein Barr Virus) was also examined. Dose-response curves showed that viral infectious processes were limited, even though the replication of viruses in chronically infected cells was not impaired. Again, this effect seemed to be mediated by a limitation of the pro-inflammatory consequences of the infection due to increased oxydo-redox protection of the cells. The production of free radicals and pro-inflammatory cytokines by the activated PBMC was downregulated, while the synthesis of anti-oxydant enzymes, normally impaired during viral infection, was stimulated. LC-derived products also regulated the pro- and anti-apoptotic properties of HIV and EBV. These observations must, however, be reproduced in human epithelial cells to support their relevance to the in vivo situation.

The experiments outlined above demonstrate that LC or LC-derived products may protect against pathogen-induced pro-inflammatory disorders as a result of the inhibition of IL4 induced IgE synthesis and the improvement of the redox balance of cells (increase in the production of anti-oxydative molecules). Taken together, the data that were presented indicate that LC strain or LC-derived products improve the natural immune defence of the host and protect immune cells against degeneration and aging or apoptosis. This stimulation of the non-specific intestinal mucosal immunity is postulated to lead to improved protection of the gut mucosa, thus explaining the effectiveness which has been reported for given probiotic strains/foods in the treatment of various intestinal disorders.

In vitro tests have also been used to compare the immunostimulating capacity of different lactic acid bacterial strains. Human PBMC from healthy individuals were incubated with live or dead bacteria, and the stimulation of cytokine release – typically IL2, IL4, IL6, IL10, IFN γ and TNF α – was measured. Tremendous interstrain variability has been observed in these experiments but, in all studies, live bacteria were found more potent than dead ones and also than *E. coli* LPS. It is hypothesized that the cell wall structure (peptidoglycan, teichoic acid) plays a critical role in this stimulation effect, the nature of the involved element remaining unknown. An elegant way to address the latter point would be to construct mutants with altered cell wall composition by applying molecular biology techniques.

The modulation of cytokine production by yogurt consumption in anorexia nervosa patients (ANP) has, for example, been looked at using this kind of in vitro assay. Twenty seven ANP aged between 14-19 years were enrolled upon hospital admission and were divided into two subgroups. The first one received yogurt for the first 10 weeks and milk for the following 10. The second group had the opposite regimen (milk/yogurt). PBMC were collected at five different periods and tested in vitro for the stimulation of IFN γ , IL2 and TNF α secretion upon stimulation with PHA. The TNF α and IL2 levels increased towards the end of both regimens. This could be only the result of improvement in the nutritional status of ANP under treatment. However, IFN γ increased during the yogurt consumption periods and IFN γ level has been proposed as a marker of the immunomodulation effect of yogurt in ANP.

CHAPTER VIII

PROBIOTICS AND INTESTINAL DISORDERS

Probiotics may influence intestinal physiology either directly or indirectly through modulation of the endogenous ecosystem or immune system. Among the health beneficial properties attributed to probiotics, their potential to prevent or help cure intestinal disturbances has received much attention. Suggested effects for diverse lactic acid bacterial strains or yeasts include alleviation of diarrhea from various origins and of irritable bowel syndrome, prophylaxis of gastrointestinal infections, amelioration of inflammatory bowel disease (IBD) and reduced risk of colon cancer. While a wealth of information has been generated in this area, the efficacy of only a few strains has been demonstrated in randomized placebo-controlled clinical trials. Different human studies are presently being performed to examine the effect of probiotics and prebiotics on IBD and colon cancer.

It should be emphasized that probiotics may, in principle, exert therapeutic, curative (eradication of pathogens, stabilization of gut microflora, normalization of intestinal permeability...) or preventive effects (increase in specific IgA levels, modification of antigen structure and immunogenicity...). From the data collected so far, it would appear that depending whether probiotics are applied for healthy subjects or for patients with acute infection or altered immune response, they may either stimulate the immune response or reduce abnormal situations.

TREATMENT AND PREVENTION OF CHILDREN'S DIARRHEA

Different randomized controlled clinical trials have demonstrated that the strain *L. rhamnosus* GG is effective in the treatment of gastroenteritis, especially in the case of infantile rotavirus diarrhea. In one study, infants admitted to the hospital for acute diarrhea

were fed with either GG fermented milk or killed yogurt, after six hours of oral rehydration. The aim of the study was to determine the effect of the strain on the immune response to rotavirus and milk antigens as well as on the shortening of the diarrheic episode. One day after admission, the number of watery stools per day decreased rapidly in the "GG group" and ceased completely after 4 days, in contrast to the control group. The number of immunoglobulin-secreting cells was measured in blood during the acute phase of the disease. In the group of infants receiving GG, levels of IgG, IgA and IgM increased temporarily. More strikingly, anti-rotavirus IgA secreting cells were observed in much higher numbers than in the control group, leading to protection against re-infection.

In another study, 100 infants (aged 3-36 months) with acute diarrhea were treated with oral rehydration solution plus *L. rhamnosus* GG or placebo. The duration of the diarrhea was significantly shortened in the "GG group" versus the control one, the effect being more pronounced in the 61 children with diagnosed rotavirus infection. Viral shedding in faeces was also reduced in these patients for whom duration of hospital stay and weight gain course were shortened. This positive effect has also been observed in trials conducted in developing countries.

The influence of milk fermented with *L. casei* DN-114001 (LC) on diarrhea in children attending day care centers was also examined in a randomized double blind placebo-controlled study conducted in the Paris area. The aim was to determine whether regular consumption of yogurt prepared with LC in addition to the common starters had a beneficial effect on prevention and duration of diarrhea. Children consumed fermented milk (*L. casei* LC + *S. thermophilus* + *L. bulgaricus*), traditional yogurt or jellied milk (control) during three periods of one month, each followed by one month without supplementation; 287 children aged 6 to 36 months were enrolled in a period of 24 months. The severity of diarrhea over the 6 months study was significantly decreased in the group receiving LC fermented versus jellied milk.

In a second study, 928 children (6-24 months) consumed LC fermented milk or standard yogurt daily during 12 weeks. This period was followed by 6 weeks of observation, the total duration of the trial being 18 months. Of the 928 children enrolled, 148 developed at least one diarrheic episode. The incidence of diarrhea was lower in the group consuming LC fermented milk (15,9%) versus traditional yogurt (22%).

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a general term for ulcerative colitis (UC) and Crohn's disease (CD), which are chronic intestinal inflammatory disorders of unknown etiology. Whereas UC consists of a more superficial inflammation limited to the large bowel that causes bloody diarrhea, CD is a transmural granulomatous inflammation that can occur in any part of the gut from mouth to anus. Characteristically it occurs in the ileocaecal region and colon, and the inflammation is patchy and discontinuous. Surgical resection of the affected areas is often needed, but does not prevent recurrence of the disease.

Recent studies have provided valuable insights into the role of the mucosal immune system in the amplification and regulation of inflammatory responses that result in the histologic and clinical features characteristic of IBD. Both diseases, UC and CD, exhibit the classic pathological characteristic of chronic inflammation: infiltration of macrophages and lymphocytes, including large numbers of plasma cells. Inflammatory mediators and cytokines have been viewed as particularly promising for both understanding acute inflammation of the intestine and providing pathophysiological mechanisms to target for therapeutic intervention.

The production of auto-antibodies against epithelial cells has been reported, and may contribute to the epithelial damage observed in IBD. The presence of antineutrophil cytoplasmic antibodies (ANCA) in IBD patients has also been described. Studies indicating an increased level of activation of the complement pathway in IBD suggest its involvement in cell lysis and its participation in the acute effector events of tissue destruction.

One of the most remarkable characteristics of the mucosal immune response is its enormous selectivity and efficacy in excluding pathogenic antigens. Following antigen interaction with M-cells which leads to transcytosis, antigens are phagocytosed by either macrophages or dendritic cells, leading to local initiation of the immune response which ultimately induces clonal expansion of antigen-specific B and T-cells. Some phenotypic marker modifications are reported for macrophages isolated from IBD patients, such as preferential expression of the CD14 molecule (table II). An increase of TNF α has been convincingly described in the intestinal mucosa of patients suffering from active IBD, thus indicating the role of mononuclear phagocytes and granulocytes as producers of pro-inflammatory cytokines in the initiation and maintenance of the intestinal chronic inflammatory process. NF κ B family members control transcriptional activity of promoters of inflammatory cytokines, cell surface receptors and adhesion molecules involved in intestinal inflammation. Continuous activation of such transcriptional factors is observed in IBD.

Table II. Modification of markers in macrophages isolated from IBD patients

Normal macrophages	IBD macrophages
Low CD14	High CD14
CD68	CD33-CD44-CD16
Low NF κ B	High NF κ B

The binding of phagocytes to blood vessel endothelial cells followed by migration into the diseased area of the bowel is facilitated by interactions between molecules on the leucocyte cell surface and their counterparts on the endothelial cells. The process of homing into areas of disease involves adhesion molecules expressed on both leucocytes and endothelial cells. Interaction of ligands with selectins induces the expression of integrins on the leucocyte surface. Several studies indicated that the expression of primarily cell adhesion molecules (P Sel, E Sel, ICAM-1, LFA1, VLA1...) is greatly increased

in active IBD and leads to cell influx into the diseased section of the intestine. In IBD, initial characterization of immunological alterations focused on T and B lymphocyte activation. It appears that cytokine balances (between pro-inflammatory and contra-inflammatory cytokine-driven immunoregulatory processes or between Th1 and Th2 responses, respectively) are of particular importance in the maintenance of a non-inflammatory state in the mucosal compartment. Many animal models have been developed to reproduce IBD. Two distinct groups of animal models have been described: spontaneous colitis developed in genetically-modified animals or colitis induced by chemicals or by cellular transfer (CD45 T-cells in SCID mouse). IL2^{-/-} or IL10^{-/-} knock-out mice provide a chronic T CD4⁺ dependent colitis model, correlated to an increase of certain cytokines (IFN γ , IL6, TNF α). The same modifications occur in CD45RB reconstituted SCID mice. Such animals can be protected by anti-TNF α , anti-IFN γ or IL10 treatment, but also with anti-IL12 in certain cases. This underlines the role of IL12 in the activation of the signalisation cascade. The production of IL12 appears to be induced in response to bacterial products. The presence of bacterial antigens together with high levels of IL12 could lead to the differentiation of T helper cells towards the Th1 type (increased levels of IFN γ) and finally to the production of pro-inflammatory cytokines by activated macrophages. Several results indicate that both AP1 and STAT-4 are required for IL12-dependent IFN γ promoter activity and their activation has been observed in CD, but not in UC, patients. Therefore, chronic colitis seems to be associated with Th1 cells response (increased level of IFN γ and IL2). This has been confirmed in STAT-4 transgenic mice where IBD seems to be initiated by Th1 profile induction (IFN γ). An increase in certain transcription factors (NFATc) was also observed and nuclear binding activity of GATA-3 is downregulated. This seems to be correlated with down-expression of IL4 in CD.

The main theory is that the abnormal immune response seen in IBD is driven by some members of the intestinal flora and/or results from a defective mucosal barrier. Lamina propria cells from IBD patients respond with MHC class II restricted proliferation and enhanced secretion of cytokines to both autologous and heterologous fecal antigens. This strongly supports the hypothesis that disrupted tolerance contributes to the chronic inflammatory process in IBD. A better insight into the pathogenesis of IBD may provide a rationale for immunotherapy in patients with chronic intestinal inflammation. Due to their immunomodulation capacity, probiotic strains should be of potential use in strategies designed for the restoration of a normal immune balance at the intestinal mucosa level.

COLORECTAL CANCER

The definition given by Fuller for a probiotic can in theory be applied to colon cancer prevention because of the implication of the intestinal flora in the development of this pathology. However, the probiotic effect that remains the most controversial is the anticancer activity attributed to certain LAB. Two types of studies have been conducted in this field:

- in vitro and in vivo animal models studies;
- dietary intervention studies in human volunteers and epidemiological studies correlating cancer to certain dietary regimens.

Although there is no direct evidence that probiotic LAB prevent or protect humans from colon cancer, many observations based on experimental animal models suggest their potential beneficial effect.

Colon carcinogenesis has been well characterized genetically and goes through distinct stages (figure 6). The first one is the hyperproliferation of epithelium which can be correlated with deregulation of the expression of several tumor suppressor genes and oncogenes.

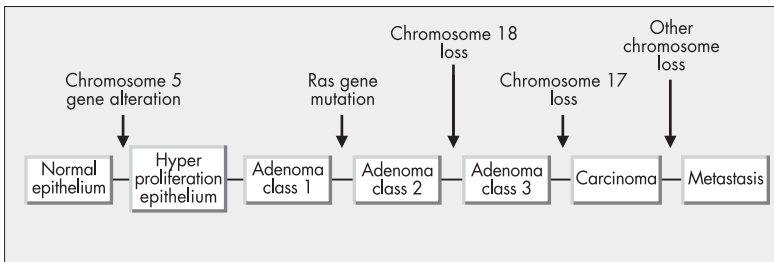


Figure 6. Stages of the colon carcinogenesis.

Some dietary factors (total fat and protein, meat intake, high caloric intake) have been associated with increased risk of cancer and there are different hypotheses as to how they might be involved in carcinogenesis (figure 7). They could participate in the initiation phase in which formation of genotoxic compounds (mutagens) occurs in the colon lumen, causing DNA damage and leading in some way to oncogene activation. In the promotion phase, cancer “promoters”, which are not considered as genotoxics, could increase irritation of the mucosa or induce cell cytotoxicity followed by compensatory hyperproliferation, thus increasing tumor growth. It is not completely clear what the genotoxic agents and the mutagens in the colon are. They could be coming from heterocyclic amines in the burned parts of fried meat. Many foreign compounds are detoxified in the liver and are found in the bile as conjugates that the intestinal bacteria are able to deconjugate thus liberating the carcinogen.

A typical exemple of dietary risk factors is fat, which increases the amount of primary bile acid going to the colon where it is converted in secondary bile acids by the microflora which will act on tumor promoters. This leads to a cytotoxic effect on colonic epithelial cells, compensatory hyperproliferation and, eventually, clonal expansion of these cells. The microflora has been hypothesized to play an important role in this process and there are different stages where it could influence its development.

On the other hand, some dietary factors have been associated with decreased risk such as dietary fibers, vegetables, fruits, vitamins A, C, D and E and calcium. The potential beneficial role of LAB supplements remains, however, to be determined.

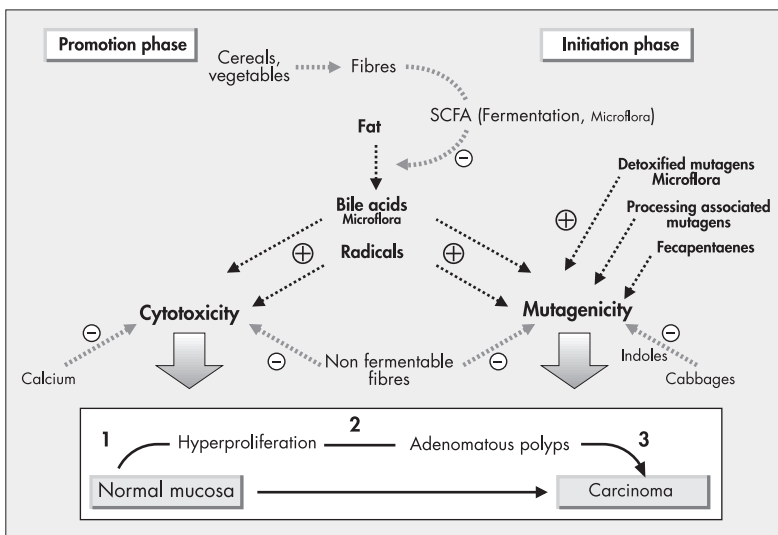


Figure 7. Hypothetical pathways for the relationship between diet and colon cancer.

In vitro studies have shown that several LAB including different lactobacilli, bifidobacteria, *S. thermophilus*, *L. lactis* and *E. faecalis* are able to bind mutagenic compounds (mainly heterocyclic amines) to varying degrees. The binding is usually instantaneous and pH-dependent, and interestingly, in this case, dead bacteria are as good as live bacteria. The binding is almost always associated with a decrease in the genotoxic potential of these compounds. How relevant this in vitro observation is for humans is not very clear, but some animal and human studies strongly suggest this. It has also been reported that given LAB can in fact degrade some carcinogens like nitrosamines, for example. Moreover, recent assays performed with the colon cancer epithelial HT29 cell line have shown that some probiotic bacteria (belonging to the species *L. helveticus*, *Bifidobacterium* spp., *S. thermophilus* and *L. bulgaricus*) or a compound they produce can interact directly with colonic epithelial cells, decreasing growth rate and induced differentiation.

In general, studies of this kind show that LAB cultures are able to counteract mutagenic/genotoxic effects and influence colonic cell kinetics.

In animal models, several fecal enzymes have been involved in the conversion of procarcinogenic compounds into carcinogens. It was the initial observation that LAB could decrease the level or the activity of these enzymes in man and animal, which raised the interest for their potential anticancer effect. Although still unknown, the mechanism of this action could be linked to a pH drop in the colon so that the faecal enzymes are in a sub-optimal environment. Additional studies showed that when food mutagens are given simultaneously with LAB supplements, the uptake and distribution of carcinogens in the animal body is decreased, possibly because of reduced bioavailability of the toxic

compounds bound to the LAB. Other studies have indicated an antigenotoxic effect of some lactobacilli and bifidobacteria in the colon of rats exposed to mutagens such as MNNG, AOM and DMH which induce DNA damage. The nature of the protective principle is not known precisely but there is some evidence that LAB may induce foreign compound-metabolizing enzymes.

Chemically induced carcinogenesis in animals can be used as a model to rapidly assess LAB effects at the level of preneoplastic lesions and colonic tumors. Aberrant crypts, similar to those observed in cancer patients, are seen in the colon of rats two weeks after exposure to a carcinogen. This constitutes a nice, relatively inexpensive, sensitive and quick assay to search for chemoprotective agents. There are at least 10-20 good recent studies reported in the literature using such models. For example, a reduction of the number of animals (40% versus 77% in the control group) showing colon cancer, after 20 weeks of *L. acidophilus* NCFM feeding has been obtained in a DMH-induced colon cancer model. The number of preneoplastic lesions was decreased as was the half-life time of one of the DNA adducts essential in tumor formation, suggesting that the LAB strain inhibited tumor formation through the induction of enzymes which removed DNA adducts. In the same way, consumption of *Bifidobacterium longum* has been associated with a decrease of AOM-induced tumors in rats. In this study, intermediate cancer biomarkers were measured and LAB were shown to inhibit cell proliferation, ODC activity and expression of ras-p21 oncogene.

In dietary intervention studies in human volunteers, a LAB-mediated decrease in fecal enzymes that may be involved in carcinogen generation has been observed. This effect needs a continuous consumption of LAB containing products, as the fecal enzymes go back up when feeding is stopped. The strains tested in this study included different *L. acidophilus*, *B. bifidum*, *L. delbrueckii* and *S. thermophilus*.

The effect of LAB on fecal and urinary mutagenicity has also been assessed. When human subjects are fed well-done hamburgers, a huge increase in the levels of genotoxic compounds (mostly heterocyclic amines) is observed after 48 h in their urine and feces. In one trial, healthy subjects on a standardized diet consumed fried beef twice daily for three days. Diets were supplemented with either *L. lactis* spp. cremosis or *L. acidophilus* NCFB1748 fermented milk. A significant decrease of the mutagen excretion in urine and feces was obtained in the group consuming *L. acidophilus*. A marked suppressing effect of orally administered *L. casei* on the urinary mutagenicity arising from ingestion of fried minced beef has also been reported in another study. This might indicate that the LAB are binding mutagenic agents in the lumen, making them less bioavailable. Both types of studies are interesting provided that fecal enzymes or heterocyclic amines play a role in human colon cancer, which is possible but not proven.

Bile acids are considered to act as tumor promoters. However, only the fraction that is in solution in the human fecal water interacts with the colonic epithelial cells to exert cytotoxic and proliferative effects. This fraction corresponds only to a tiny part of the total fecal bile acids which are used as biomarkers for colon cancer as they are increased in

these patients but also in subjects at high risk. One study has demonstrated that consumption of a fermented dairy product, containing *L. acidophilus* NCFB1748, for 6 weeks, markedly decreased the amount of bile acid levels in the fecal aqueous phase in colon cancer patients, perhaps by decreasing colon pH. It was shown independently that abnormal colonic crypt proliferative activity in colonic adenoma patients returned to normal after 3 months administration of *L. acidophilus* and *B. bifidum*. Here again, fecal pH was significantly reduced.

The effect of a yogurt enriched with *B. longum* and lactulose on fecal bacterial flora and various risk markers for colon carcinogenesis has been tested in a large group of healthy volunteers. The only observed effect was an increased excretion of bifidobacteria.

Very few good epidemiological studies have been conducted in recent years and most of them were not specifically designed to look at probiotics or fermented milk products and colon cancer. However, in a case-control study conducted in the Netherlands, a reverse association was observed between breast cancer and fermented dairy product consumption.

In summary, the mechanisms by which LAB might inhibit colon cancer are unknown. Nevertheless, certain hypotheses have emerged. They may:

- suppress the growth of intestinal microflora incriminated in producing putative mutagens/carcinogens and promoters;
- produce antimutagenic and antitumorigenic compounds in the colon;
- bind potential mutagens and carcinogens;
- alter physiological conditions in the colon (lowering of pH) affecting the metabolic activity of intestinal flora, the action of bile acids and causing quantitative and/or qualitative alterations in the bile acid degrading bacteria;
- enhance the host's immune system.

In conclusion, great care must be exercised in extrapolating from the results obtained *in vitro* and in animal studies. It will be necessary to correlate these with carefully designed epidemiological studies in humans. Specific strains with anticancer effects need to be identified, and their mechanism of action will have to be well-characterized. This will allow for further development in the field of probiotics as related to cancer prevention.

CHAPTER IX

PUBLIC AND LEGAL IMPLICATIONS

Regulation of food by law is intended to protect public health and the safety and interest of consumers while not hampering innovation and the food trade. Creating products with optimal probiotic activity is thus the responsibility and task of food science and nutrition research and ultimately of the manufacturer.

Whereas the term “probiotic” is very well defined in a scientific context (cf. the definition given by Fuller in 1989), there is no special legislation and prerequisites for practical use. In consequence, it is at least possible, if not to be expected, that there are foods of varying quality on the market and even some with no demonstrable probiotic activity whatsoever. Food control agencies will, however, be at most able to analyze the type of microorganisms and their counts in a food sample, but cannot verify the claimed probiotic effects of that food. Manufacturers often do not provide any information apart from the bacterial strain added. At most, they refer to literature on that particular microorganism and on clinical or *in vitro* studies carried out with it, sometimes included in different kinds of foodstuff. However, it is known that the same bacterial strain added to different foods can have quite different effects, giving rise to a consensus at the “Symposium on probiotics and prebiotics” in Kiel (1998) that the probiotic activity or effect of a given food should be demonstrated in studies performed with that same food. The heavy burden of such proof rests with the manufacturer.

It is forbidden by law to mislead consumers as to the true nature of a product, as to properties which the food by agreed scientific standards does not have, or by attributing to it properties which all similar foods also have. There are very few investigations into the consumer’s understanding of what a probiotic implies. Although most consumers will not know precisely what “probiotic” means scientifically, it is apparent that they expect some special properties in a food so labeled, especially some health-promoting or health-sustaining activity for which they may be ready to pay a higher price. Interestingly, there

has been an enormous increase in the market of yogurt with probiotic microorganisms (for example, by 100% in Germany from 1996 to 1997) in the recent years.

At present, most products are presented in the market as ordinary foodstuffs for all kinds of consumers with general claims in the sense of “contributing to your health” or “influencing your immune system”, in addition to labeling indicating inclusion of probiotic microorganisms. There are some exceptions, such as products intended for infants and young children which, by definition, are foods for particular nutritional use. In the European Union, there are no specific legislative requirements for foods with probiotic activity, apart from the rules in general food law concerning the safety of foods, truthful labeling and regulations on food labeling. The European Directive on food labeling requires the listing of all ingredients, including the strains of microorganisms (irrespective of which category they are considered to belong to) and food additives added for non-technological purposes.

Manufacturers, however, apparently wish to be allowed to mention in their advertising the more specific effects of their products, and not just a general claim on “positive effect on health”. This is especially true when effects have been ascertained by scientific investigations, for instance on the modulation of reactions of the immune system, on functions of the body (e.g. digestion of the lactose), on so-called disease-related conditions (e.g. elevated blood cholesterol levels), on the course of gastro-intestinal infections, etc.

In the General Guidelines on Claims Codex, the following claims are prohibited:

- Claims which cannot be substantiated.
- Claims as to the suitability of a food for use in the prevention, alleviation, treatment or cure of a disease, disorder, or particular physiological condition unless they are:
 - in accordance with the provisions of Codex standards or guidelines for foods, and follow the principles set for these guidelines
 - or
 - in the absence of an applicable Codex standard or guideline, permitted under the laws of the country in which the food is distributed.

The idea and intent behind this rule are that consumers of foods labeled with medicinal claims should not be tempted to self-treatment with food and eventually be endangered by lack of or tardy medical advice. This rule constitutes one of the main differences between food and drugs, which becomes less significant in the presentation of foods like dietary supplements. In the same way, some nutrients can be ingredients for both drugs and food, with no well-defined rules on dosage to mark the difference. There is, however, a difference in the effects of the chosen ingredients, and this effectiveness must be substantiated in the case of drugs. The effects or benefits of the latter are weighed against risks or undesirable effects, whereas foods must bear no risk of an adverse effect on healthy consumers. It is expected that foods for particular nutritional purposes or dietary supplements will fall under different regulations in different countries.

As already mentioned, the type of claims permitted respectively for food and drugs are clearly different: no medicinal claims for foods, only nutrient content and nutrient

function claims as well as claims on health are permitted, versus approved medicinal claims for drugs. This prohibition of medicinal or disease-related claims is also valid for foods for particular nutritional uses which are marketed as covering the particular nutritional requirements of infants and young children or of persons with special physiological and pathophysiological conditions, except in those cases where a directive, guideline or standard provides for exceptions from this rule.

There is no European harmonization on claims, and a lively discussion presently surrounds claims, especially health claims and their definition. The original definition, "Health claims means any representation that states, suggests or implies that a relationship exists between a food or a nutrient or other substance contained in a food and a disease or health-related condition" (Alinorm 97/22A, App. VII), was deleted in 1996 from the Guidelines for Use of Nutrition Claims Codex. This definition allowed for assumption of a direct and causal relationship between a disease and the consumption or non-consumption of a food, and thus was deemed as misleading to consumers. However the Codex Committee on Food Labeling decided to pursue the discussion on health claims.

The United States of America in particular are very much in favor of a rule similar to their rules as laid down in the Nutrition Labeling and Education Act of 1990. In this act, certain so-called health claims that "describe the relationship between a nutrient and a disease or health-related conditions" are allowed for in a food when it complies with specified requirements on nutrient content and following authorization by the Food and Drug Administration. The wording of the claim must follow prescribed rules, and must avoid the term "prevention" and use "reduction of risk" instead, for example. "Prevention" is forbidden, as it would suggest the food to act as a direct and insurmountable obstacle to the occurrence of a disease or a health-related condition. Health-related condition here refers to a patho-physiological change that might lead to disease.

The number of permitted health claims for food in the United States is restricted. In contrast, dietary supplements are allowed under the Dietary Supplements Health and Education Act of 1994 to bear in their labeling statements that "describe the role of a nutrient or dietary ingredient intended to affect structure-function in humans" or that "characterize the documented mechanism by which a nutrient or dietary ingredient acts to maintain such structure-function".

There is in fact an agreement between European and American food law that medicinal claims are not permissible for foods. The main difference is the authorization of a restricted number of so-called health claims that allow a reference to a disease in the United States.

It would appear that one of the main reasons for the current disagreement on so-called health claims is their definition. Often "health" is described as an absence of disease. It may be necessary to differentiate between foods and drugs by their purpose or intended use, and consequently by differences in permitted claims. The Netherlands' Code of Practice on the assessment of health claims on food and drink products from 1998 gives an interesting definition which is: "A health claim is a direct, indirect or implied claim that

a food carries special qualities which improve or maintain the user's health". In fact, three categories may be distinguished:

- normal foods that maintain or promote health (nutrition/growth/pleasure);
- foods for special populations or special conditions (claims to be substantiated);
- drugs for specific diseases (effect to be proven).

In conclusion, producers of probiotic foods are not currently allowed to inform consumers by labelling of beneficial effects on diseases or disease-related conditions. The implications of a potential revision of the rules on claims for food, especially one that introduces the possibility of mentioning a disease on food labels, should be carefully assessed.

Immunity and Probiotics

The beneficial effect of lactic fermenting agents was proved over a century ago. Since that historic discovery, scientific research has continually moved forward and increased our knowledge in this field.

Thus, over the past few years, the concept of probiotics has emerged. These living micro-organisms exert a beneficial influence through the intestinal flora, allowing people to enjoy the best possible health simply by watching what they eat.

Now research has reached a new stage: identifying and understanding the inter-relationships between probiotics and the body's immune system:

- How do those inter-relationships work?
- Which immune cells are involved?
- How do the inter-relationships change as we grow older?

This book, at the leading edge of current research, provides the essential replies that will enable us to define better the role of nutrition, and particularly that of probiotics, which already make up a growing share of consumer purchases.

«Immunity and Probiotics» bears witness to the impact of scientific research on our food and the new image it gives the subject of nutrition. A legal framework needs to be established in the field, and the book ends by offering the basis for such a framework.

It is a book to be read carefully, both for its scientific interest and for the totally new prospects it opens up for the nutrition of the future.



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