



NUTRITION AND HEALTH COLLECTION

# Yoghurt: Eighty Years of Active Research for Health



AN INITIATIVE OF THE DANONE RESEARCH CENTERS

Yoghurt:  
Eighty Years of Active Research  
for Health



**Éditions John Libbey Eurotext**

127, avenue de la République  
92120 Montrouge, France  
Tél. : 01 46 73 06 60

John Libbey & Company Ltd  
13, Smiths Yard, Summerley Street  
London SW18 4HR, England  
Tel. : 1947 27 77

John Libbey CIC  
Via L. Spallanzani, 11  
00161, Rome, Italie  
Tel. : 06 862 289

© John Libbey Eurotext, Paris, 1999  
ISBN : 2-7420-0278-2

Il est interdit de reproduire intégralement ou partiellement le présent ouvrage  
sans autorisation de l'éditeur ou du Centre Français d'Exploitation du Droit de Copie (CFC),  
20, rue des Grands-Augustins, 75006 Paris.

## **Scientific Committee**

Juan-R. MALAGELADA, Hospital Vall d'Hebron, Spain  
Alfredo MARTINEZ, University of Navarra, Spain  
Joseph RAFTER, Karolinska Institute, Sweden  
Dennis SAVAIANO, Purdue University, USA  
Jose Antonio MATEOS, Danone SA, Spain

## **Contributors**

F. AZPIROZ, Barcelona  
G. BOUDRAA, Oran  
Y. BOUHNİK, Paris  
P. BOURLIOUX, Paris  
D. CARASSO, Barcelona  
A. FAZEL, Paris  
A. GONZALEZ, Pamplona  
S. KERNEIS, Paris  
A. MARCOS, Madrid  
A. MARTINEZ, Pamplona  
L. MORELLI, Parma  
D. O'SULLIVAN, Minnesota  
G. PERDIGON, Tucuman  
B. POOL-ZOBEL, Karlsruhe  
J. RAFTER, Stockholm  
I. ROWLAND, Coleraine  
J. ROBLES, Barcelona  
M.E. SANDERS, Colorado  
D. SAVAIANO, Indiana  
J. SCHREZENMEIR, Kiel  
F. SUAREZ, Minnesota  
A. TOMKINS, London  
M. TOUHAMI, Oran  
R. VONK, Groningen  
M. de VRESE, Kiel

## **Scientific Coordination**

Irene LENOIR-WIJNKOOP, CIRDC, Paris

## **Summary Redaction**

Francisco GUARNER, Barcelona

## CONTENTS

<b>Introduction</b> .....	9
<b>1. The functional properties of yoghurt</b> .....	11
Epidemiological studies.....	11
The functional components of yoghurt.....	12
<b>2. The fate of lactic acid bacteria in the gastrointestinal tract</b> .....	17
Survival of lactic acid bacteria in the human body.....	17
Intestinal transit.....	19
The contribution of molecular biology.....	20
Key questions.....	21
<b>3. The study of M-cells as targets of the interaction of bacteria with the immune system</b> .....	23
<b>4. Yoghurt in the prevention and treatment of human diseases</b> .....	27
Diarrhoeic disorders.....	27
Anorexia nervosa.....	30
<b>5. Yoghurt in lactose malabsorption and intolerance</b> .....	33
Clinical significance of lactose malabsorption.....	33
Lactose digestion and tolerance from live and heat-killed yoghurts.....	36
Intestinal and colonic factors in lactose intolerance.....	37
<b>6. The role of lactic acid bacteria in colon cancer prevention</b> .....	39
Early events associated with colon carcinogenesis.....	39
Modulation of DNA damage.....	42
Late events associated with colon carcinogenesis.....	43
Mucosal immunostimulation by yoghurt in the prevention of colon cancer.....	45

# INTRODUCTION

In the beginning, yoghurt was sold in pharmacies.

Since the early days, and up to now, research in this domain has been continuous.

A lot of work has been done to define what is yoghurt in terms of microbiology, process of fermentation, and the standard of identity of yoghurt.

Yoghurt is a defined product covered by different international standards of identity: Codex, I.D.F. and others have defined yoghurt as a milk fermented by specific cultures. In most countries, *L. bulgaricus* and *S. thermophilus* together are the two basic yoghurt cultures.

However, in other countries, other probiotic cultures may be added to these basic cultures, and the product may still be called yoghurt.

What is important for all of us, to maintain the health benefits associated with yoghurt, is that yoghurt must be alive and active during its shelflife. In other words, that post-fermentation treatments that will reduce the amount of live and active cultures, should not be part of the yoghurt definition.

Way back, a journalist asked me a question: "Do you think that yoghurt, such a traditional product, still is and will be a modern food, a part of our diet and food habits in the future?"

This is an excellent question, since yoghurt has been used throughout the ages, centuries, thousands of years. But what is unique about this product, is that we are still unraveling, discovering about its health benefits.

If we just try to answer this question: what are the basic principles that govern the modern food behaviour we all have? I would say there are four major important aspects.

1) Our food pattern is toward a decrease in the energy intake: we were all eating 2,700-3,000 calories. Today the average is 1,800-2,200, so the energy intake has decreased.

2) Consumers are demanding more and more: "If I eat this food, is it good for me? and why is it good for me?" Food is considered as a source of well being. I want to take this food because I want to feel good, or because I want to have strong bones, or to age successfully. Food is playing an important role in the well being and successful ageing of the population.

- 3) The time we spend for preparation and intake of food has been reduced.
- 4) We eat everywhere: at home, in restaurants, in snack bars, everywhere we go we eat.

How does yoghurt fit in this pattern? Perfectly well, in fact:

### 1) **Calorie intake**

As we are taking less and less energy each day, it becomes critical to derive from our food the necessary amount of nutrients that we need in order not to suffer any deficiency.

Yoghurt, given its content of calories, is a very rich product. It's an excellent source of protein needed for growth. If we want to have strong bones, it will deliver a good quantity of calcium and phosphorus that are necessary. It's also a good source of potassium and selected vitamins.

### 2) **Well being**

The benefits of yoghurt in case of lactose intolerance are well known today. Yoghurt allows people who cannot take milk, to benefit from all the nutrients present in milk. A good percentage of the population – more than 50% in Spain probably – lack the specific enzymatic machinery to be able to consume milk without any problem: for them yoghurt is an alternative. That has been demonstrated.

Other researchers have explored the domain of diarrhoea: it has been shown – notably by studies in Algeria – that yoghurt will help reduce the incidence of diarrhoea, even may prevent the diarrhoea. This is specifically important for children because we don't want them to be malnourished. The World Health Organization has recommended that, when possible, milk should be replaced by yoghurt, so to reduce the deficiencies associated with diarrhoea.

Other researchers have been pioneers in demonstrating the impact of yoghurt on specific immune response systems (this topic has been largely covered in the second Danone Symposium in Bonn).

Yoghurt could also help prevent some sorts of risk associated with cancer. A single product, yoghurt, is capable to do all that.

### 3) **Convenience**

Yoghurt is a very convenient type of food: you can take it and eat it wherever you go.

## **Yoghurt does perfectly fit in the modern food pattern.**

A lot of research has been done on yoghurt: the topic of lactose intolerance has been largely addressed. As for immunity and cancer, what we have so far are hypotheses, that need to be further established. For the future research we need rapid and reliable methods, but what's more, we need consensus on the methods that are needed to clearly establish the link between yoghurt consumption and some health benefits.

**Akram Fazel**  
**Director of Research**  
**Danone Dairy Division**

## CHAPTER I

# THE FUNCTIONAL PROPERTIES OF YOGHURT

Functional foods hold a great promise for future trends in human nutrition, and yoghurt is certainly one of the most exciting players in this field. Modern consumers are interested in foods which will keep them healthy and prevent disease. However, the interaction between food and health is a very complex one. Research aimed at identifying specific effects of food on health and at a better understanding of the mechanisms involved in these effects will be required to provide consumers with a real understanding of the value of food. Proof of efficacy is a huge challenge facing both nutritional science and the food industry.

Compared to milk, yoghurt has some interesting properties when considered from a physiological point of view. Yoghurt certainly seems to have a beneficial effect on gastric emptying time. Yoghurt also contributes to a better digestion of lactose. The availability of calcium is increased after yoghurt ingestion. A beneficial influence on the microflora of the gastrointestinal tract has been shown by a number of studies in recent years. Finally, yoghurt offers interesting effects on the immune system. All of these effects can reveal themselves in an overall positive influence on human health.

## EPIDEMIOLOGICAL STUDIES

Epidemiology is often considered as the first approach used to identify a relationship between diet and health. Most modern epidemiology studies that have been carried out with yoghurt are in the area of cancer. Between 30 to 60 per cent of the environmental risk of cancer can be attributed to diet, and therefore dietary habits represent a major influence on cancer incidence rates. A case-control study performed in France found

a negative association between yoghurt consumption and the risk of breast cancer, based on data from 1,010 breast cancer patients and 1,950 controls. Again, a study performed in the Netherlands showed lower consumption of fermented milk among 133 breast cancer patients as compared to 289 population controls. In Los Angeles County, a case-control study showed that calcium intake was associated with decreased risk of colon cancer and it was also shown that yoghurt consumption was protective. Finally, a large epidemiological study in the Netherlands showed a weak inverse association between colorectal cancer and the consumption of fermented milk and dietary calcium. It can be concluded that epidemiological evidence suggests that yoghurt consumption may have beneficial effects on health, at least in the area of cancer.

## **THE FUNCTIONAL COMPONENTS OF YOGHURT**

The main nutritional and functional characteristics of yoghurt have been summarised in table I. Certainly, yoghurt is an important source of high quality protein, calcium, and vitamins. In addition, a variety of functional components that may be active at a level beyond the basic nutritional value of yoghurt have been identified in recent years. The list includes bioactive peptides, conjugated linoleic acid, sphingolipids, butyrate and probiotic bacteria.

**Table I.** Nutritional and functional components of yoghurt.

<b>Nutritional components</b>	<b>Functional components</b>
<ul style="list-style-type: none"><li>• High quality protein</li><li>• Calcium</li><li>• Vitamins</li></ul>	<ul style="list-style-type: none"><li>• Probiotic bacteria</li><li>• Bioactive peptides</li><li>• Butyrate</li><li>• Conjugated linoleic acid</li><li>• Sphingolipids</li></ul>

Functional peptides are produced during the fermentation process from casein and other proteic fractions of milk. As part of a larger protein, they are inactive but become bioactive when released. Their size ranges from 3 to 64 aminoacids and they tend to be hydrophobic. These peptides can resist digestive hydrolysis within the gut. When absorbed as intact bioactive peptides, they have a physiological effect, either locally in the gastrointestinal tract or systemically once entering the circulatory system. Very few human studies

on these peptides are currently available, and our knowledge on their biological effects relies mainly on data obtained by in vitro and animal studies. Table II shows a list of some of the functional peptides that have been isolated from milk proteins. Effects on the immune system were described. Some of these peptides are able to modulate gastrointestinal motility. Cardiovascular effects include antihypertensive and antithrombotic activity. Other peptides act on the nervous system as opiate agonists or antagonists.

**Table II.** Bioactive peptides from milk proteins.

• Casomorphins	}	_____	Opioid agonists
• Lactophorins			
• Lactoferroxins	}	_____	Opioid antagonists
• Casooxins			
• Casokinins	_____	Antihypertensive	
• Casoplatelins	_____	Antithrombotic	
• Immunopeptides	_____	Immunostimulants	
• Phosphopeptides	_____	Mineral carriers	

Other interesting components of yoghurt or milk fat are the conjugated linoleic acids (CLA). This is a collective term to describe one or more positional and geometric isomers of the essential fatty acid, linoleic acid. Linoleic acid is converted into CLA by intestinal bacteria, or bacteria found in the ruminant. Animal fat is the principal dietary source of CLA, including milk fat and meat. Between 75 to 90% of the CLA in dairy foods is of the cis-9, trans-11 isomeric form. CLA is absorbed from the gut and makes its way to cell membranes, fat tissue and blood lipids. In vitro and experimental animal studies indicate that CLA inhibits the development of a variety of tumors, particularly mammary tumors. Diet supplementation with CLA at 0.1 to 1% decreases the carcinogen-induced formation of some cancers in experimental animals. However, there is no direct evidence that CLA protects against cancer in humans, including colon or mammary cancer. An interesting effect which has been observed is that CLA can decrease body fat and increase the muscle mass in mice.

The sphingolipids are a group of phospholipids that are found in milk fat globular membrane. The sphingomyelin is the most common species in milk, at concentrations ranging from 39 to 119 mg per litre, which are physiologically active concentrations. Dairy products are the main dietary source of sphingolipids. Interestingly, they can enter the aqueous phase of milk through processing effects on the milk fat globular membrane, which means that they can also be found in low-fat dairy products. The physiological effects of

sphingolipids include anticarcinogenic effects that have been shown in animal research. They are thought to influence cell regulation and thereby influence carcinogenesis. They may inhibit the growth and metastasis of tumour cells and transform precancerous cells towards normal cells. They have been shown to increase calcium release from intracellular stores, thereby eliciting calcium protective effects against colon cancer.

Butyrate is another important component of milk and milk fat. It is a four carbon, short-chain fatty acid that is found uniquely in milk fat, at an average of 3 to 4% by weight. No other common food contains butyrate. However, the primary source of butyrate in the colon is the bacterial fermentation of fibre and not dietary butyrate. A variety of important physiological effects of butyrate have been described. Butyrate inhibits epithelial cell proliferation and induces differentiation and programmed cell death. It is associated with down-regulation or inactivation of the expression of oncogenes. It may also inhibit tumour invasiveness and metastasis. In experimental animal studies, butyrate may protect against colon cancer.

Finally, the bacteria associated with yoghurt fermentation as well as additional lactic acid bacteria added as probiotic bacteria are thought to promote health via a variety of mechanisms. Oral probiotics can be described as “living microorganisms which, upon ingestion in certain numbers, exert health benefits beyond basic inherent nutrition”. Primarily, probiotic bacteria are associated with fermented dairy products. In more recent years, the trend has been to isolate probiotic candidates from the intestinal habitat, the rationale being that they may have the opportunity to influence the gastrointestinal tract microflora. Some strains such as *Lactobacillus acidophilus* and *Lactobacillus casei* have both an intestinal source and the ability to survive in the intestine, and they are also associated with fermented dairy foods. Other organisms are primarily associated with fermented dairy foods, including *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Some other strains have an intestinal source but they are not associated with traditionally

**Table III.** Activities of probiotic bacteria.

- Intestinal health
- Anti-cancer
- Immune system modulation
- Milk tolerance
  - Lactose intolerance
  - Milk allergy
- Vaginal/urinary tract health
- Stomach health
- Pathogen translocation (barrier effects)
- Hypertension
- Cholesterol lowering

fermented dairy products, such as bifidobacteria and some *E. coli* strains. The activities of probiotic bacteria are summarised in table III, and they will be discussed in more detail in the following chapters. They can play an interesting and potential role in intestinal health, including anti-cancer effects. They modulate the immune system and have an influence on milk tolerance, both lactose intolerance as well as milk allergy. Vaginal and urinary tract infections may be prevented by probiotic bacteria both with intravaginal suppository applications as well as with oral consumption. Gastritis can be treated with some probiotics. Pathogen translocation may be prevented by probiotics which provide a barrier effect. Arterial hypertension as well as hypercholesterolaemia are also targets for probiotic applications. Of course, only one or a few strains have been specifically identified to play a role in each of these particular indications.

There are important issues surrounding the development of products containing probiotic bacteria, including the essential one of defining what the active principle is, and what the mechanisms leading to a beneficial health effect are. We also need to define what doses of specific probiotic strains are required to elicit specific health benefits in target populations. Well-conducted human studies are warranted.

## CHAPTER II

# THE FATE OF LACTIC ACID BACTERIA IN THE GASTROINTESTINAL TRACT

## **SURVIVAL OF LACTIC ACID BACTERIA IN THE HUMAN BODY**

Lactic acid bacteria can influence physiology and health through direct or indirect effects occurring in the gastrointestinal tract. For instance, lactic acid bacteria can deliver active constituents such as enzymes to target segments of the intestine. Knowledge of their pharmacokinetics is needed to determine the optimal conditions of their consumption to obtain a physiological effect. It is usually accepted that live bacteria in the human gastrointestinal tract need to reach a minimal concentration of  $10^5$  units per mL in the small intestine or  $10^7$  units per mL in the colon to express potential probiotic activities.

Host factors known to influence the pharmacokinetics of lactic acid bacteria are gastric acid secretion, intraluminal bile acids, gastrointestinal motility, microbial interactions and the immune system, which controls the intestinal flora by IgA secretion, among other mechanisms. The vehicle is an important point in the delivery of viable bacteria. The survival rate of lactic acid bacteria is substantially higher when they are administered in yoghurt rather than in a liquid medium with the same pH.

Pharmacokinetics of probiotics are also studied by *in vitro* models. For instance, *in vitro* experiments can provide information on bacterial resistance to acid and bile, adhesion properties of a strain to epithelial cells, etc., though these data differ greatly from those from *in vivo* pharmacodynamic experiments. Recently, multicompartmental dynamic models which reproduce the environment and motility of the gastrointestinal tract have been developed. They may predict results of *in vivo* situations and may provide a useful screening tool. However, human studies are the best way to establish the pharmacokinetics of lactic

acid bacteria. Bacterial recovery in faeces after ingestion of a specific strain is a simple non-invasive method. Intestinal intubation is a better approach to investigate the fate of bacteria in the gastrointestinal tract, since samples can be collected at different levels of the intestinal lumen. These methods are subjected to the reliability of the bacteriological techniques used to detect and identify the test strain. As mentioned further on in this chapter, the use of molecular biology techniques allows the detection of specific strains and the distinction of the given strain from endogenous strains of the same species. The use of transit markers such as spores of *Bacillus stearothermophilus* is a valuable tool to investigate the potential ability of lactic acid bacteria to colonise the intestinal tract. Persistence of the given strain for a longer period than that of the transit marker suggests actual colonization.

Using a triple lumen tube that was introduced orally up to the terminal ileum in human volunteers, *Streptococcus thermophilus* was detected in the jejunum, and *Lactobacillus bulgaricus* both in the jejunum and ileum. However, *Lactobacillus casei* and a *Bifidobacterium* sp. were detected throughout the entire intestinal tract and in the faeces (table IV). After ingestion of yoghurt, duodenal aspirates showed viable *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, but after ingestion of heated yoghurt no viable bacteria of these species were obtained from the duodenal lumen. About 1 to 10% of *Lactobacillus acidophilus* ingested in fermented milk were found to survive up to the terminal ileum, and so did 30% of ingested *Bifidobacterium bifidum*. Exogenous bifidobacteria ingested in a fermented dairy product reached a mean fecal concentration of  $10^9$  colony forming units per gram of faeces and remained at this high level during the ingestion period. However, after cessation of oral administration, the kinetics of the bifidobacteria gradually dropped and paralleled those of the bacillus used as a transit marker. This observation indicates that the bifidobacteria strain did not colonise the human colon. So far, colonisation of the human intestine with exogenous lactic acid bacteria has not been proven. However,

**Table IV.** Detection of ingested exogenous microorganisms in the intestinal tract.

	Jejunum	Ileum	Feces
<i>S. thermophilus</i>	+	-	-
<i>L. bulgaricus</i>	+	+	-
<i>L. acidophilus</i>	+	+	+
<i>L. casei</i> (GG)	+		+
<i>Bifidobacterium</i> spp	++	++	++
<i>S. faecium</i>	+		+
<i>S. boulardii</i>			+

in this study, the concentration of bifidobacteria in faeces during the period of consumption achieved levels above those required to obtain a significant metabolic activity provided by a probiotic.

## INTESTINAL TRANSIT

The fate of lactic acid bacteria within the gastrointestinal tract is influenced by the secretory and motility functions of the gut. Stable isotope methods are currently used to assess the gastrointestinal function. The most obvious advantage of methodologies based on stable isotopes is safety. They are environmentally safe. The use of stable isotopes is feasible even with pregnant women and new-born children. Simultaneous and repeated use of several tracers are possible in the same subject. Most tests are based on the exhalation of  $^{13}\text{C}$  and are easy to perform and painless to the patient. Lactose- $^{13}\text{C}$ -ureide measures intestinal transit time. Lactose-ureides are barely absorbed and after oral ingestion they will pass through the small intestine into the caecum. Bacteria in the colon hydrolyse the molecule; all individuals harbour bacteria capable of splitting the urea bond that is followed by the breakdown of urea to  $\text{CO}_2$  and ammonia. The  $^{13}\text{C}$  label can therefore be measured in breath or urine. This test can be used to measure oro-caecal transit time and is an alternative to the lactulose hydrogen breath test. Gastric emptying is also measured using  $^{13}\text{C}$ -labelled substrates. Lactase deficiency can be studied with the oral administration of  $^{13}\text{C}$ -lactose. Bacterial overgrowth in the small intestine can be evaluated by tests based on breath exhalation of  $^{13}\text{CO}_2$  after oral ingestion of either  $^{13}\text{C}$ -glucose or  $^{13}\text{C}$ -xylose.

Small and large bowel transit can be studied with relative simplicity and tolerability using the standard lactulose breath test to measure oro-caecal transit time and the radio-opaque pellet method to measure colonic transit time. The lactulose breath test is based on the oral administration of lactulose, a sugar which is not absorbed in the small bowel, but reaches the caecum and is fermented by the flora. Hydrogen produced by its fermentation can be detected in end-expiratory breath samples. Oro-caecal transit time is defined as the interval between ingestion of lactulose and first breath sample showing an increase in hydrogen concentration above a baseline of at least 3 parts per million, and this being maintained in three subsequent breath samples. This test presents a good correlation with scintigraphic measurements of small bowel transit. The radio-opaque pellet method is used for the measurement of colonic transit time. Gelatine capsules containing cubic radio-opaque pellets are administered three times a day at 8-hour intervals for three consecutive days. On day 4, an abdominal X-ray film is taken. The pellets are identified in the different colonic segments and counted. Markers located to the right of the vertebral spinous process and above a line from the fifth lumbar vertebrae to the pelvic outlet are assumed to be in the right colon. Markers to the left of the vertebral spinous process and above a line from the fifth lumbar vertebrae to the anterior superior iliac crest are assumed to be in the left colon. Markers located below the two aforementioned lines are assumed to be in the rectosigmoid colon. Colonic transit times in each segment and throughout the

entire colon are calculated by a standard formula. This method is accurate and gives a good correlation with the radiosciintigraphic method.

## **THE CONTRIBUTION OF MOLECULAR BIOLOGY**

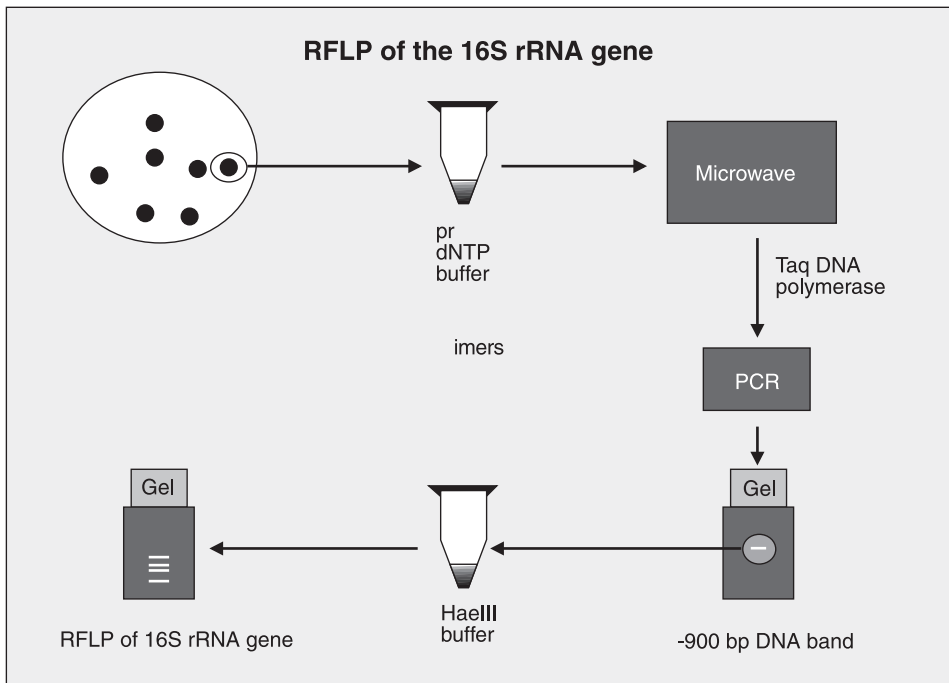
Probiotic properties are strain related, rather than species related. Conventional methods to detect the presence of a given probiotic strain in samples from a feeding trial are time-consuming and often ambiguous. Specific primers based on sequences of the 16S ribosomal RNA gene will provide a rapid method for the detection and identification of lactobacilli isolated from faecal samples by means of polymerase chain reaction (PCR) amplifications. In a multinational European Union project, strain-specific primers were developed and successfully used to detect bacterial cells by PCR analysis. Faecal samples were taken from human volunteers fed with specific probiotic lactobacilli. Colony forming units grown in Lactobacillus selective media were used for the analysis. The detection analysis was done in a double-blind fashion. A universal band was used as control for the amplification protocol and a strain specific band to detect the probiotic strain. The technique allowed the identification of all volunteers treated with the probiotic strains, without any misidentification of the untreated volunteers. These results clearly suggest that PCR-based detection of enteric strains will be of great value for future human research with probiotics.

The challenge facing us at present is to understand the diversity of the species and strains comprising the genera *Bifidobacterium* and *Lactobacillus*, that may potentially have probiotic attributes. It is necessary to identify a subset of strains with probiotic potential. The advent of molecular tools has shown tremendous promise for unraveling the mysteries of the true diversity of lactic acid bacteria within the human intestine. These tools comprise genetic fingerprinting, specific probes, molecular speciation and techniques for the in situ analysis of specific microbial groups in the intestine. Essentially, molecular biology tools for studying the gastrointestinal ecology can be divided into three different types:

- (a) tracking tools used to track the passage of microbes through the intestine;
- (b) molecular speciation tools which are clear and effective as opposed to the classical approach to speciation, which is often subjected to contention and debate, and
- (c) in situ tools which give a direct picture of the activity of micro-organisms in the intestine without having to culture them on plates.

Using PCR with universal primers targeting the bacterial 16S ribosomal RNA gene, a fragment of the gene can be amplified and isolated. Thereafter, the fragment is restricted by a restriction enzyme to obtain a characteristic sequence from a polymorphic area of the gene (figure 1). The restriction fragment can be used as a fingerprint for a particular strain. This PCR-based technique was used to track the fate of a bifidobacteria strain in six volunteers. The fingerprint of the ingested probiotic strain was different from the resident strains in the six individuals. During the eight-day feeding trial, the ingested strain quickly attained dominance over endogenous bifidobacteria among all individuals up to about sixty percent. When cessation of feeding occurred there was a wash-over effect,

and after another eight days the probiotic strain disappeared, which is an indication that this probiotic strain was transient in these individuals.



**Figure 1.** Restriction fragment length polymorphism of the 16S rRNA gene. (From O'Sullivan.)

The molecular tools for tracking, speciation and in situ analysis will be used to give us a better understanding of the microbial ecology of the gastrointestinal tract. This will allow a directed approach to probiotic strain selection.

## KEY QUESTIONS

Some questions are currently important for our knowledge of the fate of lactic acid bacteria in the digestive tract (for this purpose, the term lactic acid bacteria includes the genera *Lactobacillus* and *Bifidobacterium*, and also *Lactococcus* and some streptococci). The first question is how can we differentiate efficiently the resident lactic acid bacteria from the in-transit lactic acid bacteria? According to the data so far, lactic acid bacteria ingested with fermented milks cannot colonise the gut. They are in-transit flora. However, efficient methodologies to differentiate ingested bacteria from endogenous strains are needed to clarify this point.

It is generally accepted that lactic acid bacteria must survive within the gastrointestinal tract in order to be efficient for a particular probiotic activity. How do they survive? Does there exist only a transit or does there exist also a colonisation in the mucosa? Perhaps,

some lactic acid bacteria can come close to the mucosa and perhaps this close interaction with the mucosa is responsible for the function that we observe. Other bacteria may be metabolically active in the gut lumen during the intestinal transit. If this were true, we would need to know at which level they are active. Is it at the level of the small intestine or is it in the colon? From stomach to rectum the number of lactic acid bacteria in transit declines gradually. In the colon, we may need a large bacterial load to get an effect in the presence of the resident microflora.

To be efficient and exert a physiological function, lactic acid bacteria must be present in a sufficient number. This is the third key question, how many lactic acid bacteria must be alive and active to exert a physiological function and at which level of the gastrointestinal tract? We should be able to determine the number of living lactic acid bacteria, their location in the gut during transit and their viability. Molecular techniques give information on the presence of strain-specific DNA, but these methodologies do not tell us if the bacteria are alive.

Finally, we need to define the most appropriate method of answering each of these questions. Some investigations may be properly conducted using in vitro or animal experiments, but which kind of experiments should be done in humans? Current methodologies, as described in this chapter, may provide some answers. However, it is often difficult to compare experiments from one laboratory to another one. We need to standardise the current methods and we also need a consensus on their validity and implications. Certainly, new developments will also be required.

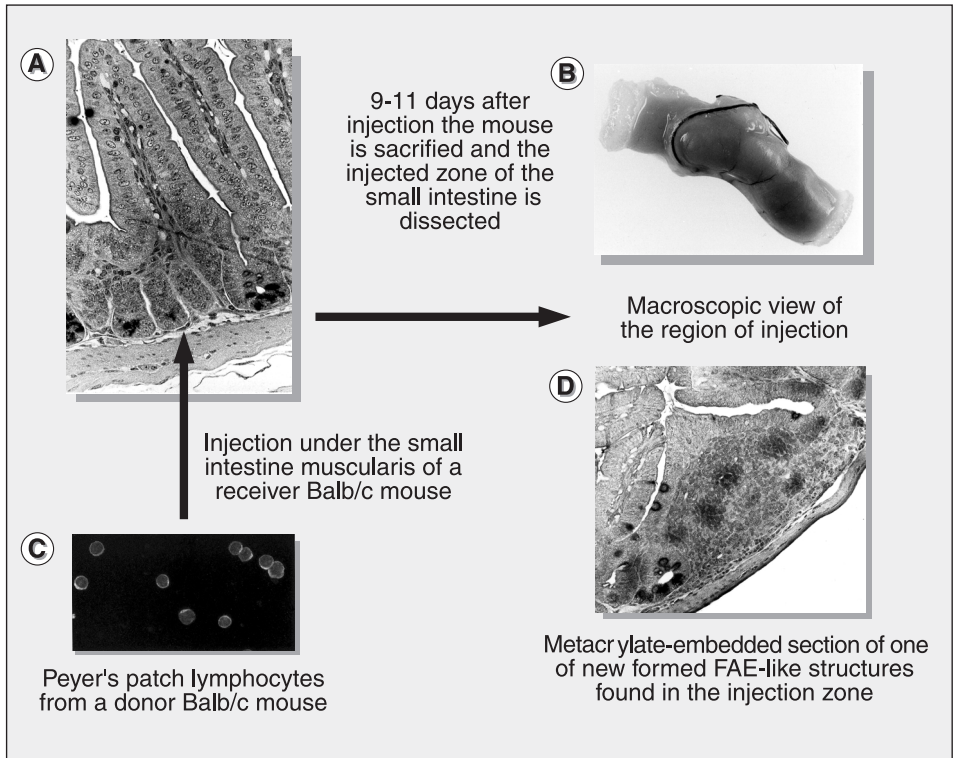
## CHAPTER III

# THE STUDY OF M-CELLS AS TARGETS OF THE INTERACTION OF BACTERIA WITH THE IMMUNE SYSTEM

The human body is in permanent contact with bacteria. Most interactions with bacteria are positive and beneficial, like, for example the relationship of the microflora with the intestinal mucosa, but interactions with pathogen bacteria may lead to an infectious disease. Some of these pathogens enter the body through the digestive tract. There are two types of protection against infection. One type of protection is non-specific and includes non-immune mechanisms such as the physical barrier provided by the mucus layer (glycocalyx and mucins), and the secretion of chemicals with bactericidal activity (lysozyme, lactoferrin, defensins) by Paneth cells in the intestinal epithelium. The second type of protection is specific for a particular pathogen and includes antibody-mediated and cell-mediated immune responses against a specific antigen.

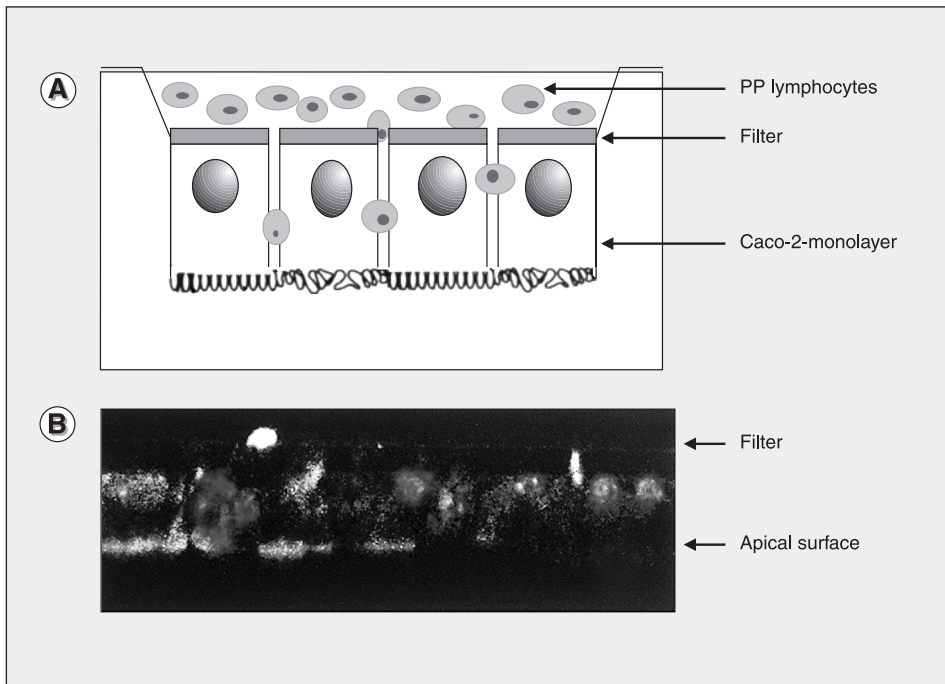
To develop a specific immune response, contact between the pathogen and the host is required. In the intestinal epithelium, there are specialised sites to sample antigens and microbes. These are M-cells. These cells are present in special structures called Peyer's patches. The interaction between microbes and the M-cells is facilitated by the fact that there are almost no goblet cells around to secrete mucus and there is no chemical defence against the micro-organisms. After sampling by M-cells, antigens or micro-organisms are internalised and directed to the underlying lymphoid follicle. Lymphoid follicles consist of a germinal centre containing mostly B-lymphocytes surrounded by CD4 positive T-lymphocytes. Macromolecules or particles are actively transported by M-cells and delivered to the lymphoid tissue. However, several pathogens including bacteria and viruses are able to exploit this pathway and use M-cells to cross the epithelial barrier and induce infection.

Follicle-associated epithelium (FAE) is lacking in immunodeficient mice. M-cells are absent, suggesting that the lymphoid cells of the follicle are essential for differentiation of the epithelium. When isolated lymphoid follicles from donor mice were injected in an intestinal segment without Peyer's patches from a recipient mouse, microscopic formation of a true Peyer's patch was observed in the same place 9-11 days after injection (figure 2). Morphological and histochemical characteristics of the epithelium in de novo – formed patches were similar to those of normal FAE. This experiment suggested that M-cells can originate from crypt cells and that lymphoid tissue has a key role in the differentiation of these cells.



**Figure 2.** De novo formation of FAE-like structures in the intestinal mucosa after injection of Peyer's patch lymphocytes. (From Kerneis, Kraehenbuhl and Pringault.)

An in vitro system for coculture of a human colonic epithelial cell line (Caco-2) with Peyer's patch lymphocytes was developed using a transwell device (figure 3). Phenotypic conversion of enterocytes into cells that express M-cells properties was observed after a few days of coculture. Lymphoid cells were found to enter the epithelial cell monolayer. The presence of lymphoid cells did not affect the monolayer, since transepithelial resistance did not change. In some areas morphological changes were observed, such as a decrease in the thickness of the apical surface, sometimes with complete disappearance of the typical



**Figure 3.** In vitro system for coculture of colonic epithelial cells and Peyer's patch lymphocytes. (From Kerneis and Pringault.)

enterocyte staining and with real modification of the surface. Transformed cells were able to transport inert particles, such as latex beads. These are fluorescent, and their adhesion and transport by the monolayer can therefore be monitored. In coculture at 4 °C, beads were not transported, but when the temperature of the system was raised to 37 °C, a high rate of passage of the beads was observed, indicating that the cells were actively transporting the beads.

The interaction between M-cells and microorganisms was investigated using the in vitro coculture system. Some bacteria like *E. coli* are able to interact with the apical surface of M-cells without being transported, whereas certain pathogens interacting with M-cells are transported by different mechanisms, and use this path to cross the epithelial barrier. Interestingly, *Vibrio cholerae* incubated with Caco2 cells only interacted with the apical surface of cells but was not internalised inside the cells. However, in the coculture system with Caco-2 cells and lymphocytes (i.e. with transformed M-cells), *Vibrio cholerae* was found inside the monolayer. This finding was confirmed by transmission electron microscopy that revealed the presence of vibria in cytoplasmic vacuoles. Bacteria like *Shigella flexneri* and *Vibrio cholerae* were efficiently transported by this system, but *Listeria monocytogenes* was transported at a lower rate, suggesting that there are at least two different mechanisms. Adhesion of *Vibrio cholerae* to apical surfaces was similar in the single monolayer culture of Caco-2 cells compared to the coculture system. However, other pathogens

did not adhere to Caco-2 cells in a single culture but showed high adherence to the apical surface of the same cells in the coculture system, suggesting the expression of new surface molecules that allow the interaction after coculture.

The coculture system is a useful model to learn about the nature of the interactions between microorganisms and M-cells, including the characterisation of surface receptors. This model also gives information on the nature of lymphoid components which are able to induce conversion of enterocytes into M-cells.

## CHAPTER IV

# YOGHURT IN THE PREVENTION AND TREATMENT OF HUMAN DISEASES

Lactic acid bacteria are able to modulate the immune system according to a large number of studies. Some of these microorganisms are capable of crossing over the gastrointestinal tract, and they can interact with other bacteria from the microflora and other cells within the intestinal tract. Some of the immunomodulatory properties consist of an increase in the production of intestinal immuno-globulines by mucosal B-cells. In vitro, lactic acid bacteria increase the release of gamma-interferon. Most research in this field has been carried out in animal or in vitro models, regarding both cellular or humoral immunity. However, there are still lots of questions to be answered about the effects of yoghurt intake in humans. Some data suggest that yoghurt is useful in the prevention and treatment of some pathologic conditions, such as diarrhoeal diseases, certain tumours and allergies.

## DIARRHOEIC DISORDERS

Despite the introduction of oral rehydration, many children die from diarrhoea every year, and many more suffer episodes of malnutrition which is precipitated by diarrhoeal disease. In 1990, the WHO predicted a reduction of the number in children dying from diarrhoea from 5 million to 1.5 million per year. However, estimates at the present time are that at least 3 million children die every year from diarrhoea-related illness. Between two and ten episodes of diarrhoea per year is the normal experience for many children in sub-Saharan Africa and in poor areas of Asia.

Fermented food products may decrease the impact of diarrhoea on public health in Third World countries. Management objectives to reduce death by diarrhoea include several aspects: first of all, to reduce the number of germs in the diet; secondly, to reduce

the incidence of diarrhoea; thirdly, to reduce the duration of diarrhoeal episodes, and fourthly, to reduce the severity of diarrhoea. As a result of these strategies, a lower rate of fatalities is expected, and also an improved nutritional outcome of diarrhoea sufferers.

Food fermentation induces a low pH and inhibits the growth of diarrhoeal pathogens in food. Fermentation of cereals in Ghana by a traditional procedure showed a pH reduction from 6 down to below 4. The food children eat in poor communities is grossly contaminated. Porridges are usually prepared in the morning and left for up to twelve hours later before being consumed by the children. The number of coliform bacteria in food at 0,6 and 12 hours after cooking was estimated by microbiological culture. Among samples of unfermented food – this is food that is freshly prepared and boiled – and fermented food, it was found that there were quite a lot of organisms in the freshly – prepared, unfermented food. It is a fallacy to think that all bacteria are destroyed by cooking. Six hours later, the number of food samples that were free of bacteria was greater in fermented food than in unfermented food. Twelve hours later, there were many more coliforms in the unfermented food as opposed to the fermented food. Thus, fermentation protects the growth of potentially harmful microorganisms in food.

In a study performed in Mexico, either a probiotic mixture including *Lactobacillus reuteri* or a placebo was given to children of a low socio-economic group for 14 weeks. Regular administration of the *Lactobacillus* reduced the incidence of diarrhoea from 0.42 to 0.27 episodes per child. Prevalence was reduced from 35 in the placebo group to 24 in the treatment group. No difference was found in the severity of the diarrhoea episodes among both groups. In this study, etiology of the diarrhoea was not investigated or considered for stratification. Most of the previous studies investigated the effect of probiotics in diarrhoea induced by rotavirus, since this is the main cause of diarrhoea among children in Europe, and a positive effect was established. There is not much evidence, so far, on the effect of probiotics in the prevention of diarrhoea induced by parasites or bacteria, but the Mexican study may suggest a positive health effect in this area.

A study in Pakistan looked at the effect of yoghurt on acute diarrhoea. Children in the control group were treated with a commercial preparation, known to be effective in diarrhoea. Traditional yoghurt was given to another group. At 48 to 72 hours after onset, traditional yoghurt had a clinical advantage over the most expensive commercial preparation.

Intestinal permeability can be assessed by the lactulose/mannitol test. After oral ingestion of the sugars, urine is collected during a 5-hour period to measure the urinary excretion of both sugars, and the lactulose/mannitol concentration ratio is calculated. In healthy control children the ratio is low, about 0.2, but it is high in children with intestinal mucosal damage, which occurs with diarrhoeal diseases. A group of Tanzanian children with severe diarrhoea were rehydrated, and then allocated to receive one of the two types of diet: either a control diet, which was the normal porridge made out of freshly prepared cereals, or a special porridge that had been fermented. Then, the impact of diet on intestinal permeability was measured by the lactulose/mannitol test. After feeding, there was an

improvement in intestinal permeability in both groups as compared to the baseline. Interestingly, the improvement was much greater in children receiving fermented food than in controls. Metabolic products of fermentation, such as butyrate and propionate, have trophic properties in the intestinal epithelium and they might account for the positive effect.

The effect of yoghurt as a treatment of acute diarrhoea in children was recently investigated in Oran (Algeria). Children admitted for acute diarrhoea were randomised to receive either an infant milk formula or yoghurt. They were stratified depending on the presence of rotavirus in their stools. Yoghurt was prepared with the same infant milk formula by fermentation with freeze-dried starters of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Clinical characteristics were comparable between the two groups at inclusion. Rotavirus was found in 35 out of the 46 children in the infant milk group and 37 out of 47 in the yoghurt group. The mean duration of diarrhoea was higher in the milk group (66 hours) than in the yoghurt group (44 hours). At 48 hours after inclusion, total remission was achieved in 64% of children receiving yoghurt, but only in 35% of children receiving milk. The beneficial effects were most apparent in subgroups of children with rotavirus: 68% cured in the yoghurt group versus 27% in milk group.

A second study compared the clinical efficacy of yoghurt feeding in children with persistent diarrhoea. Persistent diarrhoea denotes an episode of diarrhoea that begins acutely but persists beyond the expected time period for the usual self-limited disease. After a two-day period of observation, 78 children with persistent diarrhoea were randomly assigned to receive either milk or yoghurt for a period of 5 days. Both groups were similar on inclusion in terms of age, nutritional status, severity of diarrhoea and lactose hydrogen breath test. Clinical failure was defined as a 5% loss of body weight in 24 hours, absence of body weight gain during 3 consecutive days or persistence of diarrhoea after 5 days. Clinical failure rate was 43% in the group of children receiving milk, but only 12% in children receiving yoghurt. The advantage of yoghurt feeding was already apparent at 24 and 48 hours after inclusion. These results indicate that yoghurt can be used satisfactorily for nutritional rehabilitation in children with persistent diarrhoea.

Another study compared the value of fermented milk in the prevention of diarrhoea. The trial concerned Algerian infants of less than 5 months old living in an urban area, without previous history of diarrhoea and regularly fed with infant formulas but no breast milk. At entry, each mother was offered a batch of unlabeled boxes containing either infant formula or fermented infant formula (*Streptococcus thermophilus* and *Bifidobacterium brevis*). Fermented infant formula was similar to infant formula except for its taste, low pH and the presence of galactose and lactic acid resulting from partial hydrolysis of lactose. A total of 98 infants entered the trial, 49 per group. At inclusion, both groups were comparable in terms of age (a mean of 45 days in both groups). After 21 days follow-up, body weight gain was similar in both groups. In the formula group, 25 infants suffered from diarrhoea, as opposed to 17 in the fermented formula group. The total episodes of diarrhoea were 45 in the formula group and 17 in the fermented formula group. Calculated rates were 4 episodes per child per year with the infant formula and 2 episodes per child per year in children

receiving the fermented formula. These results confirm the preventive effect of fermented milk on diarrhoea. They further suggest that the fermented formula is well tolerated and can induce a clinically significant positive effect in well nourished, healthy children of less than 5 months old.

Fermentation technology has an enormous benefit to offer in the prevention and treatment of diarrhoeal diseases. Further clinical studies looking at fermented vs non-fermented foods, with or without probiotics, are needed. The specific role of probiotics as part of the treatment of diarrhoea remains to be defined.

## **ANOREXIA NERVOSA**

Malnutrition has always been associated with developing countries. However, in spite of the existing resources nowadays in industrialised countries, there is an increasing number of cases of malnutrition. Unfortunately, eating disorders are becoming more and more frequent. In this sense, anorexia nervosa is a syndrome that affects a considerable part of the female population during adolescence. These patients exhibit an altered behaviour with abnormal dietary patterns. They have phobias about their food intake, especially towards foods containing fat or high levels of carbohydrates. These patients chiefly eat fruit and vegetables. In addition, they may use some compensatory means to avoid weight gain, such as self-induced vomiting, and compelling use of drugs (diuretics, laxatives and also anorexigens). This behaviour involves weight loss leading to a final situation of malnutrition.

The production and release of cytokines is known to be affected by nutrient intake. A study carried out in patients with anorexia nervosa aimed at investigating the possible beneficial effect of yoghurt intake on cytokine production. A cross-over study was designed including 27 anorexia nervosa patients divided into two groups. Patients were recruited upon hospital admission and followed for 20 weeks. During the first 10-week period, a group of patients received yoghurt and the other group received fresh milk. During the second 10-week period, patients were changed to the alternative treatment (either yoghurt or milk). The patients received either 3 yoghurts per day or an iso-caloric volume of milk. Diet was similar in both groups of patients throughout the 20-weeks of study. Cytokine production by peripheral blood mononuclear cells was assessed in vitro after phytohaemagglutinin stimulation.

The group who started nutritional therapy with yoghurt showed a rapid and significant increase in the release of gamma-interferon that persisted during the milk intake period. The group who started nutritional recovery with milk showed a decrease of gamma-interferon production, but there was an increase during the second period (shift to yoghurt) just at the end of the study, between the 16th and 20th week. Thus, both groups showed an increase in gamma-interferon secretion during the period of yoghurt intake. Regarding interleukin-2, there were no significant changes in patients who started with yoghurt. However, the group that started nutritional therapy with milk showed significant interleukin-2 production during the period of yoghurt consumption (20th week). A similar result was

found when looking at the release of tumour necrosis factor alpha ( $\text{TNF}\alpha$ ). A certain degree of previous nutritional recovery may be needed to observe a positive effect of yoghurt on the production of interleukin-2 and  $\text{TNF}\alpha$ . Production of interleukin-6 showed an early response during yoghurt consumption, that persisted during the first weeks of consumption of milk but finally dropped. Data on interleukin-1 were similar to those observed with interleukin-6.

In summary, modulation of cytokine production in patients with anorexia nervosa can be promoted by yoghurt intake. The effects vary according to the cytokine tested. In general, inclusion of yoghurt at an early stage of nutritional therapy may be of value in improving a deficient immunocompetence.

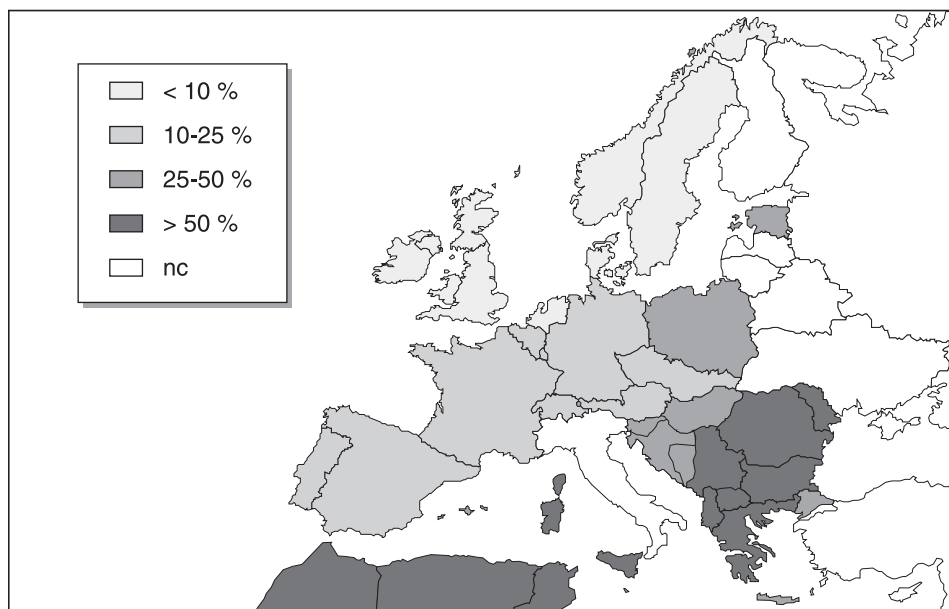
## CHAPTER V

# YOGHURT IN LACTOSE MALABSORPTION AND INTOLERANCE

## CLINICAL SIGNIFICANCE OF LACTOSE MALABSORPTION

Lactose is a disaccharide composed of glucose and galactose, that is mainly found in milk and dairy products. To be absorbed in the small intestine, lactose must be hydrolysed into glucose and galactose by a specific enzyme, lactase or beta-galactosidase which is present in the brush border of the small intestinal epithelium. In the early 60's, it was demonstrated that most of the world adult population have a genetically-determined decrease in lactase activity after weaning, and this was the key that opened different areas of investigation regarding lactose malabsorption. Thus, prevalence of lactase deficiency is relatively high, with variations between different geographical areas: 10% in Northern Europe, 25% in the United States, 50% in Mediterranean countries and above 50% in Africa and South America (figure 4). Lactose malabsorption is the result of lactase deficiency and means that a fraction of the ingested lactose is not absorbed in the small intestine and will therefore end up in the colon. This can lead to gastrointestinal symptoms such as diarrhoea, flatulence, abdominal bloating and pain. Lactose intolerance is the symptomatic complex that may result from lactose malabsorption, but lactose malabsorption does not necessarily imply lactose intolerance.

To illustrate the concrete implications of this phenomenon, let's examine the following example. If 12.5 g of lactose (the amount present in one cup of milk) are malabsorbed and reach the colon, the colonic bacteria will ferment the disaccharide, and will produce 120 mmols of organic acids that will associate with electrolytes leading to an osmotic load that will induce severe diarrhoea. Moreover, fermentation



**Figure 4.** Prevalence of lactose malabsorption. (From Martinez.)

of 12.5 g of lactose will produce 2,600 ml of CO<sub>2</sub> and approximately 4 litres of hydrogen. However, studies using labelled lactose have shown that such an amount of gases is not produced, which means that the lactose is either hydrolysed by a remnant lactase or consumed by the colonic bacteria. Bacteria ferment the lactose, and the products can be absorbed into the colon. Thus, even when lactose is malabsorbed, fermentation and absorption of the products can prevent diarrhoea. Moreover bacteria not only produce hydrogen, but also consume it. The balance between the production and consumption of gas determines the amount of gas present in the colon lumen.

Therefore it is difficult to know the quantity of lactose that will induce symptoms. In double-blind studies, ingestion of up to 18 g of lactose induced a similar rate of symptoms in lactose malabsorbers to those in controls. It was only after ingestion of 50 g of lactose (4 cups of milk) that 80% of the subjects complained of symptoms.

In addition to people with diagnosed lactase deficiency, some subjects with apparently normal lactase activity will describe themselves as being lactose intolerant. In a group of 30 subjects self-described as severe lactose intolerants, it was found that only 21 subjects were lactose malabsorbers. Regardless of the lactose absorption status, they were included in a double-blind crossover study in which they consumed one cup of either regular milk or lactose-hydrolysed milk with breakfast for one week. There was no significant difference in any of the symptoms (bloating, flatulence, abdominal pain and diarrhoea) between those taking regular milk and those taking hydrolysed milk in the malabsorber group. Another important point was that the symptoms were mild. In general people who

identify themselves as severe lactose intolerants can drink a cup of milk without experiencing significant problems.

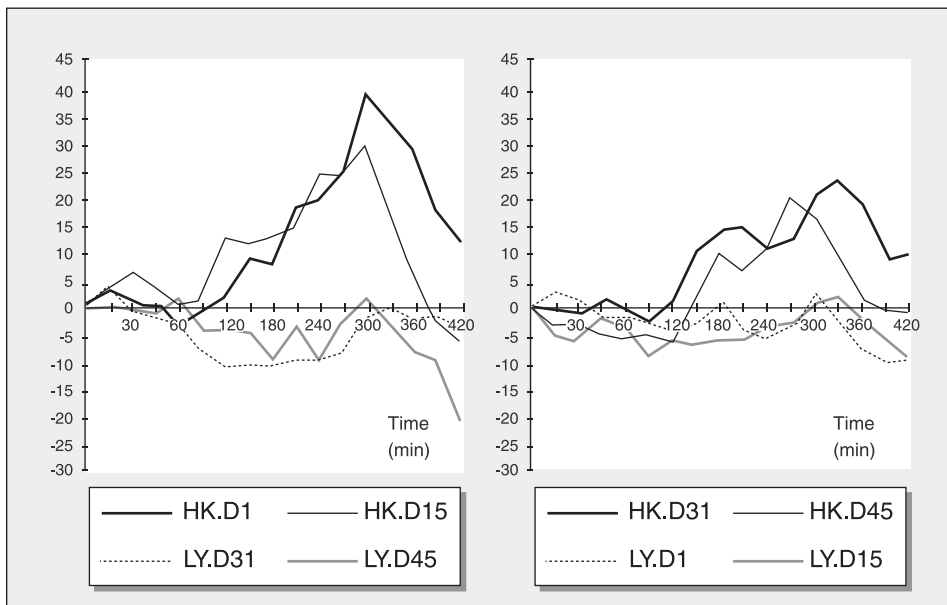
Nevertheless, self-described lactose intolerant subjects will behave as though they are diagnosed lactose malabsorbers by eliminating dairy products from their diet, consequently losing the nutritional benefits of dairy food. In order to avoid the nutritional consequences of the exclusion of dairy foods from the regular diet, several studies have demonstrated that yoghurt is a good alternative for lactose maldigesters. Further investigations have also stressed that the presence of living lactic acid bacteria in yoghurt is essential to overcome symptoms and signs of lactose malabsorption, as mentioned later in this chapter.

A critical aspect in this context is the possible loss in terms of calcium intake. Given the relationship between osteoporosis and calcium intake, a National Institute of Health Consensus Conference in the United States recommended a daily intake of 1,500 milligrams of calcium for post-menopausal women. Dairy products are the main natural rich source of calcium (table V), however 4 to 5 servings of dairy products per day would need to be consumed to obtain this amount of calcium which could cause lactose malabsorbers to develop symptoms. A study was conducted with 31 lactose malabsorbers and 31 lactose absorbers, 50% being pre- and 50% being post-menopausal women. In a double-blind randomised fashion, the diet was supplemented with milk, cheese and yoghurt, provided either as conventional products (34-g lactose/day) or as lactose hydrolysed products (2-g lactose/day). There was no significant difference for symptoms in the absorbers group. In the malabsorbers group, lactose hydrolysed products induced similar symptom scores to those for conventional products for abdominal pain and bloating, but there was a significant increase in flatus frequency with the conventional products. However, the general well being of the subjects was not affected, and there were no significant differences between the two groups. In conclusion, this study suggests that symptoms resulting

**Tableau V.** Lactose and calcium: content of various foods.

<b>Food</b>	<b>Serving</b>	<b>Lactose (g)</b>	<b>Calcium (mg)</b>
Cow's milk	240 ml	12.5	300
Ice cream	120 g	8.3	100
Yoghurt	240 ml	12	400
Cottage cheese	120 g	3	78
Other cheese	30 g	0.7	200

from lactose malabsorption do not represent a major impediment to the ingestion of dairy-rich diets, containing around 1,500 milligrams of calcium per day.



**Figure 5.** Lactose malabsorption as assessed by hydrogen breath test (HK = heat-killed yoghurt; LY = Live yoghurt; D= day). (A. Martinez)

## LACTOSE DIGESTION AND TOLERANCE FROM LIVE AND HEAT-KILLED YOGHURTS

Preliminary data were presented from human studies using the same product in three different countries with essentially the same protocol. Yoghurt as a live culture compared to heat-killed yoghurt was tested in a randomised crossover protocol in lactose malabsorbers from Germany, Spain and the United States. Both products contained the same amount of lactose. One group of subjects consumed 500 g of live yoghurt per day for 14 days. This was followed by a two-week wash out period. Subjects followed the same protocol with the heat-killed product. The other group started with the heat-killed product, then after the wash out period they consumed live yoghurt for another 14-day period. Lactose digestion was determined by the hydrogen breath test on specific test days. Gastrointestinal symptoms were recorded throughout the study by a validated questionnaire.

In all three studies, hydrogen production increased after ingestion of the heat-killed product, but there was no increase in breath hydrogen after ingestion of live yoghurt (figure 5). This finding clearly indicates that live yoghurt as compared to heat-killed yoghurt improved lactose digestion in lactose malabsorbers. Gastrointestinal symptom scores were

lower during the period of consumption of live yoghurt than during consumption of heat-killed yoghurt. In addition, yoghurt consumption over several weeks increased lactose tolerance in malabsorbers. Finally, there was a trend towards less hydrogen production after lactose ingestion at the end of the study, suggesting that repeated exposure to lactose from either live or heat-killed yoghurts induced adaptation of the organism.

## INTESTINAL AND COLONIC FACTORS IN LACTOSE INTOLERANCE

Live yoghurt can influence lactose maldigestion and alleviate complaints of lactose intolerance. Nevertheless, all the mechanisms involved in the apparition of symptoms are not known. Further research is needed to identify which factors play a role in the generation of the symptomatic complex associated with lactose intolerance, since this knowledge will lead to the development of new healthy foods based on dairy products.

A recent investigation has pointed out that some subjects are free of symptoms despite the fact that they suffer from lactose malabsorption. Lactase deficiency is usually assessed by the lactose-hydrogen breath test. Lactose reaching the colon is fermented by bacteria and an increase in hydrogen production can be detected in breath samples. However, the increase in breath hydrogen results from two processes: malabsorption of lactose in the small intestine and fermentation in the colon by bacteria, so, the absence of a peak of hydrogen in breath might be due to changes in the fermentation process rather than the actual absorption of lactose. Therefore, false negative hydrogen tests are common. A method has been developed based on the ingestion of  $^{13}\text{C}$  labelled lactose. Absorption is assessed by the levels of  $^{13}\text{C}$  labelled glucose in serum. The label with the stable isotope discriminates between endogenous and ingested glucose.

The  $^{13}\text{C}$ -lactose test was validated in a population of healthy Chinese medical students with a genetic background of low lactase activity: 73 subjects ingesting 25 g of lactose were screened; 68 tested hydrogen positive, and 5 hydrogen negative. The  $^{13}\text{C}$ -lactose test was then applied to 5 hydrogen negative subjects and 12 hydrogen positive subjects by the oral administration of 25 g of  $^{13}\text{C}$ -lactose. Interestingly, there was no significant difference in peak serum levels of  $^{13}\text{C}$ -glucose between hydrogen positive and hydrogen negative subjects, suggesting that those individuals who tested hydrogen negative were true malabsorbers with a false negative breath test. The cut-off value of  $^{13}\text{C}$ -glucose in serum was estimated as 2.0 mmol/l, 1 hour after lactose ingestion. Subjects with values below this level would be lactose malabsorbers.

The  $^{13}\text{C}$ -lactose test was also performed on 48 Dutch medical students. Again, correlation of the outcome of the hydrogen breath test with the  $^{13}\text{C}$ -lactose test showed areas with low concordance. It was found that 25% of the Dutch students showed serum  $^{13}\text{C}$ -glucose levels below the cut-off value, whereas only 17% of the students had a positive

hydrogen breath test. In addition, only 13% of the students manifested complaints of lactose intolerance. Subjects with low serum  $^{13}\text{C}$ -glucose levels and no complaints after consumption of 25 g of  $^{13}\text{C}$ -lactose are considered as non-symptomatic malabsorbers. Likewise, in Chinese population of malabsorbers, it was found that students with symptomatic complaints after the consumption of lactose showed similar  $^{13}\text{C}$ -glucose levels to those students without complaints.

The study demonstrated that some subjects with poor absorption of lactose do not develop symptoms after a challenge with oral lactose. This finding suggests that besides digestion, there must be other factors involved in the genesis of symptoms after lactose ingestion. These factors are presumably related to the fermentation process in the colon.

## CHAPTER VI

# THE ROLE OF LACTIC ACID BACTERIA IN COLON CANCER PREVENTION

## EARLY EVENTS ASSOCIATED WITH COLON CARCINOGENESIS

Many studies support the role of the human intestinal microflora in tumour development in the colon. By definition, probiotic bacteria can beneficially affect the host by improving its intestinal microbial balance. Hence, probiotics could have anti-cancer effects. In general, in vitro studies and in vivo studies in laboratory animals give strong support to the protective role of probiotic bacteria in colon cancer progression. Human studies reported so far are interesting, but they are certainly not as supportive as the laboratory studies. Although there is no real direct evidence to show the protective effect of probiotic bacteria in human tumour development of the colon, there clearly is a lot indirect evidence.

Colon cancer develops through several distinct stages, from normal epithelium to hyper-proliferating epithelium, adenoma, carcinoma and metastasis. In the last ten years, huge progress has been made in identifying the genes that are responsible for or contribute to the progression of these sequential stages. On the other hand, epidemiology indicates that there are dietary factors associated with increased risk, for example fat, and with decreased risk including lactic acid bacteria, fibre and fruit (table VI). Much effort in current studies is directed towards environment interactions with gene expression/suppression. The question today is how dietary components, including lactic acid bacteria, interact with genes that contribute to tumour development. For instance, butyrate, a metabolite of yoghurt, has been shown to directly affect the expression of genes which are important in tumour development in the colon.

**Table VI.** Diet and colon carcinogenesis. Epidemiology.

<p>Dietary factors associated with increased risk</p> <ul style="list-style-type: none"> <li>• Total fat</li> <li>• Total protein</li> <li>• Meat intake</li> <li>• High caloric intake</li> </ul>
<p>Dietary factors associated with decreased risk</p> <ul style="list-style-type: none"> <li>• Dietary fiber</li> <li>• Vegetables, fruit</li> <li>• Vitamins A (beta-carotene), C, E, D</li> <li>• Calcium</li> <li>• Lactic acid bacteria</li> </ul>

Some hypotheses on the relationship between diet and colon cancer are currently under investigation. The diet-derived components that may influence colon cancer are generally divided into initiators or promoters. Initiators are genotoxic compounds which can induce DNA damage and may give rise to genetic alterations. Most genotoxic agents are detected with short-term assays, for example the Comet assay, and are called mutagens. Mutagens are genotoxins that have been shown to induce tumours in animals, and in this sense they are also carcinogens. Where human colon cancer is concerned, however, we do not know which are the actual mutagens involved. Free-radicals produced in the colon or coming from dietary components could contribute to genotoxicity. Fecapentaenes produced by the intestinal microflora are also strongly mutagenic and are believed to be involved in producing human colon cancer. Another group of compounds, the heterocyclic amines, are formed when burning meat at a high temperature. They are mutagenic and carcinogenic in animals. Another hypothesis concerns detoxified mutagens. Environmental contaminants like polyaromatic hydrocarbons or benzopyrenes are detoxified in the liver, like many foreign compounds, and excreted in bile as conjugates. Bacterial enzymes in the intestine can deconjugate them and liberate the mutagenic moiety which could contribute to tumour development.

Promoters are not genotoxic; they do not damage genes. They contribute to tumour progression by other methods, for instance by inducing cell proliferation. At high cell proliferation rates, a damaged cell will have a greater statistical chance of developing into a tumour. In addition, cells that are highly proliferative are much more sensitive to genotoxic agents. Fat is a well-known tumour promoter, because it contributes to a hyperproliferating effect in the colonic epithelium that is believed to be mediated by bile acids. Dietary fat increases the secretion of bile containing primary bile acids that may leak down into the colon. Secondary bile acids produced by colonic bacteria are believed to be tumour promoters, since they are cytotoxic to epithelial cells and induce proliferation. Fibre may

protect by inducing a low pH that prevents the enzymatic transformation of primary bile acids into secondary bile acids, and calcium may protect against the cytotoxic effect of secondary bile acids on epithelial cells.

Probiotics can act on early events of the process. First of all, probiotic bacteria can alter enzyme activity of the indigenous flora and avoid the production of genotoxic compounds. Secondly, probiotic bacteria may interfere with mutagens and prevent genotoxic damage to the colonic epithelial cells. A considerable amount of studies clearly show that probiotics can produce an *in vivo* decrease of faecal enzymes which play a part in the conversion of pro-carcinogens into carcinogens. Indeed, it was the observation that probiotic bacteria influence these enzyme activities that opened up the interest in probiotics as anti-carcinogenic agents. In animal models, several strains of probiotic bacteria are very efficient in decreasing the activity of several enzymes that are known to be active by producing potential initiators of colon cancer.

On the other hand, a huge amount of studies have shown that probiotics are able to inhibit mutagenic activity. *In vitro* studies of mutagenic compounds and lactic acid bacteria actually demonstrate that they can decrease genotoxic activity, i.e. they prevent damage to DNA sequences. Some other studies have shown that lactic acid bacteria have the ability to physically bind mutagenic compounds, such as heterocyclic amines derived from cooked meat. Absorption of mutagens from the gut can be suppressed. There are strain differences, and there are different effects with different mutagens. A few studies have shown that some lactic acid bacteria strains can have direct effects on colonic cells in culture. These strains lower growth rate and induce differentiation in a colon carcinoma cell line. Growth arrest or growth differentiation can be associated with protection.

Dietary intervention studies in human volunteers have shown that several strains of lactic acid bacteria can decrease the enzymes that may be involved in carcinogen generation. A continuous consumption of the active strain is necessary to maintain the effect. Still, it is very important to confirm that these enzymes play an essential role in human colon carcinogenesis. Healthy subjects on a standardised diet consumed fried beef patties twice daily for 3 days and a significant increase in mutagenicity was observed in urinary and faecal samples. Simultaneous supplementation of the diet with a strain of *Lactobacillus acidophilus* in fermented milk showed no increase in the excretion of urinary and faecal mutagens. Likewise, another study showed that oral administration of *Lactobacillus casei* suppressed the urinary excretion of mutagens arising after the ingestion of fried ground beef in humans.

Further studies have shown the effects of certain lactic acid bacteria on soluble faecal bile acids. Consumption for six weeks of a fermented milk product containing *Lactobacillus acidophilus* markedly decreased bile acid levels in the aqueous phase of faeces in colon cancer patients. This change can be associated with reduced cytotoxicity and reduced epithelial cell proliferation. In fact, an Italian study has shown that the abnormally high proliferative activity that is observed in the colonic crypts of patients with adenoma was returned to normal after a three months administration of *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. Thus, lactic acid bacteria can be seen to influence the levels of

soluble bile acids in the colon, which could indirectly reduce proliferative activity in the epithelium.

However, two recent studies on the effect of lactic acid bacteria supplements on risk markers for colon cancer, failed to confirm these findings. Yoghurt enriched with *Bifidobacterium longum* significantly increased the excretion of lactic acid bacteria in the stools of human volunteers, but there was no effect on risk markers such as oro-anal transit time, stool weight and pH, faecal short chain fatty acids, faecal bile acids and faecal neutral sterols. Again, another study looked at the effect of a prebiotic (a fructo-oligosaccharide) on faecal bifidobacteria and a number of risk markers for colon cancer. Prebiotic ingestion led to a significant increase in faecal bifidobacterial counts, but no effect was observed on faecal pH, activities of nitroreductase, azoreductase or beta-glucuronidase.

## **MODULATION OF DNA DAMAGE**

Damage in DNA sequences of important genes, such as proto-oncogenes, tumour suppressor genes and DNA repair genes, will cause an increased cell proliferation and a high probability of cell transformation. The Comet assay is an interesting method to use for monitoring DNA damage. Isolated cells are embedded in agarose, where the cells are lysed and the DNA is liberated and subjected to electrophoresis. Thereafter, DNA is stained with fluorescent dyes and damage can be recognized by a comet shape and quantified in different ways. This method can be used in animals, in in vivo studies, and also in cultured cells.

In rats fed with carcinogens (dimethyl-hydrazine, DMH or methyl-nitro-nitrosoguanidine, MNNG) and pretreated with lactic acid bacteria, such as *L. acidophilus*, *L. gasseri*, *B. longum*, *B. brevis* or *Streptococcus thermophilus*, there was a significant decrease in the genotoxic effects on colonic epithelial cells compared to those in rats receiving the carcinogens only. However, heated lactic acid bacteria were not protective for either carcinogen. The effects were strain specific, as observed with different strains of *Lactobacillus delbrueckii*, sub-species *bulgaricus*, and *Streptococcus thermophilus*. Some of the strains were active but others had no effect.

In vitro incubation of colonic cells with MNNG and different cellular fractions of the effective lactic acid bacteria strains showed that whole cell preparations, cell wall fractions and isolated peptidoglycans of the cell wall induced a significant reduction in genotoxicity, but that cytoplasm fractions had no effect. In addition, genotoxicity by MNNG on isolated colon cells was prevented by both the supernatant and pellet of *Lactobacillus acidophilus* culture in logarithmic growth. However, heat treated pellets had no effect, suggesting that anti-genotoxic activity was mediated by relatively short-lived products generated by viable bacteria. For instance, acetate can prevent DNA damage induced by hydrogen peroxide in colon cells in vitro. Another protective compound was found to be the aminoacid cysteine, that is released from cell wall proteins by proteases.

Carcinogen induced DNA damage can be prevented in the rat by prebiotic carbohydrates. A group of rats treated with saccharose and the carcinogen MNNG showed higher DNA damage than a group of animals receiving lactulose with the carcinogen. Lactulose is fermented in the gut and butyrate could be responsible for the observed protection. In fact, colonic cells incubated with butyrate showed less DNA damage induced by hydrogen peroxide than cells incubated without butyrate. Similar effects were also observed after inducing genotoxicity with MNNG. Further studies suggested that protection by butyrate could be mediated by the release of mucus. Butyrate induces the secretion of mucus and it has been shown that mucus itself decreases the genotoxic effects of chemicals by a scavenging mechanism.

Another protective mechanism is the induction of chemopreventive enzymes against oxidative stress, notably the enzyme glutathion-S-transferase (GST), which is abundant in colonic epithelial cells and can be induced by dietary factors. Some non-digestible carbohydrates can induce GST activity in colonic cells. Interestingly, GST is also induced by the microflora. Colon cells from germ-free rats showed lower levels of GST than cells from conventional rats.

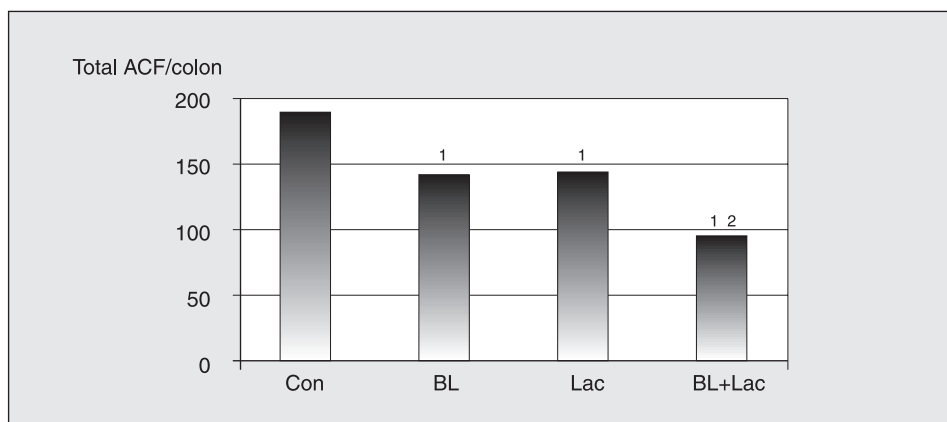
## LATE EVENTS ASSOCIATED WITH COLON CARCINOGENESIS

Early events in colon carcinogenesis are related to the initiation phase. They are molecular events where the carcinogens interact with DNA causing damage, mutations and chromosome aberrations. Late events are related to the promotion phase, and are usually recognised by morphological cell changes that can be observed under the microscope or eventually as macroscopic changes in the colonic mucosa. Protective effects of probiotics at this stage consist of the suppression of precancerous changes and the suppression of tumours in animals.

Aberrant crypt foci (ACF) are induced in the colonic mucosa of rats and mice by colon carcinogens such as azoxymethane (AOM), dimethyl-hydrazine (DMH) or imidazoquinolines (IQ), which are the carcinogens formed in fried meat. In these animals, ACF do not develop spontaneously but only after exposure to a carcinogen. Aberrant crypts are characterised by increased size and a thicker area around the crypt. Some ACF may progress to adenomas and malignant carcinomas. They are also found in human colonic mucosa, but their precise significance in humans has not been fully established.

Numerous animal studies have clearly documented the protective effect of different probiotic strains and of some prebiotics (lactulose, inulin, fructo-oligosaccharides) against the induction of ACF by exposure to carcinogens. Some studies have tested the effects of probiotics, prebiotics or both throughout the whole process of initiation and promotion of tumours. In such studies, animals are fed with the probiotic/prebiotic for about a week, dosed thereafter with the carcinogen, and then they continue the probiotic/prebiotic treatment for the remainder of the study. Other studies have concentrated on the effects on promotional events by giving the carcinogen first and then feeding the probiotic/prebiotic

2 or 3 weeks later, up to the end of the study. Induction of ACF is usually assessed after 2-4 months. In a recent study, three probiotic strains (*B. longum*, *L. acidophilus*, *L. casei*) were tested after the carcinogen (promotion phase design). There was a decrease in the number of ACF in probiotic-treated groups as compared to controls, suggesting that there had been protection by the probiotic during the early promotional phase. Another study with *B. longum* and lactulose showed that both the probiotic strain and lactulose on their own significantly decreased the number of ACF, but the combination of the two was much more effective (figure 6). Interestingly, the activity of glutathion-S-transferase in the colon was found to be significantly higher in animals treated with *B. longum*, lactulose or both, than in controls. Another study has shown that the combination of *B. longum* and inulin is extremely effective at reducing the number of ACF after exposure to AOM. Again, the protective effects were being exerted at the promotional phase, because treatments were administered after the carcinogen.



**Figure 6.** Effect of *B. longum* and lactulose on ACF induction by AOM in rats (<sup>1</sup> $p < 0.05$  vs control; <sup>2</sup> $p < 0.05$  vs BL or Lac alone) (From Challa et al.)

There have been a number of studies showing the suppression of tumours experimentally induced in animals. A very early study found a decrease in the number of colon tumours and also a corresponding decrease in bacterial enzyme activity in animals fed with a *L. acidophilus* strain. In another interesting study, rats received IQ over a period of about nine months. When compared with the incidence of tumours in rats that were given IQ alone, the rats simultaneously receiving a freeze-dried preparation of *B. longum* showed a significant decrease in liver tumours (approx. 50%) a decrease in mammary tumours, and no incidence of colon tumours. In this study, treatment with *B. longum* completely suppressed the induction of colon tumours by IQ. This is particularly interesting because IQ is found in the human diet, and is thought to be a human colon carcinogen. Lactobacillus GG was also shown to significantly reduce the incidence of colon tumours in animals exposed to DMH, when the probiotic was given during the initiation and promotion phases. Moreover, the stage of the tumours in the control group was further advanced since some had

penetrated the colonic wall, whereas none in the probiotic group had. However, no significant effect was observed when the probiotic was given only after the promotional phase.

In summary, animal studies have shown that probiotics and non-digestible carbohydrates can decrease precancerous lesions in the colon of rats. Combinations of probiotics and prebiotics seem to be highly effective. Probiotics can also suppress tumours in the colon, and indeed might suppress tumours in other tissues of rats as shown by one experiment. They may be acting at the early promotional stage of carcinogenesis.

## **MUCOSAL IMMUNOSTIMULATION BY YOGHURT IN THE PREVENTION OF COLON CANCER**

A group of mice treated with yoghurt did not develop colonic tumours after exposure to DMH. Yoghurt as well as *L. bulgaricus* and *S. thermophilus* are known to enhance immunity. The role of mucosal immunity as a mechanism in the prevention of tumourigenesis was investigated in the mouse. Treatment with yoghurt was associated with an increase of CD4<sup>+</sup> T lymphocytes in the intestinal mucosa as shown by histological studies. The effect of yoghurt was observed in normal mice and in mice exposed to DMH. By contrast, exposure to DMH without further treatment enhanced counts of CD8<sup>+</sup> T lymphocytes in intestinal tissue, but the effect of DMH was blunted by treatment with yoghurt. Interestingly, the amount of mucosal IgG-secreting cells was increased in the colon of mice exposed to the carcinogen, and again yoghurt treatment prevented an increase in the number of these cells. Moreover, the amount of IgA-secreting cells was markedly increased in colon samples from mice treated with yoghurt. Further studies also suggested that yoghurt consumption increases the number of apoptotic cells in animals exposed to DMH.

Taken together, these data suggest that yoghurt increases gut mucosal immunity in the host by increasing CD4<sup>+</sup> cells and shifts the response towards an anti-inflammatory pattern with predominance of IgA over IgG production. Yoghurt may promote mechanisms of cell apoptosis.



---

Achévé d'imprimer par Corlet, Imprimeur, S.A.  
14110 Condé-sur-Noireau (France)  
N° d'Imprimeur : 40046 - Dépôt légal : août 1999  
Imprimé en U.E.

# Yoghurt: Eighty Years of Active Research for Health

A source of life, well-being and good health, yoghurt's highly positive image has persisted down the ages. At the dawn of the third millenium, its value has been confirmed by epidemiological studies which have shown its effectiveness in preventing serious illnesses.

This dairy product, which has been produced in the same way since the beginning, is both authentic and perfectly modern in its simplicity and effectiveness.

The aim of this book is to bring together a body of scientific knowledge about yoghurt : its composition and the role of the bacteria it contains, its use in cases of milk intolerance, its curative action – particularly for treating diarrhoea and its probable role in preventing cancer of the colon.

This accessible and practical book is vital reading for anyone working in the health field – bacteriologists, public health officials and nutritionists – as well as for anyone interested in maintaining good health.



ISBN : 2-7420-0278-2

