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NUTRITION NEWSLETTER 5

N 1011 - 9027

Editorial

The principal theme in the 1996 IDF Nutrition Week was handled in a Symposium on Dairy Microorganisms as Probiotics and there were special addresses on other topical issues, marketing strategies for Europe, calcium & osteoporosis and the oxidation hypothesis of coronary heart disease, as well as the customary meetings of groups of experts.

The Programme Committee of the Symposium, led by Prof. S. Salminen (Finland) and hosts, led by Prof. Chr. Barth (Germany) are to be thanked for an event that was successful both for the knowledge imparted and for the background organization.

E. Hopkin
Secretary General of IDF

IDF's "Nutrition Newsletter" aims at periodically presenting and stimulating exchange of information in the field of human nutrition. The articles provided are derived primarily from the IDF Groups of Experts concerned with nutrition, or constitute the account of special IDF events in the field, such as the Nutrition Week.

The newsletter is issued in English and is sent to the National Committees of IDF, to subscribers of the IDF Bulletin, to IDF groups of experts and other interested parties.

The International Dairy Federation (IDF) is a non-profit, non-governmental organization, created in 1903 in order "to promote, through international co-operation, the solution of scientific, technical and economic problems in the dairy field". Thirty-three countries, in all parts of the world, are members of the IDF.

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ASPECTS OF NUTRITION

FOOD MARKETING STRATEGIES FOR THE CONCERNED CONSUMER IN EUROPE

A. Nienhaus
General Manager of CMA, Centrale Marketing, Gesellschaft der deutschen Agrarwirtschaft mbH, Bonn, Germany

The title given to me for my paper quite rightly focuses attention on the consumer, for he or she is the most important person, particularly in the food sector. By buying certain products, consumers set new priorities almost every day and force producers, processors and retailers to follow their course. Food marketing seeks to discover their preferences and cares, to influence their behaviour through Public Relations and advertising and to win their acceptance of new products and packagings.

DO WE HAVE ENOUGH FOOD FOR ALL ?

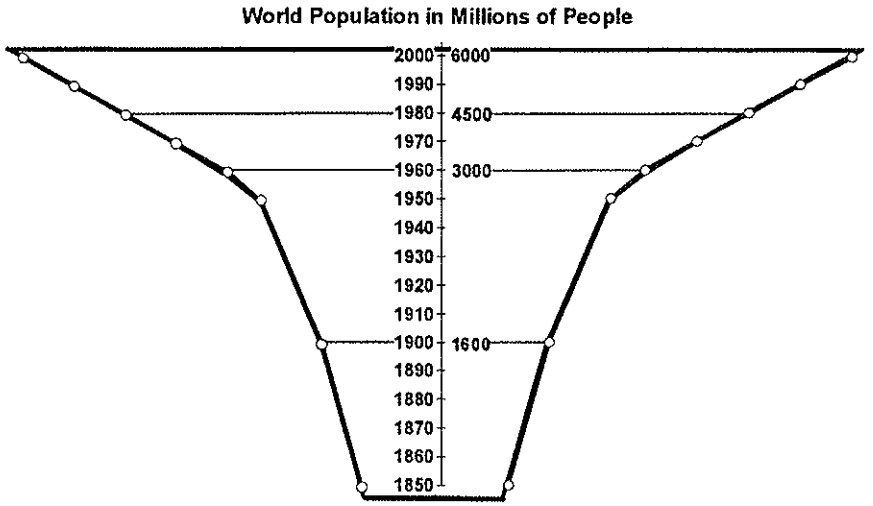
Modern media and means of transport have made the world shrink. The explosive growth in world population makes people anxious and makes us ask ourselves whether there will still be enough for us all to eat in future and whether we will still be able to control the ecological problems.

The population will grow most strongly in the so-called developing nations, particularly in Asia and Africa, and not in the industrialized countries. China and India are two countries in which the per capita production of food has been successfully increased and largely solved the problem of hunger.

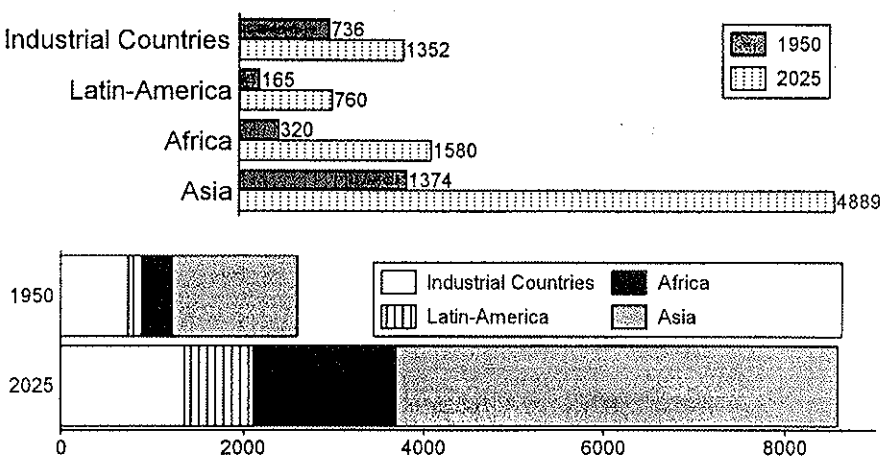
Boosting the production of vegetable and animal food is based on the use of modern breeding methods and fertilizers in intensive farming. In this way, it helps to increase our consumption of energy and the concentration of carbon dioxide in the atmosphere, two factors which have a tangible effect on the world climate.

European farmers are helping to reduce the concentration of carbon dioxide by growing regenerative raw materials. What is far more important, however, is to reduce the per capita consumption of energy in the highly developed western industrial nations in order to restore a worldwide balance.

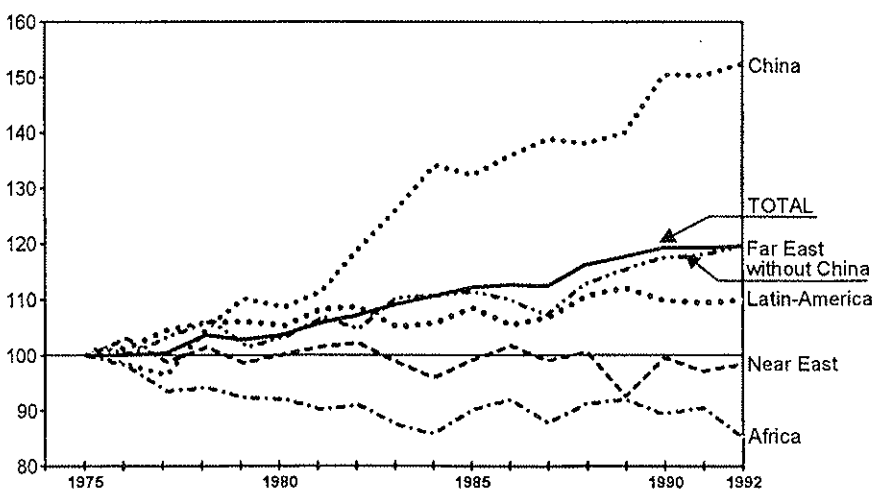
Development of the World Population During the Last 150 Years



Portions of the World Population According to Regions 1950–2025 in Millions of People



Production of Food Per Capita (1975 = 100)



FAO, Agriculture

CONSUMERS ARE MORE ECOLOGICALLY AWARE

According to relevant surveys, around 60% of German consumers claim to act in accordance with ecological needs. The core group of consumers who actually take ecological aspects into account when buying food, for instance by buying products with ecologically compatible packagings or which are produced locally, accounts for around 30% of the total in Germany.

The proportion of ecologically aware consumers has declined for several years in all countries of Europe as political parties and the food industry have paid more attention to consumers' cares and wishes. The German-speaking part of Switzerland is a good example of just how strongly language, the media and habits can influence our ecological awareness : this area has the largest proportion of ecologically aware consumers (34%) as compared with only 13% in French-speaking Switzerland. This is the second-smallest figure, the lowest being accounted for in the new German Federal states where fundamental problems worry the people, particularly job-finding.

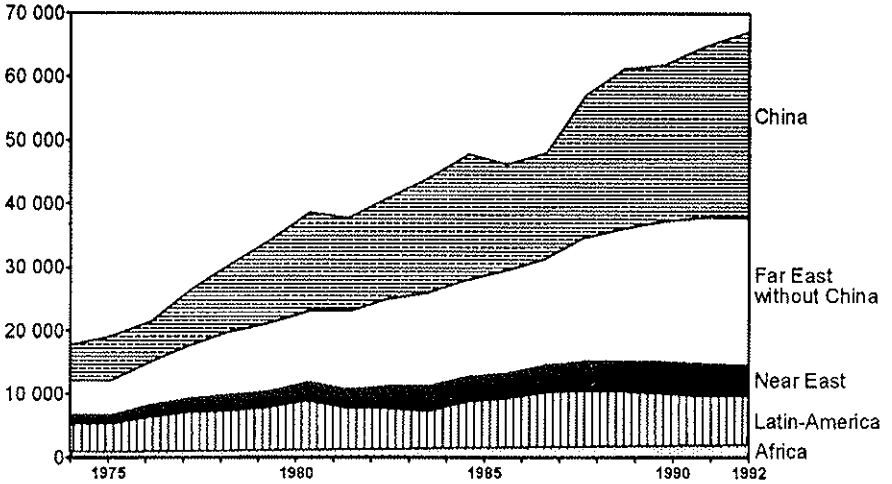
The agricultural and food industry can use consumers' concerns about preserving a pleasant environment, for example by producing organic food or integrated grown food. Representative surveys show that the highest marks are accorded to such products. Unfortunately, however, 50% of German consumers are not prepared to pay more for such products, despite the greater input required. And the other half too are not prepared to pay more than about 15% or 20% extra. This is a hurdle which is not easily overcome.

NATURAL AND FRESH LOCAL PRODUCE

People have never been more uncertain than they are today; 53% of German consumers believe that food can cause ill health. Their fears as to whether our food is really healthy will increase now that the possibility of Bovine Spongiform Encephalopathy (BSE) being transmitted to man can no longer be ruled out entirely.

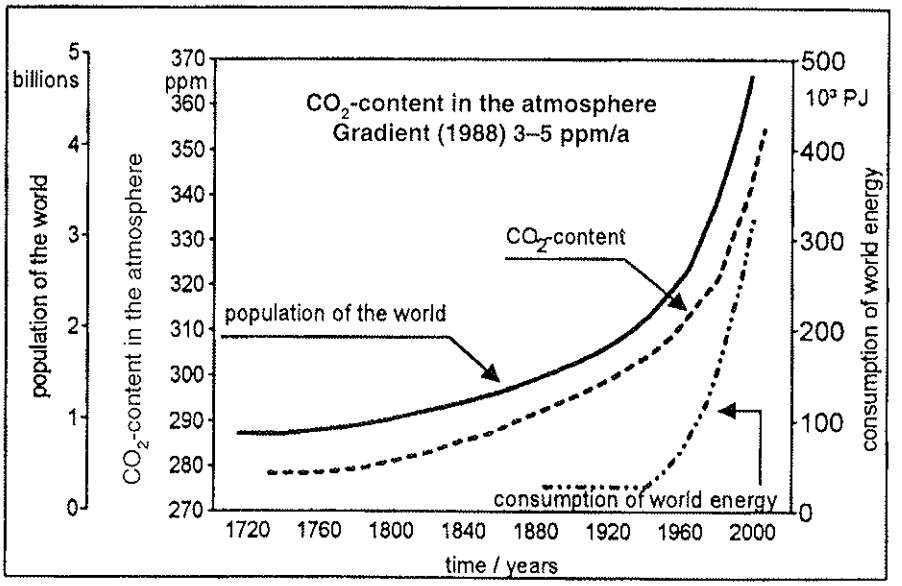
The origin of our food is more important today than ever before; 95% of German consumers now want to know where their fresh meat was produced. If the producer were known personally, 15% of consumers would buy more poultry and around 11% more eggs, fresh meat and vegetables. In a European market without borders, concerned consumers are turning back to their local region and preferably to the

Application of Fertilizer in Developing Countries 1975–1992 (total pure nutrient in 1000 ts)



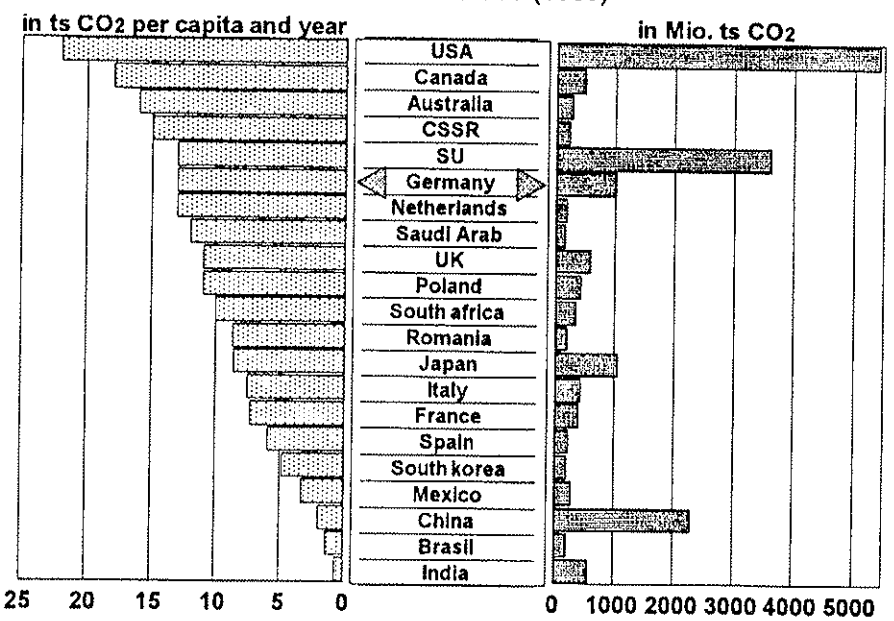
FAO, Agriculture

Population, Consumption of Energy, CO_2-Content



Dr-Ing. M N Fisch

Energy Conditioned CO_2-Output of the World in absolute values (1989)



producers themselves, thus rapidly boosting the importance of direct food sales.

Food retailers must also take consumers' wishes into account and include local produce in their ranges. In this way, they can simultaneously display their ecological awareness and cut transport costs. However, this trend towards local produce is limited by what are frequently excessively small production volumes which simply do not fit into the national or even European distribution networks. A mixture of around 70% products traded on a national or European scale and a special range of around 30% local produce would consequently seem ideal.

ETHICAL STANDARDS ARE RISING

The domination established by Genesis no longer corresponds to the growing ethical sensitivity of our modern consumers. Their feelings are better reflected by Genesis 2,15 "The Lord God took the man and put him in the garden of Eden to till it and keep it" than by Genesis 1,28 "Be fruitful and multiply, and fill the earth and subdue it".

Our duty to protect the animals is stronger than our duty to protect the plants which surround us. The domestication of farm animals has greatly improved the reliability of our food supply, but it has also made us responsible for the animals. Animals are increasingly becoming the companions of lonely consumers for whom slaughter is taboo and who question our right to eat meat. It is consequently not surprising to find that the per capita consumption of meat has declined by around 10% since 1988.

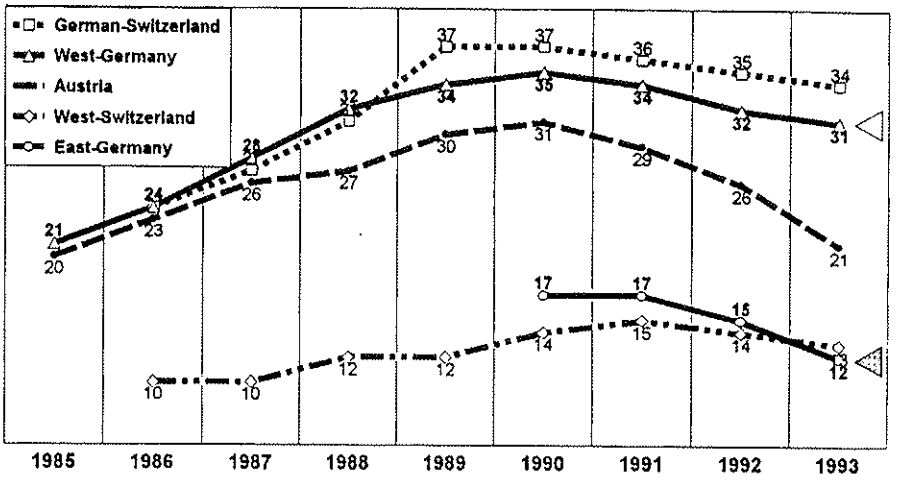
The great advantage of milk and dairy products is that they are not associated with slaughter in any way. While meat consumption in Germany drops from one year to the next, the consumption of milk and dairy products is growing steadily, particularly where the numerous cheese specialities are concerned.

The hitherto undisputedly healthy image of milk and dairy products could be jeopardized by the official approval of Bovine Somatotropin (BST). German and European consumers are more willing to accept a performance-boosting additive in animal feedstuffs than a weekly or monthly injection of BST which they generally believe would overtax the dairy cows' natural production capacity and which would not be in keeping with our responsibility for the animals.

The dramatic moment of the situation is evident. Although the world has

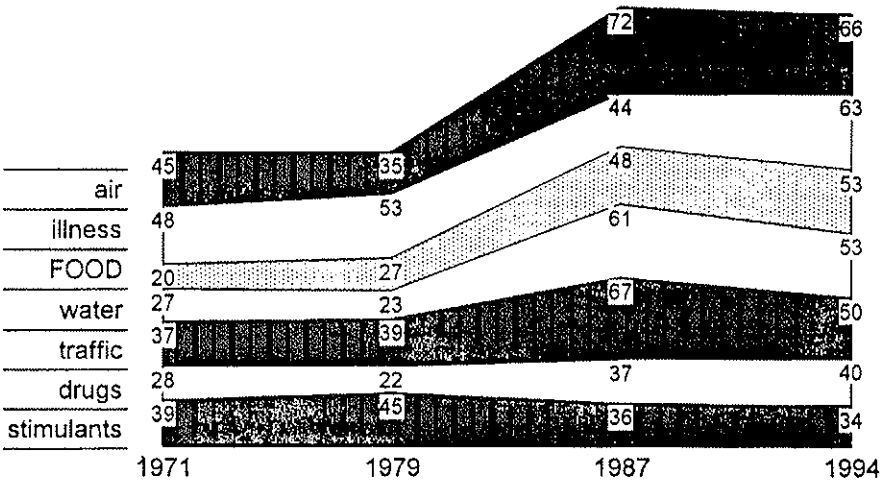
Environmental Awareness in Country Comparison

Quota of the extremely environment-conscious (core group) of all households in %



G&J; GfK Handelsforschung GfK Euro-Scan

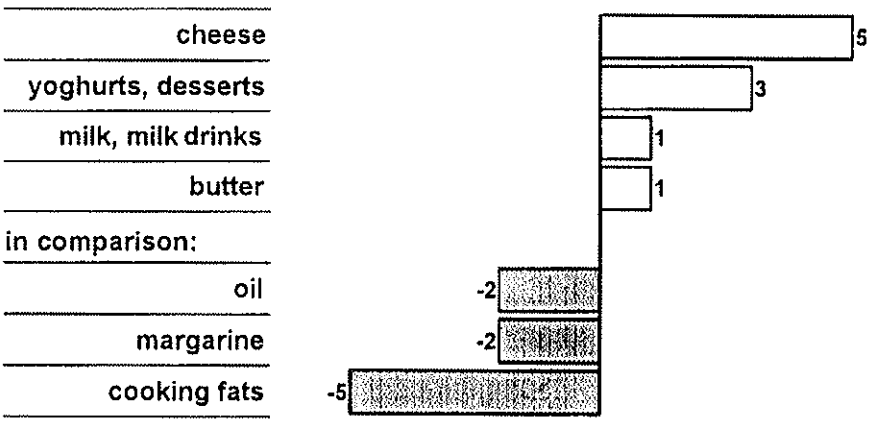
Anxieties of Health Risks



ifak, n=2000 resp. 1000 7 items (Splitz)

Private Demand for Dairy Products purchasing quantity, growth rate in %

1995 versus 1994



GfK, 7000er HH-Panel

become very small and is interconnected by free worldwide trade, the consumers' opinions prevailing in the USA, for example, are completely different from those in Europe and particularly in Germany. Can free world trade really take absolute priority over the consumers' desire for natural milk products and their efforts to influence the future of the food industry's largest branch through their buying habits ?

Consumers could naturally be allowed to decide for themselves if a declaration were specified by law or made voluntarily. In all cases, however, this would inevitably lead to discrimination of the product range, with the result that the untarnished natural image of milk would become a matter for public debate and threatened. It is an important basic marketing principle that even mistaken consumer opinions must be taken seriously as long as they exist.

SCIENTISTS POINT THE WAY

At a time characterized by daily new findings on the composition, pollution and recommended intake of foods, the importance of scientific statements becomes almost inestimable and is indeed not always fully appreciated by the scientists themselves. Consumers have no alternative but to trust the scientific statement because they can only acquire the necessary knowledge in exceptional cases to judge the food and also the ingredients and its importance in our daily diet.

Consumers are deeply shaken by contradictory statements on an existing risk and the possibilities of containing it, as has been highlighted by the British case of Bovine Spongiform Encephalopathy (BSE). They are not prepared to engage in Russian roulette when enjoying their German beef roulade (a type of beef olive).

The food industry must also rely on the scientists' advice, particularly in the case of newly composed foods. This can mean that a traditional natural product, such as butter, the composition of which can hardly be changed, must compete against a new industrial product, namely margarine, which is highly adaptable depending on the components and processing methods selected.

CMA must defend the traditional natural products, from the unsaturated oil in rapeseed to butter with its relatively large proportion of saturated fats which are believed to increase cholesterol levels. CMA has entered the debate with a publication entitled "Cholesterol – the other opinion" in order to keep the door open for new scientific findings and prevent the discrimination of butter as a natural product. The image of butter as the "healthiest fat" has also risen from 33 to 47 percentage points in the course of only 5 years.

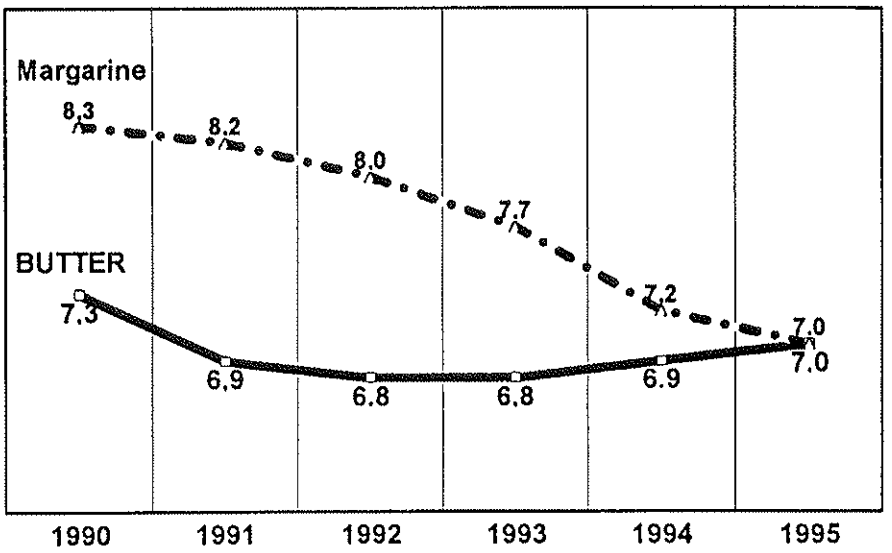
Success is on the butter side, at least in Germany, where butter consumption has stabilized while consumption of margarine is declining.

The so-called spreads which are made up of vegetable oil and some butter fat are another example of a new product line. In some European Union countries, such as rural Ireland, they now account for over 40% of the market. In Germany, on the other hand, they have effectively been withdrawn from the market by the trade due to the lack of consumer acceptance for these products following the opposition of the dairy industry and CMA.

Successfully – from CMA's point of view – influencing consumer opinion in this way requires a strong marketing institution which must be able to strengthen its own statements with the advertising lines of the food processing industry. This has been admirably demonstrated in Germany. The German food industry bears around 15% of the total cost of around DM 35 billion for advertising and sales promotion.

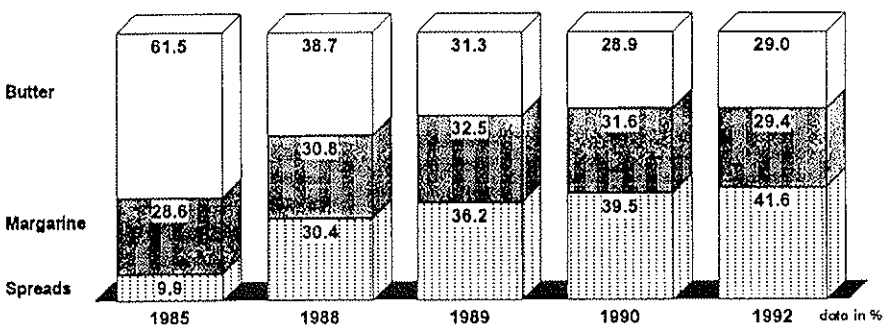
As the German example has shown, the consumers' appreciation of food can be effectively improved if product safety, quality and advertising go hand-in-hand. However, consumers can also be extremely choosy, as shown by the different image of vegetables or German cheese, for example.

Per Capita Consumption of Butter and Margarine in kg



ZMP, Eurosat + own calculation

Spreadable Fat Consumption in IRELAND



	– in 1000 ts –				
Butter	31.0	19.1	15.1	13.8	13.7
Margarine	14.4	15.2	15.7	15.1	13.9
Spreads	5.0	15.0	17.5	18.9	19.7
Total	50.4	49.3	48.3	47.8	47.3

EG-Kommission 1991, Wasserfuhr 1991 Richarts & Braun 1991, Richarts 1994

POLARIZATION IN THE FOOD TRADE

The exceedingly strong competition prevailing in the German and European retail trade has led to more concentrated structures. Events in Europe today are now dictated by only a small number of strong retail chains which give around 5000 of the 50 000 available specialities a chance of being listed and thus appearing on the shelves and then in shoppers' baskets.

The highly concentrated food trade is experiencing a polarization of consumer wishes and markets.

Around 37% of the foods traded make up the premium segment of high-quality premium products, while another 37% are found in the discount line which bring customers into their shops with low prices in particular. The good old medium quality is losing ground from year to year, so that every producer is forced to choose either the premium segment or the discount segment in order to survive.

The discount line is also benefiting from the declining average income of European consumers. This is surprising, considering that food only accounts for between 15 and 20% of household expenditure in Germany and many other member countries of the European Union. Rents, transport and leisure activities head the list of expenditures.

The agricultural industry itself has ultimately helped to make our food less expensive. In 1950, one farmer only grew enough food to feed 10 consumers : by the year 2000, that figure will have risen to almost 100. The GATT negotiations have also helped to make food less expensive by aiming to bring European farm prices into line with world prices.

Compensatory payments to farmers are intended to make up at least part of the decline in prices, but this makes the European farmers more and more dependent on national and European funds which are classified and misunderstood as new subsidies by the general public.

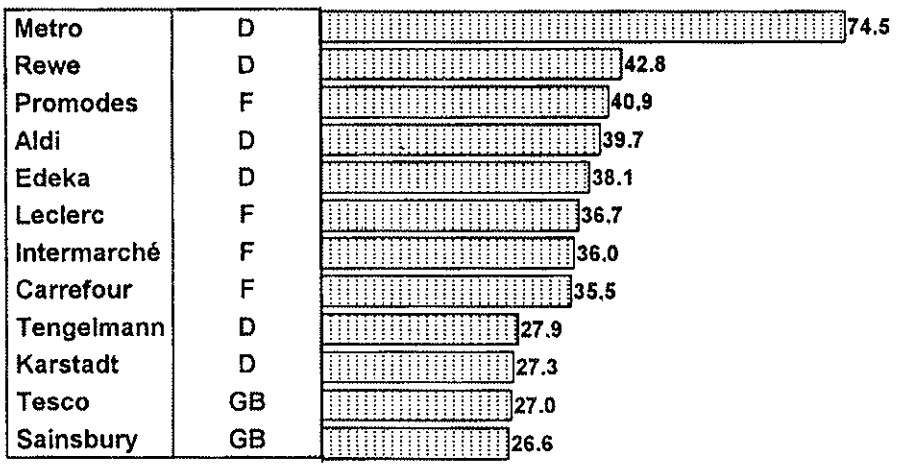
FUTURE MEGATRENDS

Three megatrends will shape the food market between now and the turn of the century.

The food industry will also be increasingly characterized by the growing trend to individualize all areas of life. By the end of the century, 65% of consumers will live in single households with their own individual lifestyle, guided by their own justified or unjustified cares and spontaneous decisions. Only those

Food Trade in Europe

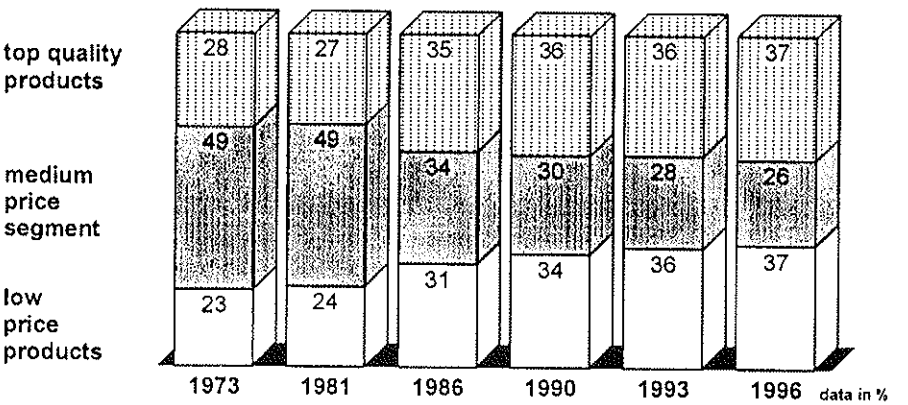
The 12 biggest companies/turnover in billions of DM 1994



M + M Eurodata, Frankfurt

Polarization of Markets

Range of items: 44 products (food and non food)

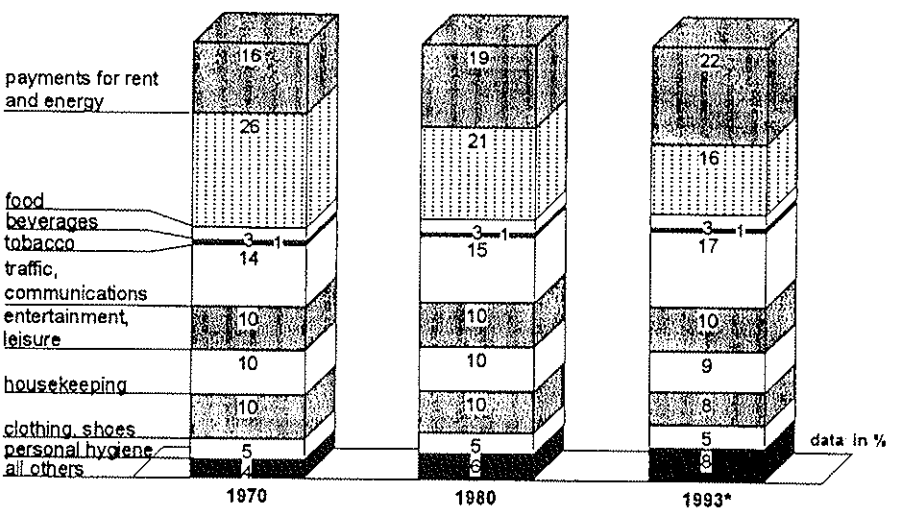


GfK and own calculations

Private Expenditure of Households

- range of application -

1970 – 1993



*non final result

Statistisches Bundesamt

who can offer competent market research and the required products with which to respond quickly to these consumers' changing emotions and demands will also be able to take advantage of their high purchasing power.

The safety of our food will become increasingly important. Nothing is of more interest to the media than nutrition, for food is something we have to buy every day and every real or assumed food scandal boosts the circulation or viewing figures. "Transparent food production" with continuous control throughout the entire process will increasingly come to replace inspection and control of the finished product. Quality assurance throughout the entire process avoids all possible errors from the outset and guarantees that certain consumer wishes are met, such as the demands for ecological or integrated crop farming or natural animal farming.

Considerations based purely on weighing up costs and benefits, as in the past, will increasingly be superseded by a more ecological way of thinking in continuous production cycles. The importance of preserving the fertility of the soil and an attractive, natural landscape is growing in exactly the same way as protecting animal rights and animal welfare.

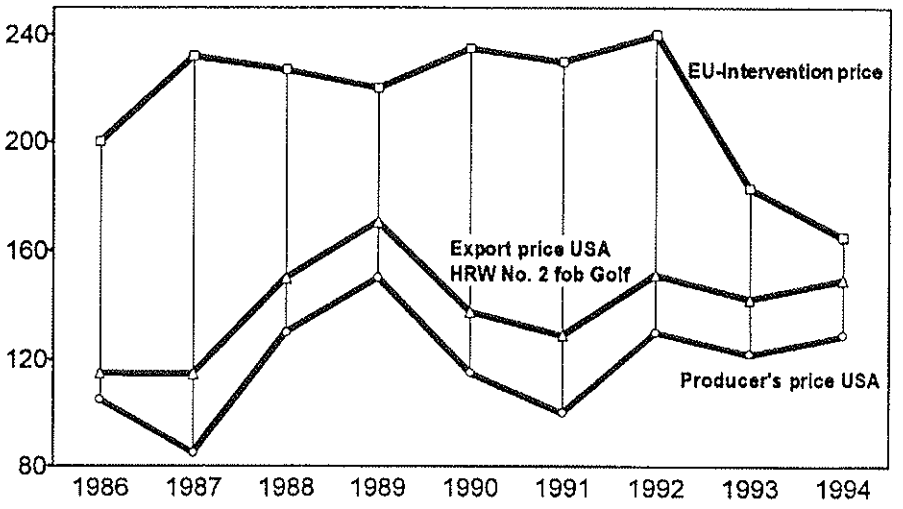
It will be the basic element for consumers' preference for meat and meat products, particularly among younger consumers who currently account for no more than 70% of the average consumption. So-called "eco-products" or organic products, which now account for only 5% of the food basket, will be able to expand, but a good marketing concept will be required in order to help them establish preferences by emphasizing their own advantages without discriminating the large range of standard products.

RECOMMENDATIONS FOR A FUTURE FOOD MARKETING STRATEGY

- Consumers are self-assured adults, but they are also both uncertain and worried. Their secret desires and often irrational cares must be investigated and taken into account in the marketing.
- The origin of food and its certification are two factors which have become unexpectedly important in our modern Europe without borders. They can help to stop consumers' fears and are an effective means of differentiating products, also as a counterpart to anonymous mass-produced articles.

Market Price for Wheat in EU and in USA

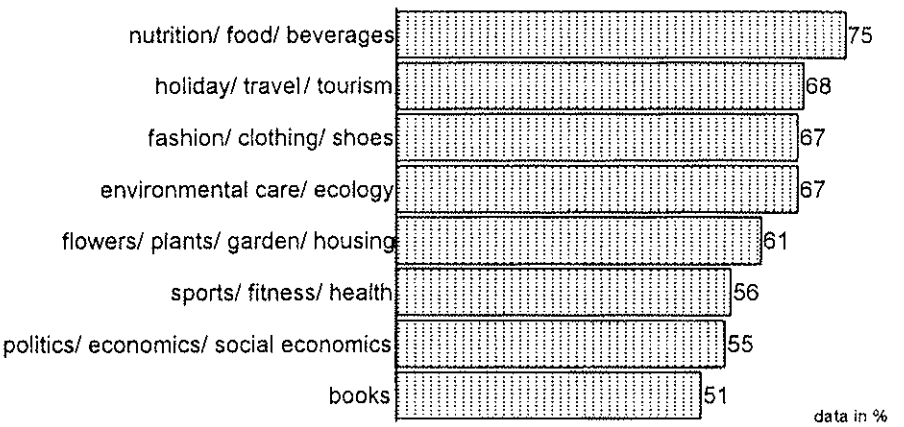
(US-Dollar per t)



Stat. Bundesamt/ZMP
USDA - EU-Commission

Which Topics are attractive ?

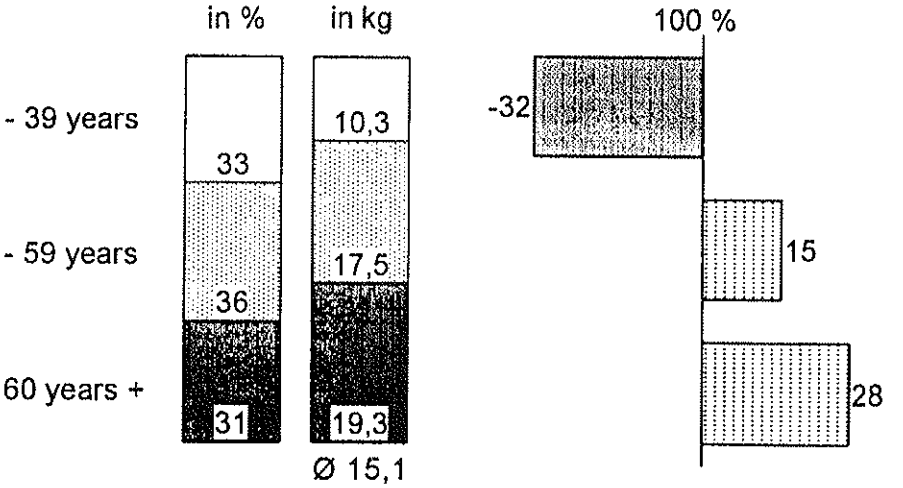
"very interesting : interesting



Regional press 1991
all interviewees

Fresh meat

Per capita consumption in German households acc. to age groups



GfK - HH Panel 1993
n=5000

- Quality assurance will also become more important and more stringent. Inspection and control of the end product with an up- or down-grading of the products will increasingly be replaced by quality assurance throughout the entire production process in a form of "transparent production". It will prevent a deterioration in quality from the outset and can meet buyers' and consumers' precise requirements.
- Scientists must help in drawing up quality programs and documenting these in certified systems, as well as in assessing the possibilities for genetic engineering which many consumers consider to be nothing more than manipulation. More and more often, consumer acceptance is outstripped by technical progress. Balanced decisions and advanced information based on scientific findings are therefore essential.
- Natural products will also be able to defend their traditional terrain against the technically possible new compositions. Man's ability to adapt to the food available, which has developed over the millennia, is increasingly being seen as a form of protection against allergies and nutritional mistakes. It is naturally important to reduce the amount of food available in line with the declining demand.
- Although scientific differences of opinion and debates between so-called scientific schools may encourage new research, they do not help to reduce the fears of concerned consumers. For this reason, we need a supreme scientific organ to discuss different theories and then to put forward and publicize the valid scientific finding in each case.
- The official Food Report published in Germany every 4 years could be taken as an example here. It provides a sound basis for consumers and advisory organizations, as well as for food marketing. In addition, it also presents the scientific advances which are particularly immense in the field of nutritional physiology, as this is a relatively new field of research. The vast number of questions and problems that remain to be solved is demonstrated by the large scope of this "German Institute for Nutrition" to which we have been invited.

CALCIUM IN NUTRITION

NUTRITIONAL POSSIBILITIES TO PREVENT OSTEOPOROSIS

G. Schaafsma

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It is now well recognized that in western counties the frequency of primary osteoporosis types I and II is increasing at a faster rate than could be expected merely on the basis of ageing of the population. Ageing can only explain about 40% of the increase in osteoporosis frequency. In the Netherlands the annual incidence of hip fractures increased from 4590 in 1972 to 11 820 in 1990, and it is expected that in the year 2010 the total costs related to osteoporosis will be more than 1000 million Dutch guilders.

A low bone mass is a dominant risk factor for osteoporosis development. According to the WHO definition, osteoporosis is present if the T-score of bone mass is lower than 2.5 times the standard deviation, whether or not fractures are present. The T-score is defined as the deviation (expressed in standard deviations) from the average Peak Bone Mass (PBM). Prevention of osteoporosis should aim at optimizing conditions for PBM formation and decreasing the rate of bone loss after the age of 40–50 years.

Several variables are known to influence the bone mass. The main variables are genetic background, hormonal changes (for example menopause), physical activity, and nutrition. It is likely that the increase in age-adjusted osteoporosis incidence is caused, at least in part, by decreased physical activity, associated with western life styles. However, there are no quantitative data to support this relationship. It is believed that sufficient axial burdening (weight bearing activity) of the skeleton is a prerequisite for maintenance of bone integrity. Nutritional measures will not have much effect in the absence of adequate physical activity.

With respect to dietary measures aimed at osteoporosis prevention, the most important dietary variables are calcium, vitamin D, sodium, alcohol and caffeine.

CALCIUM

Calcium intake should meet current recommended intake levels at all ages. There is no evidence that intake levels in excess of these recommendations have any long-term beneficial effects on the bone mass. The best natural sources of dietary calcium are dairy products. If no dairy products are consumed at all, calcium supplements are required.

Preliminary results of the Dutch part of the multi-center EU-sponsored CALEUR study on the effect on bone of calcium intake levels in young girls and young adult women, a study which is coordinated by TNO, indicate a positive effect of calcium on bone, in spite of the fact that mean calcium intake levels in the Netherlands compare favourably with recommended intakes.

In addition to calcium intake, calcium bioavailability is a relevant issue [1]. A recent investigation on the bone mass of Dutch adolescents fed a vegan-type diet [2] showed a lower bone mass in these children which could not be related to calcium intake levels. One explanation could be the lower bioavailability of calcium from vegan diets, being rich in phytate and fibre. Calcium supplements should be taken with the meal in order to improve absorption. Among the different calcium salts that can be used as a supplement, those with the higher solubility characteristics, for example calcium citrate and calcium citrate-malate, show a slightly better absorption than calcium carbonate or calcium phosphate. The practical importance of this difference, however, is probably small.

VITAMIN D

Adequate blood concentrations of 25-hydroxycholecalciferol throughout life are required to optimize the active intestinal calcium transport system. Vitamin D supplementation may be necessary in elderly people who are housebound and do not have sufficient exposure to the sun. It should be taken into account also that at an older age the sensitivity of the skin to synthesize vitamin D is lower than that at a younger age. Generally, it can be said that oral amounts of 400 IU of vitamin D are required daily for those categories of the population at risk of developing vitamin D deficiency (young children, pregnant women, elderly and sick people). Since adequate intake levels of vitamin D by these categories of the population cannot be obtained from natural sources, there are interesting opportunities for the dairy industry to fortify specif-

ic dairy products with vitamin D. Unfortunately this is not allowed in all European countries, because of the risk of vitamin D overuse. For instance, fortification of foods with vitamin D is not authorized in the Netherlands and people there have to depend on pills for vitamin D supplement.

SODIUM, CAFFEINE AND ALCOHOL

Sodium, caffeine and alcohol may increase the urinary excretion of calcium and therefore increase calcium requirements. Moderation of sodium intake and limitation of caffeine and alcohol intake are warranted in view of osteoporosis prevention. However, data on long-term effects of high intake levels of sodium, caffeine and alcohol on bone are not available.

OTHER NUTRITIONAL FACTORS

A high protein intake has long been considered as a risk factor for osteoporosis because of its calciuric action, but since natural sources of protein are also rich in phosphorus, the calciuric action of protein is almost entirely balanced by the antagonistic action of phosphorus on urinary calcium excretion. There is no evidence for adverse long-term effects of protein on bone mass. On the contrary, the possibility of a positive IGF-1 mediated anabolic role of supplementing the diet of the elderly with high quality proteins justifies further research. Fluoride has a positive effect on bone mass. A good natural source of fluoride is tea. There is no evidence for long-term beneficial effects on bone of increasing the magnesium content of the diet to levels in excess of current recommended intake levels. Borium has been speculated to influence calcium and bone metabolism via effects on the hormonal status. The significance of borium supplementation for bone, however, is uncertain and no recommendations can be made. The same statement is valid for vitamin K. There is no evidence of beneficial effects on bone of vitamin K supplementation, in spite of the defined role of this vitamin in gamma-carboxylation of bone proteins.

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THE POSITION OF DAIRY PRODUCTS IN THE PREVENTION OF OSTEOPOROSIS

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Osteoporosis has been recognized as a bone disease of multifactorial genesis with increasing relevance also for developing countries where profound changes or a "westernization" of life style factors occur.

In contrast to genetic and biological factors, diet as one life style factor can be modified individually or by public health activity. The debate on calcium (Ca) intake in the prevention of osteoporosis has continued over the years, although a lot of new data are published every year. Obviously new results hardly contribute to the solution of the questions of whether and how Ca is a potent factor in preventing or postponing loss of bone mass, density and function. The so-called "evangelists" underline the necessity of life-long adequate Ca intake, "adequate" often being regarded as higher than the RDAs. The "nihilists" deny the scientific basis for emphasizing the importance of Ca (Kanis, J.A., Eur. J. Clin. Nutr. 48: 757–767 (1994)). Interestingly, both groups rely on the same literature to underline their statements. It becomes very obvious that the author's point of view affects his interpretation of results more than vice versa.

In the past, emphasis was put on investigations on Ca and osteoporotic or postmenopausal women. Data lined out that although Ca cannot rebuild bone that has already been lost, obligatory age-related bone loss can be postponed. Furthermore, estrogen, physical or vitamin D therapy in osteoporotic women are effective only in the presence of sufficient amounts of Ca. There is agreement on the importance of Ca in tertiary prevention, especially in combination therapy.

In postmenopausal healthy women, dietary Ca has not consistently been found as a predictor for bone mineral content or density. However, most of these findings are based on cross-sectional studies, which have their limitations and provide the weakest body of evidence. A rise in the number of participants cannot compensate for the general problems of inaccuracy in assessing Ca intake by questionnaires. Recent intervention studies have underlined the potential of dietary Ca in postponing bone loss, an effect being more obvious in total body bone and femoral density than in the metacarpal cortical area. In lumbar spine, Ca supplementation was more effective in early perimenopausal and late postmenopausal than in early postmenopausal women. Ca supplementation was significantly efficient when added to habitually low and high Ca intakes, although the effect was more pronounced in the former.

Recently, emphasis has been put on studies investigating Ca in primary prevention, that is, effects on peak bone mass development. For children and adolescents Ca supplements were effective in improving an increase in bone density more than bone content. This was true even if habitual Ca intake was equivalent to the RDA. A predominant effect on defined skeletal sites was not consistent.

The relevance of dairy products with respect to prevention of osteoporosis is closely related to the effect of Ca supplied in the form of highly soluble salts since there is no pronounced difference in absorbability. Intervention suites with dairy products and their influence on bone density support the beneficial effect of dietary Ca on peak bone mass development.

CORONARY HEART DISEASE

THE OXIDATION HYPOTHESIS OF CORONARY HEART DISEASE (CHD)

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SUMMARY

Coronary heart disease (CHD) is thought to begin with an injury to the lining of a coronary artery. Around the injury, an atherosclerotic plaque develops, narrowing the artery. Later a thrombus forms, starving the heart of blood and oxygen and resulting in a heart attack. The dominant theory of how CHD develops has been the idea that diets rich in saturated fatty acids result in an excessively high concentration of cholesterol in the blood. High blood cholesterol in the form of low density lipoproteins (LDL) was regarded as the main factor contributing to atherosclerosis.

The oxidation hypothesis is a further development. Polyunsaturated fatty acids, which are key components of biological membranes, are susceptible to damage by oxygen free radicals – highly reactive products of oxygen metabolism. The resulting oxidized lipids are themselves free radicals and cause damage to proteins, one of which is the apoprotein of LDL, the main carrier of cholesterol in human blood. The 'modified' LDL protein is no longer recognized by the LDL receptors on cell surfaces which regulate LDL metabolism. Instead, the modified LDL are engulfed by macrophages and these become the cholesterolrich 'foam cells' characteristic of atherosclerotic plaques.

Damage caused by lipid peroxidation, and therefore the resulting pathological change, is normally prevented or minimized by adequate defence against oxidation. This comprises a range of antioxidants in tissues, some of which are enzymes and some of which are the nutrients, vitamin E, vitamin C and carotenoids. High density lipoproteins (HDL) may also act as antioxidants.

Epidemiological evidence that CHD is associated with oxidant stress resulting from poor antioxidant defence is now substantial and has been reinforced by several intervention studies in which dietary antioxidant supplements were given. However, it still remains to be proved conclusively that lipid peroxidation in the living body is a major cause of the development of atherosclerosis and CHD.

A major consequence of this hypothesis has been an increasing emphasis on dietary antioxidant intake, provided mainly by fruit and vegetables.

BACKGROUND

There is uncertainty about the initial cause of coronary heart disease (CHD) and indeed whether there is a single 'cause' or many. It is generally accepted that the disease is initiated by an injury to the wall of an artery. Once the wall has been injured, repair mechanisms are brought into play but during the repair process there are opportunities for many different factors (some of which may be dietary or diet dependent) to influence the course of the disease. This is why the disease is frequently referred to as 'multifactorial' and why it is impossible to say that there can be a single hypothesis for CHD. The antioxidant hypothesis should not, therefore, be viewed as an explanation for the disease that is totally independent of all other hypotheses. Rather, is it an attempt to explain certain aspects of the disease that were hitherto puzzling and it should be viewed as being complementary to many other ideas about the natural history of CHD.

Nevertheless, one hypothesis has tended to dominate all others and that is the so-called 'lipid' or 'cholesterol' hypothesis in which it is postulated that an elevated concentration of cholesterol in the blood either causes or exacerbates the chronic degenerative condition of atherosclerosis. At a certain stage, the atherosclerotic plaque may rupture, giving rise to thrombi (blood clots) that may block the artery. If this 'thrombosis' occurs in a major artery supplying the heart muscle, then a fatal or non-fatal heart attack may occur.

This is a much simplified scenario. Over the years, the hypothesis has been refined. Thus, once the structure and metabolism of lipoproteins and their receptors was understood, it was stated that LDL was really the 'atherogenic particle' and later that both LDL and HDL played important roles, the former as an active agent in the disease process, the other offering 'protection' by transporting cholesterol away from the site of the arterial lesion. Despite these refinements, certain observations did not fit the theory. For example, LDL did not seem to be taken up readily into lipid-filled cells that are characteristic of atherosclerotic plaques but LDL that had been chemically changed by oxidation were taken up. Furthermore, epidemiological evidence began to show that concentrations of antioxidants in the blood were more strongly associated with coronary heart disease mortality than the concentration of cholesterol. These findings led to the elaboration of the concept that coronary heart disease had something to do with the susceptibility of lipids and proteins in the body to oxidation and that these adverse effects could be counteracted by improving the body's defence against oxidation. This is the subject of this review.

FREE RADICALS AND LIPID PEROXIDATION IN THE BODY

Oxygen is essential to life, but its use in biology poses certain problems. During oxygen metabolism, forms of the element and its compounds are produced (Figure 1) that are much more reactive than O_2 ('triplet oxygen'). With the exception of singlet oxygen and hydrogen peroxide, these are normally free radicals and have one or more unpaired electrons (Table 1). Collectively they are termed "reactive oxygen species" (ROS) [1].

Table 1: Principal reactive oxygen species (ROS) produced in the body

Non-radicals:	Hydrogen peroxide	H_2O_2
	Singlet oxygen	$^1O_2$
Free radicals:	Superoxide	$O_2^{\cdot-}$
	Hydroxyl	$OH^{\cdot}$
	Lipid peroxyl	$ROO^{\cdot}$
	Lipid alkoxyl	$RO^{\cdot}$

ROS are produced in the body in diverse metabolic pathways (Figure 1). These include the normal oxidative metabolism of absorbed food components in the mitochondria and peroxisomes, and the metabolism of drugs and environmental contaminants in the endoplasmic reticular membranes of cells by oxidase enzymes involving cytochrome P_{450}. Bacterial killing by macrophages is also reliant on the production of ROS underlining the importance of controlled free radical reactions to the body's defence. However, cigarette smoke, abnormal oxygenation and radiation also contribute to the ROS flux.

The enzymic conversion of polyunsaturated fatty acids into eicosanoids

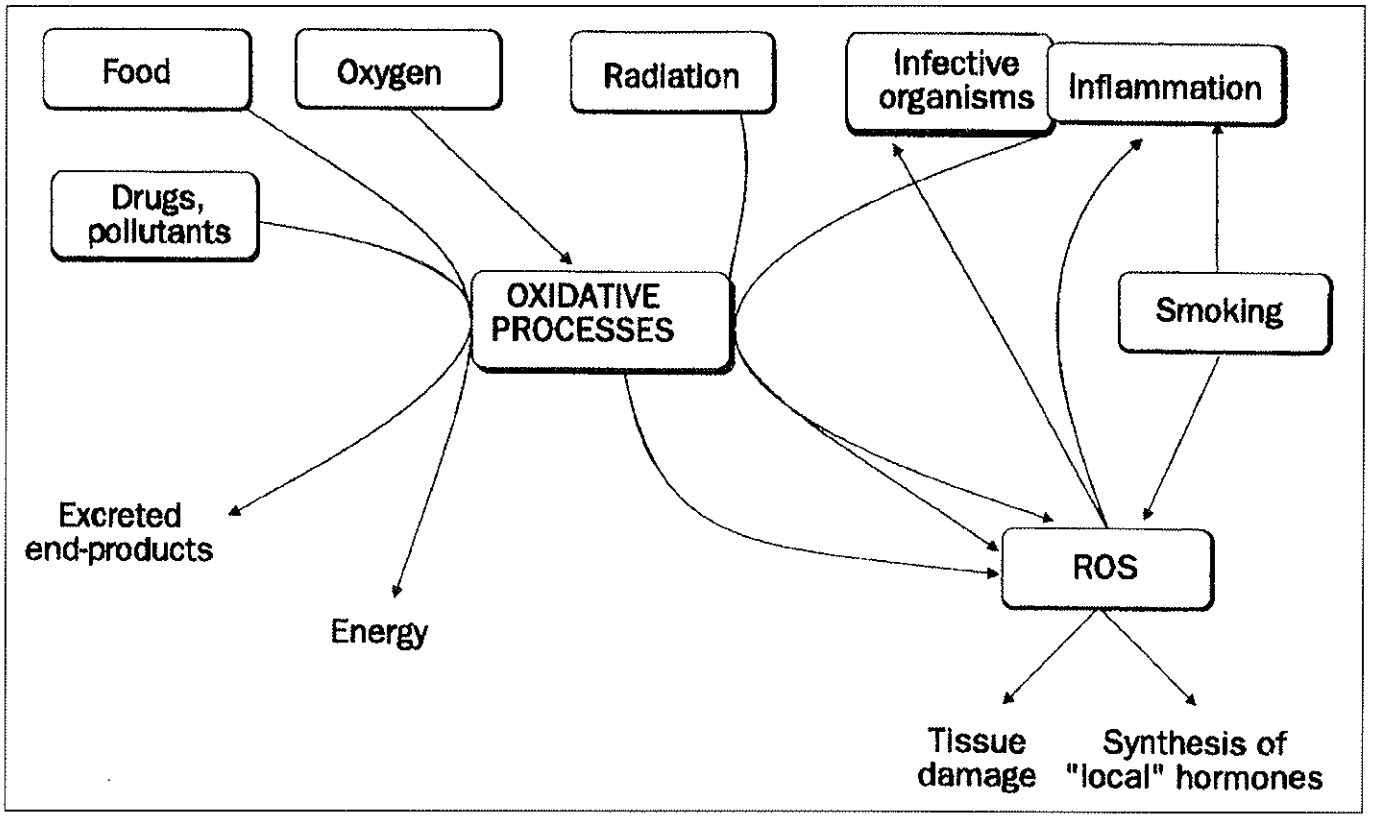

Figure 1: Production of reactive oxygen species (ROS) in the body.

and leukotrienes involves free radical mechanisms that generate lipid peroxide intermediates [2]. Lipid peroxidation is *initiated* when a free radical abstracts a hydrogen atom (which itself is a free radical) from the hydrocarbon chain of a fatty acid, or the ring system of steroids to produce a lipid free radical (Figure 2). Many ROS can initiate lipid peroxidation but it is thought that the most important initiating species in the human body are the highly reactive $OH^{\cdot}$ (hydroxyl) radical and singlet oxygen [1]. Hydroxyl radicals are formed by irradiation of water or by the scission of hydrogen peroxide (which is a product of several metabolic pathways) catalysed by iron or copper ions (Figure 2). Singlet oxygen is formed when certain photosensitizing compounds absorb light in the presence of oxygen. Such compounds include the naturally occurring porphyrins, bilirubin and riboflavin, as well as some dyes and drugs. Singlet oxygen may also be formed when two lipid peroxyl radicals interact with each other.

Any fatty acid or steroid can be the target for peroxidation but the methylene-interrupted double bond sequences of polyunsaturated fatty acids (PUFA) are particularly sensitive because of the reactivity of the methylene groups. Once a lipid free radical has been formed it undergoes molecular rearrangement to form a *cis–trans* conjugated diene system followed by attack of the carbon-centred rad-

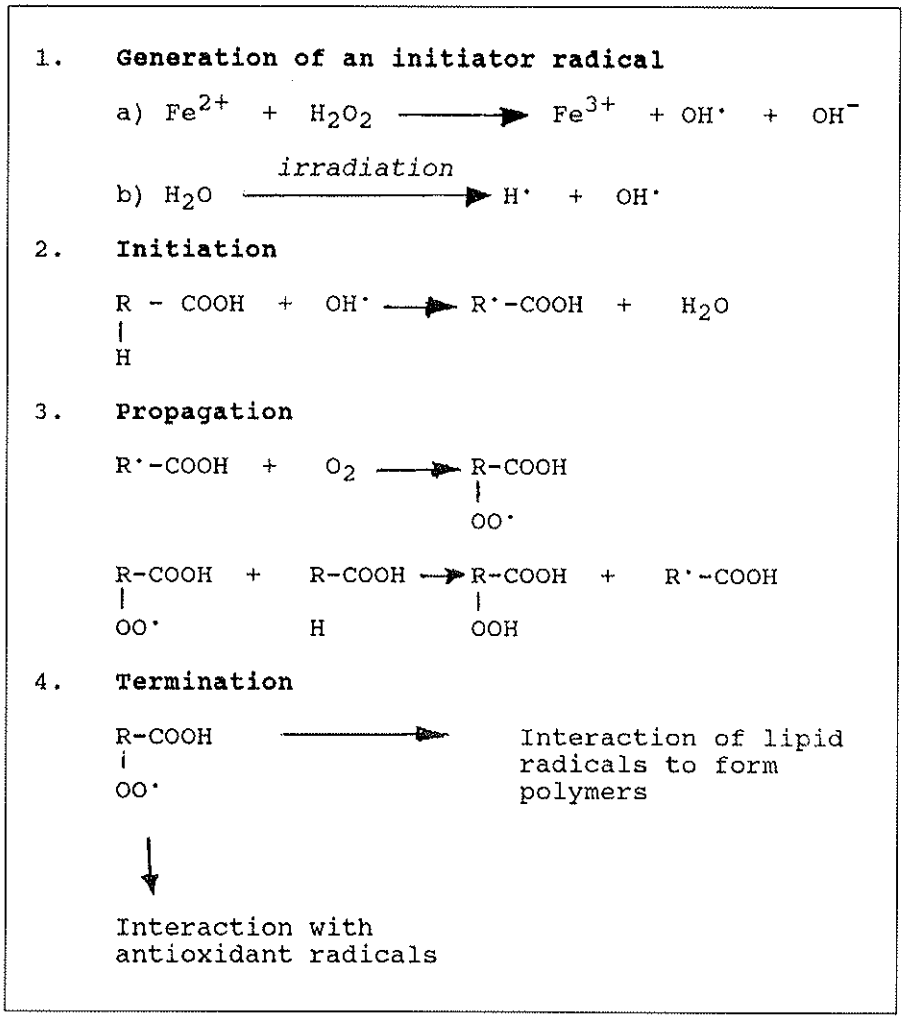

Figure 2: Mechanism of lipid peroxidation.

ical by oxygen to produce a lipid peroxyl radical. The latter may then abstract a hydrogen atom from an adjacent lipid chain to produce another lipid free radical, itself being converted into a hydroperoxide. Additionally, singlet oxygen, generated by the interaction of two lipid peroxyl radicals, can also initiate the production of further lipid free radicals. Thus the *propagation* of a potentially endless supply of lipid free radicals can continue until the chain reaction *is terminated* by destruction of the radical by one of several forms of antioxidant defence [1,2].

ANTIOXIDANT DEFENCE AGAINST DAMAGE BY LIPID PEROXIDES

Metabolic reactions that involve lipid free radical intermediates, such as those of the cyclooxygenase and lipoxygenase reactions [2] occur within the confines of an organized biological membrane. Membrane-bound enzymes ensure that reactive and potentially damaging intermediates are rapidly and locally metabolized into harmless end products. Antioxidants, like *alpha*-tocopherol, built into the structure of the membrane, help to maintain the integrity of the membrane and protect the PUFA against peroxidation.

Once a tissue has been damaged, by whatever cause, and membranes have been disrupted, several factors contribute to uncontrolled lipid peroxidation. These are:

- separation of the unsaturated chains from the vicinity of the antioxidant
- release of catalysts of free radical formation, especially iron and copper ions
- release of hydrolytic and oxidative enzymes that may otherwise not have come into contact with lipid substrates

Lipid peroxides, unless quickly destroyed, have the potential to cause damage to the genetic material of the cell, DNA, and to many important proteins, giving rise to disease states as will be discussed later. Three types of biological defence systems have evolved to limit inappropriate exposure to ROS [1, 3]. These are summarized in Table 2. Briefly:

- *Primary* (sometimes called 'preventive') antioxidants inactivate free radicals before they have a chance to initiate peroxidation chains. They may be small molecules like vitamin A, but are often complex enzyme systems, like *catalase,* that decomposes hydrogen peroxide, or *superoxide dismutase,* which destroys superoxide radicals, thought to be the most abundant ROS in the body. The requirement of the antioxidant defence enzyme, *glutathione peroxidase* for selenium explains the role of this element as an antioxidant nutrient.
- *Secondary* (sometimes called 'chain-breaking') antioxidants trap radicals that are formed in anoxidation chain once it has started. These may be small molecules that are themselves able to form free radicals, combining with chain propagating radicals to terminate the chain. Many are essential nutrients: the so-called antioxidant nutrients, ascorbic acid (vitamin C), tocopherols (vitamin E) and the carotenoids.
- *Tertiary* defence systems are those devoted to the repair of damage already caused to proteins and DNA. Many enzymes involved in repair processes are dependent for their activity on essential nutrients including zinc, folates and cobalamin (vitamin B_{12}).

Table 2: Types of antioxidant defence

Primary	'Preventive'	Catalase (Fe) Superoxide dismutase (Cu/Mn) Glutathione peroxidase (Se) Transition metal binding proteins Vitamin A Riboflavin
Secondary	'Chain-breaking'	Vitamin E Vitamin C Carotenoids Riboflavin
Tertiary	'Repair systems'	Enzymes for repair of protein and DNA damage with Mg, Zn, folate, vitamin B_{12} as cofactors

Antioxidants often have multiple roles in the defence system. Thus, glutathione peroxidase has a primary role in the conversion of H_2O_2 into water but can also act in a chain-breaking manner in concert with vitamin E in detoxifying organic peroxyl radicals. Riboflavin plays a role in secondary defence as a cofactor for the enzyme *glutathione reductase,* required for the regeneration of glutathione and a primary role in maintaining tissue integrity. Vitamin C can remove newly formed ROS in the aqueous phase as well as its more widely recognized role in acting synergistically with lipid-soluble vitamin E in regenerating the active form.

A principal aspect of primary defence is in the sequestration of transition metal ions by various binding proteins, in which form they are incapable of catalysing the conversion of H_2O_2 and superoxide into $OH^{\cdot}$ radicals.

Whereas almost all attention has been directed towards the well-known antioxidant nutrients, vitamins E and C and *beta*-carotene, it is becoming increasingly apparent that foods contain a multiplicity of compounds with antioxidant activities. These include other carotenoids such as lycopene, other tocols such as the tocotrienols, flavonoids, and many phenolic compounds (for example gallic acid and catechins) [3].

THE CONCEPT OF OXIDANT STRESS

As detailed above, ROS are produced in the body continually as part of normal metabolism, and a complex and generally efficient defence system exists to protect against their potentially damaging effects. However, the body may become vulnerable to damage from ROS under certain conditions [1]:

- When the defence system is inadequate, either because intakes of antioxidant nutrients are low, or malnutrition or disease has resulted in an inability to synthesize the enzymes needed to destroy ROS or repair existing damage.
- When exposure to oxygen is particularly large. This may occur in the reperfusion of cardiac muscle after an infarct or in premature babies who need ventilation with extra oxygen yet whose lungs are too immature to cope with the stress.
- When exposure to other pro-oxidants is particularly large, for example, in the condition of iron overload.
- When tissues are injured, physically or during infectious diseases or inflammatory reactions. This leads to disruption of cellular organization,

and release of enzymes, pro-oxidants and the generation of abnormal concentrations of ROS.

OXIDANT STRESS AND ARTERIAL WALL DAMAGE: ROLE IN ATHEROSCLEROSIS

In atherosclerotic plaques, a characteristic finding is a large number of cells (called 'foam cells' from their appearance) engorged with droplets of lipid, mainly cholesteryl esters. Foam cells originate from macrophages whose main task is to engulf anything that can be regarded as a 'foreign body'. It has long been recognized that low density lipoproteins (LDL) are the main sources of the cholesteryl esters of foam cells. Normally LDL enters cells by first interacting with receptors on the cell surface, whereupon receptor and LDL particle are sucked into the cell [2]. It came as a surprise to cell biologists to find, however, that macrophages in culture were not very adept at taking up normal LDL and this turned out to be because they did not have normal LDL receptors on their surfaces. Instead they have 'scavenger receptors' and it was soon learned that, while not recognizing normal LDL, these scavenger receptors recognized LDL that had been modified in some way [4]. One of the first biological clues that lipid peroxidation might be involved in atherosclerosis was the finding that peroxidized LDL (in which the apoprotein moiety is modified by interaction with lipid peroxidation products) were avidly taken up by macrophages via the scavenger receptor to become the foam cells characteristic of the plaque [5].

Whereas the oxidation of LDL and uptake of modified particles by macrophages can be routinely demonstrated by experiments in vitro, evidence for the involvement of this sequence of events in human atherosclerosis is indirect. Oxidation of LDL in vitro is delayed by a number of antioxidants [6], principally vitamin E, and such antioxidants also inhibit the development of 'fatty streaks' (thought to be the forerunners of atherosclerotic plaques) in the arteries of experimental animals. Oxidized lipids, and indeed modified LDL, are found in human atherosclerotic plaques [7], and antibodies to modified LDL have been detected in human plasma [8, 9].

An important recent development is the discovery that high density lipoproteins (HDL) are powerful antioxidants [10]. HDL inhibits LDL oxidation by endothelial and smooth muscle cells and prevents cytotoxicity after LDL oxidation. Thus HDL seems to have multiple roles in protecting against the pathogenesis of atherosclerosis. Another study found that bovine lactoferrin inhibited cholesterol accumulation in macrophages that had been initiated by acetylated or oxidized LDL [11].

Long before the current explosion of interest in the role of free radical reactions in normal biology and their relevance to disease, some people had argued strongly that the process of aging resulted from decay of cells as a result of continual attack upon their nucleic acids and proteins by free radicals [12]. CHD can be regarded as a 'disease of aging'. A major problem in the future for this area of research will be to decide whether ROS cause the pathological changes or whether ROS play a role on the sidelines simply because they are the products of tissues that are already dying from some other cause.

OXIDANT STRESS AND PLATELET FUNCTION: ROLE IN THROMBOSIS

Compared with research into the role of lipid peroxidation in atherosclerosis, little work has been done to examine its role, if any, in the thrombotic phase of cardiovascular disease. The events leading to the formation of a thrombus are exceedingly complex but the aggregation of blood platelets plays a contributory role. Platelet aggregation is stimulated by thromboxanes derived from arachidonic acid via the cyclo-oxygenase pathway [2].

There are several reports that the antioxidant nutrients, vitamins C and E, inhibit platelet aggregation in vitro, that animals depleted of vitamin E produce less thromboxane from arachidonic acid and that production is restored by vitamin E repletion. Platelet aggregation can be enhanced in the presence of LDL and this effect is amplified if the LDL are oxidized [13].

In patients with CHD, blood platelets contain higher concentrations of lipid peroxidation products and lower activities of antioxidant enzymes than in control subjects without CHD. Their platelets are more susceptible to the effects of aggregating agents [14]. Oxidized LDL also inhibits fibrinolysis – the process by which a thrombus, once formed, is dissolved and removed [15].

EPIDEMIOLOGICAL EVIDENCE FOR THE OXIDATION HYPOTHESIS OF CHD

A prospective epidemiological study in Finland found that the concentration of autoantibodies against oxidized LDL was an independent predictor of the rate of progression of atherosclerosis in the carotid artery [9]. High concentrations of pentane, which is one of the end products of lipid peroxidation, have been detected in the breath of patients with acute myocardial infarction [16]. Higher lipid peroxidation products and lower activities of protective enzymes have been found in the blood and aeries of patients with coronary artery disease [17, 18].

Other indirect epidemiological evidence comes from studies of antioxidant vitamins. Gey et al. [19] found a highly significant inverse correlation between CHD mortality in 16 European communities and plasma concentration of vitamin E. About 62% of the differences in CHD mortality were explained by plasma vitamin E and about 17% by total plasma cholesterol concentration. In a case-control study in Edinburgh, patients with angina were found to have significantly lower plasma concentrations of vitamin E than healthy controls [20], and in epidemiological studies involving over 120 000 people in the USA, high intakes of vitamin E (from supplements) were associated with significantly reduced risk of CHD [21].

In the Zutphen Elderly Study [22], intake of dietary flavonoids was significantly inversely associated with mortality from CHD, and in the EURAMIC Study [23] high intakes of *beta*-carotene (as measured by the concentration in adipose tissue) were associated with reduced risk of a first myocardial infarction.

People with diabetes mellitus, a metabolic disorder involving poor control of glucose metabolism, are more likely to develop CHD than non-diabetics. There is evidence for elevated plasma concentrations of lipid peroxidation products and decreased concentrations of antioxidant nutrients in diabetics [24]. Another feature of diabetes is the presence of increased concentration of plasma proteins linked to glucose. It is known that glucose in solution or conjugated to proteins can generate oxidants in the presence of transition metal ions [25] and this suggests one possible mechanism linking diabetes with CHD. However, it is difficult to assess whether apparent involvement of oxidative stress in diabetes is primary or secondary.

INTERVENTION STUDIES

There have been relatively few intervention studies in relation to the oxidation hypothesis of CHD. One study [26] found that daily supplements of vitamin E for 10 weeks protected LDL from oxidation even in diabetic subjects. In

another study [27], supplementation of acute mycocardial infarction patients with vitamins C and E for 14 days suppressed free radical production by neutrophils and suppressed the rise in blood concentrations of lipid peroxidation products seen in unsupplemented patients.

THE NEED TO ASSESS LIPID PEROXIDATION IN THE BODY

In summary, the free radical (oxidation) theory of disease states that, in the absence of adequate antioxidant protection, reactive oxygen species (ROS) cause damage to key biological molecules – proteins and DNA – resulting in disruption of cellular structure and metabolic control and eventually leading to pathological change. If this occurs in major arteries supplying blood to the heart, heart disease may result. ROS may cause these changes directly or, more usually, by initiating peroxidation of PUFA in biological membranes or plasma lipoproteins. Much of the evidence has come from experiments in vitro or from epidemiological associations.

To make further progress it will be necessary to demonstrate that lipid peroxidation is taking place in the living body and to quantify it.

ASSESSMENT OF PEROXIDATION DAMAGE IN VIVO

Products of lipid peroxidation

Intermediate products of oxidation, such as lipid peroxides, hydroperoxides, hydroxyacids and conjugated dienes, have all been measured in human plasma and tissue samples by physical and chemical methods.

Abbey et al. [28] measured the production of conjugated dienes in isolated human LDL as an indicator of peroxidation because they are simple to measure spectrophotometrically. Freshly isolated LDL were incubated with cupric ions to catalyse peroxidation. Diene formation proceeds in three distinct stages: a lag phase of approximately 40 min in which no dienes are formed. This equates with the utilization of endogenous reserves of antioxidant and provides information about antioxidant status of the tissue. When these reserves are depleted, diene formation is rapid and occurs at a roughly linear rate for about 20 min before reaching a plateau when formation is balanced by decomposition. The 'oxidizability' of LDL increased in a roughly linear fashion with the concentration of linoleic acid in the LDL, which was in turn a function of the linoleic acid content of the diet.

Interpretation of methods involving the measurement of concentrations, or rates of formation, of intermediate products of lipid peroxidation requires care. Because these products are undergoing rapid turnover, determination of their concentration at a single arbitrary time may give a distorted picture of the degree of oxidation; continuous measurement over a period of time is therefore an advantage. Measurements of UV absorption, chemiluminescence or even total peroxide or carbonyl value are non-specific and may include compounds that have little to do with the process of lipid oxidation. Many currently used techniques are unreliable indicators of peroxidation in human tissues [29] and methods, such as HPLC or GC in combination with MS, that identify specific structures originating from peroxidation of lipids are needed. End products of lipid peroxidation, for example malondialdehyde, also need similar rigorous identification since the commonly used colour reaction with thiobarbituric acid is non-specific. More recently, a number of compounds resembling prostaglandins have been detected in human urine and plasma [30]. Since they appear to have originated from lipid peroxidation in vivo, they have been used as markers of this process. A major problem with all such methods is that they assume that what is measured is representative of overall lipid peroxidation in the body. However, certain end products may arise only from certain types of precursors (for example, specifically from n-3 polyunsaturated fatty acids rather than unsaturated fatty acids in general) or from constituents of the diet, and so cannot be regarded as proxies for the general process of lipid peroxidation in vivo.

Products of peroxidative damage to macromolecules

An important consequence of lipid peroxidation is the secondary damage that can be inflicted upon proteins and DNA. Specific methods for the detection of whole protein or amino acid modification are now being developed [31]. Specific assays for various end products of DNA damage (for example 8-hydroxydeoxyguanosine, 8-hydroxyguanosine and 8-hydroxyguanine) in urine and plasma are also now well established [32].

Antioxidant status

Another approach to the assessment of oxidant stress is to measure the antioxidant status of tissues or the whole body. Free radical reactions are taking place continuously as a result of oxidative metabolism and a certain level of oxidation products can be regarded as normal. A level of oxidation products equated with 'damage' will arise when protective antioxidant concentrations fall below a certain threshold. At the present time, quantitative information about such 'thresholds' is limited. Much research, therefore is devoted to measuring normal concentrations of antioxidant nutrients in biological tissues as well as changes in concentrations that may be predictive of increased risk of the occurrence of oxidative damage.

HPLC is now generally the method of choice for determining the principal compounds: the tocopherols, carotenoids and ascorbate. A major problem is that, while it is relatively simple to monitor plasma concentrations of these compounds, the relevance of such measurements to antioxidant status in individual tissues is questionable. Another important question concerns the range of antioxidants that should be measured.

In addition to those listed above, should other substances with known antioxidant properties – glutathione, uric acid, the tocotrienols, minor carotenoids, flavonoids and polyphenols also be measured ? Measurement of the activities of antioxidant enzymes, for example superoxide dismutase, glutathione peroxidase and catalase, as well as the adequacy of supply of nutrient cofactors such as Zn, Cu and Se is also recommended. One research group has adopted the approach of measuring 'total antioxidant activity' as distinct from concentrations of individual compounds. The method measures spectrophotometrically the ability of antioxidant substances to scavenge 2,2'-azinobis- (3-ethylbenzothiazoline-6-sulfonate) radicals [33].

The measurement of antioxidant content and activity of foods and diets is also now of interest to nutritionists. However, little or nothing is known about the bioavailability of antioxidant nutrients, that is, how much of what is consumed in the diet actually finds its way to sites in the body in which it is needed. Progress towards this objective is being made by the use of deuterium labelled tocopherols to assess the absorption and metabolism of vitamin E in man [34]. Further developments with this and other antioxidant nutrients, using deuterated and ^{13}C labelled compounds can be expected in the future, providing much needed background information for understanding the role of dietary antioxidants in protection against free radical damage.

IMPORTANCE OF LIPID PEROXIDATION IN FOODS

This review has concentrated mainly on the pathological effects of the products of lipid peroxidation in vivo. Some authors have argued that severe antioxidant stress may also result from the ingestion of lipid peroxidation products in foods. Although there is little evidence that lipid peroxides of long chain unsaturated fatty acids are absorbed from the gut into the blood [35], there is no doubt that smaller molecular weight products of peroxidation, for example aldehydes, are readily absorbed and can be toxic. It is unlikely that there will be gross pathological effects of the ingestion of peroxidized foods since the palatability of food deteriorates long before the peroxide value reaches a level indicative of toxicity. Nevertheless, recent reviews of the toxicity of peroxidized foods urge caution that the consumption of only minor quantities of the lower molecular weight products of lipid peroxidation may seriously deplete the body's antioxidant defence system, and this needs more research [36,37].

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DAIRY MICROORGANISMS AS PROBIOTICS

BASIC ASPECTS

THE ROLE OF THE INTESTINAL MICROFLORA IN HEALTH AND DISEASE AND NOVEL METHODS TO STUDY IT

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The human intestine is a very complex ecosystem containing several hundred species of predominantly anaerobic microorganisms, the so-called intestinal microflora. The microflora has a high catalytic potential and has been implicated in both beneficial and detrimental effects on health and well-being of the host. Effects or functions ascribed to the microflora are immunostimulation, pro- and anticarcinogenic activity, resistance to infection, prevention of bacterial translocation, control of ion concentration and colonic pH, and formation of short chain fatty acids such as acetate, propionate, and butyrate. The latter has been shown to play an important role in the regulation of colonic cell growth and differentiation.

In order to prevent deleterious effects and to promote beneficial effects of the intestinal microflora, it has been suggested to manipulate its composition and metabolic activity, for example by introducing live bacteria or stimulating certain population groups assumed to be beneficial. To evaluate the efficacy and usefulness of such interventions and to gain insight into the mechanisms that rule the activity and composition of the microflora, it is absolutely crucial to really understand the interactions between the various microbial population groups and the host. To reach this ambitious goal it is not sufficient to study only the overall activity of the microflora, but it is necessary to approach the open questions at various levels. We need to identify the microorganisms responsible for a process or a reaction observed in vivo and to study the diet- and host-related factors that influence this activity. We need to know the spatial organization of the microbial communities in the various sites of the gut as well as the mutual metabolic dependencies of the microbial population groups involved.

The advent of molecular biology has revolutionized our knowledge of the phylogenetic relationship of bacteria. Sequence comparison of 16S and 23S ribosomal RNA has led to evolution-based classifications of microorganisms. The use of oligonucleotide probes directed at these molecules enables the microbiologist to study the biodiversity and the dynamics of the microbial population residing in the intestine.

LACTIC ACID BACTERIA IN DAIRY PRODUCTS

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Since ancient times, Lactic Acid Bacteria (LAB) have been an important constituent of fermented milks and cheeses.

Most fermented dairy products are rooted in accidental microbial and/or biochemical changes. The type of changes which happened to take place depended on the type of microbes and enzymes present, climatic conditions, together with the type of domestic animals and the way in which the milk was handled and treated in different geographical locations.

As examples, we know fresh cheeses from the Near East and Mongolia. No doubt this type of cheese originated through the habit of nomadic people to transport milk and other liquids in the stomachs from ruminants. Such stomachs would contain rennet and other enzymes together with microorganisms of which different types of LAB would often be dominating in a milieu with ruminants and milk.

A slightly different sequence has probably taken place in the Middle East where milk was stirred with branches of papaya trees containing the milk coagulating enzyme "papain". Being a vegetable, lactobacilli probably accommodated to vegetables like *Lb. plantarum*, and other thermophiles would dominate the flora and add organoleptic characteristics to such products. Most certainly local flora and the traditional handling of milk at different geographical locations are to be appraised for the great variety of fermented milks and cheeses we know and appreciate today.

Another example of how local conditions have been decisive in the characteristics of a dairy product is a fermented product produced on small farms in the northern part of Scandinavia called "Tätte Mjölk". This product was originally produced from the milk of reindeer by adding a small amount of the meat-eating spice or flower "*Pingicula Vulgaris*". Together with this flower the milk was supplied with coagulating enzymes as well as mesophylic and psychrotrophic LAB that produced a lot of extracellular slime (polysaccharides) and contributed a very special viscosity and flavour to the product. In many of such special products, we are dealing with a very delicate kind of symbiosis, and as far as Tätte Mjölk is concerned, many dairies have tried to produce this product industrially with only little success. After a few propagations the starter culture collected from the local farms in northern Scandinavia lose their characteristics and as nobody knows the secret of these starters, nobody could solve the stability problem. Basically, the same situation exists more or less for other geographic specialities of fermented products, like Kumiss produced from mare's milk in Russia, and Skyr produced from sheep and goat milk in Iceland.

Originally, the production of cheese and other dairy products was the job of women. To master production of good quality fermented dairy products was more than good craftsmanship; it was regarded as a real art, surrounded by a lot of mystery and superstition as microbiology was not known until the later part of the 19th century.

Today, microbiology and biochemistry are the bearing elements in the production of fermented dairy products. A lot has been learned during the past century of research, but even though pure starter cultures are used in many productions, I would assert we are still in a situation that even though we have learned a lot, the most important thing we have learned is that there is even more to be learned.

No doubt the main reason for using LAB is as shown in Figure 1.

I. Preserve

II. Contribute better organoleptic properties to the food
- a) Improve taste and flavour
- b) Improve consistency and mouthfeel

III. Contribute dietetic properties

Figure 1: Purpose of using starter cultures.

The LAB used when producing dairy products have their name from producing lactic acid as their main product. Beyond LAB, other microorganisms may be used in the production of various products, like yeast and moulds and bacteria closely related to LAB, as for example Bifidobacteria.

In the following phylogenetic tree (Figure 2) the LAB are grouped together, with closely related microorganisms often connected with milk as spoilage or pathogens. In my opinion, Bifidobacteria are too distant to be included in this phylogenetic tree.

Throughout the world a huge number of different types of dairy products exist and it is beyond the scope of this presentation to aim at a comprehensive review.

In Figures 3 and 4 an attempt is made to group some major cheese types and fermented milks according to physical characteristics and type of LAB used in their manufacture.

To produce dairy products on an industrial scale and with the consistency and variety in characteristics that modern consumers require, raw material, technology and starter cultures must be controlled. Furthermore, product development and product innovation in today's modern dairy industry are based on scientific knowledge.

As far as starter cultures are concerned, the traits listed in Figure 5 are among the most important to be controlled and governed if controlled and reproducible product development is to be aimed at.

Phage resistance is an obvious requirement for a modern industry producing on a large scale. Phage attack is most often feared due to the possibility of "dead vats" and thereby economic loss for the dairy here and now. In my opinion the greatest risk involved with phage attack is the more moderate and even minor attacks as they often result in changes in composition and performance of the starter culture.

Carbohydrate metabolism is the basis for acidification power, formation of flavour components, gas and polysaccharides. As such, control over activity, flavour and viscosity of fermented milks and to a great extent eye formation in continental and Swiss type cheeses takes the start in controlling carbohydrate metabolism.

Concerning protein metabolism, this is decisive for flavour as well as consistency development in all types of cheeses.

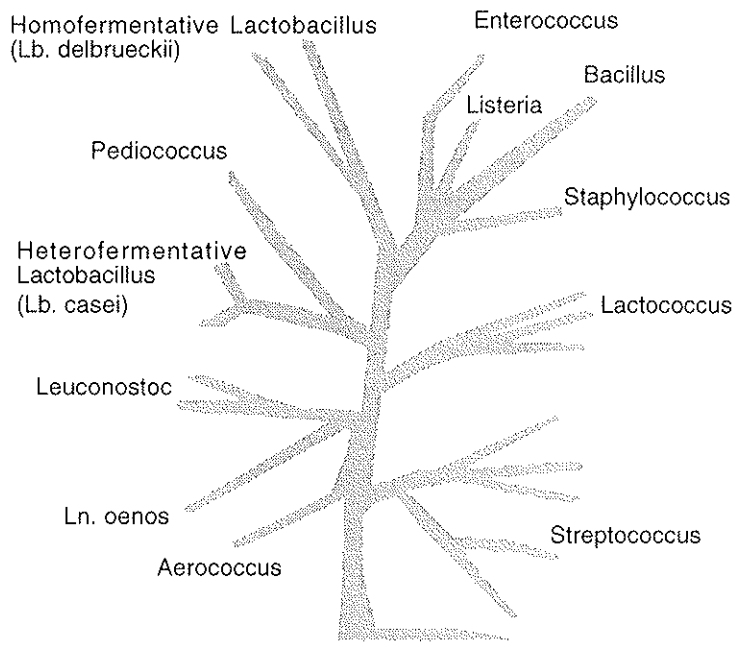

Figure 2: Phylogenetic tree of LAB and other important genera of G+ bacteria for dairy products.

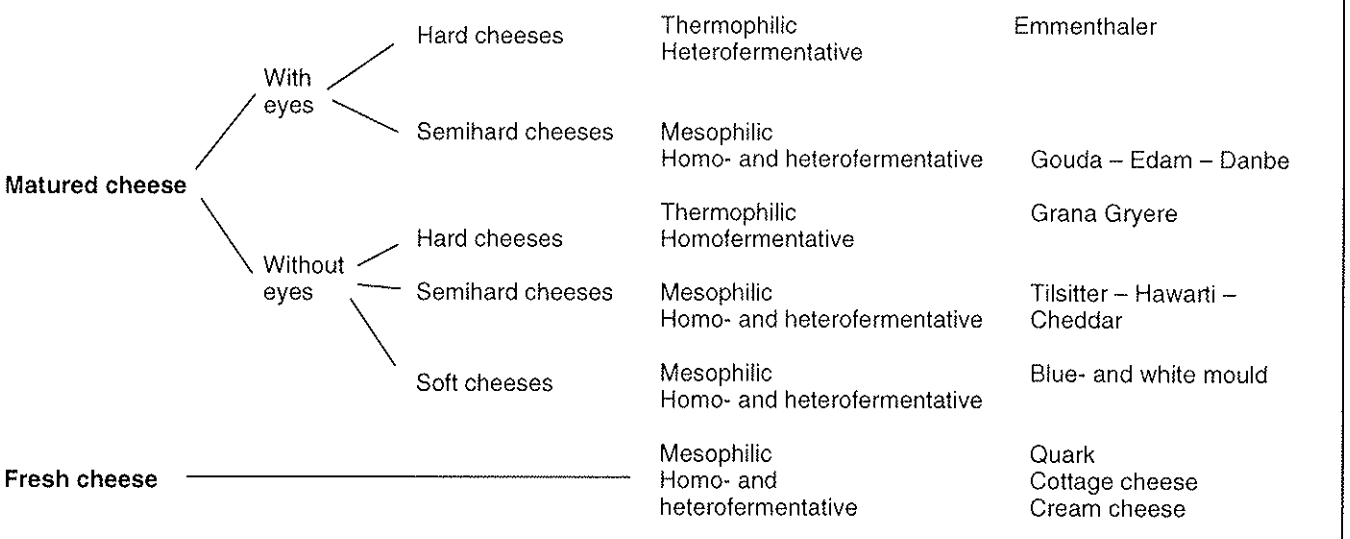

Figure 3: Classification of some cheeses according to physical structure and lactic acid bacteria used.

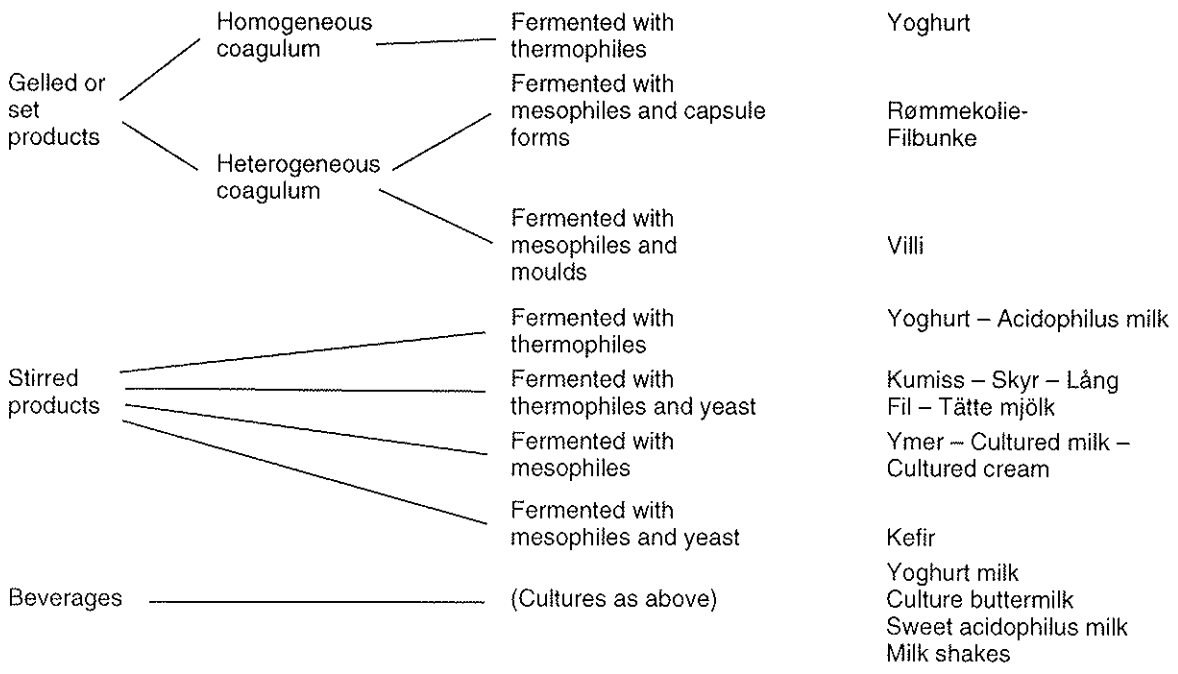

Figure 4: Classification of cultured milk products according to physical structure and microorganisms used during fermentation.

Securing a well-balanced proteolytic degradation of the milk casein in cheese is regarded as one of the greatest challenges for improving cheese quality. Proteolytic activity is not only important for cheese maturation, it is also essential as a tool for the LAB to provide sufficient amounts of low molecular nitrogen for their protein anabolism, and is thereby decisive for their speed of acidification. Too high a proteolytic activity of LAB is not desired as it may lead to the formation of bitter peptides from the milk proteins.

From a nutritional point of view, many positive attributes are related to products containing LAB. Combining probiotic LAB and good organoleptic properties of dairy products is a challenge for the dairy industry in the years to come.

No doubt the trend in the market is changing from "Reduced in something" towards "Added value", beyond general nutrition and pleasure of eating which today is taken for granted by the consumer.

Even though the nutrition and health aspects of using LAB and Bifidobacteria as probiotics are the main theme of this Nutrition Week, the objective of this presentation is to give an impression of the importance of LAB in the production of fermented dairy products from a general point of view. As many participants this week are nutritionists, microbiologists and/or medical professionals, the presentation is kept in general terms, with the aim of providing an overall impression rather than a detailed account.

- PHAGE RESISTANCE
- CARBOHYDRATE METABOLISM
  - Activity
  - Aroma and gas formation
  - Polysaccharide formation
- PROTEIN METABOLISM
  - Proteolysis (flavour and texture)
  - Peptidolysis (flavour and texture)

Figure 5 : Important traits to be controlled for tailoring starter cultures.

UNIQUENESS OF PROBIOTIC STRAINS

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INTRODUCTION

A probiotic is a live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host. Health benefits must be demonstrated by proper clinical trials and nutritional studies.

The bulk of evidence on probiotic cultures and foods is based on anecdotal reports and poorly controlled studies, making the work inconclusive and general health claims impossible. However, evidence is now accumulating from well-designed, randomized and placebo-controlled double-blind studies indicating that a few well-characterized lactic acid bacteria strains have documented probiotic health promoting effects when defined doses are administered [1].

SUCCESSFUL PROBIOTIC STRAINS

Probiotic bacteria with these properties and documented clinical effects include *Lactobacillus acidophilus* (NCFB 1478), *Lactobacillus casei* Shirota strain, *Lactobacillus* GG (ATTCC 53103) and *Lactobacillus acidophilus* LA1 [1]. A large number of published studies exist on each preparation documenting their health effects. All of these are currently further tested for different intestinal disorders. New strains emerge and are likely to be included in our diet. These include *Lactobacillus reuteri, Bifidobacterium animalis. Lactobacillus casei* Danone 001, *Bifidobacterium bifidum*, and strains such as *Enterococcus faecium*. Work on cholesterol lowering properties of probiotic strains is extensive, but no conclusive evidence from studies to promote one strain as a unique strain in this area is available as of yet.

PROPERTIES OF GOOD PROBIOTICS

Table 1 lists some properties common to many probiotic strains. However, not every one of these properties is necessarily needed for specific probiotic effects. Thus, this list forms a basis for assessing the important char-

Table 1: Desirable properties of probiotic bacteria (according to Salminen et al. [2])

Probiotic strain characteristic	Functional and technological properties
Human origin	Species dependent health effects and maintained viability; applicability to fermented foods
Acid and bile stability	Survival into the intestine, maintaining adhesiveness; maintenance of flavour and aroma profiles during processing and storage
Adherence to human intestinal cells	Immune modulation, competitive exclusion of pathogens: maintenance of mild acidity throughout storage time, good acidity profile
Colonization of the human intestinal tract	Multiplication in the intestinal tract at least temporarily, immune modulation; maintenance of this ability throughout processing and storage
Production of antimicrobial substances	Pathogen inactivation in the intestine, normalization of gut flora; good storage stability and shelf life in functional food products
Antagonism against cariogenic and pathogenic bacteria	Prevention of dental decay and pathogen exclusion, prevention of pathogen adhesion, normalization of gut flora, ability to keep the properties after freeze-drying, drying and other processing methods
Safety in food and clinical use	Accurate strain identification (genus, species), documented safety
Clinically validated and documented health effects	Dose–response data for minimum effective dosage in different products

acteristics. What then makes a probiotic strain unique ? The uniqueness depends on characteristics verified in carefully conducted clinical studies repeated in several places (Table 2). Only documented effects can establish unique strains. Each strain must be studied on its own merit, no two strains are identical.

Table 2: Requirements for good clinical studies of demonstration of unique probiotic properties for functional food use

Each strain documented and tested independently, on its own merit.
Extrapolation of data from closely related strains not acceptable.
Well-defined probiotic strains, well-defined study preparations.
Double-blind, placebo-controlled human studies.
Randomized human studies.
Results confirmed by several independent research groups.
Publication in peer-reviewed journals.

PROPERTIES THAT MAKE A STRAIN UNIQUE

Several properties could be listed here. Examples include documented properties such as adherence to a specific area of the intestinal tract (targeted effects), immune enhancing effects facilitating rapid recovery from acute gastroenteritis or protection against enteritis, immune effects slowing down or preventing tumour development, and mucosal effects to facilitate improved antigen handling and transport in food allergy. All these effects should be well documented, preferably by two or more independent research groups. This way, unique properties can be generally accepted.

CONCLUSION

The unique properties described in this paper indicate that there are some common background characteristics for many intestinal disturbances which facilitate effective use of probiotic bacteria. It is clear that probiotic bacteria have potential in the treatment of clinical conditions with altered gut mucosal barrier functions. Unique documented properties of probiotic bacteria offer new dietary alternatives for the stabilization of the intestinal ecosystem. Provided that the properties are proven in proper clinical or nutritional studies, they can be used for immunotherapy to counteract local immunological dysfunctions and to stabilize the natural gut mucosal barrier mechanisms. It is important that the probiotic properties of each strain are demonstrated separately in carefully planned and controlled human studies since even closely related strains many have different mechanisms of action.

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SELECTION OF PROBIOTIC LACTIC ACID BACTERIA (LAB) ON THE BASIS OF SOME PHYSIOLOGICAL ACTIVITIES

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INTRODUCTION

The microorganisms used in the preparation of fermented milks have shown a balancing activity on the intestinal microecosystem, with positive effects on human health. As underlined by some authors [1, 2], the effects observed may be due to the interaction of these microorganisms and not only their direct influence on the metabolism of the host itself.

Controversial results, quite often obtained in various experiments carried out to establish the probiotic effect of LAB, have been attributed to the strains utilized [3]. During research on transit

and/or colonization of LAB in yoghurt, we observed that the *Lactobacillus delbrueckii* subsp.*bulgaricus* strain used, taken from the faeces (both from children and germ-free mice) was able to ferment N-acetyl-glucosamine, leading to the supposition that it has a capacity to break away the hexosamine from the mucin, and to hydrolyse it through inducible enzymes [4]. Therefore in this report we would like to underline the importance of the evaluation of some physiological characteristics used as criteria in the selection of the strains of some eu-probiotic species of fermented milks, correlating them according to their behaviour at the intestinal level, with the intent of better defining the strains in the complex system of the study of probiosis.

For these reasons, the selection of LAB should be carried out both through examination of their adaptability to technological production, maintaining cells in their best physiological state, and trying to establish the physiological characteristics that could be used as indices of their assumed behaviour at the gastrointestinal tube level.

The fermented milks, on sale at present, that have been most carefully studied for their eu-probiotic properties can be subdivided into 3 groups based on the type of flora they have [5]:

- Group A: yoghurt, mesophilic fermented milks prepared with specific dairy bacteria having eu-probiotic properties: *Streptococcus thermophilus* and *L. delbrueckii* subsp. *bulgaricus,* lactococci;
- Group B: fermented milks prepared with intestinal LAB having probiotic properties and suitable for dairy preparation: *Lactobacillus casei* and *Lactobacillus acidophilus*;
- Group C: bacteria exclusively of intestinal origin with highly acclaimed probiotic properties but with little adaptability to dairy preparation: bifidobacteria (*Bifidobacterium bifidum, Bifidobacterium brève, Bifidobacterium longum, Bifidobacterium infantis).*

SELECTION OF STRAINS

This is carried out on the basis of the usual cultural, biochemical and technological tests [6].

Physiological characteristics for evaluation of technological production adaptability such as:

- capacity to grow in milk and acidify it according to the thermal cycle production [7];
- resistance to fermented milk acidity so as to guarantee viability of the cells during shelf life of the product, so that the LAB cells can reach the gastrointestinal tract in their best physiological state [6].

Physiological characteristics involving probiotic aspects

With the aim of colonization the common tests carried out are:

- resistance to gastric juices and bile salts [8];
- transit capacity, resistance to peristalsis, colonizing capacity in the human gut, verifiable first in germ-free animals [9].

Moreover, it would be useful to know:

- growth capacity at 37°C, especially for species with optimal temperature different from that of the human body;
- growth speed at 37°C in similar conditions of each intestinal tract (for example pH, rH);
- bacteria capacity to utilize endogenous and exogenous nutrients through their biochemical activity.

The nutrients available in the large intestine are starch (15–20%), non-starch polysaccharide complexes of plant cells, and proteins that escape digestion and absorption in the small intestine, and also, in the absence of dietary substrate, endogenous materials such as exfoliated epithelial cells and mucins [10–13].

In this condition it is supposed that also probiotic bacteria are able to break down these nutrients into useful substances for both their growth and autochthonous bacteria. The mucins, acidic glycoprotein secreted by the goblet cells, play an important role in maintaining colon functions. They contain a large quantity of N-acetyl-glucosamine, N-acetyl-galactosamine and a smaller quantity of fucose [14]. The mucins of the small bowel are broken down by the lysozomal enzymes; however, studies on germ-free mice have shown that they are also broken down by bacterial enzymes [15]. In the large intestine, the enteric bacteria (only a few Bifidobacteria and Bacteroides), in fact, break down both carbohydrates and the protein moieties of mucin glycoprotein [16–18]. The hydrolysis of macromolecules in smaller subunits that are more quickly assimilated are due to a variety of hydrolytic enzymes (constitutive and inducible), like protease, peptidase and glycosidase produced by intestinal bacteria, whose yield is controlled by the substrate. Type of substrate and their metabolisms by intestinal bacteria are described in the literature [19]. In an experimental model that produces the various colon tract conditions, the influence of porcine mucin on bacteria growth and production of cell-associated enzymes in the human colon was shown: precisely for N-acetyl-D-glucosaminidase, N-acetyl-D-galactosaminidase, α- and β-D-galactosidase, α-D-glucosidase, α-L-arabinofuranosidase and α-D-xylosidase, but not for β-L-fucosidase [13].

It has been reported that the subdominant microflora at levels inferior to 10^7/g of faeces is the one producing the highest quantities of β-D-galactosidase, N-acetyl-D-glucosaminidase, sialidase and α-fucosidase [14]; and only a few anaerobe strains isolated in the human colon ferment porcine mucin, among which are *Ruminococcus* and *Bifidobacterium bifidum* [20, 21]. Since during administration of fermented milks the referred LAB are at a level close to that of the subdominant microflora (10^7–10^9), it may be deduced that they can intervene in the colon through their metabolic activity. On the basis of this knowledge, since in the presence of mucin glycosidases are stimulated, it would be interesting to compare the extracellular enzymatic activities of probiotics with those involved in mucin degradation through the intestinal microflora. Thus a desired strains selection would be possible. The glycosidases are produced by strains of Lactococci, *S. thermophilus, L. delbrueckii* subsp. *bulgaricus* [22], *L. casei, L. acidophilus* and bifidobacteria (of CSL collection and Bahaka et al. [23]) as shown in Table 1. Therefore, for probiotic purposes the strains having positive activities, for example α-galactosidase, which hydrolyses specific sugars, such as α-D-galactosyl-oligosaccharides, allowing selective proliferation of bifidobacteria in the intestinal tract, are selected, and also strains of α-D-glucosidase, N-acetyl-D-glucosaminidase or β-L-fucosidase positive.

Other enzymatic activities, especially of yoghurt strains capable of intervening in the dietary wastes of intestinal content, such as α-D-xylosidase and α-maltosidase, β-D-mannosidase and α-D-mannosidase are produced, but not β-D-glucosidase. Confirming this, both surnatant faeces (free bacterial cells) of germ-free and oloxenic mice treated with *L.delbrueckii* subsp.*bulgaricus* α-D-galactosidase positive strain (in our Laboratory) contained the enzyme, whereas those treated with the α-D-galactosidase negative strain did not (as in control mice). Two of the above strains were β-D-galactosidase positive, and in fact in the same experiment this enzyme was found in the surnatant faeces of both germ-free and oloxenic mice, while it did not exist before treatment (unpublished data) (Table 2).

Table 1: Enzymatic characteristics of LAB and bifidobacteria species used in fermented milks

ENZYMES	% OF FREQUENCY					
GLYCOSIDASES	LACT. (40) API 20 Z	S. THERM. (30) LRA ZYM	L. BULG. (18) LRA ZYM	L. CASEI (20) API 20 Z	L. ACID. (20) API 20 Z	BIFIDOBACTERIA (20) API 20 ZYM
In mucin degradation						
α-D-galactosidase	0	40	60	20	100	100
α-D-glucosidase	80	60	60	100	30	100
α-L-arabinosidase	nd	100	90	nd	nd	[23] 0 *B. brève* [23] 96 *B. longum* [23] 96 *B. infantis*
α-D-xylosidase	nd	90	90	nd	nd	nd
α-L-fucosidase	0	20	10	0	0	0
β-D-fucosidase	nd	100	100	nd	nd	[23] 100 *B. brève* [23] 90 *B. longum* [23] 90 *B. infantis*
β-L-fucosidase	nd	0	11	nd	nd	nd
N-Ac-α & β-D-glucosaminidase	10	0 or weak	0 or weak	10	100	10 *B. bifidum* *B. infantis*
Versus other substrata						
β-D-galactosidase	100	100	100	100	100	100
β-D-glucosidase	30	0	0	90	90	100
α-maltosidase	nd	93	83	nd	nd	nd
β-D-mannosidase	nd	43	35	nd	nd	nd
α-D-mannosidase	nd	7	4	nd	nd	nd
PROTEASES	LACT.	S. THERM.	L. BULG.	L. CASEI	L. ACID	BIFIDOBACTERIA
Trypsin	0	0	0	0	0	0
Chymotrypsin	0	13	0	0	0	0

LACT. = Lactococci; S. THERM. = Streptococcus thermophilus; L. BULG. = Lactobacillus delbrueckii subsp. bulgaricus; L. CASEI = Lactobacillus casei; L. ACID. = Lactobacillus acidophilus.
( ) = number of strains/CSL. nd = not determined.

Table 2: Glycosidases in surnatant faeces (free bacterial cells) of germ-free and oloxenic mice treated with two strains of *L. delbrueckii* subsp. *bulgaricus*

SAMPLES	α-D-galactosidase	β-D-galactosidase
Germ-free faeces (6 mice)	–	–
Oloxenic faeces (6 mice)	–	–
L. BULG. 1	–	+
Germ-free faeces L.BULG. 1	–	+
Oloxenic faeces L.BULG. 1	–	+
L. BULG. 2	+	+
Germ-free faeces L.BULG. 2	+	+
Oloxenic faeces L.BULG. 2	+	+

L. BULG. = L.delbrueckii *subsp.* bulgaricus.
P.Camaschella et al. (CSL), unpublished data.

Regarding β-D-galactosidase activity, important in lactose intolerant subjects [24, 25], all strains of all LAB species and bifidobacteria are positive. But a considerable divergence of this activity between strains was observed (for example in *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus*) by some authors [26, 27]. According to Sasaki this activity in *L. acidophilus* strains is only one-quarter to one-fifth of that in *L. delbrueckii* subsp. *bulgaricus* strains NIAI B-6, and for this reason, he considered the last strain more suitable as a dietary adjunct for lactose maldigesters [26].

At the same time it should be pointed out that galactose, fucose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine, the four oligosaccharides released by hydrolysis of the mucin, and other sugars derived from dietary products, could represent an energy source for LAB and bifidobacteria at the intestinal level. Thus, organic acid derived from them can provide a source of energy for the other saccharolytic anaerobe microorganisms or for the host [28]. They may exert a significant influence over the composition of the intestinal microflora, particularly near the mucosal surface [15]. On the basis of this hypothesis, sugar fermentation (in particular those derived from mucins) is also considered as a useful parameter for the selection of probiotic bacteria. The sugars fermented by LAB and bifidobacteria are shown in Table 3.

Since many colon bacteria species can utilize either dietary components (for example fibre soluble) or mucin as energy sources, one strain can dominate another of the same species, depending on their enzymatic activities and the sugar fermented. In mucin degradation the proteolytic activity is

Table 3: sugar fermentation of species used in fermented milks (CSL collection)

SUGARS	LACT.	S. THERM	L. BULG.	L. CASEI	L. ACID.	BIFIDOBACTERIA
D-Glucose	+	+	+	+	+	+
Lactose	+	+	+	+	+	+
Galactose*	+	+	±	+	+	+
D-Fructose	+	+	±	+	+	+
Saccharose	(V)	+	-	+	+	+ { *B. longum* +; *B. brève* +; *B. infantis* +; *B. bifidum* ±
D-Mannose	+	-	+	+	+	(V) *B. brève* +
NA Glucosamine*	-	-	(V)	+	+	(V) { *B. bifidum* +; *B.brève* ±
L-Arabinose	-	-	-	+	+	(V) *B. longum* +
D-Raffinose	-	-	-	-	-	+ { *B. longum* +; *B. brève* +; *B. infantis* +
D & L-Fucose*	-	-	-	-	-	-

LACT. = Lactococci; S. THERM. = Streptococcus thermophilus; L. BULG. =Lactobacillus delbrueckii subsp. bulgaricus; L. CASEI = Lactobacillus casei; L. ACID. =Lactobacillus acidophilus.

+ = positive
- = negative
** = sugars involved in mucin degradation.*
± = weak positivity (max. 10% of strains)
(V) = variable (11–89% positive)

principally due to cystein, serin, chymotrypsin-like protease, and to a lesser extent metalloprotease, aspartic-protease and trypsin-like protease produced by mass colon bacteria. Within the LAB groups under consideration only 10% of the *S.thermophilus* strains show chymotrypsin-like activity (Table 1).

CONCLUSION

At the intestinal level the described enzymes produced by cells (extra- or endocellular) of tested LAB and bifidobacteria may be able to exhibit their own activities either on the mucin or on the basis of the diet (meat and vegetables), and therefore the availability of different substrates, affecting the nutritional system. Consequently it emerges that LAB are important from the probiotic point of view, not only in comparison with other intestinal microbial species but also when compared to the host physiology and dietary contents.

Thus, the strains that produce glycosidase which attack the fucosides, mannosides or xylosides can involve fibre digestion, leaving carbohydrates available as an energy source for their growth or that of other species.

On the basis of this hypothesis some interactive processes between probiotic bacteria and autochthonous microflora can be explained (for example in the case of an increase of bifidobacteria following the intake of yoghurt or of LAB yoghurt washed cells) [29]. Therefore they can be considered as key microorganisms for the growth of autochthonous anaerobic species, intervening favourably in the host's health and counter-balancing the negative effects of some diets, such as those lacking fibre. The attempt to correlate the enzymatic activities, expressed by the microbial mass of the colon, to those of the strains of species used for fermented milk production seems to provide some valid information useful in selection of strains having probiotic properties, as resulted from our experiments on animals. Research on this subject requires further study to establish whether the behaviour of selecting strains on the basis of the afore-mentioned criterion can be verified at the intestinal microecosystem complex level of humans.

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STATE OF THE ART CONCERNING PROBIOTIC STRAINS IN MILK PRODUCTS

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During the LABIP (Lactic Acid Bacteria Industrial Platform) Workshop on the application of lactic acid bacteria as probiotics, held in Frankfurt am Main, 13–15 November 1995, the state of knowledge with respect to the benefits and risk of the use of probiotics in human nutrition was critically discussed by a group of more than 20 scientists, including independent experts from research institutions and universities and representatives of industry. To facilitate discussions, three questions were raised in advance:

1. What kind of effects should be present to call something a probiotic ?
2. What kind of evidence is necessary ?
3. What is the state of the art of the benefits and risks ?

Answering the first question resulted in a new definition for probiotics, which is more in line with the concept of functional foods. Previous definitions were considered to be too restricted to the improvement of the intestinal ecology. According to the new definition "oral probiotics are living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition". Probiotics may be consumed either as a food component or as a non-food preparation. It is recommended to know as much as possible about the mechanism underlying the beneficial effect.

Answering the second question resulted in the opinion that, in general, well-controlled studies in humans are required to obtain evidence for a health claim. The kind of study depends on the particular claim one has in mind. A case-by-case consideration is appropriate since standard protocols for nutritional studies are lacking. Generally accepted rules for sound scientific principles are applicable. No specific legislation exists in Europe about the use of health claims for foods. Medical claims for foods are not allowed. The use of health claims, which are not scientifically based, will be considered as disseminating misleading information to consumers. It was concluded that the company using a health claim should be able to present evidence in favour of the claim upon request. It was strongly recommended to publish the scientific evidence in peer-reviewed journals. It was also concluded that any change of the food matrix could have an effect on the probiotic activity. Therefore it is required to study probiotic effects in the commercial food matrix. Sometimes it may not be necessary to perform studies in humans, as could be the case when the probiotic effect of an almost identical strain has to be confirmed. In those cases validated models could be used (in vitro, in situ or animal models) to compare the original strain (already tested in humans) with the new strain.

Regarding the benefits and risks of probiotics, a distinction was made between *established effects, potential effects and potential risks.*

The following *established effects* were identified:

- Reduction of signs of lactose intolerance
- Reduction of several types of diarrhoeal diseases
- Reduction of bacterial enzyme activity
- Effects on the immune system

It was stated that the physiological benefits of the effects on bacterial enzyme activity and the immune system with respect to disease prevention remain to be established.

The following *potential effects* were identified:

- Lowering of LDL cholesterol levels
- Competitive exclusion of intestinal pathogens
- Cancer prevention

– Increased resistance to infection

As *potential risks* the following issues were noted:

– Adverse effects in infants, who do not yet have an established stabile intestinal flora
– Adverse effects in patients with autoimmune diseases
– Transfer of gene coding for antibiotic resistance (for example from specific strains of *Enterococcus faecium*).

In view of these (possible) benefits and risks, the nutritional significance of dairy products which are now on the market in several European countries can be discussed. This concerns products with strains (of human origin) of *Lactobacillus acidophilus, Lactobacillus casei* and *Bifidobacterium bifidum*.

As far as these products are aimed to exert beneficial effects on the intestinal flora, the question still to be answered is what these products mean for normal healthy people.

Recently three studies have been completed on the *hypocholesterolaemic effects in humans of selected probiotic strains*:

(1) a Danish parallel study with a fermented dairy product of MD foods (Gaio) containing a strain of *Enterococcus faecium* [1];

(2) a cross-over study by TNO in Holland with a fermented yoghurt-like product of the Danone company (Actimel), containing *Lactobacillus acidophilus* and a fructo-oligosaccharide [2];

(3) a cross-over study in the USA on a fermented yoghurt of the Mona company in Holland, containing a *Lactobacillus acidophilus* strain [3].

Each of these controlled studies showed a significant lowering of the LDL-cholesterol level. The effect varied between 4.2 and 10.5% and was obtained within 3 to 10 weeks of ingesting the test products, if compared to the control treatment. The results of the Mona study strongly suggest the existence of a carry-over effect of the *Lactobacillus acidophilus* into the second period of the cross-over, limiting the significant cholesterol-lowering effect to the first treatment period. The results of these three studies are encouraging and provide a breakthrough in the field of cholesterol-lowering properties of fermented dairy products, which remained a matter of debate since the studies in the early seventies by George Mann in the Masai tribe. However, further research on the mechanism of the hypocholesterolaemic action, the dose–effect relationship and the persistence of the effects with time and in various population groups should be performed to assess the relative importance of these products in the diet compared to other generally accepted measures to lower the blood cholesterol level, such as lowering the intake of saturated fat and cholesterol.

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PHYSIOLOGICAL AND SAFETY CONSIDERATIONS

SAFETY PROPERTIES OF LACTIC ACID BACTERIA: SET-UP OF AN EXPERIMENTAL MODEL

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Lactic acid bacteria (LAB) and in particular, lactobacilli, are used extensively in food fermentations. These are also commensal inhabitants of the oral cavity and the gastrointestinal tract. They are considered as harmless saprophytes, non-toxigenic and non-pathogenic microorganisms, and sometimes as probiotic agents because of beneficial effects for health. However, some cases have been described where LAB were isolated from clinical samples in severe human infections such as septicaemia, meningitis and endocarditis. Infective endocarditis (IE) is the most frequently encountered infection with lactobacilli.

The present study was carried out to evaluate the safety of *Lactobacillus casei* and *Lactobacillus rhamnosus* strains by using the experimental endocarditis model of Perlman & Freedman in rabbits. This model was chosen because it is used to assess the virulence of pathogenic strains. For this purpose, the bacterial ability to adhere to a fibrin-platelet matrix of cardiac vegetations is considered to be a major pathogenic trait in the progression of IE. We compared the adhesion of strains isolated from dairy products (industrial strains) and from patients with IE (so-called clinical strains).

Investigations were performed on 2.5 kg female New Zealand White rabbits. A polyethylene catheter was inserted through the right carotid artery into the left ventricle to induce the formation of vegetations, and was left in place throughout the experiment. Twenty-four hours later, 1 ml of various inoculum sizes (10^4, 10^6 or 10^8 CFU/ml in 0.9% NaCl) was inoculated by the marginal ear vein. Five days after bacterial inoculation, the animals were sacrificed. The heart was removed and examined. All vegetations from each rabbit, were excised, pooled, weighed and quantitatively cultured onto MRS agar plates at 37°C for 72 h to detect viable bacteria inside.

Results have shown that all strains adhered to cardiac vegetations in a concentration-dependent manner. At 10^4 CFU/ml, only clinical strains were able to adhere. At 10^6 CFU/ml, the adhesion was variable but remained lower for industrial strains. The attachment of bacteria to fibrin-platelet matrix was almost identical for all bacteria at 10^8 CFU/ml. Furthermore, at 10^6 CFU/ml, the mean bacterial counts into vegetations, ranged from 4.7 to 6 $\log_{10}$ CFU per gram of vegetations, and were significantly lower than those obtained by other authors with pathogenic strains.

In conclusion, a bacterial adhesion to cardiac vegetations was observed with all tested strains. An inoculum size of 10^6 CFU/ml allows discrimination of different adhesion between strains. This adhesion represents the first step of endocarditis with pathogenic strains. To determine the safety of lactobacilli, it is now necessary to test the consequences of their adhesion on the outcome of the infection as a function of time.

IMMUNE SYSTEM STIMULATION BY LACTOBACILLI

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In previous works using mice as the experimental model, we demonstrated that the following LAB – *Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* – can stimulate the systemic immune response, but only some can activate the secretory immune response with an increase in the s-IgA levels.

From these studies we chose the strain of *L. casei* to determine the beneficial effects: (a) in prevention of enteric infections, (b) in an immunodefficiency process such as malnutrition or immunosuppression by corticoid therapy, and (c) on a nonintestinal tumour such as a fibrosarcome.

We demonstrated that the previous administration of *L. casei* was effective in the prevention of an infection against *Salmonella typhimurium*, improved the mucosa immunity in a malnutrition process, and showed antitumoral activity against a fibrosarcome.

In a corticoid immunosuppression model we observed that some LAB such as *L. casei* and *L. delbrueckii* ssp. *bulgaricus* were able to reverse the state.

The use of LAB for therapeutic purposes is still limited and it is necessary to know the mechanisms by which LAB stimulate the immune system. Thus it is very important to determine:

- how long the optimal dose lasts.
- which type of immune cells are stimulated.
- why the given dose is so important.
- why some LAB can stimulate the secretory immune system and others can not.

We demonstrated that at optimal dose *L. casei* maintains the IgA secreting cells and s-IgA level only for 3 days. We also determined IgA, $CD4^+$ and $CD8^+$ T cells. An increase in the IgA cells and $CD4^+$ T cell and maintenance of the relation $CD4^+/CD8^+$ were observed. If this relation is altered there is not a good mucosal immunity.

In the immune system activation by LAB, we observed the importance of avoiding bacterial translocation and the increase in the number of intraepithelial lymphocytes (IEL).

ADHESION AND PROLIFERATION OF PROBIOTIC STRAINS IN THE INTESTINE AND THEIR IMPORTANCE FOR THE PROBIOTIC EFFECT

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INTRODUCTION

Probiotics have invaded human consumption and can be found in infant food, in fermented milks and in pharmaceutical products. They have been defined by Fuller (1989) as "live microorganisms which benefically affect the health of the host by improving its intestinal balance". Recent advances in probiotic research have confirmed the health benefits of some probiotic lactic bacterial strains. Lactobacilli and bifidobacteria are the microorganisms the most frequently studied.

BIFIDOBACTERIA AND *LACTOBACILLI* IN THE ECOLOGY OF THE DIGESTIVE TRACT

Bifidobacteria are normal resident bacteria in human microflora. They can become established during the first 2 weeks of life and are present in the dominant intestinal human adult microflora at about $1 \cdot 10^9$ bacteria/g. *Lactobacilli* are found occasionally in the intestinal microflora of infants and are detected in the subdominant microflora of human adults. *Lactobacilli* are able to adhere to the stomach mucosa of mice, rat [1] and pig [2], and the crop mucosa of chicken [3]. They have been shown to adhere to intestinal biopsies of human jejunum or colon [4].

ANTIMICROBIAL EFFECTS OF LACTIC PROBIOTICS

One of the best documented effects observed in clinical and experimental studies is the antidiarrhoeic activity of lactic probiotics: they shorten the time diarrhoea in infants; this has been observed with: *L. acidophilus* LB [5], *L. casei* GG [6–8], and *Bifidobacterium sp.* [9, 10]. In human adults, the strain LGG has been shown to dimínish the risk of travellers' diarrhoea [11]. Another clinical effect shown recently is the antagonistic effect against *H. pylori* exerted by the spent-culture supernatant of *L. acidophilus* LA1 in humans [12].

IMMUNOLOGICAL PROPERTIES OF LACTIC PROBIOTICS

Lactic probiotics stimulate the non-specific immunological response. The ingestion of *L. acidophilus* LA1 increases the level of non-specific IgA antibody response in healthy volunteers [13]. LA1 and LGG are also responsible for the augmentation of the number of intraepithelial lymphocytes in germ-free mice inoculated with these strains [14]. Several strains of *Lactobacillus* have also been shown to increase the secretion level of IFN α and γ [15]. Lactic probiotics stimulate the specific immunological response to antigens [16] and pathogens. The ingestion of LGG increase the titre of specific IgA to rotavirus in convalescent infants after diarrhoea [17] or after vaccination against rotavirus [18]. Ingestion of fermented milks containing LA1 associated or not to *Bifidobacterium* sp. is able to increase the level of specific IgA to *Salmonella* after vaccination by an attenuated strain of S. *typhi* in healthy volunteers [19]. Perdigon et al. [20] showed that the ingestion of *L. acidophilus* or *L. casei* decrease the infection rate of *Salmonella* in mice and simultaneously increase the specific IgA response to *Salmonella*.

The mechanisms of the beneficial effects of fermented milks or probiotics are not yet well understood. One of the interesting questions is: is it possible to improve scientifically the choice of lactic bacteria for the use of fermented milks in particular situations ? Our laboratory has tried to answer this question.

ADHESION OF LACTIC BACTERIA TO HUMAN INTESTINAL CELLS

The first point we investigated was the adhesion of lactic bacteria to human intestinal cells. The models used were Caco-2 cells and HT29-MTX cells, issues of human colon carcinomas and showing the characteristics of mature enterocytes of the small intestine and goblet cells, respectively. Among a panel of 9 dairy and 16 human *Lactobacillus* strains tested, only a few were able to adhere to the cells (LA1 [21], LB [22], BG2FO4 [23]). The adhesiveness was not related to a particular species of *Lactobacillus*. Moreover, 13 human *Bifidobacterium* strains were tested for their ability to adhere onto human enterocyte-like cells [24]. A high level of calcium independent adherence was observed for 5 of them (Bbr4, Bl1,

B28, B20, B29). As observed by scanning electron microscopy, adhesion occurs at the apical pole of the Caco-2 cells and the mucus secreted by the HT29-MTX cells.

IN VITRO ANTIMICROBIAL PROPERTIES OF THE LACTIC PROBIOTICS.

We investigated the antimicrobial properties in vitro of adhering human lactic bacterial strains against colonization of cultured intestinal cell monolayer by a variety of diarrhoeagenic bacteria: *Salmonella typhimurium* and 3 strains of pathogenic *E. coli*: enterotoxinogenic, enteropathogenic, and diffusely adhering. The human *L. acidophilus* strains LA1 [21], LB [25] and the human *Bifidobacterium brève* 4 strain were shown to inhibit the cell association of these pathogenic bacteria to the enterocytic Caco-2 cells, in a concentration-dependent manner [24]. Heat-killed *L. acidophilus* LB were slightly less active than living bacteria [26].

SECRETION OF ANTAGONISTIC SUBSTANCES BY LACTIC BACTERIA

We have suspected the secretion of antagonistic substances against pathogenic bacteria in the culture of some lactic bacterial strains. When *S. typhimurium* is incubated with the spent-culture supernatant of LA1 in the ratio 1/1, the number of viable cells decreases 100 fold after 1 h of contact. We demonstrated that lactic acid is not responsible for the antagonistic activity of LA1. After treatment by proteasic enzymes, the antagonistic activity is only slightly decreased but does not disappear. This antagonistic activity modifies the ability of *Salmonella* to invade the enterocyte-like cells. When *Salmonella* is treated during 1 h or 2 h with the spent supernatant of LA1, then centrifuged and washed before being incubated with the Caco2 cells, the number of *Salmonella* associated to the cells is not modified but the number of the intracellular bacteria is strongly reduced. The same result was obtained with the LB and B28 strains. Apparent alteration of the surface of *Salmonella* and diffusely adhering *E. coli* could be seen by transmission electron microscopy after they were subjected to the spent-culture supernatant of LB, LA1 and B28.

IN VIVO ANTAGONISTIC EFFECT BY LACTIC PROBIOTICS

The different antagonistic effects exerted by lactic probiotics have been demonstrated in vitro on intestinal cultured cells. We investigated if they were effective in vivo in animal models. We have developed an animal model of C3HOuj/co mice infected by a strain of *S. typhimurium*. Two situations were studied: conventional mice and germ-free mice. In conventional mice the lactic bacteria are given daily to the animals: like probiotics, they transit along the digestive tract without colonizing the gut. On the contrary, in germ-free animals, the effect of a strain colonizing the different segments of the digestive tract was studied. The strain is given once and becomes established in the gut. Conventional C3H/HE/oujco mice received fresh cultures of *L. casei* GG or *L. acidophilus* LA1 daily after the inoculation of *S. typhimurium* C5 [27]. The number of faecal *Salmonella* was reduced significantly in mice treated daily with each *Lactobacillus* strain. These results show that the LA1 and the LGG strains, given as probiotics to C3H mice, are able to decrease the number of *Salmonella* detected in the faeces.

In germ-free C3H mice, the population levels of GG were similar in the stomach and in the distal digestive tract ($1 \cdot 10^9$ cfu/g). LGG was found associated with the stomach mucosa and the intestinal wall increasingly from proximal to distal intestine. LGG was also detected in the mesenteric lymph nodes at about 100 bacteria/g of organ, indicating a slight translocation of the strain. Two days after the inoculation of the strain C5 to the LGG monoassociated mice, the *Lactobacillus* strain was detected at a low level between 10^4 and 10^5 and then at 10^3 bacteria/g during 2 to 4 and 9 days post-inoculation, respectively. The establishment of LGG in the gut was able to delay the mortality of the animals, since 100% of the germ-free mice were dead at 9 days post-inoculation, while all the LGG monoassociated mice were still alive. However, the LGG did not protect the monoassociated animals completely since all of them died at 15 days post-inoculation. These results obtained in vivo show that the adhesion of LGG and the low remaining level of this strain is able to delay the mortality of the animals. The mechanism of action remains unknown. It could be related to the immunological properties of LGG, to its adhesion properties, or to the antagonistic substance of the LGG.

CONCLUSION

Upstream to the clinical studies, the selection of lactic bacteria is crucial. Several biological and microbiological criteria in selection and preparation of *Lactobacillus* strains for use as dietary adjuncts, have been originally listed by Klaenhammer [28] and have been more recently updated by a consensus panel of experts who established the list of requirements for the health attributes of lactic cultures. Our results show that some, but not all, the strains of *Lactobacillus* or *Bifidobacteria* can adhere to the human intestinal cells. The adhesiveness properties have been observed mostly for strains of human intestinal origin. Enteroadherent lactic bacteria inhibit the adherence and cell-entry of enterovirulent bacteria to human enterocyte-like cells. In infected conventional and axenic mice, the enteroadherent lactic bacteria develop antagonistic activity against *Salmonella* infection. Antibacterial activity is present in the spent culture supernatant of several enteroadherent lactic bacteria. Finally, the results presented here give information on the mechanisms of action of lactic bacteria and other new criteria for the upstream selection of probiotic bacteria strains, together with the industrial criteria

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SAFETY CONSIDERATIONS REGARDING PROBIOTIC STRAINS

Conclusions of two specialist workshops

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INTRODUCTION

Lactic Acid Bacteria (LAB) have been used since antiquity in a large number of food fermentations, where their activity is of prime importance for the keeping quality, taste and texture of the final product. The long history of their safe use in traditional fermented foods means that LAB, isolated from these sources, are regarded as non-toxic, food-grade microorganisms. Reports have, however, appeared in the recent literature that some strains of LAB species might have been involved in clinical infections and it is important that some common opinion is reached on the significance of this. Therefore LABIP, the Lactic Acid Bacteria Industrial Platform, an organization of a group of EU industries involved in the use and production of LAB and especially interested in following closely all EU-funded research in this area, convened a workshop on "The Safety of Lactic Acid Bacteria" (Frankfurt am Main, November 1994) during which about 20 leading European scientists in the fields of food- and medical microbiology, gastroenterology and nutrition shared their ideas on this topic. In November 1995 a second workshop was held on "Lactic Acid Bacteria as Probiotics". Both workshops have been sponsored by the European Commission, DG 12.

The key objectives of the workshop were:

(1) to bring leading EU food- and medical microbiologists and gastroenterologists together to discuss the safety/the probiotic properties of LAB
(2) to arrive at a clearly written consensus document on the safety / probiotic properties of LAB.

The document of the first workshop has meanwhile appeared. The workshop appeared to be the first event where medical- and food microbiologists discussed this topic together.

RISK GROUPS AND RISK FACTORS FOR PATHOGENICITY DUE TO LACTIC ACID BACTERIA

We are dealing with extremely low risks because of the very low numbers of infections as compared to the high number of people exposed to LAB. The following conclusions can be drawn from literature.

(1) Concerning risk factors linked to the host
- All cases of infections with LAB published to date were observed in people with underlying conditions, especially abnormal valves for endocarditis and decreased defence in all other cases [1–3].
- Other risk factors known for opportunists such as extremes of age or pregnancy have not been identified as risk factors for infections by LAB and there is thus no recommendation to be made in this particular area.
- No case of infection with LAB has been observed in otherwise healthy people or pregnant women.
- No case has been observed in people working with LAB and therefore challenged with higher numbers of LAB than the general population.
- No case has been linked to the consumption of fermented foods, probiotics or drugs containing LAB.

(2) Concerning the risk depending on LAB
- *Lactobacilli*
  Most of the strains (but not all) isolated from clinical cases belong to the species *L. rhamnosus, L. casei* (or *L. paracasei*) and *L. plantarum* (for a review see Gasser [3]). These species are also among the most frequently found in man's indigenous flora. There is only limited information concerning the risk linked to potential virulence factors present in *Lactobacillus* strains. In strains isolated from human endocarditis, some factors possibly linked to virulence (adhesion, platelet aggregation, enzyme content) have recently been identified by the group of Knox in Australia, but these factors are also present in the majority of the strains of the general population of oral lactobacilli [4–6]. Infections involving lactobacilli are extremely rare and considered to be of endogenous origin. The risk of infection of exogenous origin is negligible. The "Berufsgenossenschaft der Chemischen Industrie" in Germany has recently assigned the various microbial species to four different risk groups according to the individual hazards that may be involved in handling these organisms (group 1: no risk; group 2: small risk; group 3: moderate risk; group 4: high risk). Although *L. rhamnosus* has been placed in group 2, based on the information mentioned, it was the general conclusion of this workshop that all lactobacilli should belong to group 1, but that the species mentioned warrant further careful surveillance.
- *Lactococci and leuconostocs*
  Strains from these genera can occasionally be found in clinical samples but most often are found as co-isolates without any evidence of a primary role in the infections. They consequently can be considered as safe and can be classified in group 1.
- *Enterococci*
  Enterococci are normal human commensals well adapted to life conditions of most of the septic compartments of the human body. They represent, for example, the predominant Gram-positive cocci in the human faeces (10^5–10^7 CFU/g). However, strains of *E. faecalis* and *E. faecium* are frequently involved in various infections and vancomycin resistant strains are emerging. Several virulence factors have been described, including adherence to and invasion of host tissues, modulation of host immunity and secretion of lytic products [7]. The distribution of these factors among the commensal population of enterococci is unknown. Therefore the potential risk presented by enterococci from endogenous as well as exogenous origin cannot be quantified but the frequency of *Enterococcus* infections shows that it is far higher than for other LAB. Enterococci can readily be isolated from most food processing lines, and foods and probiotic products containing such strains, which are used without established risk, have been on the market for a very long time. Therefore it is concluded that, if someone, at this moment, intentionally wants to use such microorganisms, it must be done in the knowledge of this background and that there should be demonstrable positive effects.

RECOMMENDATIONS FOR FURTHER RESEARCH

(1) To increase the knowledge on the intrinsic properties of the LAB introduced in food or used as probiotics. This includes the antibiotic resistance profile, contents of transmissible plasmids, some enzymatic activity including the abilities to deconjugate and/or to dehydroxylate bile salts or to metabolize some amines.

(2) To study the pharmacokinetics of ingested LAB. This can be done in vivo in man but also in vitro, using models that are either static (test tubes containing acid or bile) or, even better, dynamic, simulating the human intestinal tract.

(3) To assess some risks linked to metabolic properties of some LAB strains (quantification of the transfer of plasmids, extent of bile salt metabolism) which can be done using in vitro models simulating the small intestine or the colon.

(4) Virulence factors of LAB are not yet clearly identified and validated methods to assess them are still lacking. Adhesion properties, binding to fibronectin, etc., are currently being studied in connection with the risk of endocarditis by the group of Knox. Systematic studies on the presence of properties that can be described as potential virulence factors have to be done in endogenous strains of LAB and it has to be established in animal studies whether these factors are needed for colonization. Also animal models have to be found to test the validity of the properties that have been described as virulence factors in vitro. For endocarditis, animal models are known.

(5) "Surveillance" of people ingesting LAB and the occurrence of infections (phase IV studies of drugs) seems a very good approach to increase our knowledge. This should include a clear identification of LAB isolated from infections by reference laboratories and methods.

(6) To study the effects of the massive ingestion of LAB on the transient composition of the digestive flora and on the possible translocation of the ingested bacteria into the bloodstream.

(7) Studies on interactions of probiotics with the immune system of immuno compromised people.

(8) Studies on selection of low- or non-adjuvant strains.

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PROBIOTIC STRAINS AND HEALTH

PROBIOTICS FOR FARM ANIMALS

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ABSTRACT

The use of probiotics for farm animals is still increasing. Time and cost considerations tend to limit the amount of experimental work which is undertaken. The work which has been done has yielded inconsistent results but the positive research findings which have been obtained continue to confirm that under the right conditions probiotics can have beneficial effects on growth rate, feed conversion and resistance to disease. The recent results obtained in chickens, pigs and cattle with probiotics based on lactic acid bacteria and fungi are discussed and possible mechanisms considered.

INTRODUCTION

The on-farm use of probiotics for cattle, pigs and chickens continues to expand but experimental work aimed at improving our knowledge of how they work, what are the optimal conditions for activity and development of more effective strains, has not received the funding required to underpin the practical applications of probiotics. Consequently, few field trials have been done. Because of this lack of fundamental knowledge about the mechanism of the probiotic effect, those trials which have been done have yielded variable results.

ANIMAL TRIALS

A typical example is a trial done in Spain by Tortuero et al. [1]. They compared two probiotic preparations containing (a) *Enteroccus faecium* and Lactobacillus casei and (b) the two yoghurt starter organisms, *Streptococcus salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. In the first experiment the preparation containing *E. faecium* and *L. casei* increased the weight gain during the experimental period up to 21 days of age. The yoghurt-starter cultures also gave a positive response but only between 12 and 21 days of age. However, when the preparation containing *E. faecium* and *L. casei* was retested, it failed to improve the weight gain. The authors draw attention to the fact that the second test was done with pigs from split litters, whereas the first experiment was carried out with piglets distributed at random on to the two treatments. The significance of this is difficult to assess at the moment and no doubt there were other factors which varied between the two trials.

This sort of result is helpful in the sense that it confirms that under the right conditions a significant growth response can be obtained. But it also illustrates the kind of confusion that exists in this type of experimentation where attempts to repeat a positive result fail and, because of the difficulty of ensuring that all the relevant factors other than supplementation are constant, it is impossible to explain such a failure.

There was a larger decrease in coliform count from $\log_{10}$ 8.10 down to 6.85 in the group, showing the better improvement in growth rate. This group also showed an increase in the concentration of interleukin 2 (from 3.74 ng/g up to 7.43 ng/g).

Neither of these differences was statistically significant; however, they do agree with other studies showing effects on coliform count and immune status.

An extensive and well conducted poultry trial was published earlier this year [2]. This measured a wide range of features, including growth rate, feed conversion and egg production. The feed supplement used was a lactobacillus but no further information regarding species identification was given. The trial looked at effects occurring during the pullet phase (7–19 weeks) and during the egg-laying phase (20–59 weeks).

During the pullet phase, feed consumption and weight gain increased as a result of feeding the lactobacillus supplement, but for the layers there was no effect on weight gain. However, there was increased daily feed consumption and increased egg size. The quality of the eggs was not affected. In the past, several studies have shown positive effects on egg production but they were not statistically significant. To my knowledge, this is the first paper to describe an effect on eggs which is significant.

An interesting new approach to probiotic administration to chickens has been used recently. Embrex, in the States, have developed a device for inoculating eggs with vaccine. In collaboration with Probiotics International Ltd, UK, trials have been done with the multistrain probiotic, Protexin. Eggs at 18 days' incubation were inoculated with Protexin into the airsac or amnion. This procedure had no effect on hatchability; in fact, the injected eggs had slightly increased hatchability and accelerated hatch date. The results so far available are very preliminary, but the mean figures from two trials indicate that there is an increase in body weight at 2 weeks of up to 8.7%, depending on the dose and the site of inoculation.

The work on probiotics for cattle has increased in recent years. In calves, studies have been conducted using *Saccharomyces cerevisiae, Aspergillus oryzae*, various species of lactobacillus and *Enterococcus faecium*. In the last 10 years, positive effects (not always statistically significant) have been found for feed intake, weight gain, earlier weaning, decreased scouring, decreased faecal coliform count and reduced demand for antibiotic treatment.

It is interesting to note that benefits need not always be measurable in terms of increased growth rate or feed efficiency; as in the case of a study by Seymour et al. [3] last year, the effect may be demonstrated by monitoring the days of fever experienced by the animal and the number of antibiotic treatments required to maintain it in good health.

In adult cattle the studies have used mainly the fungal probiotics. Using this type of preparation for beef cattle, recent trials have shown improvements in feed efficiency and dry matter intake. Analysis of all the published data on this subject indicated that the average increase in daily gain of cattle fed yeast culture was 7.3%. The corresponding figure for feed efficiency was 6.0% (J.T. Huber, unpublished data, 1996).

Numerous studies have been done with lactating cattle. Over several years the average increase in milk yield of cows treated with *Aspergillus oryzae* was 2.5%. Increases in milk yield have also been obtained by supplementation with yeast. Both types of supplementation have induced improved butterfat concentrations in milk.

MECHANISM

Some information is now available on the mechanism of probiotic activity in cattle and some informed speculation can be made. In vitro and in vivo studies with fungal probiotics have shown an improvement in digestion of fibre. One of the curious features of the fungal probiotic effect is that it occurs without the probiotic organisms being able to multiply in the rumen. In vitro studies with rumen liquor suggest that the probiotic will survive for about 6 h. With the limited amount of knowledge available it has been suggested that fungal probiotics may produce their beneficial effects in the following ways:

(a) by stimulation of indigenous rumen fungi. There is some evidence that this might be occurring from in vitro studies which showed that *Aspergillus oryzae* can improve the growth of the rumen fungus *Neosallimistic frontalis* [4];

(b) by increasing the number of cellulolytic bacteria in the rumen. There is also experimental evidence for this; *Saccharomyces cerevisiae* stimulated the growth of the rumen bacterium *Fibrobacter succinogenes* [5];

(c) by improved rumen metabolism resulting form decreased concentration of lactic acid which, in turn, is due to stimulation of lactic acid fermenting bacteria such as *Selenomonas ruminantium* and the consequent reduction in pH. However, this effect on pH is small and is considered by some workers to be unlikely to have any significant effect on rumen metabolism;

(d) by removal of sugars, toxic metals or molecular oxygen, all of which can inhibit the growth of cellulolytic bacteria.

PREBIOTICS

A recent development which, although not strictly covered by the definition of probiotics but is closely related conceptually, is the use of specific substances to stimulate the growth of desirable bacteria already present in the gastrointestinal tract, particularly of the colon. The approach has been used for many years in the form of lactulose supplementation, but recently the emphasis has shifted to oligosaccharide substances based on various sugars and derived from various substrates. Although not totally specific, they tend to stimulate the bifidobacteria much more than other groups of intestinal bacteria. Most of the published work relates to humans, and significant changes have been obtained with respect to both composition and activity of the colon microflora [6]. This type of product has been given the name Prebiotic.

A mannan oligosaccharide has been used in studies with turkey poults [7]. Inclusion of the supplement at a rate of 0.11% of diet resulted in significant weight gains and improvement of feed conversion on poults up to 8 weeks of age. There were also significant increases in plasma IgG and bile IgA, indicating an effect on the immune system [8]. However, whether this is sufficient to provide protection against infection has yet to be determined.

The so-called prebiotics are an attractive development because they are non-viable. This removes from the manufacturers the problem of maintaining viability in a product which may have to tolerate large variations in moisture and temperature during its use on the farm. Several studies have shown how it is not always possible to rely on the product description which appears on the label. Not only may the viable count be low, the specification of bacterial content may also be misleading, with the claimed component replaced by a completely different microorganism. A non-viable preparation would remove these problems and would allow the use of genetic manipulation to improve yields without the attendant problem of release into the environment of genetically modified viable microorganisms. However, it may not be possible, even when we know enough about the biochemical basis of the probiotic effect, to reproduce the probiotic effect with a non-viable supplement. The unique quality of the probiotic is that it is designed to colonize and produce the probiotic agent at the required target site in the gut. For example, it can grow and metabolize in the colon and produce a substance that would be readily digested if administered by mouth.

CONCLUSION

There is an enormous potential for the use of probiotics in farm animal feeds but, as with probiotics used in other contexts, we need to know more about the fundamental mechanisms of probiotic activity. The most effective way of achieving this end is by a thorough understanding of the gut microflora and its interaction with the host animal.

This is a very time-consuming and expensive project and, in the present economic climate, is unlikely to be funded adequately. In the meantime, we must continue to gain information on the optimal conditions for the probiotic effect and attempt to produce more consistent results in field trials with farm animals.

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POTENTIAL OF PROBIOTIC STRAINS IN STABILIZING INTESTINAL MICROFLORA TO PREVENT GASTRO-INTESTINAL INFECTION

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Since Metchnikoff in 1908 suggested that consumed milk fermented with lactobacilli may displace toxin producing microorganisms, and thereby prolong life in man, several studies have been carried out to determine the beneficial effects of probiotics. A wide range of microorganisms have been used as probiotics, ranging from Lactobacillus species and Bifidobacterium species to Enterococci, Clostridium, Coli and Yeast. The ability of probiotics to prevent gastrointestinal infections and other beneficial health effects may be influenced by several characteristics of the microorganisms: (1) the ability to survive and colonize; (2) production of enzymes; (3) production of bacteriocins and other antibiotic-like metabolites; (4) stimulation of the immuno-system.

Beneficial effects have been shown concerning Traveller's diarrhoea, antibiotic associated diarrhoea and prevention of Rota-virus shedding and infection. Treatment of recurrent *Cl. difficile* infection has been demonstrated in some smaller studies. Other studies have failed to reproduce these findings.

Concerning colonization, some promising work has been done, but so far it is very difficult to compare the studies, and it is important to carry on similar studies with standardized concentrations of identical probiotics for different places in the world in different populations. Finally, it is very important to find out how a beneficial probiotic functions in the intestine, where the target is and under what conditions probiotics may work.

Studies remain to be done to evaluate which probiotic to choose, and for what reason: should it be yeasts, lactobacilli, enterococci or perhaps *E. coli*, not to mention which subspecies.

LACTOBACILLI IN THE TREATMENT OF GASTROINTESTINAL DISORDERS IN CHILDREN

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GUT MUCOSAL BARRIER

The intestinal mucosa is an important organ of defence, providing a barrier against the antigens encountered by the enteric route, and most foreign antigens are excluded by the intestine's mucosal barrier. Apart from the barrier function, the intestinal mucosa is efficient in assimilating antigens. In health, antigen transfer is well controlled and aberrant antigen absorption does not occur. Intestinal antigen handling determines subsequent immune response to the antigen [1].

The barrier functions are incompletely developed in early infancy. The binding of antigens to immature gut microvillus membrane is increased compared to the mature mucosa, which has been shown to correlate with the increased uptake of antigens [2]. An increased antigen load may evoke aberrant immune responses and lead to sensitization [3]. Intestinal permeability can be secondarily increased as a result of inflammation in the intestinal mucosa induced by viruses, bacteria or dietary antigens. A greater amount of antigens may thus traverse the mucosal barrier and the routes of transport may be altered.

PROBIOTIC BACTERIA

Gut microflora is an important constituent in the intestine's defence barrier. It has been shown that in the absence of the intestinal microflora antigen transport is increased [4]. Earlier studies have indicated that specific strains of lactobacilli have a beneficial effect on the clinical course of rotavirus diarrhoea [5, 6]. Oral introduction of probiotic bacteria has been associated with alleviation of intestinal inflammation and normalization of increased intestinal permeability, and promotion of the intestine's immunologic barrier, particularly intestinal IgA responses [1, 7].

The contribution of intestinal microflora in modification of dietary antigens to tolerogens was investigated. Part I of the study consisted of lymphocyte proliferation tests in 7–9 healthy adults to assess the mitogen-induced proliferative responses of peripheral blood mononuclear cells to cow milk proteins with and without the in vitro degradation by *Lactobacillus* GG (ATCC 53103). Part II of the study included 14 infants fed an extensively hydrolysed whey formula (group WF) and 13 given an extensively hydrolysed whey formula containing *Lactobacillus* GG $5x10^8$ cfu /g (group WF-GG). The patients were clinically examined and the severity of the skin condition (SCORAD) was graded before and 1 month after commencing the therapy. The concentrations of tumour necrosis factor-α, eosinophil cationic protein and α-1 antitrypsin in faeces as indicators of intestinal inflammation were determined.

Part I of the study indicated that the hydrolysis of α_{s1}- and κ-caseins with *Lactobacillus* GG-derived proteases reversed the stimulation of proliferation by κ-casein and enhanced the suppression by α_{s1}-casein in healthy controls [8]. Without hydrolysation, casein increased the production of interleukin-4 in cultures of patients with atopic dermatitis. *Lactobacillus* GG-hydrolysed casein reduced the production of interleukin-4 in cultures from healthy controls and patients with atopic dermatitis [9].

Part II of the study indicated a more rapid alleviation of intestinal inflammation in group WF-GG: a reduction in SCORAD scores was seen after 1 month of therapy, indicating a significant improvement of atopic eczema [10].

These results indicate that by promoting endogenous barrier mechanisms, probiotic bacteria might have a role in the treatment of gastrointestinal disorders associated with clinical conditions with impaired mucosal barrier function. The capability of intestinal bacteria to modify immunoactivity to food antigens may introduce an immunotherapeutic model for food allergy.

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ROLE OF CULTURED AND CULTURE-CONTAINING DAIRY PRODUCTS AND PROBIOTIC BACTERIA IN HEALTH AND DISEASE

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INTRODUCTION

A large number of studies on probiotic lactic acid bacteria and bifidobacteria have appeared in the literature again. We have tried to summarize most of the studies, and a compendium on human study results is presented in Table 1. Animal and in vitro studies are discussed.

PROBIOTIC AND PREBIOTIC EFFECT ON GUT MICROFLORA

The effect of the prebiotics of transgalactosylated disaccharide containing a mixture of β-1-4 linked galactooligosaccharides (4'-GOS) was investigated on the faecal microflora and their metabolism in 20 healthy adults. The consumption of 4'-GOS (2.5 and 10 g/day) for 3 weeks showed a significant increase in the number of bifidobacteria. Although not significant, 4'-GOS feeding did result in a decrease in the secondary bile acids, deoxycholic acid and lithocholic acid (Ishikawa et al. 1995). The probiotic effects of *Bifidobacterium thermophilum*, *Enterococcus faecium* and *Lactobacillus acidophilus* on weight gain and prevention of diarrhoea were investigated in newborn piglets and calves. The average weight gain of piglets in the probiotic-fed group (n=16) was higher than that of control group (n=15), with a tendency to decrease in the frequency of diarrhoea. In the trial of newborn calves (n=137), administration of probiotics was also effective in increasing the weight gain and decreasing in the frequency of diarrhoea (Abe et al. 1995).

IMMUNE RESPONSE

A double-blind trial was conducted in 138 patients with superficial transitional cell carcinoma of the bladder following transurethral resection to evaluate the prophylaxis of recurrence by an oral *Lactobacillus casei* (BLP) preparation. The study reported effective prevention for recurrence and the effect was related to immune enhancement (Aso et al. 1995). A preparation of peptidoglycan (PG) of *Bifidobacterium thermophilum* of swine was administered orally to SPF-C57BL/6CrSLc mice in order to confirm the enhancement of the cytotoxic activity of natural killer cells (NK), intraperitoneal cytotoxic T lymphocytes (CTL) and lymphocytes stimulated concanavalin A (Con A-stimulated lymphocytes). A significant higher rate of cytolysis of the NK cells from the spleen and the mesenteric lymph node (MLN) was observed in mice fed PG-mixed feed for 3 weeks. However, a single oral administration of PG had no significant effect on NK activity. The PG-mixed feed administered group showed a higher CTL activity and cytotoxic activity of Con A-stimulated lymphocytes as compared with that of the control group. These results indicate that the cytotoxic activity of mice was enhanced by oral administration of PG (Sasaki et al. 1994). The antitumour mechanisms of cell wall preparation of *Bifidobacterium infantis* (WPG) was investigated (Sekine et al. 1995). In brief, WPG enhanced the in vitro antitumour activities of mouse peritoneal exuadate cells elicited with proteose-peptone and thioglycolate broth determined by cytostatic and cytolytic assays. Tumour necrosis factor-α (TNF-α) and reactive nitrogen intermediates (RNI) were responsible for such augmented cytotoxicity. Oral administration of *Bifidobacterium brève* YIT 4064 augmented anti-rotavirus IgA production in the mouse mammary gland and intestine(Yasui et al. 1995). Thus, mouse pups born to and nursed by dams fed *B. brève* YIT 4064 and immunized orally with rotavirus were more strongly protected against rotavirus-induced diarrhoea than those born to and nursed by dams immunized against rotavirus only.

GASTROINTESTINAL DISEASES

A new study on traveller's diarrhoea was reported by Katelaris et al. (1995). The efficacy of *Lactobacillus acidophilus* (NFCB 1478) and *Lactobacillus fermentum* was studied in a randomized placebo controlled trial involving British soldiers deployed to

Table 1: New studies on specific probiotic strains

Strain	Effect	References
Lactobacillus acidophilus LA1	Enhancement of anti-infective mechanisms, in vitro inhibition of *Helicobacter pylori*	Schiffrin et al. (1996)
Lactobacillus casei Shirota	Absence of 7α-dehydroxylase activity, Shortening of rotavirus diarrhoea	Takahashi & Morotomi (1994) Sugita & Togawa (1994)
Lactobacillus GG (ATCC 53103)	Reduction in the duration of rotavirus diarrhoea, stabilization of mucosal barrier, increased rotavirus specific IgA response at convalescence, degradation of food antigens, down-regulation of T-cell responses, treatment of *C. difficile* colitis in children	Raza et al. (1995) Majamaa et al. (1995) Salminen et al. (1996) Sütas et al. (1996) Billar et al. (1995)
Lactobacillus reuteri *Lactobacillus casei* (Danone 001)	Reduction in the duration of rotavirus diarrhoea Survival in an in vitro model of gi-tract, Reduced duration of infant diarrhoea, increased resistance of mice to cholera and *Salmonella typhimurium*	Casas et al. (1995) Paubert-Braquet et al. (1996) Portier et al. (1993) Reinert et al. (1995)
Lactobacillus acidophilus NFCB 1748	Reduction in radiotherapy related late effects	Salminen et al. (1995)
Bifidobacterium 173010	Good survival, recovery from terminal ileum, decreased colonic transit,	Grimaud et al. (1995)
Lactobacillus plantarum	Not effective in preventing traveller's diarrhoea	Katelaris et al. (1995)
Bifidobacterium animalis	Prevention of rotavirus diarrhoea	Saavedra et al. (1995)

Belize. Overall, 282 subjects were randomized to receive one or the other of the lactobacilli strains or placebo beginning the day before travel and continuing for 3 weeks after arrival. There were no significant differences in the incidence of diarrhoeal episodes between subjects in any of the three groups after 3 or 4 weeks, indicating that these lactobacilli preparations were not protective in this geographical area (Katelaris et al. 1995; Katelaris 1996)

Lactobacillus reuteri has been shown to decrease the duration of rotavirus diarrhoea in children (Casas et al. 1995). *Lactobacillus* GG has been proven effective in watery diarrhoea in several studies in Asia (Raza et al. 1995).

Different lactic acid bacteria were compared for their effects on the immune response to rotavirus in children with acute rotavirus gastroenteritis (Kaila et al. 1992; Majamaa et al. 1995). Serum antibodies to rotavirus, total number of immunoglobulin-secreting cells (ISC) and specific antibody-secreting cells (sASC) to rotavirus were measured at the acute stage and at convalescence. The treatment with *Lactobacillus* GG was associated with an enhancement of IgA sASC to rotavirus and serum IgA antibody level at convalescence. Another study was made to compare the immunological effects of viable and heat inactivated lactic acid bacteria (Majamaa et al. 1995). *Lactobacillus* GG administered as a viable preparation during acute rotavirus gastroenteritis resulted in a significant rotavirus specific IgA response at convalescence. The heat inactivated *Lactobacillus* GG was clinically as efficient, but the IgA response was not detected. This result suggests that viability of the strain is critical in determining the capacity of lactic acid bacteria to induce immune stimulation. Another placebo-controlled study on treatment of acute diarrhoea in children with *Lactobacillus* GG is ongoing in Peru. The first report indicated that colonization of children was observed (Sheen et al. 1995).

The capacity of *Lactobacillus* GG to degrade food antigens, and thereby modify their immunoactivity was investigated (Sütas et al. 1996). For this purpose, the immunoactivity of caseins and *Lactobacillus* GG-degraded caseins was assessed in lymphocyte transformation tests in healthy adults. Casein, α_{s1}-casein, β-casein suppressed the lymphocyte proliferation capacity, whereas κ-casein induced it. Degradation of casein, α_{s1}-casein and β-casein by *Lactobacillus* GG enhanced the suppressive effect of these caseins on the lymphocyte proliferation capacity. Degradation of κ-casein by *Lactobacillus* GG reversed its inducive effect to profound suppression. These results indicate that *Lactobacillus* GG can degrade food antigens such as bovine casein, and downregulate T-cell responses. The results were identical in healthy children and in children with atopic dermatitis (Sütas et al. 1996).

RADIOTHERAPY SIDE EFFECTS AND CULTURED MILKS

In a randomized study, it was earlier reported that patients receiving pelvic radiotherapy and a fermented milk containing viable *Lactobacillus acidophilus* NCFB 1748 had a significant decrease in diarrhoea (Salminen et al. 1988). In a 5-year follow-up study there was a trend to less late serious intestinal complications (Salminen et al. 1995). It has been shown that *Lactobacillus acidophilus* NCFB 1748 has the ability to colonize human colon mucosa in vitro even though adhesion to Caco-2 cells is relatively small. In a Swedish study (Henriksson et al. 1995) fermented milk intake decreased the severity of late effects caused by pelvic radiotherapy. This indicates an important role for lactic acid bacteria in the intestinal tract following radiotherapy.

ANTIMUTAGENIC PROPERTIES

Antimutagenic activities of fermented milks on the mutagenicity of Trp P-2, MNNG, B[α]p, AF2 and AB1 were investigated by the Ames test (Tamai et al. 1995). The fermented milks produced by mixed cultures with various kinds of lactic acid bacteria and yeast (*Lactococcus, Streptococcus, Leuconostoc, Lactobacillus, Bifidobacterium* and *Saccharomyces*) showed more effective antimutagenic activity over a wider range of mutagens (18–75%) than those cultured with a single strain of lactic acid bacteria (0–79%). Although the mechanism remains to be solved, the antimutagenic activity against mutagens was proportional to the amount of the fermented milks.

In a new study, *Lactobacillus* GG was shown to cause a reduction in DMH induced tumour formation in rats. It was reported that *Lactobacillus* GG inhibited the initiation and early phase promotion on the tumorigenesis process (Goldin et al. 1996). However, the organism was not able to prevent the growth of tumours once they had been established (Goldin et al. 1996).

NEW STUDIES ON HEALTH EFFECTS

In a Dutch study it was observed that calcium in milk and fermentation by yoghurt bacteria (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) increased the resistance of rats to salmonella infection (Bovee-Oudenhoven et al. 1996). It was observed that when rats were given low calcium milk followed by *Salmonella enteriditis* challenge they had reduced colonization resistance when compared to high calcium milk or pasteurized yoghurt.

The effects of dietary undigestible oligosaccharides (lactulose, fructooligosaccharide, gentioologosaccharide, maltooligosaccharide, glucomanooligosaccharide and galactooligosaccharide) on the development of galactosamine hepatitis in rats was investigated (Wang et al. 1995). Briefly, 4-week-old Wister rats were fed diets containing 10% oligosaccharides or monosaccharide, besides the standard diet, for 2 weeks after 1 week of prefeeding with the standard diet. On the last day of feeding, the rats were injected intraperitoneally with D-galactosamine solution (800 mg/kg body weight), and 20 h later the activities of GOT (glutamic-oxaloacetic transaminase), GPT (glutamic-pyruvic transaminase) and LDH (lactate dehydrogenase) were determined. As a result, the rats fed the galactooligosaccharide group were significantly lower than in other groups, suggesting that the development of galactosamine hepatitis is depressed by dietary galactooligosaccharide in addition to the constitutive saccharides of lactose or galactose.

Antihypertensive effects of extracts of *Lactobacillus casei* cell lysate (LEx) was demonstrated in patients with mild hypertension (Nakajima et al. 1995). Although patients (n=28, x=71 yr.) were already on hypertensive medication, 400 mg of LEx given twice a day for 2 months resulted in a significant decrease in systolic blood pressure, diastolic blood pressure and heart rate (beats/min) in a double-blind, placebo-controlled study. In addition, in the LEx-treated group, a significant reduction of both total cholesterol and fasting plasma glucose was observed. These findings support the role of LEx in the treatment of hypertension, with beneficial effects on glucose and lipid metabolism.

SAFETY OF LACTIC ACID BACTERIA

During the recent years, discussion on the safety of lactic acid bacteria and other probiotic bacteria has continued. Donohue & Salminen (1996) discussed the status of safety studies and the requirements for safety assessment for probiotic bacteria. Similarly, Adams & Marteau (1995) reviewed the safety of lactic acid bacteria. Saxelin & coworkers (1996) conducted an epidemiological study in Finland on bacteremia caused by lactobacilli. No cases of bacteremia with organisms of dairy or pharmaceutical origin were observed during the period 1989–1992. Altogether, the

number of bacteremias caused by lactobacilli was a little higher (0.241 versus 0.101%) when compared to that reported in France (Gasser 1994).

REVIEWS

One of the largest summary reports and new results on probiotics, lactic acid bacteria and fermented milks was published as the Proceedings of the First Australian Symposium on Intestinal Microflora and Health (Asia Pacific J. Clin. Nutr. 5 (1996). This special issue summarizes several aspects of intestinal microecology, influence of probiotics and future research directions.

The effects of probiotics in the stabilization of the gut mucosal barrier and mechanisms related to this barrier were discussed by Salminen and coworkers (1996). The assessment described the use of probiotics in the treatment of disturbed intestinal microflora and altered gut permeability which are characteristic of many intestinal disorders. Saavedra (1995) described the general situation of clinically tested probiotics and the gaps in our knowledge on well-studied lactic acid bacteria.

Tanaka (1995) reviewed the clinical effects if *Bifidobacterium brève* and *Lactobacillus casei*. On the clinical effects of diarrhoea, administration of *B. brève* preparation (BBG-01; 10^9 cells/g) to the patients with *Campylobacter enteritis* (n=133) showed the enhanced eradication of *Campylobacter jejuni* along with the recovery of normal flora. In the case of infantile intractable diarrhoea primarily induced by antibiotics (n=15), the stool frequency and appearance were dramatically improved within 3–7 days after administration of BBG-01, from chronic watery diarrhoea (mean 25 days), with normal flora predominating resident *Bifidobacterium* or administered on *B. brève*. On the clinical effects, *Lactobacillus casei* preparation (BLP; 10^{10} cells/g) is useful for the prevention of the recurrence of superficial bladder cancer: the 50% recurrence-free duration was prolonged significantly by BLP treatment (n=23, 350 days) to 1.8 times that in the control group (n=25, 195 days). BLP administration was also effective in the decrease in urinary mutagenicity derived from the ingestion of cooked meat. The blood pressure lowering effects of extracts of *L. casei* (LEx) were demonstrated in patients with hypertension as well as in spontaneously hypertensive rats (SHR).

Lee & Salminen (1995) reviewed the state of the art of probiotic bacteria and their stability in fermented milks. A current status on all successful probiotics is given and the research needs discussed.

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LACTOBACILLUS GG PRODUCTS WITH CLINICAL DOCUMENTATION

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Health benefits of *Lactobacillus* GG (ATCC 53103) reported in international refereed journals

(1) **Tolerant to intestinal conditions**
- adhesion to intestinal cells, transient colonization

(2) **Known daily doses for intestinal colonization**
The lowest number of bacteria needed for colonization depends on the administration medium.
≤ 10^8 cfu in milk
≤ 10^9 cfu in fermented milk and enterocoated tablets
≥ 10^{10} cfu in freeze-dried powder and gelatine capsules

(3) **Balancing effect on intestinal microecology**
- increase of lactobacilli and bifidobacteria and decrease of certain clostridia
- normalization of faecal flora during treatment of shigellosis
- normalization of faecal urease activity during intestinal disorders: rotavirus infection, Crohn's disease, juvenile chronic arthritis

(4) **Reduction of risk factors for intestinal tumour formation**
- decrease of formation of chemically induced intestinal tumours in rats
- decrease of harmful enzymes in the human colon

(5) **Stabilizing effect on the gut mucosal barrier**
- stabilization of permeability of the gut to macromolecules
- enhancement of the local immune response in rotavirus diarrhoea, adjuvant effect in oral vaccination
- suppression of allergic reactions in vitro and in vivo (milk allergy)

(6) **Competitive effects on potentially pathogenic microorganisms**
- production of an antimicrobial substance in vitro

(7) **Prevention and successful treatment of diarrhoea**
- prevention of antibiotic associated diarrhoea
- prevention of traveller's diarrhoea
- treatment of rotavirus diarrhoea and other acute watery diarrhoeas
- treatment of *Clostridium difficile colitis*
- treatment of shigellosis

(8) **Safe in use**
- no acute toxicity in mice
- increase in the longevity of irradiated mice
- reduction of endotoxaemia and alcoholic liver disease in rats
- no degradation of the intestinal mucosa
- well-tolerated in all studies: preterm infants, full-term infants, children with rotavirus and other infections, adults, the elderly
- used in dairy products in Finland since 1990
- safe in epidemiological studies

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ANALYSIS OF THE BIFIDOBACTERIAL AND LACTOBACILLUS POPULATIONS OF HUMAN FAECES USING GENETIC FINGERPRINTING OF BACTERIAL ISOLATES

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Fundamental studies of the normal microflora of the intestinal tract of humans are a prerequisite to understanding the influences of probiotics on the consumer, and in predicting ways in which improved probiotic products could be derived. An important topic that has not been adequately investigated is the impact of the consumption of a probiotic on the composition of the microflora. To begin to address this topic, the composition of the normal microflora in the absence of the administration of a probiotic must be studied. It has generally been concluded that the composition (genus, species) of the intestinal microflora of an adult human is stable. We have wondered, given the omnivorous habits of humans and marked variations in life style, whether the supposed stability of the microflora would be true if its composition was studied in terms of bacterial strains. Two genetic fingerprinting methods (ribotyping, pulsed field gel electrophoresis) have been used in our work to differentiate between strains of bifidobacteria and lactobacillus obtained from human faecal samples. Both of these methods differentiate between bacterial strains on the basis of restriction fragment length polymorphism of DNA.

Our study of the strain composition of bifidobacterial and lactobacillus populations began with an investigation of the normal microflora of two human subjects over a 12-month period. Faecal samples were collected at monthly intervals and total populations of bifidobacteria, bacteroides, lactobacilli and enterobacteriaceae were enumerated. Over the 12-month period, populations of obligate anaerobes (bacteroides, bifidobateria) were stable, populations of lactobacilli were slightly less stable, and enterobacterial numbers fluctuated dramatically. To study the strain composition of the bifidobacterial and lactobacillus populations, we subcultured 10 colonies, chosen randomly, from each appropriate selective medium that had been inoculated with a dilution of faecal homogenate that gave about 100 colonies per plate. Thus we sampled only the numerically predominant lactobacilli and bifidobacteria of the faeces. In the case of Subject 1, one bifidobacterial strain (G1) predominated throughout the 12-month period. In contrast, the bifidobacterial microflora of Subject 2 was considerably more dynamic. The composition of the bifidobacterial population of this subject was always complex, and strains appeared, disappeared and occasionally reappeared. Strain A6 was nevertheless detected frequently. For both subjects, for much of the 12-month period, a single lactobacillus strain was present in faecal samples. It appeared, initially, that both subjects harboured the same strain because they had identical ribotypes. Pulsed field gel electrophoresis, however, showed the strains from the two subjects to be different.

We are now investigating the composition of the bifidobacterial and lactobacillus microfloras of additional human

subjects. Based on the examination of a single faecal sample from four further subjects, the bifidobacterial and lactobacillus populations are either simple or complex, depending on the individual. As well as informing us of the composition and stability of these bacterial populations, our research permits the detection of numerically predominant strains of bifidobacteria and lactobacilli with which to investigate the impact of the normal microflora on the host.

The impact of the microflora that we are beginning to study concerns the immunological system of humans. We are utilizing two tests: the lymphocyte transformation assay as an overall measure of T-cell activation by bacterial antigens, and quantification of IgG antibodies as a measure of B-cell activation. Both methods require the collection of peripheral blood samples. Six subjects have been examined using these tests. When exposed to heat-killed bifidobacterial or lactobacillus cells, the lymphocytes of none of the subjects were stimulated to a degree considered biologically relevant. All subjects had serum IgG antibodies reactive with their indigenous bacteria. This can be taken as evidence that bifidobacterial and lactobacillus antigens have access to the immunological system and that each individual has B-cells with receptors that recognize antigens associated with their own normal microflora bacteria.

How can the serum IgG results be reconciled with the non-responsiveness of T-lymphocytes in the transformation assay ? The bifidobacteria and lactobacilli are inhabitants of the intestinal contents. Lymphocytes with receptors that recognize the bifidobacterial and lactobacillus antigens are probably sequestered in the gut associated lymphoid tissue: the site where first contact with such bacterial antigens by the immunological system would occur. Therefore, reactive T-lymphocytes are not present in detectable levels in the peripheral blood. T_2-lymphocytes interacting with appropriate B-cells in the gut associated lymphoid tissue would result in the synthesis of antibodies that would eventually spill over into the systemic blood circulation. Therefore antibodies are present in the blood at detectable levels. The antibody titre was higher in some subjects compared to others. Perhaps this reflects differences in individuals as to the degree in which a particular arm of the immunological system (T_1: cell mediated immunity or T_2: humoral) is stimulated by bifidobacterial and lactobacillus antigens. There is clearly a great deal of fundamental research relating to lactic acid bacterial members of the intestinal microflora and their interaction with the human immunological system that must be carried out. All of the phenomena associated with the normal microflora and described in this presentation may have implications for the development and use of probiotics.

Acknowledgement

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PRACTICAL CRITERIA FOR SELECTION AND JUDGEMENT OF LACTIC ACID BACTERIA AS PROBIOTICS

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The present discussion around probiotics is increasingly focusing on questions related to those properties considered as desirable. In addition, attention is given to practical problems encountered with the screening and selection of the most favourable candidates for special purposes. Existing controversies result from the fact that no agreement has yet been reached on objective criteria for assessing probiotics properties claimed for novel product preparations.

Relevant criteria may be categorized with respect to functional, biotechnical or safety aspects. Safety assessment should also take into consideration modern taxonomic approaches. A statement of being non-pathogenic or "GRAS" should be based on a long safety record in the use of the particular probiotics strain. Considerations of governmental regulation are presently under discussion. Finding additional criteria for in vitro/in vivo "safety assays" is considered as a special future challenge.

Biotechnical aspects are determined by industrial and practical considerations such as fermentation patterns, phage resistance and biological stability, sensory properties and growth behaviour in typical substrates (for example milk).

Functional properties include the ability to survive in the gastrointestinal tract (for example related to gastric juice and bile resistance), immune stimulation (for example enhanced phagocytosis), adhesion to intestinal mucosa, cholesterol lowering activity and antigenotoxic activity.

A preliminary minipig feeding trial was conducted in an attempt to evaluate some of these properties. For this purpose approbiate strains were selected from ca. 300 LAB isolates from pigs, using criteria such as acid tolerance and bile resistance and also the specific functional aspect of the hypothesized cholesterol lowering effect on account of the bile salt hydrolytic activity (BSH).

The small number of animals allowed only the observation of some trends with respect to the probiotic function, such as resistance to adverse environmental conditions, a not unequivocal cholesterol lowering effect, decrease of two procarcinogenic faecal enzymes(β-glucuronidase and azoreductase) and improvement of defecation by an increase of the moisture content of the faeces (but no diarrhoea). We saw no significant shifts among the 12 main groups of the faecal flora and no change in the faeces pH. The attempt for faecal recovery of the administered strains by antibiotic resistance as a marker failed due to the multiple antibiotic resistance of the minipigs autochthonous lactobacilli flora. The use of gene probes and, for example, in situ hybridization may solve the recovery problem.

Further studies should serve to improve the methodology, also with a view to the selection and development of new functional probiotics. A further goal should be the safety assessment of commercially available cultures and evaluation of claimed functional properties.

BIFIDOBACTERIUM LONGUM AND LACTULOSE SUPPRESS AZOXYMETHANE-INDUCED PRENEOPLASTIC LESIONS IN RAT COLON

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Lactulose is a substrate for preferential growth of *B. longum* which has been shown to inhibit colon tumorigenesis. The role of lactulose (L) and possible synbiosis between lactulose and *B. longum* (B1) in suppressing colon tumorigenesis was studied in a 2 x 2 factorial experiment using azoxymethane-induced (AOM) rat colonic aberrant crypt foci (ACF; preneoplastic lesions) as a model.

Groups of 15 male Fisher 344 weanling rats were assigned to one of the four diets for 13 weeks: (1) AIN76A (control=C); (2) C+0.5% B1 BB536 (100 million viable cells/g feed; a gift from Morinaga Milk Industry, Japan); (3) C+2.5% L or (4) C+0.5% B1+2.5% L. All rats received s/c injection of AOM @ 16 mg/kg body wt. at 7 and 8 weeks of age. Colons from each group were assayed for ACF (10 rats) and glutathione-s-transferase (GST, 5 rats). There was no significant difference in feed intake or weight gain among treatments ($P>0.05$). Number of ACF for diets, C, C+B1, C+L, and C+B1+L were: 187±9, 143±9, 145±11 and 97±11, respectively ($P<0.0001$). Interactive effect between L and B1 was not significant ($P>0.05$), pointing to an additive inhibitory activity for L and B1. Colonic mucosal GST levels were significantly higher in B1 and L groups compared to C group. Results of this study indicated that *Bifidobacterium longum* and lactulose exert additive effect in suppressing colon preneoplastic lesions.

LACTIC ACID BACTERIA – A NEW APPROACH TO DETOXIFY AFLATOXINS

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Aflatoxins are a group of a growing list of fungal secondary metabolites of economic and health importance. They are potent carcinogens in several species of animals, and epidemiological studies have implicated them as acute toxicants as well as hepatocarcinogens in man. Aflatoxins have been found in many food items, including milk and milk products. The production of aflatoxin in such commodities can be influenced by several factors, including temperature, a_w, pH, available nutrients and competitive growth of other microorganisms.

It is known that different acid producing bacteria possess antimicrobial activity against pathogenic organisms. Strains of lactic acid bacteria have been found to inhibit both the growth of *Aspergillus flavus* (mould producing aflatoxins) and aflatoxin production.

This study examined the in vitro potential of selected strains of probiotic lactic acid bacteria to degrade aflatoxins. Our results indicate the ability of some strains to degrade aflatoxins. The degradation of aflatoxins may be a result of metabolic transformation by the bacteria and/or binding of aflatoxins to the bacterial cell wall. The potential of using these strains by the food industry for the purpose of detoxication of aflatoxins is promising and should reduce the risk of human exposure to these extremely toxic and carcinogenic compounds.

COLONIZATION OF *LACTOCOCCUS LACTIS* L1A ON HUMAN INTESTINAL TRACT AND EFFECT ON THE BACTERIAL FLORA

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In vivo colonization by *Lactococcus lactis* L1A was studied in nine ileostomy subjects and 21 healthy volunteers. The effect on the intestinal bacterial flora was also studied in the ileostomy subjects during two intervening 3-week periods when they drank 250 ml low-fat milk or fermented low-fat milk (VERUM HÄLSOFIL®, Norrmejerier, Umeå, Sweden). The ileostomy effluents were also tested for β-glucoronidase. *Lactococcus lactis* L1A was found in the faeces from the healthy volunteers 8 days after administration was terminated. In the ileostomy subjects the *Lactococcus lactis* L1A was found in the effluents already 90 min after administration (10^7 cfu/ml). Some effect could be seen on *Streptococcus bovis* and *E. coli* during this period.We could also see an increase in Lactobacilli and Lactococci during administration of *Lactococcus lactis* L1A.

LACTOCCOCUS LACTIS L1A REDUCES ANTIBIOTIC ASSOCIATED DIARRHOEA

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Twenty healthy volunteers participated in a double-blind randomized study concerning the effect of *Lactoccocus lactis* L1A on antibiotic associated diarrhoea during phenoxymethylpenicillin treatment. L1A has a marked capacity to inhibit the growth of a variety of Gram-negative bacteria which are potentially pathogenic in man.

The volunteers received phenoxymethylpenicillin (12.5 mg/kg weight, b.i.d.) for 10 days together with 500 ml fermented milk daily (VERUM HÄLSOFIL®, Norrmejerier) or placebo (Lättfil). Both groups continued with the fermented milk for another 5 days after antibiotic treatment.

Volunteers receiving VERUM HÄLSOFIL® with L1A had significantly fewer episodes of diarrhoea than those taking placebo during antibiotic treatment, 11% compared with 43%. Also during the 5 days following antibiotic treatment there was a significant difference, 55 and 31%, respectively. The faecal flora was found to be relatively constant during the observation period. No significant differences were observed between the two groups with regard to the number of lactobacilli and lactococci. Among the volunteers receiving L1A, the bacteria were recovered in faecal specimens – also during penicillin treatment – in seven out of 10 persons.

To conclude, the ingestion of L1A in a fermented milk product significantly reduces diarrhoea caused by peroral administration of phenoxymethylpenicillin.

VERUM HÄLSOFIL®

A.-K. Karlsson
Norrmejerier, Umeå, Sweden

A collaborative project between researchers at Umeå University and Norrmejerier was initiated in 1987. In the early 1970s, Professor Stig Holm collected traditional milk fermentation cultures from farms in northern Sweden. The bacterial strains isolated were analysed for inhibitory effects on potentially pathogenic enteric bacteria. A certain bacterial strain, *Lactococcus lactis* L1A, with outstanding inhibitory effect in vitro was isolated and selected.

A novel milk fermentation starter culture containing *Lactococcus lactis* L1A was developed, and a new probiotic milk product, VERUM HÄLSOFIL®, became commercially available. Clinical studies with VERUM HÄLSOFIL® have shown that *Lactococcus lactis* L1A survives the passage through the gastrointestinal tract and that it can be recovered from faecal samples up to 15 days after the consumption of VERUM HÄLSOFIL® has stopped. Other studies have shown that VERUM HÄLSOFIL® can decrease antibiotic associated diarrhoea and normalize the consistency of faeces of constipated patients. Furthermore, the product has been shown to reduce the cholesterol absorption in the small intestine of ileostomy operated persons.

ARE VIABLE MICRO-ORGANISMS ESSENTIAL FOR THE ENHANCEMENT OF INTESTINAL HYDROLYSIS OF LACTOSE BY THE β-GALACTOSIDASE OF FERMENTED MILK PRODUCTS ?

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To investigate whether improvement by fermented milk products of lactose digestion in lactose malabsorbers requires viable bacteria, we fed each of the following experimental diets to 14 conventional Göttingen minipigs for 1 week in randomized order:

(1) a kefir-based product (1 l/d) with viable lactobacilli (≈ 4 x 10^8 cfu/l) and 70 g/l lactose added (active microbial β-galactosidase ≈ 800 U/l; o-NPG)
(2) the same product, but with lactobacilli killed by γ-irradiation (intact cell walls and active β-galactosidase)
(3) the same product, but with lactobacilli killed by shear-force (in part broken cell walls but active enzyme)
(4) the same product with lactose, but no lactobacilli added (no β-galactosidase activity) (control).

Lactose digestion was estimated from postprandial plasma galactose concentrations. When lactobacilli in the kefir were viable or killed by irradiation (diets 1 and 2) plasma galactose peak concentrations and area-under-the-curve values were practically identical and significantly higher than control values. But there was almost no improvement of lactose digestion when lactobacilli cell walls were damaged (diet 3).

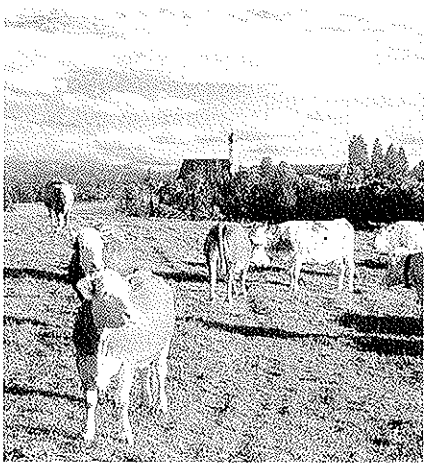
In a similar experiment, six gnotobiotic piglets were fed fermented pasteurized milk, fermented milk with viable lactobacilli or fermented milk with γ-irradiated lactobacilli; the latter two diets containing active β-galactosidase. Only fermented pasteurized milk led to a significant faecal lactose loss and almost no β-galactosidase activity in the faeces.

When human malabsorbers consumed fermented milk containing active microbial β-galactosidase but killed lactobacilli with cell walls in part broken, the H_2 exhalation response was intermediate between that towards a pasteurized milk product (no β-galactosidase activity, high response) and towards a native fermented milk product (high response).

It is concluded that lactose digestion in lactose malabsorbers can be improved efficiently if the milk product contains active microbial β-galactosidase. The bacteria need not be alive, but they need (largely) intact cell walls as a mechanical protection during the gastric passage.

„Gute Milch ist kein Zufall."

„Für beste Milch muß das Umfeld stimmen", sagt Landwirt Rudolf Deschler aus Weilheim in Oberbayern. Daran hat der 54jährige Bauer Interesse, denn sein Einkommen richtet sich nach der Qualität der Milch. Seine 50 Kühe erhalten frisches Gras, Heu sowie Getreide-, Mais- und Grassilage.



Im Sommer kommen die Kühe auf die Weide, aber auch im Winter erhalten sie nur bestes Futter.

Zweimal täglich wird gemolken, wobei besonders auf Hygiene geachtet wird. Nach einer gründlichen Euterreinigung wird das Melkgeschirr angelegt, und die Milch gelangt sofort über geschlossene Leitungen in den Kühltank. So ist sie hygienisch einwandfrei und vor äußeren Keimen geschützt. Die Rohmilch wird täglich vom Kühlwagen der Molkerei abgeholt und geprüft.

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IDF NUTRITION WEEK

POTSDAM, GERMANY, MAY 1996

SUMMARY REPORTS OF MEETINGS OF GROUPS OF EXPERTS

GROUP F20 –
CULTURED AND CULTURE-CONTAINING DAIRY PRODUCTS IN HEALTH

Chairman: S. Salminen (FI) – Deputy Chairman: D. Farr (CH)

Decisions:

1) Symposium Proceedings will be published, as recommended by the organizing Committee, in the Nutrition Newsletter.
2) Working Group established for the preparation of draft monograph. S. Salminen (FI), C. Barth (DE), R. Tanaka (JP), R. Fonden (SE) & G. Mogensen (DK).
3) Deadline for draft monograph: next Nutrition Week.
4) Report to Annual Session agreed upon.
5) Definition of a probiotic suggested to PC.
6) Annual Review will be continued (Salminen/Tanaka).
7) Assistance in the area of F20 will be given to US National Committee in organizing a meeting/symposium on Nutraceuticals.

Next meeting: in Zürich, March 1997.

GROUP F22 –
DIETARY CALCIUM AND HEALTH

Convenor: G.J. Schaafsma (NL)

The current status of the preparation of the IDF Monograph on Calcium was discussed. Delegates and observers of the meeting were asked to send any comments on the available chapters to Prof. Schaafsma before the end of May 1996. This concerns the chapters prepared by Dr Goulding, Prof. Gurr, Dr Sieber, Prof. McIntosh and Prof. Nordin. IDF will be asked to reproduce the latter three chapters and to send them to all group members and the observers of the meeting. The chapter on Calcium and Hypertension by Dr Miller will be available within a few weeks and remaining chapters (Ca–caffeine and Ca–protein interrelations) will be prepared by Prof. Schaafsma. It is aimed to send all chapters in final form to IDF before the summer holidays.

Next meeting: will be held in association with the Nutrition Week in 1997 in Switzerland.

GROUP F48 –
NATURAL BIO-ACTIVE SUBSTANCES IN MILK AND COLOSTRUM

Rapporteur: H. Korhonen (FI)

1) Regarding the need to establish a new group on natural bioactive substances in milk and colostrum, the following arguments were discussed and unanimously agreed upon:
   a) There is an increasing interest in the development of the so-called functional foods which possess health promoting properties. Milk and colostrum provide an excellent source of substances which have biofunctional (bioactive) properties with a good potential for industrial utilization.
   b) There is a need to improve the image of milk as a multifunctional nutritive foodstuff due to a number of negative health claims related recently to consumption of milk or certain dairy products.
   c) There is a need to compile continuously increasing scientific research data about the biofunctional components and activities of milk and colostrum and forward this information to the scientists in the field as well as to the dairy industry.
2) The field of substances (topics) to be covered by the prospective Group F48 was discussed using as a guideline the list drafted by the rapporteur.

   The list was approved as a whole provided that a priority list of the topics will be produced before submitting the list to PC/F. Also, it was decided that the rapporteur will discuss the topic "Bioactive peptides" with the Chairman of Group F20 (Seppo Salminen) and F24 (Christian Barth) in order to avoid overlap. It was decided that the bacteriocins and other metabolites of lactic acid bacteria will not be included in the work of Group F48.
3) A few amendments were made to the objectives and functions of the Group F48 and will be recorded in the final draft to be submitted to PC/F.
4) The rapporteur noted that he had received a favourable response from all experts (10) from different countries who were asked to comment on the drafted list of topics and objectives of the Group F48.