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European Research towards Safer and Better Food

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Preface

Research is more than ever before an international task for the advancement of science and for building up networks among scientists which allow fast and fruitful communication.

With its framework programmes the European Commission has created powerful tools to support both goals. Its AIR and FAIR programmes are addressing especially sensitive areas of consumer interests and demands as food safety and food quality. To review the outcomes of these programmes and to promote their transfer to industry and into public knowledge, the Federal Research Centre for Nutrition, Karlsruhe, together with the European Commission and the European Federation of Food Science and Technology, has organized the Congress *European Research towards Safer and Better Food* in continuation of its series of Karlsruhe Nutrition Symposia.

Special features of the congress are: Food Safety and Monitoring Aspects; Food, Nutrition and Well Being; Technological Methods to Improve Food Quality; Consumer Perception and Transfer Strategies. A special session is devoted to Meat – Safety Aspects and Nutritional Quality. As the Congress is organized on the threshold of the Fifth Framework Programme, special lectures are presented on the background and strategies of this new scientific venture.

The Congress Proceedings comprise two parts plus a supplement, which are considered as a unit. Part 1 contains contributions presented as lecture; contributions in the form of posters are contained in part 2. In the supplement appearing after the Congress those contributions are collected which were not available at the time of printing. The supplement offers also an authors index along with other congress-relevant information.

Walter E.L. Spieß

Chairman

European Research towards Safer and Better Food

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3rd Karlsruhe Nutrition Symposium: European Research towards Safer and Better Food
Review and Transfer Congress, Congress Centre, Karlsruhe, Germany
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Session 1:

Food Safety and Monitoring of Safety Aspects

Are genetically modified food products safe? What do consumers think?[1] [2]

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[1] The research presented in this paper is funded by the European Commission through the project 'Consumer Attitudes and Decision-Making with regard to Genetically Engineered Food Products', contract number FAIR-PL96-1667. Co-ordinator of the project is Professor Klaus G. Grunert, The MAPP Centre at the Aarhus School of Business. Participating organisations are the Technical Research Centre of Finland; Oy Panimolaboratorio-Bryggerilaboratorium AB, Finland; Chr. Hansen A/S, Denmark, Universität Potsdam, Germany; ISIDA, Italy and Institute of Food Research, Great Britain.

[2] For reasons of simplicity, the term 'genetically modified food products' is used in this paper as a general designation of foods and food ingredients which contain or consist of genetically modified material or which are produced from, but do not contain, genetically modified material.

Abstract

The introduction of genetic engineering in food production has received considerable public attention in many European countries. Understanding the concern that consumers express over such new technologies and their application in food production is crucial to legislative authorities, industry and other organisations with vested interests in the issue. In this study, means-end chain theory was used as a theoretical basis for investigating consumer perceptions of two genetically modified food products (yoghurt and beer). By means of the laddering technique, 400 consumers in four countries were interviewed about their perceptions of risks and benefits of consuming various conventionally manufactured products and of consuming genetically modified products offering tangible consumer benefits. Results show preference for the genetically modified product alternatives to be low, and point to perceived risks in relation to self and the environment as major explanatory factors along with moral considerations and food neophobia. Some interesting cross-national differences are found as well.

Introduction

These years, still more advanced breeding and product modification techniques are developed based on gene technology and other kinds of modern biotechnology. Most scientists regard these new techniques simply as a natural extension of traditional breeding methods and regard the prospects of using gene technology to change product properties for financial, environmental, health- and quality-related reasons as practically unlimited. By arguing that genetic engineering is actually safer than traditional breeding techniques because of the tight control procedures that are imposed by the authorities specifically in this area, these people do not hesitate to advocate that gene technology be used in the food domain.

The success of using gene technology both in primary production and processing of food products is, however, contingent on consumer acceptance. While most technological experts celebrate the advantages of gene technology and dismiss the possibility of serious risks arising from its

application (Scholderer, Balderjahn & Will, in press), consumers seem to associate considerable risk with this technology (eg Frewer & Shepherd, 1995; European Commission, 1997). Since consumers' risk evaluation are based more on subjective criteria, their risk perception with regard to genetic engineering in food production is likely to differ fundamentally from experts' risk evaluation.

Consumer attitudes towards genetic engineering in food production

A number of attempts have been made to explain consumer attitudes towards genetic engineering, and part of these studies have looked specifically into consumer attitudes towards genetic engineering in the food domain (for a review see Bredahl, Grunert & Frewer, 1998). Deriving general conclusions from this research is complicated by large variations in research methodology and level of abstraction, but perceived risks and benefits of genetic engineering seem to be major determinants of attitudes towards applying genetic engineering in the food domain (Frewer & Shepherd, 1995; Hamstra, 1995). It is also likely that the relationship between the two is compensatory, meaning that perceived risks of genetic engineering can to a certain degree be offset by greater perceived benefits of applying the technology in food production, as has been shown to be the case with other technological activities (Fischoff, Slovic, Lichtenstein, Read & Combs, 1978). Furthermore, it seems that with genetic engineering consumers not only consider the consequences of the technology for themselves, as is generally assumed in multi-attribute attitude models (eg Fishbein, 1963), but also take into account perceived consequences for other outcome groups such as their family or the environment. Consumers' attitudes towards genetic engineering in food production can also be expected to be influenced by more general attitudes held by consumers such as attitude towards technology or food neophobia (Hamstra, 1995; Sparks, Shepherd & Frewer, 1994), and by knowledge domains pertaining to gene technology or food production in general (Frewer, Howard & Shepherd, 1997). Considering that genetic engineering is sometimes simply used as a processing aid that does not change the product characteristics from those of a conventional product, and where the genetically modified material is perhaps not even present in the final product, it also makes sense to expect consumer attitudes to be influenced both by beliefs relating to the production process itself and by beliefs about the quality and consequences of consuming the resulting product.

Thus, information about the *beliefs* which consumers hold about the attributes or consequences of applying the technology becomes essential for explaining consumer attitudes towards genetic engineering in food production.

Means-end chain theory provides a sound theoretical frame for investigating these subjective associations by explaining how consumers mentally link perceptions of product attributes to the attainment of basic life values through self-relevant consequences (Gutman, 1982; Olson, 1989).

Since genetically modified foods are still only rarely found in the supermarkets, consumer familiarity with these products is generally low, but as has been indicated above, risk perception is high. This gives reason to expect consumer attitude formation with regard to genetic engineering in food products to be complex and closely related to basic life values, which gives further support to applying means-end chain theory in this context.

Purpose of the study

The aim of the study was to gain insight into the beliefs which consumers have with regard to genetically modified food products, and specifically to generate information about:

- perceived risks and benefits, including the impact of tangible benefits on consumer belief structures
- possible differences in beliefs relating to different outcome groups
- beliefs held by consumers when a) the genetically modified material is part of the final product and b) when the genetically modified material is not present in the final product
- the impact of more general attitude domains on consumer belief structures
- cross-national differences in beliefs and attitudes

Method

Means-end chains can be measured by the laddering technique, which is a semi-structured qualitative interview method that allows for quantitative analysis of the collected data (Reynolds & Gutman, 1988). The technique takes its starting point in product attributes regarded as important by the respondent for choosing among a given set of products. For each relevant product attribute the interviewer uses a series of 'why is that important to you'-type questions to make the respondent reach increasingly abstract levels of explanation. As in means-end chain theory, the technique presupposes a hierarchical cognitive structure, and the idea is to uncover the entire chain from concrete and abstract product attributes over functional and psychosocial consequences to instrumental and terminal values. The elicited chains are commonly referred to as 'ladders'.

Data collection

400 laddering interviews were carried out in Denmark, Germany, the UK and Italy, using beer and yoghurt as tangible product examples.

Four product profiles were developed within each product category: two distinct, traditional product alternatives, and two distinct more modern product alternatives, of which one was claimed to be produced by means of genetic engineering.

Tangible products were created from existing products which were equipped with new labels containing the relevant product information and claims of different consumer benefits. The products were only used for visual presentation.

In the interviews, salient attributes were first elicited by a ranking procedure where the respondents were asked to rank the exhibited products according to preference and state their reasons for the order of these product preferences. The cited attributes were then used as a starting point for the laddering procedure where consequences and eventually values were pursued. In total, 2187 ladders were extracted in the interviews about yoghurt and 1874 ladders were generated in the interviews about beer. The data were collected in late 1997.

After completion of the fieldwork, all individually mentioned concepts were categorised into attributes, consequences and values, and by thorough meaning-based interpretation, all concepts were then coded into broader categories. The procedure was carried out separately for the beer and

yoghurt data. The concepts about yoghurt were finally coded into 60 broader categories, while the concepts in the beer data were coded into 61 categories.

Following this procedure, the coded data were analysed and interpreted at the aggregate level by means of hierarchical value maps, which is a form of graphic representation of the data summarised across a group of respondents. Separate value maps were produced for each product and country in order to take possible cross-national differences into account. Associations between categories are usually only presented in hierarchical value maps if they have been mentioned, directly or indirectly, by a minimum number of respondents. In this case, each hierarchical value map was produced with cut-off levels ranging from 3 to 5 respondents, before a final solution was chosen by inspecting the interpretability of the produced maps.

Results

Below, we begin by presenting results concerning product preferences before proceeding to results pertaining specifically to means-end structures with regard to the genetically modified product examples.

Product preferences

The initial ranking of the products can be used to investigate the relative preference for the products. The first preferences are shown in figure 1.

Overall, the more traditional product alternatives were clearly preferred. Thus, more than half of the respondents in all four countries ranked the medium-priced traditional beer as the product they preferred most of the four beers. Similarly, the traditional full-fat whole-milk yoghurt was reported as the most preferred yoghurt by a majority of respondents in Denmark, Germany and Italy, while in the UK, the highest preference was for the fat-free yoghurt with artificial components to ensure a smooth texture.

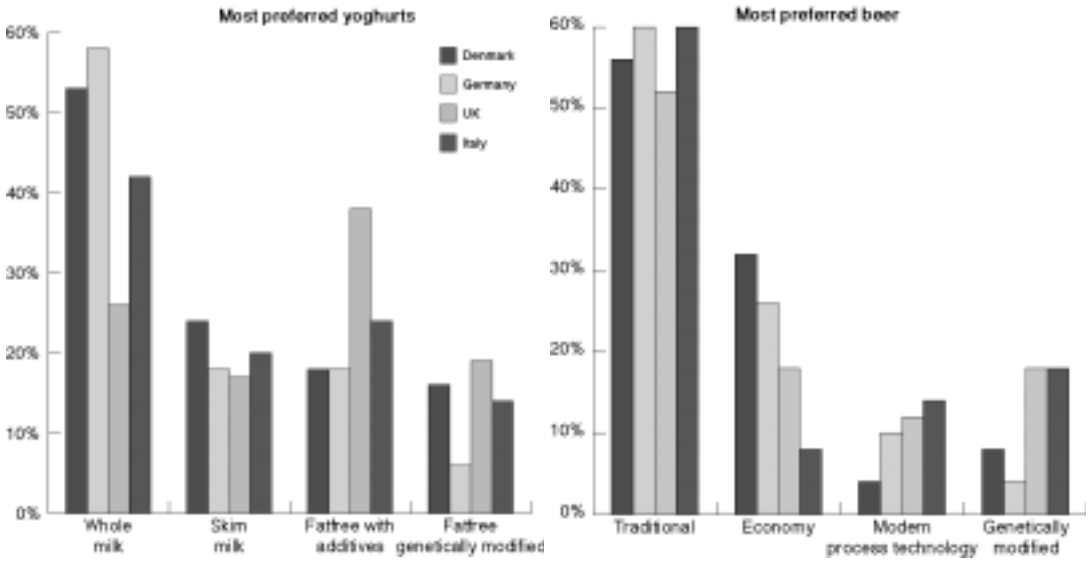

Figure 1: First preferences

As can be seen in the figure, preference for the genetically modified products was generally low, and more so in Denmark and Germany than in the UK and Italy. We expect to be able to identify the major reasons for these differences in product preferences in the subsequent analysis of the generated means-end chains.

Means-end structures with regard to genetically modified yoghurt

The fact that the one of the four yoghurt products was claimed to be genetically modified was mentioned as a salient product attribute in the elicitation task by most respondents, and in all four countries this attribute was associated with quite negative consequences, which were believed to prevent the fulfilment of important life values. The hierarchical value maps for the Danish and British respondents are shown in figures 2 and 3.

The most dominant association seems to be the belief that the application of genetic engineering will turn yoghurt into an unwholesome and unnatural product, and, judging from the perceived consequences, the belief seems also to be tied to the expressed feelings of unfamiliarity with the product. Thus, there were strong beliefs that ingestion of the product would reduce personal healthiness and that the product could not be trusted because of its perceived unknown long-term consequences on human health and the environment. These prevalent perceived consequences are crucial since they were generally believed to inhibit the achievement of important life values, such as long and healthy life, happiness and inner harmony, security and responsibility for nature and other people.

The belief that the application of genetic engineering would damage the environment was found primarily among Danish and German consumers, but there were no strong links from this belief to self-relevant psychosocial consequences or values, which indicates that perceived environmental impact was perhaps not of crucial importance.

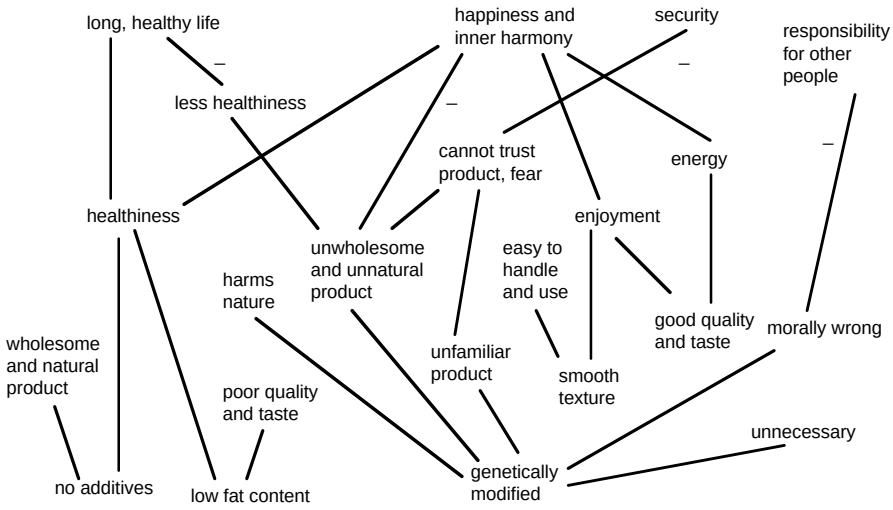

Figure 2: Genetically modified yoghurt, Danish respondents, cut-off=4

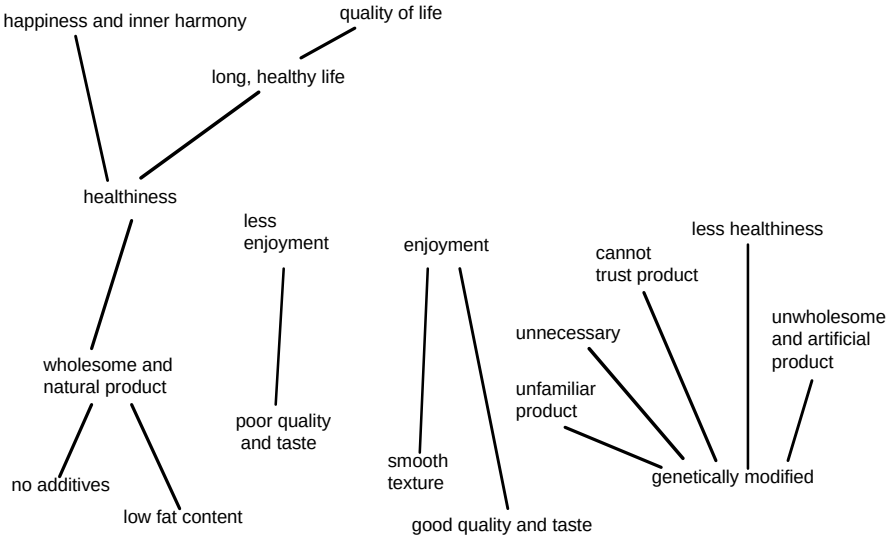

Figure 3: Genetically modified yoghurt, British respondents, cut-off=4

In addition, genetic engineering in yoghurt was perceived to be morally wrong by respondents in Denmark and Germany, and a number of respondents in these two countries as well as in the UK also opposed the application of genetic engineering in yoghurt by claiming that there was no need for this technology in food production at all.

The hierarchical value maps also show that the consumers were in fact aware of the benefits that had been added to the hypothetical products by means of the gene technology, and, judging from the perceived consequences, these benefits were highly appreciated. Thus, both the absence of additives and the absence fat were mentioned as important product characteristics, and were believed to enhance a long and healthy life via perceived increased healthiness. The smooth texture which was claimed to have been achieved by means of gene technology was also seen as desirable, at least in Denmark and the UK. Here, respondents claimed that smooth texture would increase the enjoyment of consuming the product, and, in Denmark, the smooth texture was also linked to consuming the product without spilling and increased usage possibilities (breakfast, dessert or snack). From the ranking procedure, we know, however, that these positive attributes and consequences could generally not outweigh the perceived negative and undesirable consequences of the application of genetic engineering.

As already indicated, the hierarchical value maps point to some interesting cross-national differences, though the basic pattern of the associations remains the same. In general, Danish, German and British consumers seem to perceive a far larger palette of consequences of the application of gene technology, and these consequences seem in Denmark and Germany in particular to be closely related to important personal values. This is apparently not quite so in the UK where the ladders of perceived consequences of genetic engineering do generally not reach the value level. In addition, security generally seems a more central value among Danish and Italian consumers than among British and German consumers, and the more social values, responsibility for nature and the welfare of other people, seem more central to Danish and German consumers.

Means-end structures with regard to genetically modified beer

The hierarchical value maps for the genetically modified beer basically resemble those of the genetically modified yoghurt, despite the fact that here the genetically modified material, yeast, is filtered out during the production process and therefore not present in the final product. Hierarchical value maps for the German and Italian respondents are shown in figures 4 and 5.

Again, genetic engineering was an important attribute, and a range of undesirable consequences was inferred from its application. The central associations were unwholesomeness and unnaturalness and low trustworthiness of the resulting product, which were typically believed to prevent the fulfilment of the important life values long and healthy life, happiness and inner harmony and security. Again, genetic engineering was also perceived by some as morally wrong and basically superfluous in food production. Notably, undesirable side effects of the application of gene technology on personal healthiness were also a commonly perceived, despite the absence of genetically modified material in the product.

The maps also indicate that the fact that a product is based on a modern and intensive production method in itself triggers significant negative perceptions of the product. Thus, the intensive brewing method was associated with poor quality and taste in all four countries, and these associations were then subjectively linked to less enjoyment and the prevention of happiness and personal well being.

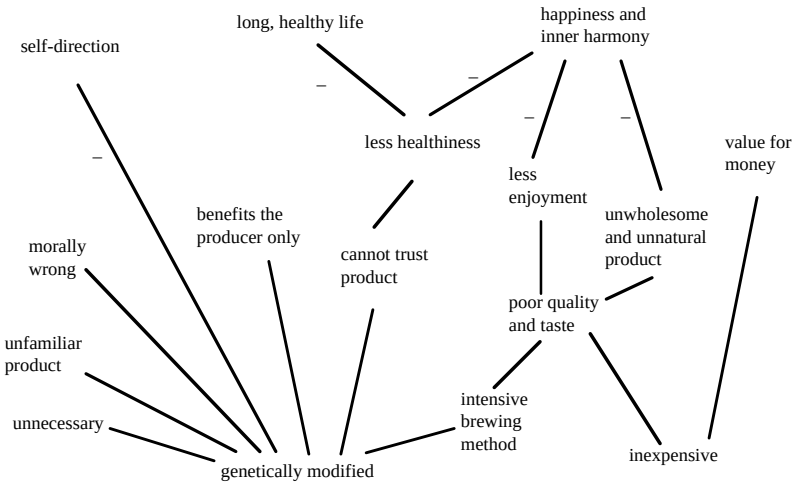

Figure 4: Genetically modified beer, German respondents, cut-off=4

Interestingly, environmental friendliness, which was one of the tangible benefits attributed to the product by means of genetic engineering, was noted and valued in Denmark, the UK and Italy, but not in Germany. In these three countries there seems to be no doubt about the significance of this perceived attribute for consumer choice as the perceived consequences eventually also reach the value level. Among the German respondents, there was a belief that the application of gene technology in this case did not benefit anyone but the producer. The relevance of the low price of the product as a consumer benefit is even more doubtful, as the respondents generally associated the low price with lower quality and taste, while on the other hand they also pointed out a desirable money aspect.

Again, the widest range of associations arising from the application of gene technology was found among Danish and German respondents, while there were particularly few associations to this attribute among the Italian respondents (note the lower cut-off level in the Italian map).

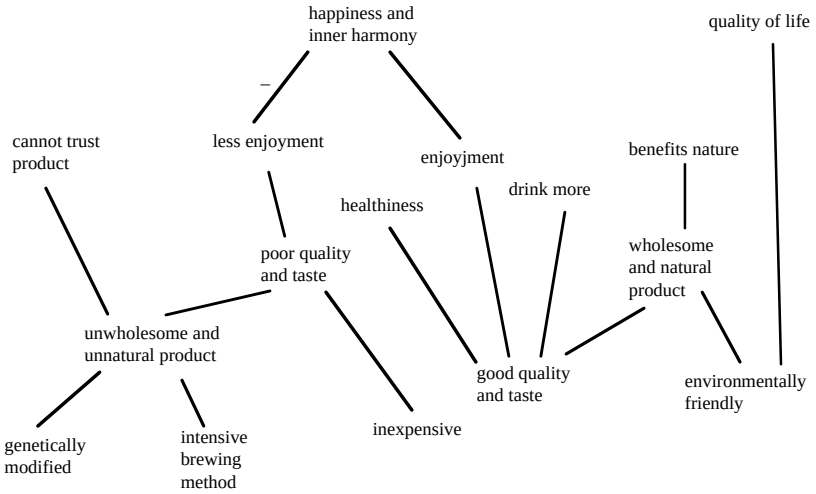

Figure 5: Genetically modified beer, Italian respondents, cut-off=3

Concluding remarks

This study has employed means-end chain theory and the laddering technique to investigate consumers' cognitions with regard to genetically modified beer and yoghurt in four countries. As expected, the study has given valuable insight into consumers' motivational structures and subjective meanings with regard to genetically modified foods.

The associations with the application of gene technology were generally found to focus more on perceived risks than benefits, with the main focus on beliefs relating to perceived unhealthiness and low trustworthiness of the resulting products. These beliefs were generally seen to inhibit the attainment of individual life values such as happiness and inner harmony, long and healthy life, quality of life and security, and the more social life values responsibility for nature and for other people.

The results also indicate that consumers consider risks and benefits of genetic engineering both in the light of perceived consequences for themselves, for other people and for the environment. At present, no differences in beliefs depending on which outcome group that is considered can be identified, however.

The importance of *relevant* consumer benefits for consumer acceptance of genetic engineering in food production was verified. Particularly the benefits which were attributed to the genetically modified beer could not compensate for the perceived undesirable consequences of the application of gene technology.

Unlike our expectations, absence of genetically modified material in the end product (the beer case) did not seem to affect the associations arising from application of gene technology in the production

process compared to the instance where the material was still present in the final product (the yoghurt case). Any effect of this circumstance presupposes some basic knowledge about food production and, in this case, specifically about beer production, which the consumers may not have.

The results indicate that food neophobia may play a significant role for the formation of consumer attitudes and, eventually, acceptance of genetically modified foods. Thus, low familiarity with the product and gene technology was often mentioned and subjectively linked to low trustworthiness of the product and eventually less security or less happiness and inner harmony. Therefore, it also seems likely that demystification of the technique by more knowledge of the basic principles of gene technology and its likely impact on self and the environment will significantly affect consumer beliefs and attitudes. Having said this, it is, however, also important to mention that many consumers apparently oppose genetically modified foods also for ethical reasons which are not very likely to be affected by increased knowledge of gene technology and its risks and benefits.

A number of cross-national differences were also identified, and generally more complex cognitive structures were found in Denmark and Germany than in the UK and Italy.

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New approaches to the microbial stability and safety of foods

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Abstract

There remains considerable concern regarding the high levels of food-poisoning disease in Europe and the fact that it is rising year by year. At the same time, preservation techniques are becoming milder in response to consumers' requirements for higher quality, more convenient foods. Mild preservation techniques can encourage microorganisms to undergo "stress reactions" that increase their resistance, and sometimes their pathogenicity.

Most food preservation techniques act by *inhibiting* the growth of microorganisms rather than by inactivating them [e.g.: reduction in temperature (chilling, freezing); reduction in a_w (curing, conserving, drying); reduction in pH (addition of acids, lactic and acetic fermentation); modified atmosphere packaging (vacuum, N_2, CO_2, O_2); addition of preservatives]. Few techniques act by *inactivating* microorganisms [e.g.: heat (thermization, pasteurization, sterilization); ionizing radiation].

New, "emerging" and potential techniques mostly act by inactivation, e.g.: "natural" systems [animal-derived (e.g.: lysozyme, lactoperoxidase, lactoferrin, lactoferricin, ovotransferrin, serum transferrins, small peptides such as histatins and magainins); plant-derived (e.g.: phytoalexins, low MW components of herbs and spices, phenolics such as oleuropein, essential oils); microorganism-derived (e.g.: bacteriocins)]. New physical techniques also act by inactivation rather than inhibition (vegetable- fruit- and carcass-decontamination dips and sprays, high hydrostatic pressure, high voltage electric discharge, high intensity laser and non-coherent light, "manothermosonication").

The new and improved techniques for the inactivation of vegetative and spore forms of food-spoilage and -poisoning microorganisms are being increasingly exploited, and must be encouraged if a substantial reduction in food poisoning is to be achieved.

Introduction

There remains considerable public concern regarding the current high level of food poisoning disease in Europe, and the fact that the level continues to rise rather than fall year by year. At the same time, there are strong and increasing demands from consumers for foods that are more convenient, fresher, more natural [1], less heavily processed (e.g.: "REPFEDs and "Sous Vide" foods that are mildy heated and distributed at chill temperatures [2]), less heavily preserved (e.g. less acid, salt and sugar [3]) and less reliant on additive preservatives than hitherto (e.g.: sulphite, nitrite, organic acids and esters [4]).

Most of these trends result in a general *reduction* in the intrinsic preservation of foods. Furthermore, many food poisoning microorganisms escape the attention of preservation techniques altogether, reaching the consumer more or less directly from contaminated foods, most often foods of animal origin. It has therefore been argued that a substantial reduction in food poisoning in the near future will be difficult to achieve unless we obtain a greatly improved understanding of the physiology of the

most important target organisms [5]. This knowledge must then be exploited in ways which effectively improve our means for the control of these hazards and reduce the risk to the consumer.

Although much physiological work has been carried out on non-food poisoning microorganisms, relatively little work has been undertaken on the physiology of the food poisoning microorganisms themselves, despite the fact that their physiology often differs greatly from that of non-food poisoning microorganisms, particularly with regard to the variety of stresses that can be imposed deliberately on microorganisms during food processing, by food preservation techniques and during distribution and storage.

AAIR Concerted Action Programme PL920630 ("Physiology of Food Poisoning Microorganisms") was therefore initiated in order to bring together research groups working on the physiology and related aspects of food poisoning microorganisms and to determine the physiological, biochemical and genetical bases of the organisms' survival of and responses to food-relevant stresses; determine the physiological and genetical factors influencing infectivity and toxinogenesis; understand the physiological bases of those synergistic systems that are already empirically applied or that have future potential; encourage the wider acceptance of modern preservation techniques. Papers by participants in the Concerted Action were published in a special edition of the International Journal of Food Microbiology [6].

Major food-poisoning microorganisms

The types of microorganisms that cause the majority of outbreaks and sporadic cases of food-poisoning are listed in Table 1. They include the infectious organisms like the *Salmonella* and *Campylobacter* species, that cause most food-borne disease in European countries, and the toxinogenic organisms such as *Staphylococcus aureus* and *Clostridium botulinum.* The latter, although infrequent, may have particularly severe consequences. Their temperature relationships are indicated in Table 1 to highlight firstly the wide range of minimum temperatures at which they may grow and secondly the range of their tolerances to thermal inactivation. In addition to the organisms listed in Table 1, other, rarer, organisms that are implicated, or suspected of being implicated, in outbreaks include *Shigella* species, *Plesiomonas shigelloides, Enterococcus* species, *Pseudomonas aeruginosa, Edwardsiella tarda, Vibrio vulnificus, Clostridium difficile, Cryptosporidium parvum,* mycotoxic fungi and viruses (e.g.: "small round" viruses and Norwalk virus). In the past, for many food poisoning outbreaks, the causative agents are unknown. Better detection methodology and public health investigation methods are helping to resolve this uncertainty and confirm some suspected pathogens as real. In particular, the extent to which viruses are implicated in food poisoning remains unclear, but it has been suggested that over 50% of suspected food poisoning cases may have a viral aetiology.

Table1: Major food-poisoning microorganisms

Minimum growth temperature	Heat resistance	
	Low[a]	High[b]
Low (0 - 5 °C or so)	*Listeria monocytogenes* (INF)[c]	*Clostridium botulinum* E and non-proteolytic B (TOX)[d]
	Yersinia enterocolitica (INF)	*Bacillus cereus* (INF andTOX)
		Bacillus subtilis (TOX)
	Aeromonas hydrophila (INF)	*B. licheniformis* (TOX)
Medium (5 – 10 °C or so)	*Salmonella* species (INF)	
	Vibrio parahaemolyticus (INF)	
	Escherichia coli entero- pathogenic and verocytotoxigenic strains (INF)	
	Staphylococcus aureus (TOX)	
(10 – 15 °C or so)		*Clostridium botulinum* A and proteolytic B (TOX)
		Clostridium perfringens (INF)
High (over 30 °C)	*Campylobacter jejuni* and *coli* (INF)	

[a] In excess of a 6 log inactivation of vegetative microorganisms by pasteurization, eg: at a temperature of about 70 °C for 2 min.

[b] In excess of a 6 log inactivation of spores at temperatures ranging from about 90 °C for most heat-sensitive types to about 120 °C for 10 min for the most heat-tolerant types.

[c] INF: organisms that may contaminate foods, and may multiply in them, and which cause food poisoning by infection.

[d] TOX: organisms that may contaminate foods and multiply in them to form toxins that then cause food poisoning by intoxication.

Adapted from ref [4]

Current and emerging preservation technologies

The majority of techniques that are currently employed to combat the microorganisms that cause food poisoning and spoilage act by inhibiting or completely preventing their growth rather than by inactivating them (Table 2). These techniques deliver stresses that the microorganisms must overcome if they are to multiply in a preserved food. Traditional techniques (cold, acid, low water activity, preservatives etc.) continue to be important but newer techniques such as the use of carbon dioxide-enriched "modified atmosphere" packaging and the wider use of lactic cultures and culture products (e.g.: bacteriocins) are adding to the armoury of inhibitory techniques. In addition, there has recently been a strong emphasis on the use of techniques in combinations that deliver the required degree of preservation but without the extreme use of any single technique, as in the successfully applied "hurdle technology" concept of Leistner [7, 8].

Table 2: Major existing technologies for food preservation

Techniques that slow or prevent the growth of microorganisms	
Reduction in temperature	
	-chill storage; frozen storage
Reduction in water activity	
	-drying; curing with added salt; conserving with added sugar
Reduction in pH	
	-acidification(e.g. use of acetic, citric acids etc.); fermentation
Removal of oxygen	
	-vacuum or modified atmosphere packaging
Modified atmosphere packaging	
	-replacement of air with CO_2; O_2; N_2 mixtures
Addition of preservatives	
	-inorganic (e.g.: sulphite; nitrite)
	-organic (e.g.: propionate; sorbate; benzoate; parabens)
	-bacteriocin (e.g.: nisin)
	-antimycotic (e.g.: natamycin)
Control of microstructure	
	-in water-in-oil emulsion foods
Techniques that inactivate microorganisms	
Heating	
	-pasteurization
	-sterilization
Techniques that restrict access of microorganisms to products	
Packaging	
	Aseptic processing

Adapted from ref. [3]

Stress reactions of microorganisms

In many cases the physiological effects of single stresses or preservatives are reasonably well understood but the physiological bases of the successful combination techniques have still hardly been studied. A potentially very important discovery has been the realisation that microorganisms can respond to mild stresses in ways that enable them to survive more severe challenges. For example, heat stress response and acid adaptation are two important physiological responses which have received recent attention and have important implications for the safety of the milder preservation technologies. For example, the heat resistance of *Salmonella typhimurium* was increased by sublethal heating such that a 10 minute, 57°C treatment that delivered a $>10^6$-fold reduction in the numbers of unshocked cells, reduced numbers of the "shocked" ones by less than 10-fold [9]. The biggest increases in heat resistance resulted from long-slow heating at steadily rising temperatures [10, 11], of obvious practical importance in the pasteurization-processing of products like large packed meat cuts and hams destined for chill storage and slicing at the point of

sale, and to Sous Vide foods that are deliberately heated slowly in order to ensure high organoleptic quality. Heat-stressed cells of *Escherichia coli* 0157 become more tolerant not only to a subsequent heat-treatment, but also to exposure to low pH [12], a reaction that is of obvious importance in the decontamination of raw fruits and other materials that may be made into juices and other acid products.

We are thus beginning to have some understanding of the mechanisms by which exposure to one stress can induce tolerance to other environmental extremes, the so-called Global Stress Response, so as to be able to more logically take account of it in assessing the efficacy and safety of particular preservation techniques and processes [13].

In contrast to the inhibitory techniques, a minority of techniques is employed to inactivate microorganisms in foods (Table 2) and, of these, heat remains by far the most-used. However, there are many recent developments that may change this situation. There is much interest in alternative, non-thermal, techniques that may be applied commercially for the inactivation of microorganisms in foods (Table 3). These include the use of enzymes, such as lysozyme, which is already used in large amounts (in excess of 100 tonnes p a [14]) to control the growth of *Clostridium tyrobutyricum* in some cheeses, and of other enzymes and non-enzymic proteins such as lactoperoxidase [15], and microbially-derived polypeptide bacteriocins [16] other than the already used and increasingly exploited bacteriocin, nisin [17]. Novel physical processes that are already applied include the use of high hydrostatic pressure to "pressure pasteurise" foods [18, 19].

High voltage electric discharges ("electroporation") can very effectively inactivate vegetative forms of microorganisms in liquid foods, and machines have been developed to do this[20]. Ultrasonication, whilst of limited efficacy alone, has been demonstrated to show a strong synergy with heat and slightly raised pressure together, and promises to allow pasteurisation or sterilisation of liquid foods to be achieved with the delivery of much reduced heating and with consequent gains in product quality [21].

Table 3: New and emerging technologies for food preservation

Natural additives		
	Animal-derived antimicrobials	
		-lysozyme
		-lactoperoxidase system
		-lactoferrin; lactoferricin
	Plant-derived antimicrobials	
		-herb and spice extracts
	Microbial products	
		-nisin
		-pediocin
		-other bacteriocins and culture products
Physical processes		
	Gamma and electron beam irradiation	
	High voltage electric gradient pulses ("electroporation")	
	High hydrostatic pressure	
	Combined ultrasonics, heat and pressure ("manothermosonication")	
	Laser and non-coherent light pulses	
	High magnetic field pulses	

There is a general framework into which one may fit the reactions of microorganisms to the major food preservation and related techniques that are applied to foods (Table 4). In most cases, the techniques are effective when they overcome the various homeostatic reactions that microorganisms have evolved in order to resist environmental stresses. These are reactions that keep some key element of cell physiology operating, constant and unperturbed, even when the environment is greatly perturbed. However, when considering all the new preservation developments, it is striking that the physiological bases of the inhibition and inactivation mechanisms that underlie the "emerging" techniques, as well as many of the traditional ones, and for the resistance mechanisms of the most tolerant microorganisms to them had, until recently, been little-studied.

Conclusions

Many of the current and likely near-future trends in food preservation, since they imply the application of less severe techniques and processes may, at first sight, make safe and effective preservation and ensurance of safety more difficult to attain. However, at the same time, the trends have introduced a scientific challenge. This is, to develop such improved understanding that we can logically create new and improved methodologies, based on a real appreciation of the microbial physiology involved. This is so with regard to the mechanisms of action of many of the traditional as well as the newer techniques that are already in use or emerging, and also with regard to the stress responses of microorganisms, that allow them to overcome the preservation techniques that are applied.

It is increasingly important to realize that cells which adapt to severe physical challenges, such as the inimical processes employed during food processing, become intrinsically more resistant not only to those particular processes, but to *other* environmental parameters, such as low pH. One linking theme is the naturally high level of resistance of cells in the stationary phase of growth [13]. New insights into the genetic control of stationary phase adaptation has allowed us to account for many of these observations and consequently predict the behaviour of cells exposed to novel processing methods.

A particular future need is to understand the physiological basis of the relationships that are employed to "model" microbial growth and survival. A further need is to research the physiological mechanisms that underlie the sometimes very effective combination technologies so as to put them on a more rational basis and foster their wider use. Another need is likewise to apply physiological techniques to the *new* food preservation methodologies that are being promoted, mostly still on an empirical basis, such as high pressure, electroporation, ultrasonics in synergistic systems, natural antimicrobials, etc.

It has become clear that most of the newer concepts for food preservation will focus on interference with homeostasis, stress reactions and multitarget preservation. The interrelation of these phenomena will lead to the even more efficient application of new "combination preservation" procedures or "hurdle technologies." The "multi-drug approach" has proved valuable in the medical field to fight bacterial and viral infections. Similarly, the "multi-target approach" should be promising in the field of food preservation too.

Table 4: Active and passive homeostatic mechanisms in microorganisms

Environmental stress	Homeostatic reaction
Active homeostasis	
Low nutrient levels	Nutrient scavenging, oligotrophy, generation of "viable non-culturable" forms
Low pH, presence of weak organic acids	Extrusion of hydrogen ions, maintenance of cytoplasmic pH and membrane pH gradient
Reduced water activity	Osmoregulation, avoidance of water loss, maintenance of membrane turgor
Low temperature - growth	Membrane lipid changes, cold shock response
High temperature - growth	Membrane lipid changes, heat shock response
High oxygen levels	Enzymic protection from oxygen-derived free radicals
Biocides and preservatives	Phenotypic adaptation and development of resistance
Ultraviolet radiation	Excision of thymine dimers and repair of DNA
Ionizing radiation	Repair of DNA single strand breaks
Passive homeostasis	
High temperature - survival	Low water content in the spore protoplast
High hydrostatic pressure - survival	Low spore protoplast water content?
High voltage electric dicharge	Low conductivity of spore protoplast
Ultrasonication	Structural rigidity of cell wall
High levels of biocides	Impermeable outer layers of cells
Population homeostasis	
Competition	Formation of biofilms, aggregates with some degree of symbiosis

Adapted from ref. [22]

However, overall, the most useful outcome of physiological investigations will be to facilitate the development, and encourage the application, of new and improved procedures for the *elimination* of food poisoning microorganisms from the most heavily or frequently contaminated types of foods, i.e.: *inactivation* techniques rather than *inhibitory* ones. This will occur, for example, through developments that improve the efficacy and practicality of acid or heat-low pH organic acid combination dips or sprays for the decontamination of plant materials, and more especially of animal carcasses prior to use as food ingredients or distribution in unprocessed form [23, 24, 25], by the steady increase in the use of natural antimicrobials in combination preservation systems, and by the application of the newer physical techniques.

Of course, control of microbial growth in foods by inhibition, through the modification of intrinsic factors will always be fundamental to food preservation and safety but, with particular regard to safety, the most significant reductions in risk would be achieved if the organisms of concern did not enter the home, shop or catering establishment in the first place. The lapses of hygiene that will always occur at some level or other would then be of little consequence.

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Lactobacilli in a healthy diet

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Abstract

The lactic acid bacteria (LAB) may be considered as perhaps the most beneficial microbial group for mankind. Both tradition, dating back to early human civilisation, and the continuing importance of lactic fermentation in human diet, bear witness to a long record of safety and acceptability. During the development of microbiology as a science in the second half of the 19th century, LAB were especially studied for their metabolism and their role in fermented foods. It is, however, only more recent that research efforts enable a better understanding of underlying mechanisms and principles contributed by the LAB in improved safety and quality of fermented foods. In an unbalanced diet (e.g. based on cereals and/or legumes), typical of several regions in Africa) lactic fermentation may play a vital role in the prevention of nutritionally related diseases such as iron and other mineral deficiencies. Strains of lactic acid bacteria (LAB), associated with fermented foods in different regions of Africa, show varying abilities to degrade antinutritive factors, and may be selected and applied more deliberately for food processing operations on different levels. Such LAB strains, isolated from fermenting cereal and legume foods, showed ability in vitro to degrade antinutritive factors such as phytic acid, trypsin inhibitor and the flatulence-causing oligosaccharides raffinose and stachyose.

In addition to their improved shelf life and safety, it is also accepted that particular fermented foods may counteract disorders of the gastrointestinal tract (GIT). In addition to contributing to an improvement of the nutritional value of raw food materials during fermentation, specific "health promoting" or "probiotic" effects of selected LAB strains are now intensively being studied. These "functional" properties of LAB associated with the human gastro-intestinal tract, are of particular interest and have become a focal point of research in recent years.

1 Introduction

Lactic fermented foods are appreciated and accepted as important part of the diet in most industrialised and developing countries. Lactobacilli represent one of the major microbial groups involved in these „desirable“ fermentations. They generally dominate the lactic acid bacterial (LAB) population, and thereby have contributed to food preservation and safety on the household level for thousands of years till the present day (Odunfa, 1985; Holzapfel, 1997). Probably no other process has had such an impact on the nutrition habits and food culture of mankind (Holzapfel, 1997). On the basis of this long tradition and the important place of fermented foods in the culture of many nations, these LAB are today generally considered as safe („GRAS“) and wholesome. This is especially true for fermentations in which lactobacilli pay a dominant role. Research progress in recent years has shed more light on the underlying mechanisms and principles related to improved safety and quality of fermented foods, and on the microorganisms involved. In addition, and as a result of increasing awareness of the close interrelationship between health and the diet, special

attention is presently given to functional properties of lactobacilli and other LAB associated with traditional and novel type fermented foods.

Traditional fermented foods, common to most developing countries, provide interesting models for studying beneficial mechanisms and microbe-food substrate interactions. Developing countries require food processing technologies that are technologically appropriate, suitable for their environments and affordable in rural and urban economies (Westby *et al*., 1997). Household-level lactic fermentation is one such traditional technology, widely practised and typical of most regions in Africa. It has been developed traditionally for a wide range of food products, including cereals and legumes, root crops, fruits and vegetables, dairy products, fish and meat (Steinkraus, 1996). As a unit operation in food processing, lactic fermentation offers numerous benefits which include: improved food safety, enhanced flavour and acceptability, food preservation, improved nutritional value, increased variety in the diet, reduction in antinutritive compounds and, in some instances, improved functional properties. Fermentation is considered an important low-cost food processing method and a common means of preservation in the tropics (Cooke *et al*., 1987), where preservation techniques such as freezing, refrigeration, canning or modified atmosphere packaging are either prohibitively expensive or not available.

Not all LAB strains associated with traditional fermented foods have „ideal“ properties in terms of adaptation to the food substrate, beneficial metabolic activities and functional properties. Industrialised countries benefit from a century of experience with starter cultures, particularly selected for large scale industrial food fermentations. In addition to the conventional, technical selection features, applied thus far, present-day approaches, however, increasingly focus on „multifunctionality“, referring to a combination of desirable properties in one single strain.

This paper highlights the significance of lactobacilli in fermentation as a safe food processing operation. Examples will be given of their potential for food safety improvement, for degrading or reducing naturally occurring antinutrients, toxins and microbial toxins in raw materials, and for the elimination or inhibition of food-borne pathogens. In addition, advances towards a better understanding of their functional or „probiotic“ properties are also addressed. Key research issues and prospects for future implementations of lactobacilli are discussed.

2 Lactobacilli to reduce food hygienic risks

2.1 Preservation and prevention of spoilage

Food fermentation has been applied for several thousand years, first and foremost for the prevention of spoilage and the extension of the shelf-life of foods. Lactic fermented foods serve as special example of ‘safe and wholesome’ foods, and also provide interesting study models on food safety mechanisms in traditional processing technologies. The production of lactic acid during fermentation results in acidification of foods to pH values generally <4.2, a level below which most undesired food-associated microorganisms may either not survive or grow. Acidification therefore constitutes a major preservation and safety factor (Holzapfel, 1997). In addition, several other antimicrobial metabolites (e.g. acetic acid, hydrogen peroxide, bacteriocins, etc.) may also be produced during fermentation, and contribute additionally to extended shelf life and safety of lactic fermented foods (Holzapfel et al., 1995). Gram-negative bacteria, including pathogens and, generally, putrefactive and spoilage bacteria, are particularly inhibited by organic acids. Conclusions on inhibitory activity may therefore be generally applicable to both spoilage microbes and pathogens. Fermented dairy

and meat products serve as examples of protein-rich, highly perishable foods with extended self-life and safety resulting from lactic fermentation. Antibacterial activity has been shown to be directly related to *Lactobacillus* strains involved in fermentation of these products (Schillinger and Lücke, 1989). Lactic fermented cereals, typical of the diet in most regions of Africa, show remarkable shelf life and safety, even under tropical conditions (Mbugua, and Njenja, 1991; Nout, 1994; Odunfa, 1995; Steinkraus, 1996). Hounhouigan *et al*. (1994) reported that lactic acid fermentation for the production of máwe (a fermented cereal in Bénin), reduced the Enterobacteriaceae population below the detection level ($<\log_{10}$ 1.7 cfu/g) after 24 h of fermentation.

A special group of bacterial metabolites, the bacteriocins, are potent antimicrobial agents of proteinaceous nature and are also produced by some LAB strains. They are intensively being studied for their potential for improved food preservation and safety. Examples are given below of the effect of bacteriocinogenic lactobacilli on typical food-borne pathogens.

2.2 Food-borne pathogens

Infants and young children in developing countries are extremely susceptible to food-borne pathogens. Consumption of contaminated foods, not uncommon, is likely to lead to infections or intoxications, especially in developing countries. This is a major cause of infant mortality in most developing countries (WHO, 1974; Motarjemi et al., 1993). The situation is enhanced by predisposition factors related to undernourishment and/or nutritional deficiencies. Food-borne diseases remain a major concern, both in industrialised and developing countries, and increased efforts are made to reduce their incidence, also by application of novel approaches such as „biological preservation“.

Food-borne pathogens implicated as causative agents of diarrhoea include bacteria such as *Escherichia. coli*, *Shigella* spp., *Salmonella* ssp., *Vibrio cholerae* 01 and *Campylobacter jejuni*; protozoa such as *Giardia lamblia*, *Entamoeba histolytica, Cryptospororidium* spp*.,* and also enteric viruses such as rotavirus (Black *et al*., 1980; Huilan *et al*., 1991; Gomes , 1991).

The hypothesis of Metschnikoff (1908), at the beginning of the century, that fermented foods such as yoghurt could inhibit enteropathogens has been confirmed also for traditional lactic fermented foods. Pathogens such as *Salmonella typhimurium* have indeed been shown to be either inhibited or destroyed by selected strains of *Lactobacillus acidophilus* and *Lactobacillus pentosus* in ogi, a traditional fermented cereal gruel (Olukoya et al., 994). The inhibition of foodborne pathogens in lactic fermented cereal gruels has also been highlighted by various workers (Mensah *et al*., 1988; Nout *et al*, 1989; Lorri, 1993; Olukoya *et al*., 1994). Fermented mixtures of water and whole flour of either maize or sorghum with a final pH of 3.8 and a lactic:acetic acid ratio of 9:1 were found to inhibit Gram-negative intestinal pathogens such as enterotoxinogenic *Escherichia coli, Camphylobacter jejuni, Shigella flexneri* and *Salmonella typhimurium* (Svanberg *et al*., 1992). Such antimicrobial activities may be termed ‘unspecific’. Antimicrobial activities against bacteria causing diarrhoea have been related to LAB involved in fermentation of uji, a Kenyan indigenous fermented cereal gruel (Mbugua and Njenga, 1991). Several studies have shown that at pH <4.0 diarrhoea-causing pathogens will be inhibited in traditional „ready-to-eat“ fermented food products (Steinkraus, 1996). Inoculation of vegetable salads with strains of (e.g.) *Lb. casei* resulted in a dramatic decrease of the otherwise dominating Enterobacteriaceae population (Vescovo et al., 1995).

Staphylococcus aureus, Bacillus cereus, and *Clostridium perfringens* are common causes of food intoxications, diseases frequently accompanied by diarrhoea. A number of bacteriocinogenic lactobacilli, associated with fermented foods, have been shown to effectively inhibit the growth of Gram-positive pathogens and toxinogens such as *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus* and different *Clostridium* spp., even under *in situ* conditions (Holzapfel *et al*., 1995). However, information available in literature generally shows a low frequency (from 0.6% to 22%) of bacteriocinogenic strains among food-associated LAB. This poses a special challenge to food microbiologists in their research efforts towards biological approaches for food safety improvement. Such bacteriocinogenic („bac+“) strains show great potential as additional safety factors, e.g. as is shown in Fig. 1 for the inhibition of *Li. monocytogenes* by a bac+ strain of *Lactobacillus sake.*

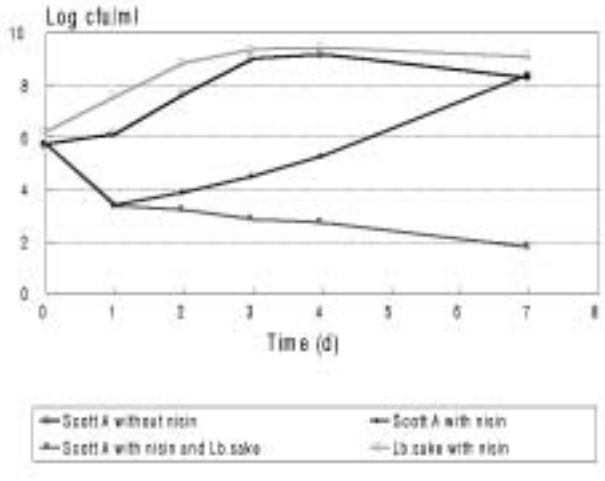

Fig. 1: Inhibition of *Listeria monocytogenes* Scott A by nisin (100 IU/ml) in combination with *Lactobacillus sake* Lb706-1a in Standard I Nutrient broth at 10°C

2.3 Toxins of microbial origin

Mycotoxins

Mycotoxins, especially aflatoxins and fumonisins, are a major risk factor in stored cereals, typical raw materials for traditional fermented foods (Holzapfel, 1997). In these raw materials, they represent the most common toxins of microbial origin, and they pose a serious safety hazard in developing countries (Westby *et al*., 1995). Mycotoxins may be controlled in foods, preferably by preventing their formation either in the field or during storage (Coker, 1995). However, even with stricter regulations on maximum tolerance levels, control mechanisms in developing countries are insufficient and very difficult to apply at the household and small scale levels to any significant extent.. The traditional production of such fermented foods from the raw materials mentioned, usually involves a number of processing steps such as cleaning, soaking, milling, dehulling and cooking, which may contribute to a reduction in contamination of the (fermented) end product. Several reports indicate the feasibility of fermentation for reducing or preventing growth or metabolism of toxin producers. It is of special significance that some mycotoxins may be degraded or inactivated during lactic fermentation of cereals. Some of these data, especially those on aflatoxin degradation, are summarised in Table 1. The reduction of aflatoxin during the preparation of some

traditional Nigerian fermented foods has been studied by Ogunsanwo *et al*. (1989 a, b). They found that fermentation of soybean flour to produce 'soyogi' reduced the initial aflatoxin B_1 level of 0.3 ppm. In another study, Adegoke *et al*. (1994) found >70% reduction in the aflatoxin B_1 level during the lactic fermentation of maize and sorghum flour for the production of ogi, a popular weaning food in West Africa.

Table 1: Examples of the reported effects of fermentation on mycotoxins in raw materials (Westby et al., 1997).

Toxin	Raw material /product	Type of fermentation	Naturally contaminated /spiked	Extent of reduction	Reference
Aflatoxin	Maize/*kenkey*	Lactic acid		None	Jespersen *et al*. (1994)
Aflatoxin	Sorghum/ogi	Lactic acid	Natural with B_1	12 - 16 %	Dada and Müller (1983)
Aflatoxin	Wheat/bread	yeast (dough)	Spiked with B_1	19 %	El Banna and Scott (1983)
Aflatoxin	Milk/yoghurt Milk/kefir	Lactic acid Lactic acid	Natural with M_1 As above	None Decreased	Wiseman and Marth (1983)
Aflatoxin	Milk/yoghurt	Lactic acid	Spiked with B_1, B_2, G_1, G_2 and M_1	None	Blanco *et al*. (1993)
Aflatoxin	Maize/*ogi* Sorghum/*ogi*	Lactic fermentation	Natural with B_1	> 70 %	Adegoke *et al.* (1994)
Alternariol and Alterna-riolmonomethylether	Pure culture isolates from *kenkey*	Lactic acid bacteria	Spiked laboratory media	Reduction >50 % by all tested strains	Holzapfel (1997)

Preliminary studies in our laboratory, showed the ability of single LAB strains from Ghanaian kenkey, to reduce *Alternaria* toxins under defined conditions. In addition, some authentic LAB strains and some isolated from fermented Turkish foods, studied in semi-synthetic medium in our laboratories, reduced patulin concentration by more than 60% (Arici et al., in preparation).

These reports strongly indicate the potential of lactic fermentation to improve the safety of some food products contaminated with mycotoxins. The underlying mechanisms of mycotoxin reduction by fermenting LAB have hardly been studied yet, and present a tremendous challenge

Bacterial toxins

Svanberg *et al*. (1992) observed that a sustained inhibition of enterotoxin producing *Staphylococcus aureus* during lactic fermentation of cereal gruels at pH <4.0 had been supported by an additional factor, probably a bacteriocin. Acid production and other antimicrobial metabolites have been suggested by Mbugua and Njenga (1991) as inhibiting factors against *Staph. aureus* and some Gram-negative enteropathogens during uji fermentation. During lactic fermentation of cereal gruel, the level of *Staph. aureus* was strongly reduced at pH values <4.16 (Nout et al., 1989), whilst Yusof et al. (1993) showed the importance of a high initial level of LAB for successful inhibition of *Staph. aureus* in a rice-based weaning food. Inhibition of *Staph. aureus* by LAB in typical fermented foods of industrialised countries, has sufficiently been documented, both for fermented dairy products (Abdallah et al., 1993; Isono et al., 1994) and fermented meat products (Barbel and Deibel, 1972; Daly et al., 1973; Niskanen and Nurmi, 1976). Even in non-fermented „ready-to-eat" foods such as salads, mere inoculation with *Lb. plantarum* strains resulted in a decline of *Staph. aureus*, especially at refrigeration temperatures (Bonestroo et al., 1993).

3 Potential of lactobacilli for improving food safety and wholesomeness

3.1 Naturally occurring toxins

Cyanogenic compounds in cassava

Cassava is an important staple food crop for at least 300 million people in developing countries. Cassava roots contain the toxic cyanogenic glucosides, linamarin and lotaustralin, which may however be hydrolysed to the corresponding ketone and glucose by the endogenous enzyme linamarinase (Nartey, 1978) and also by microbial glucosidases during fermentation. Fermented and non-toxic food products from cassava include *fuffu*, *lafun*, *gari* (Nigeria), *Kivunde* (Tanzania), *agbelima* (Ghana), *attieke* (Ivory coast). In Africa, methods of cassava processing usually involve peeling, soaking, heaping (a solid state fermentation, with mainly moulds involved), sun drying and grating followed by submerged lactic fermentation. In addition to the endogenous linamarinase enzymes present in cassava, major detoxification is achieved by microbial actions. Microorganisms important in cassava fermentation include *Lactobacillus* spp.(Amoa-Awua *et al*., 1996; Olasupo et al., 1997), *Bacillus* (Ejiofor and Okafor, 1981; Essers, 1995) and yeasts and moulds (Hahn, 1989; Essers, 1995).

Yet, not all processes yield safe products, and high dietary cyanide levels from cassava have been reported to be associated with a number of health disorders, ranging from acute poisoning (Bokanga et al., 1994) to iodine deficiency disorders like goitre and dwarfism (Tylleskar *et al*., 1992), and to the paralysis disease Konzo (Tylleskar, 1994).

Amoa-Awua (1996) reported significant detoxification during the (lactic) fermentation processing of cassava into Ghanaian 'agbelima'. The cyanogenic glucosides in cassava (119.3mg/kg) were completely removed after the fermentation process. Further clarification on the role of fermentation was provided by Westby and Choo (1994), showing microbial growth to be essential for efficient cyanogen reduction in soaked cassava roots. In our laboratories, selected strains of *Lactobacillus plantarum* were used for controlled lactic acid fermentation. As compared to the traditional „spontaneous" submerged fermentation of cassava to „kivunde" – a traditional cassava product of some regions in Tanzania – the controlled fermentation process could recently be shown to yield safe cyanide levels <10 mg/kg (Kimaryo et al., submitted)(see Table 2).

Table 2: Effect of fermentation on the cyanogenic glucoside content (mg/kg of dry weight) of cassava during processing into kivunde, a traditional Tanzanian product from submerged fermentation of cassava. The starter culture contained 4 strains of *Lactobacillus plantarum*, selected for their ability to degrade linamarin (Kimaryo, Massawe, Olasupo and Holzapfel, submitted).

Fermentation Period (day)	Fermentation type		
	Spontaneous	Back-slopping	Starter culture
0 (fresh cassava)	175.9	176.8	176.3
1	94.6	132.6	144.7
2	67.2	89.3	62.5
3	45.9	62.4	38.9
4	43.5	47.7	12.6
5	39.1	32.9	8.1
Dry kivunde	17.8	26.5	6.3

3.2 Antinutritive factors

Antinutrients such as protease inhibitors, lectins, flatus-producing sugars, tannins and metal binding agents, are present at relatively high concentrations in several food legumes. Most cereal grains contain appreciable amounts of phytate, whilst sorghum and millet are associated with significant amounts of polyphenols and tannins (Chavan and Kadam, 1989; Holzapfel, 1997). These antinutritive factors in cereal staple foods (maize, sorghum and millets)(often admixed with legumes for upgrading the nutritional value), gain special significance in an unbalanced diet in which these cereals constitute the main daily diet. This is particularly the case with weaning foods in which these factors may lead to malnutrition in developing countries. Apart from the deficiency of important amino acids such as lysine, methionine and tryptophan in cereal proteins, the availability of protein and starch is also reduced by protease and amylase inhibitors, lectin-related haemagglutinin activities in legumes and polyphenols (from millet and sorghum). Furthermore, the chelating characteristics of phytic acid (phytate), may significatly reduce the availability of calcium, iron, magnesium and zinc ions in cereal foods.

The role of lactic fermentation in the reduction of antinutritional factors in traditionally fermented foods such as cereals and legumes, has been well documented (Chavan and Kadam, 1989; Mbugua *et al*., 1992; Lorri, 1993; Steinkraus, 1996; Westby *et al*., 1997; Holzapfel, 1997). However, most reports supply only limited information on reduction kinetics and on underlying factors. Some information on the role of lactic fermentation is highlighted below.

Phytic acid

Phytates, which hydrolyse phytate into lower inositol phosphates, are present in most cereals (Irving, 1980), and are activated during germination (following traditional soaking) and fermentation. Phytate was shown to be completely hydrolysed after fermentation of germinated white sorghum and, as a result, the amount of available iron significantly increased (Svanberg and Sandberg, 1998). The reduction of phytate by lactic acid fermentation has also been reported in maize (Lopez *et al*., 1983), pearl millet (Mahajan and Chauhan, 1987; Khetarpaul and Chauhan, 1989), germinated finger millet(Udayasekhara Rao and Deosthale, 1988) and in Indian Idli (Reddy *et al*., 1986). Similarly, Reddy and Salunkhe (1980) observed almost complete elimination of phytate phosphorus during an 8-hr fermentation of rice.

Oligosaccharides (flatus-producing sugars)

Cereals and especially legumes contain oligosaccharides such as raffinose, stachyose and verbascose, which may result in flatulence, diarrhoea and indigestion. These oligosaccharides, being members of the ‘raffinose family’ are usually resistant to cooking and other small scale processing steps The a-galactosidic bonds may be hydrolysed by a-galactosidases produced by a number of microorganisms, including LAB associated with the digestive tract and with fermented foods. The typical yoghurt bacteria (*Lb. bulgaricus* and *Streptococcus thermophilis*) have been shown to reduce the stachyose content of yoghurt produced from soy milk by almost 27% (Buono *et al*., 1990). Recent studies in our laboratories on LAB strains isolated from fermented maize products in Ghana, showed the majority of the *Lb. plantarum* strains to ferment raffinose.

Protease inhibitors

Increased availability of essential amino acids including lysine, leucine, methionine, isoleucine and tryptophan has been reported to be associated with lactic acid fermentation of cereal porridges and of Ghanaian kenkey (Mbunga, 1986; Nche, 1995). Furthermore, lactic acid fermentation was also reported to improve the in vitro protein digestibility of non-tannin cereal grains (Khetarpaul and Chauhan, 1990; Lorri and Svanberg, 1993) and of high tannin varieties (Back Knudsen *et al*., 1988; Lorri and Svanberg, 1993) In children, the protein digestibility was reported to increase from a level of 47% to 73% after lactic acid fermentation of whole -grain non-tannin sorghum flour that was prepared in 'Nasha', a traditional Sudanese fermented food for infants and young children (Graham *et al*., 1986). The effect of fermentation may be related to a reduction in proteinase inhibitors (e.g. trypsin inhibitor) in legumes, a reduction of tannins and of high levels of disulphide cross linkages in sorghum prolamine proteins (Hamaker et al., 1987; Khetarpaul and Chauhan, 1989).

4 Probiotic lactobacilli and their potential for health promotion

4.1 Background and gastro-intestinal ecology

After the first hypothesis by Metschnikoff (1908) at the beginning of this century, there is now general agreement on the important role of the gastrointestinal (GI) microflora and especially the LAB in the health status of men and animals. Increasing efforts are today directed towards improvement of the health status by modulating the indigenous intestinal flora by live microbial adjuncts, also called "probiotics". An appropriate definition was suggested by Havenaar et al. (1992), according to which probiotics are defined as "mono- or mixed cultures of live micro-organisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora".

Increasing studies are focusing, amongst others, on the pharmacokinetics of different probiotic LAB in humans. In recent years it could be verified in clinical studies that LAB can induce specific immune regulators as a result of interaction with mononuclear phagocytes and endothelial cells of the host. Furthermore, particular strains of LAB were shown to have adjuvant properties by stimulation of a specific antibody response after infection with attentuated pathogenic micro-organisms. It could now also be established that certain LAB contribute to strengthen the gut mucosal barrier and thus influence gut mucosal permeability and possible diarrhoea.

4.2 Functional properties

The gut flora, and especially the LAB, due to their particular physiological functions, contribute to the ecological balance of the GIT, and thereby to the overall health and well-being of the human host. They act, amongst others, as an effective barrier against pathogenic and opportunistic microorganisms. Bacteria typical of the "normal" intestinal flora may possess a range of beneficial features, and are (e.g.) able to degrade certain food components, produce certain vitamins, stimulate the immune system and produce digestive and protective enzymes. The normal flora is also involved in the metabolism of some potentially carcinogenic substances and may play a role in drug efficacy.

Present efforts aim at manipulating the composition of the gut microflora by probiotic foods or food ingredients. Thereby the numbers and activities of those LAB suggested to possess health

promoting properties are increased. Accumulating scientific evidence in recent years tend to support the postulated mechanisms behind these beneficial effects of probiotics.

Claims of beneficial effects, suggested to result from probiotic activities in the gut, are to be substantiated by sound scientific evidence. Support of such health claims is based either on extensive *in vivo* studies or on deductions from well-founded *in vitro* "model" studies. A number of the most important functional effects from LAB, backed up by scientific evidence, has been summarised by Salminen et al. (1996). These effects include aspects such as immune modulation and strengthening the gut mucosal barrier due to: (1) gut microflora modification, (2) adherence to the intestinal mucosa with capacity to prevent pathogen adherence or pathogen activation, (3) modification of dietary proteins by the intestinal microflora, (4) modification of bacterial enzyme capacity especially of those suggested to be related to tumour induction, and (5) influence on gut mucosal permeability. Examples referring to well-characterised probiotic *Lactobacillus* strains, are shown in Table 3 (see also Salminen et al., 1996). Still, the issue of health claims connected with probiotic foods is presently heavily debated.

Table 3: Successful probiotic strains and their functional properties (modified after Salminen et al., 1996)

Property	*Lactobacillus casei* Shirota	*Lactobacillus rhamnosus* (GG) (ATCC 53103)	*Lactobacillus johnsonii* (LA1)	*Lactobacillus acidophilus* NFCB 1748
Origin	Human	Human	Human	?
Safety	Verified	Verified	Verified	Verified
Acid stability	Good	Good	Good	Good
Bile stability	Resistant	Resistant	Resistant	Resistant
Colonization	-	+	+	-
Bacteriocin production	No	Yes	Yes	No
Adherence (Caco-2)	No	Yes	Yes	No
Adherence (mucosa)	?	Yes	Yes	Yes

4.3 Modern applications

An increasing number of different product types or supplements containing viable LAB with probiotic properties is commercially available either in lyophilised form or as fermented food commodities. Strains of *L. acidophilus* and *L. casei* strains Shirota probably have the longest history among known bacterial strains for application on account of their claimed health benefits. This fact may also be related to pioneering research by Reuter (1997) during the 1960's by which valuable information on the dominant LAB in the human gut was provided. Thereby, especially the species *Lactobacillus acidophilus* (now represented by the species *L. acidophilus, L. amylovorus, L. crispatus, L. gallinarum, L. gasseri* and *L. johnsonii*) and *Lactobacillus casei*. These lactobacilli are presently the most frequent „probiotic" representatives in commercial probiotic products, and are followed by *Bifidobacterium* spp., some other LAB genera and even a few non-lactics, the latter restricted to „non-food" products. The application of „gut-associated" LAB in „novel-type yoghurts" is exemplified by the high frequency in which strains of the autochthonous *Lactobacillus* spp.(*L. acidophilus* group and *L. casei*) are applied in these products. Some information on typical lactobacilli and properties of such „novel-type yoghurts" is summarised in Table 4.

Table 4: „Probiotic“ lactobacilli detected in novel-type yoghurts (Holzapfel et al., 1998)

Manu-facturer	Origin	*Lactobacillus* viable counts (Log CFU/g)	Lactobacilli indicated by the manufacturer	Lactobacilli identified by DNA-DNA hybridization
A	Germany	7.9-8.9	*L. casei* Shirota	*L. paracasei (casei)***
B	Germany	6.4-8.1	*L. acidophilus* LA-1	*L. johnsonii*
C	Germany	8.0-8.4	*Lactobacillus casei* GG	*L. rhamnosus*
		6.3-7.8	-	*L. acidophilus*
D	Germany	5.4-6.4	BactoLab cultures	*L. acidophilus*
E	Germany	7.4-8.1	*L. casei* Actimell	*L. paracasei (casei)*
F	Germany	6.8-8.2	*L. acidophilus*	*L. acidophilus*
		6.2-7.8	*L. casei*	*L. paracasei (casei)*
G	Germany	3.9-5.7	*L. acidophilus* LA-7	*L. acidophilus*
H	Germany	4.7-6.2	*L. acidophilus* LA7	*L. acidophilus*
I	Germany	5.2-5.9	Not indicated	*L. acidophilus*
J	Germany	5.5-6.8	*L.acidophilus*	ND
K	Germany	7.1-7.8	BIOGARDE cultures	*L. johnsonii*
L	Germany	8.6-8.7	*L. casei*	*L. paracasei (casei)*
M	Germany	8.1-8.4	*L. acidophilus* LA-H3	ND
		4.7-5.3	*L. casei* LC-H2	
C	Netherlands	6.8*	*L. acidophilus* Gilliland	*L. crispatus*
		6.4*	*L. casei*	*L. paracasei (casei)**
N	Sweden	9.2	*L. acidophilus*	*L. acidophilus*
O	Germany	7.3	-	*L. acidophilus**
P	Switzerland	7.6*	*L. acidophilus*	*L. acidophilus**
		9.0*	*L. casei*	*L. paracasei (casei)**
		5.2*	*L. reuteri*	*L. reuteri**
Q	Switzerland	8.3*	*L. casei*	*L. rhamnosus**
R	France	8.3*	*L. casei*	*L. paracasei (casei)***

* acc. to Reuter (1997)

** *L. casei* suggested for *L. paracasei* by Dicks *et al.* (1996)

5 Conclusions

Fermentation still plays a major role in the establishment and maintenance of food safety, especially under traditional economies. The improvement of overall product quality and wholesomeness with respect to (i) degradation of toxic compounds in raw materials, (ii) production of antimicrobial metabolities and (iii) low-cost means of food preservation has been well established and appreciated. Moreover, thanks to increasing scientific information on microbe-substrate interactions and particularly on beneficial functional properties of LAB strains typically associated with fermentation, this potential is expected to be exploited to a much greater extent in the future. Multidisciplinary approaches will be directed more towards „multifunctionality“ of appropriate strains, including „probiotic“ features, by which recent developments will be taken into account in strain selection and application.

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Mathematical modelling of microbial growth

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Summary

Different approaches to modelling microbial growth and death responses to estimate the safety and shelf-life of foods has been the subject of much research in the past 15 years. The past will be summarised briefly and prospects for the future considered.

Introduction

For many years the safety and shelf-life of products have been estimated by “challenge tests”, or “inoculated pack experiments”. The circumstances of the challenge test often differ substantially from the exact conditions during food processing and storage, but reliance is still placed on such, relatively crude and unrepresentative, tests. Attempts are sometimes made to accelerate the results of challenge tests by incubation at higher temperatures, but the microbial response at higher temperatures does not always mimic that at lower storage temperatures. Similarly introduction of the microbes into the test food can change the local environment e.g. by introducing water, thereby facilitating local microbial growth. Perhaps most important is the common use in challenge tests of high numbers of the microbe of concern, often 10^5-10^6, justified as giving the results "a margin of safety". In the case of bacterial pathogens, such high numbers are not representative of the number normally present in raw and processed foods, and there is evidence that growth of pathogens occurs more readily from high numbers than from low numbers.

Modelling

Challenge tests have long been recognised as expensive, slow, demanding on facilities and microbiological skills. More recently it has been recognised that they provide only modest assurance that a product formulation will be safe in the food chain. Another disadvantage of challenge tests is that the knowledge acquired from them is not cumulative. When a product formulation, packaging or distribution system changes, the challenge tests should be repeated. With the large, and increasing, number of bacterial pathogens of concern to the food industry an alternative to challenge tests was required.

One option is to understand sufficiently the responses of the key microbes to the main controlling factors in the food environment, to build a cumulative store of knowledge, and to develop the means of interpolating calculated microbial responses. Microbiologists have dreamed of being able to model the growth and death of microbes in foods. The long term prospect of not having to repeat challenge tests when the responses are confidently assured was embodied in the term "predictive microbiology".

Modelling thermal death

Although modelling is regarded as novel, food technologists have for many years modelled microbial death e.g. ensuring inactivation of *C. botulinum* spores in low acid foods via the 12-D concept, the minimum heat process based on the heat resistance of spores of *C. botulinum* (1). This approach is simplistic and empirical, interpreting the response of the organism without taking into account the mechanism of spore death. Similarly, processing times for the elimination of *Salmonella* at a range of minimum internal temperatures (2) led to Good Manufacturing Practices (3) addressing the problems of cooking fresh and cured beef with respect to survival of *Salmonella.* When salmonellosis was traced to roast beef, a simple form of modelling was used to define that cook required at a range of temperatures, although eradicating the problem also necessitated careful avoidance of recontamination of the cooked product.

Modelling thermal death has long been accepted, the 12-D "botulinum cook" having been developed from a relatively small set of experimental data. The pH of the food matrix is taken into account by recognising that at low pH values the thermal process to achieve sterilisation is less severe and spores of *C. botulinum* are unable to grow and produce neurotoxin below pH values ca 4.5. Similarly the concept of *z* value, which describes the change in death rate with temperature, has proved workable, although questionable in particular circumstances.

Modelling growth

There is a long history of modelling microbial growth in the fermentation industry, with the objective of maximising biomass by optimising the effects of growth temperature and the supply of substrate(s). With the important exceptions of fermented products (milk, meat, fish, vegetables), when rapid and efficient fermentation is an essential first step towards product quality and safety, the aim of food microbiologists is usually to prevent, or minimise, microbial growth, rather than optimise it. Consequently, effects of inhibitory environmental factors and chemical preservatives have been investigated more intensively.

In food microbiology, and with the exception of fermented products, the cell concentration of interest is usually low compared with biotechnology, where it is typically 10^7-10^8 cells/ml. In food microbiology the lag phase can be important, while it is less so in a bioreactor. Models of the transition from the exponential phase to the stationary phase, like Monod's (4), are less significant in food microbiology because there are few instances where substrate limitation is important, certainly until microbial numbers reach levels that cannot be tolerated in foods. There is also less precise information about the physicochemical environment in the food than in a bioreactor. These differences have resulted in different approaches to modelling.

Food microbiologists have defined the minimum, optimum and maximum values for growth for pH, available water and temperature for most of the micro-organisms of concern. Unfortunately, some of those "minima", especially the "minimum temperature for growth", have been introduced into national or international guidelines and even legislation, forgetting that in the experimental system factors other than the single factor being investigated were optimal, which is rarely the case in foods.

Simple growth models have identified Critical Control Points and processing guidelines to give greater assurance of safety e.g. the FSIS directive (5) on time/temperature guidelines for cooling heated products, is designed primarily to control growth of *C. perfringens*. The identified control

points, minimum and maximum holding temperatures and cooling times were based on growth of *C. perfringens* in chilli (6).

The number of factors identified in the scientific literature as affecting the growth or survival of foodborne microbes is large, including temperature, pH and the acidulant, water activity and the humectant, oxygen availability, redox potential, preservatives etc. All need to be considered when assessing whether a micro-organism could pose a problem in a particular food system. The expectation of complex relationships demanding huge experimental designs had a negative effect on progress, because investigating all the factors at even a few levels was an impossible task.

Data exploring the combined effects of several controlling factors in food systems began more than 25 years ago. In the 1970's research characterising the effectiveness of sodium nitrite against *C. botulinum* in model and cured meat systems expanded due to interest in minimising formation of nitrosamines without loss of antibotulinal activity (7-11). However, little of that research was reduced to models, perhaps due insufficient systematic data or lack of availability or access to effective modelling techniques. Nevertheless that early work clarified the relative importance of the main controlling factors, and identified the need for a modelling approach. An early application of response surface analysis techniques to food microbiology (12) demonstrated that of sixteen ingredients in a soy-based ground meat analogue, only four had a significant impact on the growth of *C. perfringens*.

Approaches to modelling growth

Probability models

One approach to estimating the safety of foods with respect to *C. botulinum* was to calculate the probability that a single spore of *C. botulinum* would germinate and produce toxin. Hauschild (13) used this approach to draw together results from different authors. Other researchers systematically estimated the effects and interactions of a range of factors on the probability of germination and outgrowth of *C. botulinum* (14, 15). Alternative probabilistic models have been published for toxin production by proteolytic strains of *C. botulinum* types A and B (13, 16-18), and *Zygosaccharomyces bailii* (19).

Although only the probability of growth or toxin production was indicated, the most significant factors and interactions influencing growth were quickly identified, and the wisdom of changing a product formulation, or storage conditions, could be evaluated.

Kinetic models

Approaches to estimating growth kinetics vary in their mathematical complexity, but all have similar aims to estimate the lag phase and the maximum growth rate. Approaches include the "square root" model (20, 21), linear Arrhenius (22), non-linear Arrhenius (23), and using cardinal values from the literature (24). Estimating the effects of several controlling factors on growth at the same time has most commonly modelled growth parameters derived from growth curves against the controlling factors by a response surface approach (25-28). The empirical application of the Gompertz function to the logarithm of the cell concentration (25) should have been termed a 'modified' Gompertz model, with no deep root in population dynamics as analysed by Holgate (29), and no known mechanistic derivation. Its differential-equation-form (30) is another form of the same, 'modified', Gompertz model.

More recently dynamic mathematical models, with a sound biological and mathematical basis, have been developed. The advantages, disadvantages, and some of the pitfalls have been discussed by McMeekin et al. (31). A dynamic model by Baranyi and Roberts (32, 33) uses a simplification of the assumption of Frederickson et al. (34) that the state of a homogeneous cell population can be defined by the physicochemical environment (temperature, atmosphere, substrate etc.) and the intracellular conditions. The environment-dependence of the maximum specific growth rate and the lag (or their reparameterized forms) have sometimes been modelled independently, although it has long been recognised that there is an obvious high correlation between them. The new model (33) overcomes this problem.

In food processing and storage it is important to be able to predict the course of bacterial growth under a temperature profile that changes with time. Fu et al. (35) showed that, provided the temperature remains within the temperature range for growth, if the cells are in the exponential phase instantaneous adjustment of the specific growth rate to the temperature changes can be assumed. The new model (33) also makes it possible to predict the course of bacterial growth when the temperature changes in the lag phase during which the cells adjust to a new environment.

Predicted growth responses from some models have been compared extensively with data published by other workers (28, 36-39). The agreement between predictions of growth rate and values published by others (observed values) have, in general, been reasonable, and even good.

Being able to predict the rate of growth rate of a pathogen from measures of pH, temperature and available water indicates that, in many instances, the food itself is playing a minor role in determining the microbial response, contrary to beliefs that the food was a key determining factor.

If the predicted growth response differs from the observed (published) value, the difference is sometimes due to factors not in the model e.g. a preservative in the food, carbon dioxide in the atmosphere above the food. Several models with more than three factors have been developed to accommodate the additional controlling factors (26, 28). If the observed response still differs substantially from that predicted, it is important to try to determine the reason, and to keep a cumulative record of those reasons. Some foods contain natural antimicrobial systems e.g. eggs. There are examples where the structure of the food plays a role, perhaps by limiting diffusion of substrate or oxygen to the microbe, or acid produced away from it (40).

Non-thermal inactivation

There are sometimes conditions in foods where, although the temperature may not kill the microbe, growth cannot occur due to low pH, low water availability or other inhibitory conditions. Only recently has it been recognised that many vegetative bacterial pathogens are much more robust than anticipated, and able to withstand adverse conditions in foods for months without losing their viability. Effort has already been put into modelling those responses (41, 42). Inactivation data are also available for *L. monocytogenes* (43, 44), *Y. enterocolitica* (45) and *Staphylococcus aureus* (46).

Modelling programmes

The development of Predictive Microbiology received a huge boost when, in 1998, the UK Ministry of Agriculture Fisheries and Food reviewed possible topics for new research programmes and decided to fund a co-ordinated programme on bacterial pathogens:

- to obtain systematic data on microbial growth and death for different temperatures, pH values, water activities and preservatives;
- to treat the data mathematically to make models of the microbes' behaviour;
- to ensure that the model mimics the microbe's behaviour in a range of growth and death conditions, and
- to "validate" the model by comparison with independent published scientific data or by selected challenge tests.

In 1992, the outputs of that research programme led to a "bureau service" based on the database and the models. However, it did not prove popular with the food industry and was replaced in 1994 by commercially available software Food MicroModel™, running under Windows. This research effort prompted the USDA to initiate a similar programme modelling the growth and death responses of bacterial pathogens, leading to software Pathogen Modelling Programme (47), initially available running under DOS, and the most recent version under Windows.

Interest in Predictive Microbiology in other European countries was fostered under a Food Linked Agricultural and Industrial Research (FLAIR) Concerted Action programme on "Predictive Modelling Microbial Growth and Survival in Foods" (CA No.5, COST 905), and a FLAIR Shared Cost programme on "Computer-aided process design procedures to improve quality and safety of products with a limited shelf-life", each 1989-93.

The network of scientists in those programmes has been maintained and expanded through COST 914 "Predictive Modelling of Microbial Growth in Foods" (1994-99), within which four Sub-Groups explored topics identified as important to the improvement and further progress of Predictive Microbiology viz:

1. Validation of predictive microbiology in a wide range of European foods;

2. Instrumental methods for data capture for advanced predictive microbiology;

3. Explore mixed population effects in predictive microbiology;

4. Modelling microbial survival to eliminate pathogens from foods.

Within the EU a consortium of eight partners will extend that effort in a Shared-Cost programme "Predictive modelling in structured foods" (PREMIUM).

Future

It is not advisable to use predictions from the model where there has been no significant comparison with independent data. The user should confirm, through a few tests, that the predicted microbial response is representative of that in the foods(s) where the models are intended to be used.

Experience already indicates that models developed in laboratory media and validated against independent data offer a rapid, convenient and reasonable "first estimate" of the behaviour of foodborne pathogens in many foods. As data have accumulated for other microbes covering additional controlling factors (CO_2, nitrite, ethanol, organic acids) confidence has increased that modelling is the most cost-effective way forward. However, it is not advisable to use predictions from a model where there has been no significant comparison with independent data. Advances in

modelling spoilage have been slower because it is believed to be more complex, but prospects for rapid progress are good (48, 49).

It is sometimes difficult to chose between published models for the same pathogen, when both models have resulted from substantial laboratory experimentation. Agreement on indices of model performance would facilitate comparisons of models (50). In an attempt to help decide between models containing large numbers of coefficients and simpler models, the consequences of over-parameterization have been discussed (51). A procedure to define the useable domain of models has been suggested (51), taking account of the environmental conditions where data used in the model had been produced. Already kinetic and probabilistic models are being combined (52), with the prospect that predictions will include estimates of both the rate of growth and the likelihood of growth.

If "predictive microbiology" can be realised for thermal death, non-thermal death (survival) and growth conditions, food microbiology will advance immeasurably. Once a model has been validated in a product (i.e. a commodity and a process) (53, 54), it can be used at all stages of the food chain from harvesting, through production and processing, to distribution and retail sale. Modelling is already being incorporated into HACCP systems, product development and quantitative risk assessment (55, 56).

The history of what has come to be known as "Predictive Microbiology" was reviewed in detail by Ross and McMeekin (57). If "predictive microbiology" can be realised for thermal death, non-thermal death (survival) and growth conditions, food microbiology will advance immeasurably. Once a model has been validated in a range of products, that model can be used at all stages of the food chain from harvesting, through production and processing, to distribution and retail sale. Modelling has already been incorporated into HACCP systems, product development and quantitative risk assessment. Coupled with process engineering and knowledge of thermal properties of foods, Predictive Modelling will prove a powerful tool in the quest to optimise quality and safety (58).

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Opportunities for bacteriocins in food: Prevention and safety

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Bacteriocins of lactic acid bacteria (LAB) have been extensively studied for use as food preservatives. The two major classes of LAB bacteriocins include lantibiotics, highly modified peptides e.g. nisin, containing lanthionines and atypical didehydroamino acids (class 1), and small (< 10 kDa), heat stable unmodified peptides e.g. pediocin (class 11). Successful application of LAB bacteriocins in food preservation requires detailed information about their mode of action. Lantibiotics generally act against a broad range of Gram-positive microorganisms, whereas the specificity of class 11 bacteriocins seems to stem from the fact that these compounds recognise specific proteinaceous membrane receptors.

The efficacy of LAB bacteriocins in the prevention of pathogen outgrowth, may be compromised by the emergence of bacteriocin resistant variants in food processing situations. Emergence of nisin resistant mutants, which are generated when nisin sensitive cells are exposed to relatively high nisin concentrations, has been described for several species of Lactobacillus, Streptococcus, leuconostoc, Bacillus, clostridium, Staphylococcus aureus, and listeria, including L. monocytogenes. Nisin resistance mechanisms involve modifications of the cell wall and the membrane which result in a reduction of the efficiency of nisin pore formation.

Knowledge about bacteriocin action and resistance mechanisms may prevent misapplication and guarantee its effectiveness by managing the emergence of resistant pathogenic and spoilage strains. The use of bacteriocin cocktails or a combination with other mild preservation techniques may prevent the selection of bacteriocin resistant mutants.

Introduction

Lactic acid bacteria (LAB) play an essential role in the majority of food fermentations, and a wide variety of strains are routinely employed as starter cultures in the manufacture of dairy, meat, vegetable and bakery products. One of the most important contributions of these microorganisms is the extended shelf life of the fermented product by comparison to that of the raw substrate. Growth of spoilage and pathogenic bacteria in these foods is inhibited due to competition for nutrients and the presence of starter derived inhibitors such as lactic acid, hydrogen peroxide and bacteriocins (31,36). Bacteriocins, are a heterogeneous group of anti bacterial proteins that vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties (25). Currently, artificial chemical preservatives are employed to limit the number of microorganisms capable of growing within foods, but increasing consumer awareness of potential health risks associated with some of these substances has led researchers to examine the possibility of using bacteriocins produced by LAB as biopreservatives.

The major classes of bacteriocins produced by LAB include: I. lantibiotics, 11. small heat stable peptides, 111. large heat labile proteins, and IV. complex proteins whose activity requires the association of carbohydrate or lipid moieties (25). Significantly the inhibitory activity of these substances is confined to gram-positive bacteria. Gram-negative bacteria possess in addition the

cytoplasmic membrane and the peptydoglycan, the so called outer membrane which is composed of phospholipids, proteins and lipopolysaccharides (LPS), and this membrane is impermeable to most molecules. Nevertheless, the presence of porins in this layer will allow the free diffusion of molecules with a molecular mass below 600 Da. The smallest bacteriocins produced by lactic acid bacteria are approximately 3 kDa and are thus too large to reach their target, the cytoplasmic membrane (25,44). However, it was recently demonstrated that Salmonella species and other gram-negative bacteria become sensitive to nisin after exposure to treatments that change the permeability barrier properties of the outer membrane (37,39,41).

In 1969, the joint Food and Agricultural Organization/World Health Organization (FAO/WHO)Expert Committee on Food Additives reviewed the toxicological data for nisin and recommended its acceptance for food use. Nisin is currently approved as a food additive in over 50 countries, including members of the EU (nisin's designated food number is 234) and the USA (11).

This paper will focus on the mode of action of nisin and pediocin, and the acquired bacteriocin resistance of food pathogens. Their potentials in food preservation and control of food poisoning will be discussed, and the reader is referred to recent reviews covering similar topics (3,4,11,14,19,21,22,31,36,42,43).

Nisin action

The best studied lantibiotic is Nisin A, a 34 residue antibacterial peptide that is produced by several strains of Lactococcus lactis and strongly inhibits the growth of a wide range of gram-positive bacteria (22,23). The mature peptide displays several unusual features, such as the dehydrated residues dehydroalanine and dehydrobutyrine, which are derived from serine and threonine residues, respectively, and lanthionine and ß-methyllanthionine residues which form five intramolecular thioether bridges (23). Molecular structures similar to that in mature nisin are found in three other bacteriocins produced by L. lactis (lacticin 481), Lactobacillus sake (lactocin S) and Carnobacterium piscicola (carnocin U149) together forming the Class 1 LAB bacteriocins (25).

In gram-positive bacteria nisin has been shown to act on energized membrane vesicles to disrupt the proton motive force (PMF), inhibit uptake of amino acids, and cause release of accumulated amino acids (23,26). Nisin Z, a natural nisin variant, was isolated from L. lactis subsp. lactis strain NIZO 22186. The gene for this lantibiotic, designated nisZ, has been cloned and its nucleotide sequence was found to be identical to that of the precursor nisin gene apart from a single mutation resulting in a substitution of His^{27} for Asn^{27} in the mature polypeptide (13,34). Exposure of the food pathogen Listeria monocytogenes to nisin Z resulted in immediate loss of cellular potassium ions, depolarization of the cytoplasmic membrane, hydrolysis and partial efflux of cellular ATP (1) demonstrating in this species, the primary target for nisin Z is the cytoplasmic membrane. Nisin has been shown to associate with non energised liposomes with the greatest interaction being observed with negatively charged phospholipids. This indicated that the initial association of these positively charged peptides with the membrane may also be, in part, charge dependent (15,17). A trans-membrane orientation is not adopted prior to the application of a membrane potential (negative inside) of approximately -80 to -100 mV (23). The threshold potential might be influenced by various parameters such as the pH and the phospholipid composition of the membrane. Nisin A and Z displayed increased activity at acidic pH values and could permeabilize membranes at membrane potentials which were very low and even completely absent (1, 16,17). Nisin A can form transient multistate pores with diameters ranging from 0.2 to 1.2 nm in black lipid membranes when

trans-negative potentials are applied. Such pores would allow the passage of hydrophilic solutes with molecular masses up to 0.5 kDa. Indeed nisin A and Z have been shown to induce leakage of ATP from target cells (1,23). Two models for the mode of action have been proposed: i. a "barrel stave" mechanism (35), and ii. a "wedge" model (15).

Nisin has also been shown to act on Clostridium and Bacillus spores, but in these cases the exact mechanism of action has not been elucidated (22). Sublethal heat treatment of spores causes sufficient injury to induce sensitivity to nisin (23,36).

Pediocin action

Class 11 LAB bacteriocins are small heat stable, non-lanthionine containing membrane active peptides. The mature bacteriocins are predicted to form amphiphatic helices with varying amounts of hydrophobicity and ß-sheet structure. Klaenhammer (25) defined three subgroups within this class of bacteriocins that are: Ha, Listeria active peptides with a consensus sequence in the N-terminal of -Tyr-Gly-Asn-Gly-Val-. IIb, poration complexes formed by oligomers of two different proteinaceous peptides and, IIc, Thiol-activated peptides requiring a reduced cysteine residues for activity.

The best studied class 11 bacteriocin is pediocin PA-1 from Pediococcus acidilactici. PA-1 was recently shown to be identical to pediocin AcH (25,36). This bacteriocin shares sequence similarities with various other important anti listerial bacteriocins (Sakacin A and P, Leucocin A, curvacin A and Carnobacteriocin BM1 and B2) produced by LAB associated with meats. These (pediocin like) peptides are active against a broad range of gram-positive bacteria including L. monocytogenes. Mature pediocin PA-1 is a highly hydrophobic, positively charged peptide consisting of 44 amino acids. Pediocin PA-1 acts on the cytoplasmic membrane thereby dissipating ion gradients and inhibiting transport of amino acids, and causing ATP depletion in sensitive cells. The same activity was observed in membrane vesicles derived from these cells, whereas liposomes made from the membrane lipids were not affected (7). The specificity of pediocin seems to stem from the fact that the bacteriocin recognises a specific membrane receptor protein. A model for the mode of action of pediocin and other class 11 LAB bacteriocins is shown in Fig. 1B (3,7,46,47).

Preservation of dairy and meat products with nisin and pediocin

Dairy products. A problem often encountered in cheese production is the outgrowth of butyric acid bacteria such as Clostridium tyrobutyricum (22). In dairy practice, nitrate is commonly added to cheesemilk to prevent outgrowth of clostridia spores. This chemical preservative can be very efficiently replaced by nisin A. Outgrowth of Clostridium tyrobutyricum spores in nitrate free Gouda cheese was completely prevented when a nisin A producing strain was added to the starter culture (10% nisin A producers). Nisin A is also an effective inhibitor of L. monocytogenes, and growth of this pathogen was effectively inhibited by Nisin A in camembert (29) and in cottage cheese at 4°C as well as at 37°C (5). These results strongly suggest a potentially wider role for nisin A in the future preservation of a variety of dairy products.

The application of nisin in dairy foods which require lactic acid starter bacteria presents a problem because the wide spectrum of inhibition associated with nisin includes LAB themselves. An alternative approach which could be used to control specific pathogens or spoilage organisms in dairy foods is to employ bacteriocins with a highly specific activity range. The pediocin like, heat stable bacteriocin enterocin 1146, which is produced by Enteroccus faecium DPC 1146, is extremely

active against L. monocytogenes at levels which have no effect on lactococcal starters. E. faecium DPC 1146 was used to ferment milk, which was subsequently pasteurized. The bacteriocin is produced in milk and is unaffected by the heat treatment. This milk was mixed with fresh milk and used for cheese making. The lactococcal starters were shown to grow and produce acid normally in this mix, whereas L. monocytogenes introduced in at the same time was rapidly killed. This inhibitory effect was not observed when a variant of DPC 1146 was used which no longer produced the bacteriocin (3).

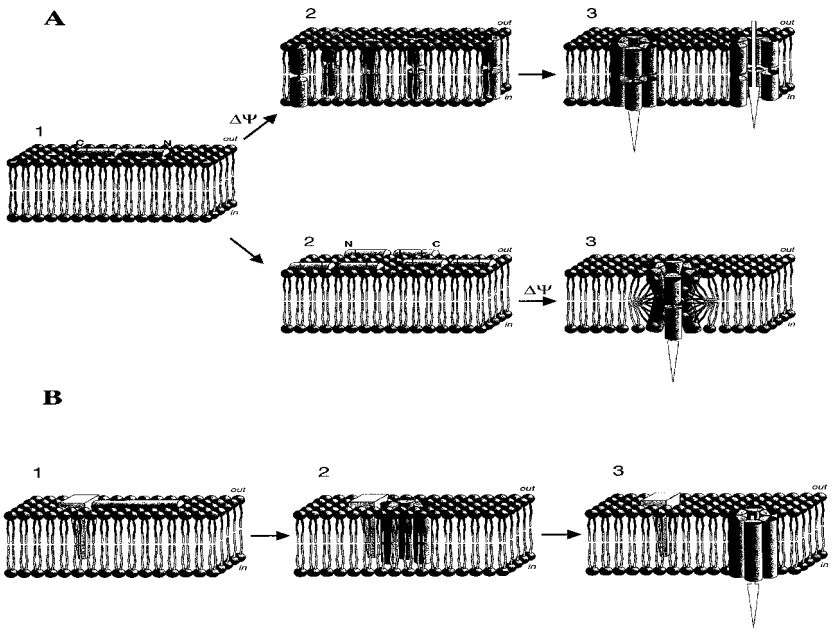

Figure 1: Models for pore formation nisin (A) and non-lantibiotic Class II LAB bacteriocins (B).

(A) The mature nisin molecule is schematically presented with the N-terminal (N) 1-19 amino acid residue part containing one Lys^+, connected via a flexible hinge region to the 2 1 34 C-terminal (C) amino acid residue part, which contains two Lys^+. The barrel stave mechanism (top) (35) involves three discrete steps: 1. binding of nisin molecules to the membrane, 2. $)P$ (inside negative) dependent insertion into the membrane, and 3. aggregation of monomers resulting in the formation of a water filled pore. In the wedge model (bottom) (15) the nisin monomers are assumed to associate at the surface of the membrane, followed by $)P$ (inside negative) dependent insertion into the membrane with the (anionic) phospholipid headgroups incorporated into the pore.

(B) Model for pore formation by Class 11 LAB bacteriocins. 1. the proteinaceous receptor is involved in bacteriocin binding, 2. PMF independent insertion of the bacteriocin into the membrane, and 3. aggregation of monomers in the membrane results in pore formation. The light and dark shaded halves represent the hydrophilic and hydrophobic regions of the amphiphilic peptides, respectively (adapted from 2).

Meat products. Over the past three decades there has been an increasing research interest in the development of nitrite free meat curing systems. The principle concern with the use of nitrite for curing of meat is the eventual formation of carcinogenic N-nitrosamines. Recently, attempts have been made to use nisin A as an alternative to nitrite. While the use of this bacteriocin alone was not successful, promising results were obtained when it was combined with reduced levels of nitrite: 100-250 ppm nisin A combined with 120 ppm nitrite was more effective than the conventional 156 ppm nitrite (40). Nisin A is apparently not the bacteriocin of choice for meat preservation. Bacteriocins produced by LAB associated with meat and meat fermentations such as Pediococcus, Leuconostoc, Carnobacterium and Lactobacillus spp. are likely to have much greater potential as meat preservatives, and the same may hold for fish products (42-44,49).

Lactobacillus sake Lb674, a mildly acidifying lactic acid bacterium originally isolated from meat, produces the bacteriocin sakacin 674, which is identical to sakacin P and very similar to pediocin PA-1 (20,27,28,45). On vacuum packed sliced Bologna type sausage stored at +7°C, L. sake Lb674 produces detectable amounts of bacteriocin and delays or completely inhibits the growth of L. monocytogenes when inoculated at levels of at least 10^{5} - 10^{6} LAB/g, while bacteriocin negative LAB had no inhibitory effect on growth of this organism. As a purified additive, sakacin 674 exhibits a marked initial effect against L. monocytogenes and reduces listerial growth during storage of this fermented meat product (3).

Yousef et al (49) investigated the growth of L. monocytogenes in packed wiener sausage, a fully cooked, cured meat product which is susceptible to contamination by L. monocytogenes before packaging. These researchers provided evidence that Pediococcus inoculants or purified pediocin can function as biopreservatives to eliminate gram-positive pathogenic bacteria in cooked meats during extended refrigerated storage.

Intrinsic and extrinsic factors affecting bacteriocin activity. The action of bacteriocins against sensitive microorganisms is influenced to a large degree by factors such as pH, cell concentration, lipid content, proteolytic enzymes, and liquid vs. solid system (3,36). Additionally, the action of nisin Z is also dependent on the temperature. The rate of Nisin Zinduced K^{+} efflux from cells of L. monocytogenes grown at 30°C was shown to be severely reduced at decreased temperatures. The ordering of the lipid hydrocarbon chains which occurs at lower temperatures resulting in a decrease in membrane fluidity are probably responsible for the reduced nisin Z efficiency observed (1). L. monocytogenes adapts to low temperature growth by increasing the proportion of short and/or branched fatty acyl chains of the lipids thereby maintaining an optimum fluidity (18), an adaptation which may well be responsible for the remaining detectable activity of nisin Z against cells grown at 4°C (1). This is in line with the observation that similar MIC values for nisin Z against food pathogens and food spoilage bacteria are found when cells are grown in BHI or in low fat milk and at high or low temperatures (Table 1) (3). The necessary adaptations at the level of the cytoplasmic membrane for growth at low temperature allows nisin Z to act efficiently against a broad range of sensitive bacteria over a wide range of temperatures.

Resistance to bacteriocins. A number of nisin resistance mechanisms have been described. Many gram-positive bacteria have been shown to be resistant to nisin due their ability to synthesize an enzyme, nisinase , which could inactivate nisin. The enzyme was isolated from several Bacillus spp. and was shown to be a dehydropeptide reductase since it specifically reduced the C-terminal dehydroalanyllysine of nisin to alanyllysine (22,36). Another resistance mechanism involves adaptation of cells to high concentrations of bacteriocins. Ming and Daeschel (32) evaluated the

spontaneous nisin resistance frequencies in eight common foodborne pathogenic and spoilage bacteria and characterized the phenotypic responses of a derivative of L. monocytogenes Scott A resistant to high levels of nisin. In BHI medium, spontaneous nisin resistance frequencies were in the range of 10^{-6} to 10^{-8} when cells were exposed to nisin at concentrations between 2 and 8 times the MIC values. Detailed characterization of a resistant mutant of strain Scott A which was obtained by a stepwise increase in exposure to nisin, revealed that changes had occurred in the bacterial membrane structure i.e. the mutant had a higher phase transition temperature, a higher percentage of straight chain fatty acids and a lower percentage of branched chain fatty acids. As a result the fluidity of the membrane was decreased which apparently resulted in a decreased efficiency of nisin pore formation in this nisin resistant mutant (32). Similar observations were reported by Davies and Adams (9).

Table 1: Minimal inhibitory concentration (MIC, µg/l) of nisin Z for food spoilage micro-organisms and food borne pathogens grown in BHI or in low fat milk at various temperatures.

	Temperature		
	7 °C	21 °C	30 °C
Growth in BHI[a]			
Bacillus cereus	400	400	800
Lactobacillus brevis	400	400	400
Lactobacillus plantarum	400	400	200
Brochotrix thermospacta	10	10	NG
Pediococcus acidilactici	5	10	25
Listeria innocua	800	800	400
Listeria monocytogenes Scott A	400	400	800
Growth in milk			
Bacillus cereus	-	800	1200
Listeria monocytogenes Scott A	400	400	1200

[a] The initial inoculum was appr. 104 to 105 cells per ml. Mic values in BHI were determined using OD measurements and the MIC values in low fat milk were determined using plate counting. NG, no growth possible at this temperature; -, not determined (3).

Alterations in cytoplasmic membrane properties may affect the association and/or penetration of nisin thereby modulating nisin sensitivity. On the other hand, alterations in the peptidiglycan may result in increased binding of nisin and/or reduced diffusion of nisin thereby lowering the number of molecules which can interact with the cytoplasmic membrane. Two nisin resistant variants of a strain of L. innocua were isolated which showed increased cell wall hydrophobicity, resistance to phage attack and three different cell wallacting antibiotics, as well as to the peptidoglycan hydrolytic enzymes lysozyme and mutanolysin, as compared to the parental strain (30). Transmission electron microscopy revealed marked thickening of the cell wall of nisin resistant cells with an irregular surface. However, after isolation of cell wall components no significant differences were observed between the parental and resistant variants. Cell wall changes in nisin resistant variants were attributed to abnormal cell wall synthesis and autolysin inhibition, the latter possibly associated with subtle changes in cell wall structures and function. Davies et al (10) reported that cell surface hydrophobicity correlated with nisin sensitivity; the wild type strain being more hydrophobic than its resistant mutant. The composition and quantity of teichoic and lipoteichoic acids were similar in the

wild type and the mutant suggesting that these compounds are not responsible for enhanced nisin resistance. Breuer and Adler (6) provided evidence that nisin resistance in Lactobacillus casei was associated with modifications of cell wall polysaccharides. The resistant variants produced larger amounts of phosphate containing anionic polysaccharides composed of rhamnose and galactose residues. Exposure of sensitive cells to nisin in the presence of these polysaccharides indeed resulted in higher numbers of survivors i.e. 50 cells per ml versus 2 . 10^4 cells per ml. As with most of the inducible resistance phenomena the exact mechanisms involved in the various processes remain to be elucidated.

Recently, Verheul et al (48) reported the isolation of a nisin resistant (NIS^R) of L. monocytogenes Scott A which was obtained by stepwise exposure to increasing concentrations of nisin. The NIS^R strain was about 12 times more resistant to nisin than the wild type (WT) strain. Comparison of the membrane fatty acyl composition revealed no significant differences between the NIS^R and the WT strain. From phospholipid headgroup composition analysis and phospholipid biosynthesis measurements during growth in the absence and presence of nisin, it could be inferred that the NIS^R strain produces relatively more phosphatidylglycerol (PG) and less diphosphatidylglycerol (DPG) in comparison with the parent strain. This could be due to decreased activity of the enzyme DPG synthase, which forms DPG by condensation of two PG molecules. Monolayer studies using pure lipid extracts from both strains showed that nisin interacted more efficiently with lipids derived from the WT strain than from the NIS^R strain, reflecting qualitatively the difference in nisin sensitivity. It has been demonstrated that nisin penetrates more deeply into lipid monolayers of DPG than those consisting of other lipids including PG, phosphatidylcholine (PC), phosphatidylethanolamine (PE), diacylmonogalactosylglycerol and diacyldigalactosylglycerol (12). The interaction of nisin with DPG is apparently much stronger which may be linked to its high charge density and to its specific charge distribution. Collectively, the mechanism of nisin resistance in the L. monocytogenes NIS^R strain is attributed to a reduction in the DPG content of the cytoplasmic membrane.

Crandall and Montville (8), showed that nisin resistance in L. monocytogenes ATCC 700302 is a complex phenotype involving alterations in the both the cytoplasmic membrane and the cell wall and a requirement for divalent cations. The Nis^R strain also showed altered sensitivities to other cell wall acting compounds, even when grown in the absence of nisin, suggesting a constitutive modification of the strain's cell wall. Notably, nisin resistance conferred cross protection against pediocin PA-1 (class 11 bcn) and leucocin S (class IV bcn).

Spontaneous sub-populations of variants (mutants) of L. monocytogenes resistant to pediocin like class II bacteriocins pediocin AcH, mesenterocin 52, curvaticin 13 and plantaricin C19 were reported by Ray and Daeschel (36) and Rekhif et al. (38). The occurrence of spontaneous resistant mutants of L. monocytogenes to the latter three bacteriocins was estimated to be in the range of 10^{-3} to 10^{-4}, and strikingly, these mutants showed cross resistance to the three bacteriocins. This resistance characteristic was stable through many generations, even in the absence of the bacteriocins (38). Whether the high frequency of resistant mutants against these pediocin like bacteriocins is due to the loss of (proteinaceous) receptor sites remains to be elucidated. Notably, all the mutants appeared to be as sensitive to nisin as the parental strains. This is important information with regard to the application of bacteriocin cocktails and the development of inducible resistance.

Bacteriocins: future prospects

The application of bacteriocins from lactic acid bacteria in combination with traditional methods of preservation and proper, hygienic processing could be effective in controlling spoilage and pathogenic bacteria, particularly human pathogens such as L. monocytogenes, in a variety of food products. However, a number of problems such as low production levels and instability in certain environments/foods need to be addressed. Some bacteriocin producing strains can be applied as protective cultures in a variety of food products. For example, well characterized, hornofermentative, mildly acidifying, bacteriocinogenic LAB are ideal canditates for biopreservation of meats where modification of the product is undesirable. However, relatively high levels of these cultures may be required for protection against some pathogens. In these cases bacteriocin producers should be selected which do not negatively influence product taste and appearance when incorporated at high numbers. These problems can be avoided if purified bacteriocins or "inactivated cultures" are used directly as natural food additives, however additional hurdles may have to be included in order to prevent bacteriocin resistant pathogens from growing.

Continued study of the physical and chemical properties, mode of action and structure function relationships of such compounds is necessary if their potential in food preservation is to be exploited. Further research into the synergistic reactions of bacteriocins and other natural preservatives, in combination with advanced technologies such as pulsed electric field (PEF) and ultra high pressure (UHP) (24,33) could result in replacement of chemical preservatives, or could allow less severe processing (e.g. heat) treatments, while still maintaining adequate microbiological safety and quality in foods. Notably, knowledge about resistance mechanisms may prevent misapplication and guarantee bacteriocin effectiveness by managing the emergence of resistant spoilage and pathogenic strains.

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Quantitative Risk Analysis of spore-forming bacteria in cooked chilled foods containing vegetables

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This communication describes some part of the work already done and to be done in the frame of the EC-funded project FAIR CT97-0125 "Research on factors allowing a risk assessment of spore-forming bacteria in cooked chilled foods containing vegetables". Ten research organisations or private companies from five EC-countries are participating to the project between 1998 and 2000.

Abstract

Cooked chilled foods containing vegetables are becoming increasingly popular throughout Europe. These foods have an excellent safety record, but potential hazards due to spore-forming bacteria (SFB) have been identified. The objective of a Quantitative Risk Analysis (QRA) is to estimate the safety level of these foods. In this communication the basic principles of a Quantitative Risk Analysis, and its application to the foods in concern will be described. The authors will discuss the uncertainty of some of the elements used to perform the QRA.

1 Why a Quantitative Risk Analysis of spore-forming bacteria in cooked chilled foods containing vegetables?

Cooked chilled foods, also known as REPFEDs (Refrigerated Processed Foods of Extended Durability) are becoming increasingly popular throughout Europe. Vegetables are frequent ingredients and are naturally and often contaminated with SFB. These foods follow the new consumer demand for more convenient, fresher, more natural foods of high organoleptic quality, which implies lighter preservation methods. Cooked chilled foods are generally processed with mild heat-treatments and rely on refrigeration for preservation. There are therefore opportunities for survival and outgrowth of Spore Forming Bacteria (SFB). Some SFB such as *Bacillus cereus*, *Clostridium perfringens* and *C. botulinum* have been implicated in food poisoning outbreaks in many different type of foods. These bacteria have been therefore identified as potential hazards in REPFEDs. In Europe REPFEDs have until now an excellent safety record.

Contamination of these foods with spore-forming bacteria may vary due to seasonal changes. Cooked chilled foods are manufactured with different processing conditions (different cooking times, different packaging conditions,…). Growth potential of bacteria may vary from vegetable to vegetables. Storage temperature may vary along shelf-life. Therefore information on the effects of these factors on the safety of foods is vital to the protection of consumer health and to the continuing success and economical expansion of the market of REPFEDs. Quantitative Risk Analysis (QRA) offers a method for the estimation of risk associated to the consumption of cooked chilled foods containing vegetables. QRA has applied to industrial operations, but application to the food industry is just starting. Some QRA on foods have been already published, one about the use of cracked eggs in Canada, an other one on pasteurized milk in the Netherlands (1,2).

2 Quantitative Risk Analysis. Some basic principles and definitions (3,4)

A Risk Analysis is a stepwise analysis of occurrence of a hazard to understand its nature and facilitate appropriate control measures. It consists of three elements : Risk Assessment, Risk Management and Risk Communication.

Risk management is the process of weighing policy alternatives in the light of the results of risk assessment, and if required, selecting and implementing appropriate control options, including regulatory measures. According to the Codex Alimentarius, there must be a "Functional separation between Risk Assessment and Risk Management".

Risk Communication is an interactive exchange of information and opinions concerning risk among risk assessors, risk managers, consumers, and other interested parties.

Both Risk management and Risk communication are preceded by Risk assessment. Our communication will mostly deal with Microbial Risk Assessment of spore-forming bacteria in cooked chilled foods containing vegetables. **Risk assessment** can be defined as **the estimation of the probability of a (foodborne) adverse effect on human health**.

Microbial risk assessment consists of the four following steps:

(i) hazard identification

(ii) hazard characterisation

(iii) exposure assessment

(iv) risk characterisation.

A Hazard is a biological agent in, or condition of, food *with the potential* to cause an adverse health effect.

A Risk is a function of the probability of an adverse health effect and the severity of that effect consequential to (a) hazard(s) in foods

(i) Hazard identification is the identification of biological agents capable of causing adverse health effects and which may be present in a particular food or group of foods.

(ii) Hazard characterisation is the qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with biological agents, which maybe present in food. For biological agents, a dose-response assessment should be performed if data are obtainable.

A Dose-response assessment is the determination of the relationship between the magnitude (dose) of a biological agent and the severity and/or frequency of associated adverse health effects.

(iii) Exposure assessment is the qualitative and or quantitative evaluation of the likely intake of biological agents via food as well as exposures from other sources if relevant.

(iv) Risk characterisation: the quantitative and/or quantitative estimation, including attendant uncertainties of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification and characterisation and exposure assessment.

3 Microbial Risk Assessment: Application to cooked chilled Foods containing vegetables and Spore-forming bacteria

3.1 Hazard identification and Hazard characterisation

In our case, it consists in the identification using the existing information of bacteria pathogenic for man and which may be present in the food. The bacteria of concern must not be restricted to those present on vegetables, while other ingredients are used in cooked chilled foods containing vegetables (meat, fish, spices, starch, …). Bacteria of concern must produce endospores to survive the process (in our case cooking and or pasteurising), be able to grow at pH of foods (from 4.5-5.0 to 7.5), must be present in the environment, must be present in foods and must have caused foodborne poisonings. Psychrotrophic bacteria must be considered because cooked chilled foods are stored at refrigeration temperatures. This is undoubtedly the case for *Clostridium botulinum* and *Bacillus cereus* for instance (5,6). In contrast *Clostridium tetani*, the agent of human tetanus, is not considered as hazardous in foods, as neither the presence of the bacteria in foods, nor cases of foodborne tetanus has ever been reported.

Hazard characterisation must determine the nature and the intensity of adverse health effects related to the identified hazardous bacteria. For instance, adverse health effects of *Clostridium botulinum* and *Bacillus cereus* are markedly different. Number of cases of *Clostridium botulinum* is relatively low (Approximately 100 cases per year in the EC), but the lethality is quite high (up to 10%) (6) and intoxicated people require intensive care. In contrast, foodborne poisonings due to *Bacillus cereus* can represent up to 20 % of reported outbreaks in some countries (5) and are probably underestimated in other. Most poisonings, consisting of diarrhoea and vomiting, are ending less than 24 hr after ingestion of the involved food, without medical treatment.

The extensive literature reviewed by the participants to the EC project and analysed at RIVM has conclude that:

- The hazardous spore-forming bacteria in cooked chilled foods containing vegetables are :

Bacillus cereus, B. subtilis, B. licheniformis, B. pumilus among *the Bacillus* sp.*;*

Clostridium botulinum, C. butyricum, C. baratii and *C. perfringens* among *Clostridium* sp.

- Hazard may be quantified as follows (Table 2):

Table 2: Quantification of hazards in cooked chilled foods containing vegetables

Quantification of the hazard based on:	*C. botulinum*	*C. perfringens*	*B. cereus*	*Bacillus* spp.
Fatality rate	high (3-11)	low (<1)	low (0)	low (0)
Outbreaks/year	high (2-15)	high (11-36)	high (4-11)	low (1.8)
Cases/year	low (4-39)	high (476-1627)	median (37-411)	low (>10-17)
Growth at low temperature	Gr. I* median Gr. II* high	median	high	low/high

* Gr. I: proteolytic *C. botulinum*, Gr. II non proteolytic *C. botulinum*

3.2. Dose response assessment

In microbial risk assessment this can be defined as the relationship between the amount of bacteria or bacterial toxin ingested and the number and the extent of the disease (number of people getting ill, number of deaths, number of hospitalised people for instance). In practical conditions this is very difficult to obtain.

- Most experiments are done on animals, but what is the translation to humans?

- There is a large variability of reaction to exposure according age, sex, health status (pregnant women, immunocompromised people).

- In many cases there is no reliable quantitative test for toxin, as for instance for the diarrhoeic toxin of *Bacillus cereus*.

- In many instances only LD50 is determined with some reliability, but this is insufficient to determine the dose-response curve (Figure 1)

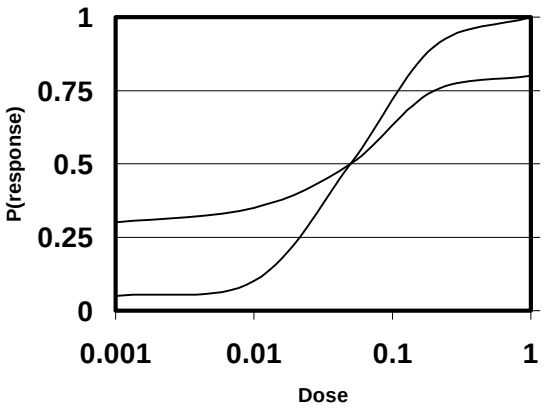

Figure 1: Two theoretical dose-response curves derived from the same LD50

3.3 Exposure assessment

In our case, the exposure assessment of the microbial risk assessment means the number of, or the amount of toxin produced by, the SFB identified as hazards in cooked chilled foods containing vegetables.

Microorganisms contaminating raw material will be subjected to different operations during manufacturing, the main being heat process(es), which will affect the survival of the bacteria, and storage, which will determine the extent of the bacterial growth (Figure 2).

Some information on contamination of raw material, survival of bacteria spores and growth of bacterial spores is already known.

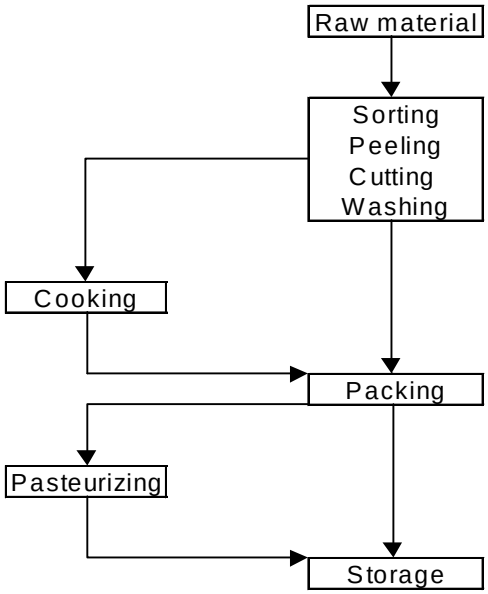

Figure 2: Flow chart of the manufacturing of cooked chilled foods

Many surveys on the contamination of foods with *Bacillus cereus* and *Clostridium botulinum* have been published (Table 3). The range of the time of decimal reduction of bacteria (called D) and of the elevation of temperature for a ten-fold reduction of D-values (called z) are well documented (Table 4). Predictive models on the growth of pathogenic bacteria, including hazardous bacteria in cooked chilled foods have been developed (Table 5). The most recently developed models including prediction on survival after heating and incubation.

Table 3: Range of contamination of foods with hazardous spore-forming bacteria

Bacteria	% positive samples	Range	Source
Bacillus cereus	2-100 %	10^{1}-10^{6} CFU/g or/ml	5
Clostridium botulinum	0-100 %	<0,1 – 2000 CFU*/kg	6

* estimated by Most Probable Number

Table 4: Range of D-values and z-values for hazardous spore-forming bacteria in REPFEDs containing vegetables.

Bacteria	Temperature	Range of D-values	z-values	Source
Bacillus cereus	95°C	1-36 min	6-9°C	5
	100°C	1-8 min		
Clostridium botulinum Gr. I	110°C	1-3 min	10°C	7
Clostridium botulinum Gr. II	82.2°C	0.1-30 min	5-10°C	8

Table 5: Predictive growth models for hazardous spore-forming bacteria in REPFEDs containing vegetables

Bacteria	Factors modelled (range)	Source
Bacillus cereus	Temperature (6-38°C), pH (5.8-8.0), a_w (0.965-0.995), glucose (0-1.8 %), Starch (0-0.625 %)	9
	Temperature (5-42°C), NaCl (0.5-5%), pH (4.5-7.5), sodium nitrite (0-200mg/l)	10
	Temperature (10°C-30°C), pH (4.5-7.0), NaCl (0.5-10.5 %), CO_2 (10-80 %)	11
Clostridium botulinum Gr. I	Temperature (15-37°C), pH (5.0-7.0), NaCl (0-3 %)	12
Clostridium botulinum Gr. II	Temperature (4-30°C), pH (5.0-7.3), NaCl (0.1-5.0 %)	13
	Temperature (4-28°C), pH values (5.0-7.0), NaCl (0-4 %), Total spores (10^1-10^5)	14

Most information is subjected to uncertainty, due to either natural variability and experimental errors, or to a lack of knowledge. Many aspects of bacterial behaviour or characteristics are subjected to natural variability. The range of contamination of foods with bacterial spores (especially with *C. botulinum* is quite wide (Table 3). $D_{90°C}$ values ranging from 5 min to more than 200 min have been observed on strains isolated from clinical samples (15). Only a proportion of *Bacillus cereus* strains can either grow at low temperature or produce toxin: 15 % of strains isolated from different foods and 53 % from milk were able to grow at 7°C (16,17). The proportion of strains positive for enterotoxin varies from survey to survey. Some strains of non-proteolytic *Clostridium botulinum* are not able to grow on vegetable substrate (18). Risk assessors can cope with this natural variability in translating available data in probability distribution.

In contrast there are several areas where we lack of knowledge. We have listed some of them:

- Influence of the history of the cells. Most laboratory experiments are done with spores prepared in the laboratory. How similar is the behaviour of these experimental spores to that of bacteria sporulating in the environment and naturally contaminating the foods?
- Effect of low inoculums. For technical reasons most laboratory experiments are done with high inoculums (more than 10^3 spores/g), while natural contaminations are expected to be quite low.
- Effect of intrinsic factors in food matrices on heat-resistance. There is some evidence that foods contain some protective compounds. Lysozyme can increase the apparent heat resistance of non-proteolytic *Clostridium botulinum* by up to 160-fold and is naturally present in sufficient concentrations in a range of foods to potentially protect spores against heat-damage (8).
- Effect of intrinsic factors in food matrices on bacterial growth. There is often a gap between the growth predicted by models and growth observed in real foods, models overestimating growth parameters. Besides the well-known effects of temperature, pH and a_w, this gap is due to either lack in nutrients or natural inhibitors. This phenomenon is easy to observe, but difficult to quantify and to predict.

- Effect of other natural contaminants. Growth of a non-pathogenic contaminant can change unfavourable conditions and therefore allowing the growth of a pathogenic contaminant (metabiotic effect) or conversely inhibit the pathogenic contaminant in favourable conditions.
- Time to toxicity versus time to spoilage. Time to toxicity is better known than time to spoilage. However consumers will probably not eat spoiled products.

Does poorly known factors have only a marginal effect on the estimation of the risk ? Does the ignorance of these factors always lead to an overestimation of the risk ?

Bacterial growth depends on environmental and intrinsic factors in the food. Environmental factors are themselves subjected to variations. These variations originate for instance from the diversity of processing technologies and from the variations of temperature along the shelf-life at both commercial and domestic levels. Data on processing are available after consultation of experts (manufacturers, professional association) and some surveys give indication on the variations of temperature along storage (Table 6).

Table 6: Variations of temperature along domestic and commercial storage of refrigerated foods

Type of refrigeration	Country	Temperature
Domestic (2)	The Netherlands	29.6% of refrigerators below < 5°C 41.6 % at 5-<7°C 26.6% at 7-<9°C 1.6 % at 9-<11°C 1.6% at 11-<13°C n = 125*
Commercial (19)	France	Foods to be stored below 4°C: 5,7 °C ± 3.3**, n=1247* Foods to be stored below 8°C: 6.9°C ± 3.5**, n=427*

*: number of refrigerators examined, **: average temperature ± standard deviation

4 Risk characterisation

The objective of risk characterisation is to bring together the information from the previous stages Hazard identification and characterisation, dose-response assessment, and exposure assessment. Risk characterisation will provide an estimate of risk to a given population, e.g. how many consumers becoming sick, how many being hospitalised, etc… Data on consumption patterns of cooked chilled foods containing vegetables are also required, assuming that some consumers are more reactive to food poisonings than other, young children, elderly people, pregnant women and immunocompromised people for instance. Risk characterisation will be the starting point of risk management strategy to be established with the parties concerned with safety, e.g. health authorities, consumers, and industries.

Conclusion

The degree of confidence in the final estimation of risk will depend on the variability and/or uncertainty in the different steps. We have shown that in many areas uncertainty can be quite significant. However microbial risk assessment has an undeniable interest. In this highly complex system, this approach may determine (i) which component has the major effect on risk and (ii) what happens to the risk when modifying some component in the system (heating longer, shortening shelf-life, introducing warnings by sensitive consumers…).

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Development of new methods for safety evaluation of transgenic food crops

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Summary of results of the AIR3-Ct94-2311 project

Summary

A common element in the designed guidelines for assessment of the transgenic food crops and derived products is that the evaluation should be based on a comparative analysis with conventionally bred products, assuming that these traditional foods have a long history of safe use (i.e. concept of substantial equivalence). It is generally recognized that animal feeding trials with whole foods is complicated by the likelihood of nutritional imbalances leading to insensitivity. On the other hand, Compositional analyses based on single compounds have limitations regarding in less well-known crops. New methods have been developed for the detection of so-called 'unintended effects' which may occur in transgenic food crops as a result of genetic modification. Both carbohydrate profiling (i.e. 2-D HPLC combined with exoglycosidase treatment and MALDI-TOF) and chemical fingerprinting (i.e. off-line LC combined with ^{1}H-NMR) appeared powerful screening tools in order to detect compositional differences in N-glycans and secondary and metabolites, respectively. Besides these methods, an appropriate experimental design essential to enable statistical analysis of various populations is proposed in order to support claims of substantial equivalence. The default statistical analysis used a mixed effects randomized block model with external factors as random block effects. A hierarchical approach was of importance by comparing the transgenic line to those of related isogenic parent lines (i.e. mean differences and a corresponding 99% confidence interval). The crop mean values, being the range of mean differences in composition across an extended population of commercial varieties of that crop including effects of external factors, were of final interest. A recommended approach to assess the likehood that some of the statistical differences in the transgenic genotype may be either false positives due to change alone or natural genetic variations not linked to genetic engineering. Application of this strategy combined with, for instance, toxicological *in vitro* profiling using a battery of endpoints to screen for possible relevant toxicity, together with the evaluation of the properties of the novel agronomical traits, may limit or even replace animal feeding studies aimed at the detection of 'unintended effects'. Results will be discussed using an insect resistant transgenic tomato expressing the *cry1Ab5* gene and an antisense RNA tomato having down-regulated levels of exogalactanase.

Introduction

Plant biotechnology (i.e. recombinant DNA technology) holds promise in developing quality crops requiring less inputs, such as fertilisers, prolonged shelf life and pesticides for (non-)food and industrial applications, thus fitting with CAP. Several of the prototypes have been moved into the phase of market introduction and therefore the evaluation of food safety aspects of engineered plants has broadly attracted scientific and social attention. In particular during the last decade the list of transgenic crops developed by US industry has increased fast, while in Europe less products are under development, of which only a few have been admitted to the market. Recently, the European

Union has issued the Regulation on Novel Foods and Novel Food Ingredients (EC/258/97) which sets the legal framework for the market introduction, and came into force May 16, 1997. Despite that the legislative situation in Europe with respect to registration requirements is not yet clear and above all there is a lack of uniform and harmonized evaluation criteria for the testing of environmental and food safety aspects of transgenic food crops. In a global context several regulatory or industry-based bodies have proposed strategies for the safety evaluation of genetically engineered foods and food ingredients. In particular the Organization for Economic Co-operation and Development (OECD), the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), have developed concepts and principles for safety evaluation of genetically modified organisms (OECD, 1993,1996,1997; FAO/WHO, 1991,1996; WHO, 1995). A common element in the designed guidelines for the assessment of food safety of, for instance, the transgenic food crops and derived products is that the evaluation should be based on a comparative analysis with conventionally bred products, assuming that these traditional foods possess a long history of safe use (i.e. concept of *substantial equivalence*). This approach is not a safety assessment *per se*, but a comparative analysis of various agronomical, (bio)chemical and nutritional parameters of the genetically modified product relative to that of existing traditional counterparts.

Application of the concept of *substantial equivalence* appeared in practise to lead to different interpretations, however, and has resulted in different data requirements for the identification of hazards and risk assessments. There exist different requirements to conduct animal feeding trials with whole foods with regards to the detection of the 'unintended effects' as a result of genetic modification. This type of testing strategy is complicated by the likelihood of nutritional imbalances leading to insensitivity and the impossibility to use large safety margins. On the other hand, compositional analysis based on single compounds as a screening method for 'unintended effects' has its limitations as well, in particular regarding unknown (anti-)nutrients and natural toxicants in less well known food crops. Also the introduction of genes of bacterial origin may theoretically influence the nature of glycosylation in plants. Additionally to the impact of the protein moiety the glycosylation pattern of a protein primarily depends on the host cell type. Especially, the N-linked oligosaccharides can have a substantial impact on the allergenicity of proteins, as N-glycans are suspected to play a certain role in adverse food reactions of hypersensitized patients (Altmann, in press). Therefore, there is a need to develop alternative methods for the detection of 'unintended effects', which may be more informative, faster and less costly.

The AIR3-CT94-2311 project has been set up aimed at setting generic approaches to study and to evaluate the food safety of transgenic food crops. In particular, methods have been developed to detect potential 'unintended effects' on plant metabolism as a result of genetic modification and to assess the allergic potency of newly introduced proteins and whole modified food. The work consisted of five main parts which contributed equally to the successful accomplishment of the project:

- to isolate newly expressed Bt-proteins (i.e. Cry1Ab5) from transgenic crops;
- to develop detection methods to assess posttranslational modification differences in newly introduced proteins as well as in transgenic crops (*carbohydrate profiling*);
- to develop of analytical methods to identify unintended metabolic changes in transgenic crops (*chemical fingerprinting*);

- to develop a battery of *in vitro* tests to screen the toxicological properties of newly expressed proteins and of the transgenic crop (*toxicologic profiling*).
- to evaluate an animal model to test the immunotoxicity and allergenic potency of newly expressed proteins and of the transgenic crop (*allergenicity testing*).

Coordinates of participants

The consortium consisted of the DLO-State Institute for Quality Control of Agricultural Products (RIKILT-DLO), Department of Food Safety and Health, Wageningen, The Netherlands (co-ordination project, biochemical, toxicological and analytical analysis); Plant Genetic Systems NV, Ghent, Belgium (plant transformation, molecular and biochemical analysis); Vrije Universiteit Brussels, Department of Protein Chemistry, St-Genesius-Rode, Belgium (protein chemistry and physical-chemical analysis); IFA Tulln, Center of Analytical Chemistry, Tulln, Austria (posttranslational modification analysis) and BIBRA International, Carshalton, Surrey, UK (immunogenicity and toxicological analysis).

Two genetically modified crops have been chosen as prototypes for the development of alternative test methods, namely: an insect resistant transgenic tomato expressing the *cry1Ab5.PGS1* gene derived from the *Bacillus thuringiensis* susp. *berliner* 1715 and an insect resistant cauliflower expressing the *cry9C.PGS2* gene derived from *Bacillus thuringiensis* serovar *tolworthi*. Antisense RNA exogalactanase tomatoes developed by Unilever Research were kindly provided by Colworth Laboratory (Shambrook, Bedford, UK) and used as 'extended' comparators for the purpose of establishing *substantial equivalence*.

Regulatory framework

In the 'Official Journal of the European Community (September 1997)' recommendations have been formulated for submission of scientific data requested for risk assessment. The concept of *substantial equivalence* is a major principle in the safety assessment of these novel products. Comparative analyses of the chemical composition of the novel product with the conventional product should be performed in order to prove equivalence. In case *substantial equivalence* can not be established, it does not necessarily mean that the product is less or even unsafe. Further toxicological and nutritional studies will be required. Also the allergenic potential of newly inserted proteins and the transfer potency of marker genes to the human microflora of the gut must be assessed. In this paper, emphasis is given to results of the AIR-project focusing mainly on the comparative chemical fingerprint analysis in order to identify changes in the overall composition of novel plant products followed by an *in vitro* toxicological profiling using relevant endpoints. Developments and results obtained in the protein isolation and allergenicity testing of novel food proteins are discussed elsewhere (H. Atkinson, this volume).

Production and characterization og transgenic tomatoes

Transgenic insect resistant Bt tomatoes

Plant Genetic Systems obtained transgenic insect resistant Bt-tomato plants from the parental San Marzano line TL001 by Agrobacterium tumefaciens mediated transformation using cotyledons as the explant. A disarmed A. tumefaciens strain containing the vector pPSO216 was used for transformation. The vector pPSO216 comprises two chimeric genes between the T-DNA border

repeats of the Ti plasmid (i.e. the coding region of the neo gene encoding neomycin phosphotransferase II (NPTII) and the coding region of the C-terminal truncated Bt2 gene derived from a Bacillus thuringiensis strain, called IAb5 derived from the coding region bt884. Based on entomological and agronomical criteria one transformant was chosen for further characterizations: the transgenic Bt-tomato San Marzano variety RLE15-0001 derived from the parental line TL001 (Noteborn et al. 1995a).

Antisense RNA exogalactanase tomatoes

Unilever produced a new tomato variety with improved rheology characteristics, by application of the antisense approach to down-regulate the endogenous exogalactanase activity, which is thought to be responsible for the cell wall alterations leading to softening. The exogalactanase antisense RNA plants were generated by transforming Moneymaker tomatoes with a LBA4404 strain of A. tumafaciens harbouring a binary antisense vector i.e. pGPTV-kan, which contained a wild type neomycin phosphotransferase (NPTII) gene flanked by an Agrobacterium nopaline synthase promotor and a pAg7 terminator. The antisense exogalactanase DNA was a partial fragment of the cDNA clone tEG1A flanked by a 35S cauliflower mosaic virus promoter and a nopaline synthese terminator (De Silva and Verhoeyen, 1998).

Characterization of unintended effects

One of the key issues in safety evaluation of transgenic food crops is whether unexpected changes may have taken place in the organism as result of the process of genetic modification. Effects which could affect the safety or nutritional status of the modified organism. Unintended alterations in agronomical traits or composition may arise from insertional mutagenesis, or as a result of metabolic effects of the new gene product(s) (Noteborn, 1995b,1998). Usually, compositional analyses have been carried out on single compounds as a screening strategy for unintended effects, but its limitations are clear with regard to (unknown) (anti-)nutrients and natural toxins, especially in less well-documented species. Detection of these secondary effects can be performed at different integration levels, i.e. DNA, mRNA, proteins including posttranslational modifications and at the level of macro and micronutrients and secondary plant products and metabolites. In this project alternative analytical methods for multi-component analysis have been explored using the (physical)-chemical or spectroscopic properties of complex food matrices, as compared to single compounds. Tools able to detect possible 'unintended effects' in transgenic food crops.

Methods of detection of unintended effects due to genetic modification
DNA-analysis
mRNA-fingerprinting
Protein fingerprinting
Carbohydrate profiling
Single compound analysis of critical nutrients and toxicants
Chemical fingerprint analysis
In-vitro toxicity screening

The application of the concept of *substantial equivalence* was considered to be starting point in developing novel, alternative methodology for a comparative product analysis. It included that the analytical devices to be applied had to allow a dynamic, analytical exercise taking into account the reference characteristics of properiate counterpart(s) and influences of environmental and processing factors. Investigations which should lead to a redesign, refinement or even replacement of, for example, the (sub-)chronic rodent feeding trials. It was considered that the claim of *substantial equivalence* can be supported by taking a hierarchy of comparisons of the chemical fingerprint of the transgenic plant to those of traditional counterparts. The default statistical analysis used a mixed effects randomized block model with external factors as random block effects. The statistics of interest were: (i) the mean of the measure, (ii) the mean difference between a transgenic line and a parental line and a corresponding 99% confidence interval, and (iii) a 1% two-sided testing for no difference between the non- and modified line. The crop mean values and mean differences were of final interest. The crop mean values referred to the range of chemical fingerprints over an extended control population of commercial varieties of that crop (i.e. tomato) including external factors such as timing, logistic and processing operations, environmental variations and a corresponding 99% confidence interval. Since in the chemical fingerprints there were typically large numbers of compositional comparisons of any variety, some false positive differences will be inevitable. Therefore on the average, about 1% of all non-differing comparisons will yield a statistically difference by chance alone. As concentrations of plant constituents will be not independent as they are metabolically linked, the frequency of false positives might be even greater than 1%. This effect was taken into consideration when interpreting the data.

Detection of unintended effects

Carbohydrate profiling of genetically modified crops

N-linked oligosaccharides were isolated, purified and characterized in order to compare transgenic and non-modified tomato fruit as well as the impact of the stages of ripening on the profiles. Both the low-temperature acetone powder method and the 'classical' ammonium sulfate precipitation method appeared to be suitable for the isolation of (glyco)proteins from tomato fruit. However, the developed acetone powder procedure was favoured because of minimal protein denaturation. The resulting (glyco)protein fraction was proteolytically digested and corresponding glycopeptides purified by cation-exchange chromatography. The oligosaccharides (N-glycans including alpha-1,2-fucosylated oligosaccharides) were released from the glycopeptides by N-glycosidase A (PNGase A) and derivatized with 2-aminopyridine (Zeleny et al., 1997). Pyridylaminated glycans were separated and structurally elucidated by 2-D HPLC in combination with exoglycosidase treatment and MALDI-TOF of individual HPLC peaks. Sixteen different N-glycans of the oligomannosidic and complex-type or truncated oligosaccharides could be detected in tomato fruit. The predominant structure in all the tomato samples was MMXF (Van Kuik et al., 1986) which accounted for approximately 50 % of the total N-glycan contents. Whereas oligomannosidic structures occurred in small amounts (3-8%) only. The majority of the glycans found were xylosylated and carried an alpha-1,3-fucose residue linked to the terminal N-acetylglucosamine. A structural element shown to be an IgE reactive determinant which contributes to cross-reactivities among non-related glycoproteins. It is proposed that this element might also be of allergological importance in some cases of food and pollen allergy (Altman, in press). The investigation of non- and transgenic Bt-tomatoes (variety: San Marzano TL001, green and red-ripe fruit) revealed no differences in the nature of N-glycans, nor were there significant differences in the relative glycan amounts. Interestingly, the relative amounts found were

quite similar across seasons and comparably to the non-modified variety Notoro used as an extended control. The carbohydrate profiling showed that as far as N-linked oligosaccharides are involved it was not possible to detect significant alterations in the alpha-1,3-fucose content of tomatoes due to genetic modification.

Chemical fingerprint analysis of genetically modified crops

Off-line Liquid Chromatography combined with High Resolution Proton(^{1}H)-Nuclear Magnetic Resonance (LC-NMR) has been used as a potential method for the detection of 'unintended effects'. A procedure was developed to fractionate tomato fruit in water-soluble and membrane-bound constituents. In order to differentiate between possible compositional changes due to genetic modification, or to genetic variability or environmental variations, chemical fingerprints have been studied in hierarchical approach: (i) isogenic lines grown under identical growth conditions, (ii) isogenic lines bred at multiple locations, (iii) the influence of downstream processing and, (iv) the transformant and the range of that crop. The results demonstrated that information on the rate of substantial equivalence between two populations may be collected by selection of those amplitudes which showed a statistically significant difference at a confidence level of 99%. Random block effects such as variations in the stage of cultivation, location, logistics or climate had a significant impact on the overall chemical fingerprints of cultivars. On the other hand, observations indicated that compositional mean differences induced by environmental factors may be diluted out if compared to the crop mean values (i.e. extended reference range of that crop including the effects induced by downstream processing). Thus, the use of several lines may help to establish a crop mean value and thereby to resolve whether the mean differences arise from 'unintended' metabolic effects or natural genetic and/or physiologic variations as has been demonstrated. It is recognized that in the agricultural practise the circumstances of culturing and of processing and storage of appropriate controls will be mostly unknown and may having a non-isogenic genotype. Notwithstanding these complicating random block effects it is recommended that the chemical fingerprint analysis should not be limited to the transgene and its isogenic parent bred under identical circumstances only, but should also include a sampling through breeding seasons and a comparing with an extended range of controls of that crop (i.e. crop mean values). Otherwise it would be a too limited approach towards the establishment of the claim of substantial equivalence and it neglects the ranges of natural variations in that crop. With regards to the results obtained and reported herein it is concluded that the method of chemical fingerprinting analysis can be used inconjunction with the conventional analysis of key nutrients and toxicants.

In case of isogenic tomato lines and approximately identical agricultural conditions there were generally minimal compositional mean differences between the parental lines and the corresponding transgenic phenotypes such as the Bt-San Marzano tomatoes. The observed mean differences were within the normal ranges of nutritional components in tomato fruit routinely found after single component analysis. As has been shown in case of transgenic Bt-tomatoes expressing the *Cry1Ab5* gene, bred under identical growth conditions but harvested across different seasons, the mean differences were randomly distributed, because no statistical significant differences could be demonstrated between combined controles versus transgenes. Therefore, it is concluded that the mean differences in chemical composition were the result of false positives due to natural genetic background variations and not due to reactions linked to genetic engineering.

Results indicated that there might be mean differences in the metabolism between antisense RNA

tomatoes and its control fruit. Because it was observed that both citric acid (TCA-cycle) and glutamic acid/glutamine, which play pivotal roles in NH_4+ assimilation into amino acids, occurred at higher concentrations in the control and transformants, respectively. Comparing the crop mean values and these mean differences is of importance, however. As shown a further increase of the numbers of varieties in the control group appeared necessary before definite conclusions could be drawn. Consequently, it was found that the levels of citric acid and glutamic acid/glutamine were non-differing variations. But it was calculated that the compound alpha-lycopene was present at significant increased levels (factor 2-4) in the antisense RNA tomatoes if compared with the crop mean values. The differences in alpha-lycopene profiles may be the result of the antisense down-regulation of the *tEG 1A* gene in tomato. Because the reduced exogalactanase enzyme activity in pink fruit correlated with a dramatic inhibition of the ripening-associated loss of cell wall galactose (De Silva and Verhoeyen, 1998). This inhibition of tissue softening is accompanied with a delayed ripening of the antisense tomato. Since lycopene concentrations correlate with fruit ripening and the tomatoes were harvested at identical timings after sowing in one site it may explain these findings.

The method of chemical fingerprinting analysis can be used in conjunction with the conventional analysis of key nutrients and toxicants. Assignment of the observed spectral changes to certain classes of compounds may indicate whether changes of toxicological importance have taken place as result of the genetic modification, which need further toxicological investigation.

In vitro toxicological profiling

The use of *in vitro* methods to screen for differences in toxicity between genetically modified and conventional foods, may be considered as a tool in order to establish *substantial equivalence* after statistical differences have been detrected in their composition. *In vitro* systems derived from organs and tissues from animals and humans, and various types of cell lines are successfully used for screening of the toxic potential of single compounds. However, their applicability to screen for potential toxicity of whole foods or extracts thereof has unsufficiently been explored up till now, and has been examined in the framework of this project.

Cytotoxicity tests have been performed with extracts of tomatoes using (i) a gastro-intestinal epithelial cell line from the rat (IEC18), (ii) a human embryonic intestinal cell line (INT 407), (iii)a human colon tumor cell line (Caco-2), which may differentiate and attain the characteristics of an epithelial cell from the proximal part of the digestive tract, and (iv) a human liver carcinoma cell line (Hep2G). Furthermore, a commercial available assay was tested, consisting of recombinant cell lines derived from Hep2G, each containing a specific stress gene promoter or response element coupled to a reporter gene (CAT-TOX(L) (Xenometrics, Boulder, US). Cytotoxicity tests have been performed with pure plant, and with extracts from transgenic and control tomatoes. IC50-values were determined for e.g. the glycoalkaloids with respect to different endpoints like such as alpha-GST-activity, MTT inhibition, NR-uptake, and LDH-release. Experiments with extracts of tomatoes indicated that only green tomatoes exhibited cytotoxic effects in these assays, with no differences between transgenic and parent lines (Noteborn et al. 1997). Results from experiments in the cellular stress responsive assays indicated some effects of extracts of green tomatoes and to a much lesser extend of red-ripe tomatoes, but no differences between modified and unmodified products were observed. More experimental work is needed in order to evaluate the potential use of these systems for safety screening of whole food products and extracts thereof, including genetically modified products.

Discussions

The concept of *substantial equivalence* designed by OECD, and further developed by WHO/FAO, is the most practical and scientifically sound approach to assess the safety of novel foods in Europe or outside the EU. Clarification of the data appropriate to approve *substantial equivalence* would be very helpful both for plant breeding companies as well as for the competent EU authorities and scientific bodies. It should be recapitulated that the OECD-concept is not a safety evaluation *per se* as it provides only an assurance that the transgenic food crop is equally (un)safe as the traditional counterpart. As demonstrated in this project *substantial equivalence* is established by a hierarchy of comparisons in order to settle that the characteristics of the transgenic tomatoes are or are not similar to the ones of the conventional nontransgenic comparators. The choice of the comparator is therefore of utmost importance, especially when 'unintended effects' of the modification are to be traced. For many commercial food crops lines isogenic with the parent line are not available, and this may complicate the interpretation of observed differences. However, the use of several closely related and current commercial varieties of that crop appeared essential in order to sort out and to decide whether the observed mean differences between the transgene and parental line were due to secondary effects, or due to natural genetic or environmental variations. More attention should be paid to these problems in order to provide a better guidance for breeders of novel transgenic crop lines.

The chemical fingerprinting techniques as have been discussed, are very promising to spot 'unintended effects' in novel food products. The methods are robust, and the extent of the generated information in conjunction with an appropriate statistical design is enormous and needs further elaboration. The experiments performed during this project illustrated that proper hierarchical comparisons between transformants and parental lines should be made. The variability of compound concentrations and mean differences between modified and unmodified lines was greater across sites than within eight replicates at a single location. Above all, only on the basis of the use of an extended range of controls of that crop (i.e. crop mean values) the 'unintended effects' could be distinguished from natural physiological variations in the contents of inherent secondary plant products and metabolites. The technique of off-line LC-NMR is of particular interest to investigate transformed species having less familiar parental or closely related lines with respect to the occurrence of side effects, but also in those cases when novel processing techniques are applied during food production. Interlaboratory testing of the technique of chemical fingerprinting at the international level should be initiated taking different genetically modified products as test cases. Standardization of pretreatment methods like extraction procedures should be part of this exercise. Moreover, an appropriate experimental design is essential to enable statistical analysis of data. Thereto, it is required that crop mean values are established that apply over the entire spatial and temporal ranges that the crop can or will be grown. Otherwise it would be a too limited approach towards the establishment of the claim of *substantial equivalence* and it neglects the ranges of natural variations in that crop. With regards to the results obtained and reported herein it is concluded that the method of chemical fingerprinting analysis can be used in conjunction with the conventional analysis of key nutrients and toxicants.

The use of *in vitro* toxicity test systems to screen for potential toxicity of compounds is well established and recognized as an important tool to obtain information on species differences in toxicity, and on underlying mechanisms of toxicity. The validity of the *in vitro* systems to be used for comparative studies of whole food products, food ingredients and extracts thereof, depends on the

sensitivity and selectivity of the toxic response, induced upon exposure to complex food matrices. During this project a number of well known cytotoxicity tests have been tested, while furthermore newly developed transgenic cell lines have been tested for their potential use in the risk assessment transgenic food crops. The cytotoxicity tests applied have shown their value, since pronounced adverse effects could be observed with the pure glycoalkaloids from tomatoes as well as potatoes, and also with extracts of green tomatoes, presumably due to the presence of high concentrations of alpha-tomatine. It is reassuring to note that the investigated *in vitro* systems did not show general matrix-related toxic effects. At the present no complete alternatives for *in vivo* studies are available to assess the unknown toxicological properties of new products, but the use of *in vitro* models is complementary to and may limit laboratory animal testing. In particular information on bioavailability, digestion, cytotoxic or genotoxic effects or organ and tissue specific biotransformation of food constituents may be obtained from these systems. As shown the use of *in vitro* toxicological test systems to screen for differences in toxic responses between modified and unmodified food products is promising and should be explored further, given the fact that the array of test systems transfected with specific biological response factors is rapidly growing. These systems should be considered as an early stand-alone warning or alert-system to identify potential changes in toxicity of the modified product as well as post-chemical fingerprinting tests to screen on toxicological relevances of detected compositional mean differences. Subsequent toxicological studies with whole animals, if needed, may be designed with more precision and a better focus, restricting in this way whole animal experimentation.

Conclusions

Data requirements for the food safety assessment of genetically modified products should be based on the type of genetic modification and related consequences. A generic approach for safety evaluation of transgenic food crops is envisaged with respect to the characterization of target and marker gene inserts and the toxicological properties of expressed products, while 'unintended effects' must be identified on a *case by case* basis, since such effects if occurring, are dependent on genotypic and phenotypic parameters. The concept of *substantial equivalence* is broadly accepted as a basis for the safety evaluation of genetically modified food crops and derived novel foods. However, the choice of comparators and of external parameters to support the claim whether the new product is substantial equivalent to its traditional counterpart may be different among EU member states, leading to different data requirements for the assessment. This creates an unclear situation for producers of transgenic food crops, and may retard their market introduction.

New methods are available for the detection of 'unintended effects', which may occur as a result of genetic modification. These methods are based on multi-component analysis of the overall composition of a product in contrast to classical methods determining single compounds. The carbohydrate profiling technique is useful for the detection of 'unintended effects' at the N-glycan level, in particular applicable to detect posttranslational modifications in newly expressed proteins as well as in the whole product. As far as glycosylated structures are concerned the method may be also applicable to detect alterations in the allergenic potential of lines (Aalberse et al., 1981; Altman, in press). The chemical fingerprinting technique, using a combination of off-line LC and proton-NMR, appears a powerful screening method for the detection of secondary effects which may be applied on a routine basis. Application of this method in combination with other measurements of, for instance, the agronomical traits, may limit or even replace animal feeding studies aimed at the detection of 'unintended effects'.

It is proposed that substantial equivalence can be demonstrated by a hierarchy of comparisons of the chemical fingerprint of the transgenic plant or derived products to those of: (i) the parental line, (ii) an appropriate closely related reference line, (iii) an extended range of controls by entering current commercial varieties and (iv) downstream processing effects. A recommended approach to assess the likelihood that some of the statistical differences in the transgenic genotype may be false positives due to change alone or arose from natural genetic and/or physiologic variations as has been demonstrated. It is recognized that in the agricultural practise the circumstances of culturing and of processing and storage of appropriate controls will be mostly unknown and may having a non-isogenic genotype. Notwithstanding these complicating random block effects it is recommended that the chemical fingerprint analysis should not be limited only to the transgene and its isogenic parent bred under identical circumstances, but should also include a sampling through breeding seasons and a comparing with an extended range of controls of that crop (i.e. crop mean values). Otherwise it would be a too limited approach towards the establishment of the claim of *substantial equivalence* and it neglects the ranges of natural variations in that crop. With regards to the results obtained and reported herein it is concluded that the method of chemical fingerprinting analysis can be used in conjunction with the conventional analysis of key nutrients and toxicants. When established that the transgenic genotype will be sufficiently different to its properiate counterpart(s) subsequent analyses might be carried out in order to identify the structure of constituents involved as has been demonstrated in this study. *In vitro* models may be useful for screening of the complex plant product for potential adverse effects and for mechanistic studies (Noteborn et al., 1997a,b). However, since these models are not yet validated, their importance should not be over-accented. The CAT-TOX(L) model showed potential utility but there is a need for validation and other biomarkers as part for future research. More research is needed in order to evaluate the potential use of toxicological *in vitro* profiling to screen for possible differences in toxicity between modified and non-modified plant products.

Recommendations

It is recommended to initiate at the European level a joint research programme, with participation of regulatory and industry laboratories, with the objective to specify safety assessment criteria, and to harmonize different assessment strategies, based on case studies of genetically modified products from different categories.

An international workshop should be organized and focused on the choice of comparators and test parameters in order to establish Substantial Equivalence for novel foods and feed. Furthermore data banks should be set up containing information on natural variations of essential plant or other product constituents (i.e. crop mean values).

The chemical fingerprinting technique, using off-line LC-NMR, should be ring tested at the international level with different categories of novel foods and feed products, and could be considered as an alternative screening method for the detection of secondary effects resulting from the genetic modification.

The ultimate test for genetically modified food crops will be the acceptance by the public. It is therefore extremely critical that the industry, the scientific community and the regulatory authorities are able to respond to queries of the public in large. Results generated by this AIR3 project will certainly contribute to a better understanding by the public of these novel plant breeding technologies and their implications.

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Assessment of allergenic potential of novel proteins in food crops using the brown norway rat model

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Abstract

The Brown Norway (BN) rat, a high IgE (reaginic antibody) responder strain [1,2], was used in these studies to develop methodologies for assessing the allergenic potential of recombinant proteins which may be introduced into food crops. In order to evaluate allergenicity within this model, it is necessary to have information on the performance of dietary proteins with known allergenic and non-allergenic potential. Three novel proteins (2 Bt proteins and 1 proteinase inhibitor) and five reference dietary proteins have been examined following intraperitoneal (ip) exposure. All the proteins induced antigen-specific reaginic antibody production. However, dose responses studies showed that they possessed different potential "inherent" allergenic potencies. Novel protein was also recognised when tested as plant extract. Although a reaginic response could be demonstrated following oral administration, further studies are required to relate this to the activity of known dietary allergens and to establish the response to non-allergenic dietary proteins (negative control) within the model. The allergenicity of plant proteins, and the confounding effects of bioactive components naturally present in plants also need to be taken into account. The model now requires validation against known protein benchmarks both in isolation and in the complete crop matrix. Once established, this type of analysis will contribute to the indicators currently adopted for determining potential for dietary allergenicity to man.

Introduction

Novel foods derived from genetically modified (GM) food crops are likely to enter the market in the near future. Questions have been raised as to how to assess their food safety in relation to allergenicity and food allergy. With regard to immunotoxicity and allergy testing of drugs and chemicals, studies and guidelines to date have been concerned mainly with assessment of contact and respiratory sensititisation potential [3]. Only recently have thoughts turned to allergenicity of food proteins [4], and this subject has been addressed by the International Food Biotechnology Council and International Life Sciences Allergy and Immunology Institute [5]. Insertion of new genes in to a plant could result in introduction of a new allergen or increased expression of an existing allergen. Therefore assay systems need to be identified and developed which can be used to assess the potential allergenic risks associated with introduction of novel foods into the diet.

Allergens are usually proteins. The epitope, or allergenic determinant of the allergen can be shared with other proteins allowing cross-reactivity between foods. IgE antibody mediated hypersensitivity is the only demonstrable cause of food allergy. Serum banks built up from patients with known allergies are useful especially in examining cross reactivity between foods and identifying the allergenic epitopes recognised by man. However, this type of approach will only provide information based upon historical exposure, and will not provide information on the allergenicity of novel foods introduced into the diet where there has been no prior human exposure. Therefore assessment of the allergenic potential of novel proteins will ultimately have to be addressed using an animal model.

In this project we have used the Brown Norway rat (BN), a high IgE responder strain, that is capable of recognising a similar profile of allergens [1] and epitopes [6] as man, and can be sensitised by the oral route [2] to develop a system for investigating the allergenicity of novel proteins and genetically modified crops. In a further refinement to the system, the adjuvant carrageenan was employed. This adjuvant is believed to act via the inhibition of IFNγ, a cytokine involved in the down regulation of IgE production, and therefore increases the sensitivity of the model.

In order to allow proper evaluation of the possible risks associated with the introduction of a potential allergen into the food supply the allergenicity of novel foods cannot be investigated in isolation and studies also need to address the existing allergenic potential of the diet. Therefore to evaluate the contribution of the newly introduced proteins in the diet it is necessary to have information on the performance of dietary proteins where the allergenic potential is known in man and can be demonstrated within the chosen animal model.

Methods

Animals

BN rats (Male 6-8 weeks old) were used for immunisation procedures, while Sprague-Dawley (SD) rats (250-400 g) were used for the analysis of reaginic antibody. Both strains of rat were obtained from Harlan Olac (Bicester, Oxon, UK). Animals received a nutritionally adequate diet, either Teklad 9608 rat and mouse diet (ovalbumin- and milk-free), supplied by Harlan Olac for BN rats, or Rat and Mouse No 1 expanded, supplied by Special Services Ltd (Witham, Essex, UK) for SD rats. Both food and water were freely available. Animals were housed in groups of three to five, dependent upon strain and experimental design. They were acclimatised for a minimum of 6 days before use.

Antibody analysis

The presence of reaginic antibody was assessed by passive cutaneous anaphylaxis (PCA). Naive (untreated) Sprague Dawley rats were clipped to remove fur on the back and flanks and injected intradermally with 0.1 ml of the test sera. Twenty four hours later each rat was injected intravenously with 0.6 ml of antigen (1 mg/ml) together with 0.4 ml Evan's Blue (2% in saline). After 20 - 30 minutes the animal was examined for positive responses. The diameter of dye extravasation at the site of serum injection was measured and the area of dye extravasation (ADE mm^2) calculated. The PCA assay is a functional assay in which any extravasation following challenge is due to presence of antigen-specific reaginic antibody and therefore represents a positive response. It is semi-quantitative giving an indication of the magnitude of the response, providing information on the level of circulating reaginic antibody, but not that antibody bound to mast cells which is responsible for the development of symptoms in sensitive individuals. The presence of antigen-specific IgE was confirmed by immunoblotting using a mouse monoclonal anti-rat IgE antibody.

Treatments

Five reference dietary proteins bovine serum albumin (BSA), ovalbumin grades II and VII (OA II and OA VII), lactoferrin (LF) and chicken egg white cystatin ((CEWC) a proteinase inhibitor) were selected for examination. The three novel proteins examined were the *Bacillus thuringiensis* crystal proteins Cry1Ab5 and Cry9C, and a proteinase inhibitor Oc-IΔD86 which have been experimentally introduced into either tomato, corn or potato respectively. For examination of crop material aqueous extracts were prepared. The ability to induce reaginic (IgE) antibody production or "inherent

allergenicity" was examined following administration of dose levels of 0.01 - 1000 μg of protein together with the IgE adjuvant carrageenan by the intraperitoneal route. Serum samples were analysed for the presence of antigen-specific reaginic (IgE) antibody responses.

Results

All the reference dietary proteins induced antigen-specific reaginic antibody formation and IgE (figure 1a). The amount of allergen administered is a critical factor in the development of an immune response. Too little or too much may result in the development of tolerance. The lowered number of responders at the highest dose levels may reflect the development of high zone tolerance. This highlights importance of carrying out dose response studies in order to compare allergenic potential. Comparison of the dose calculated to produce 50% responders (ED50) appeared to be the most effective way of comparing the "inherent" allergenic potential. Using this approach the comparative allergenic potential of the reference proteins could be ranked LF>OA II>CEWC>OA VII>BSA. The levels of protein required to produce 50% responders being in the range of 40 - 50 ng for LF, 500 - 600 ng for OA II, 2 μg for CEWC, 5 - 6 μg for OA VII and 10 μg for BSA (Figure 1a). Similarly all three novel proteins examined induced antigen-specific reaginic antibody production following intraperitoneal administration. The level of protein required to produce 50% responders for the novel proteins was 1 μg for Cry1Ab5, 30 - 40 ng for Cry9C and 5-6 μg for Oc-IΔD86 (Figure 1b).

When expressed in the plant, proteins may undergo post-transcriptional modification, such as glycosylation, that could influence allergenicity. Following intraperitoneal exposure to aqueous extracts of corn plant powder expressing Cry9C, the novel protein was recognised and reaginic antibody production induced (figure 1c). The shift in the anti-Cry9C response from an ED50 of 30 - 40 ng to 200 - 300 ng, could indicate a change in allergenicity, but more probably this reflects the effect on the immune response to the administration of a complex mixture of proteins of which Cry9C was one. Not all proteins within an aqueous plant extract will possess allergenic properties and this is illustrated by the fact that only a few of the proteins present in tomato extracts were recognised as allergenic as determined by Western blotting using antigen-specific PCA-positive sera.

Discussion

The range of reference dietary proteins investigated using the approach developed in this project were those identified as dietary allergens from human serological investigations. CEWC was selected as it would provide a suitable control for the proteinase inhibitor Oc-IΔD86. The results from the intraperitoneal studies show that a range of inherent allergenic potentials could be demonstrated both for dietary allergens and novel proteins. The application of the toxicological concept of dose response to an essentially immunological phenomenon provides useful information on the inherent allergenicity of a food protein, and will permit a ranking to be established. However in order to provide a comprehensive ranking against which novel proteins can be evaluated, the number of proteins investigated needs to be extended and to include those, if possible, with no history of allergy in man to act as negative controls within the model. The BN rat model was shown to provide a sensitive system which distinguished between the activity of OA II and OA VII which is free of the s-ovalbumin variant. Thus even without the information on background allergenicity required for assessment of novel proteins, the results from intraperitoneal administration can be used to assess the effect of a new processing step on the allergenicity of an existing product.

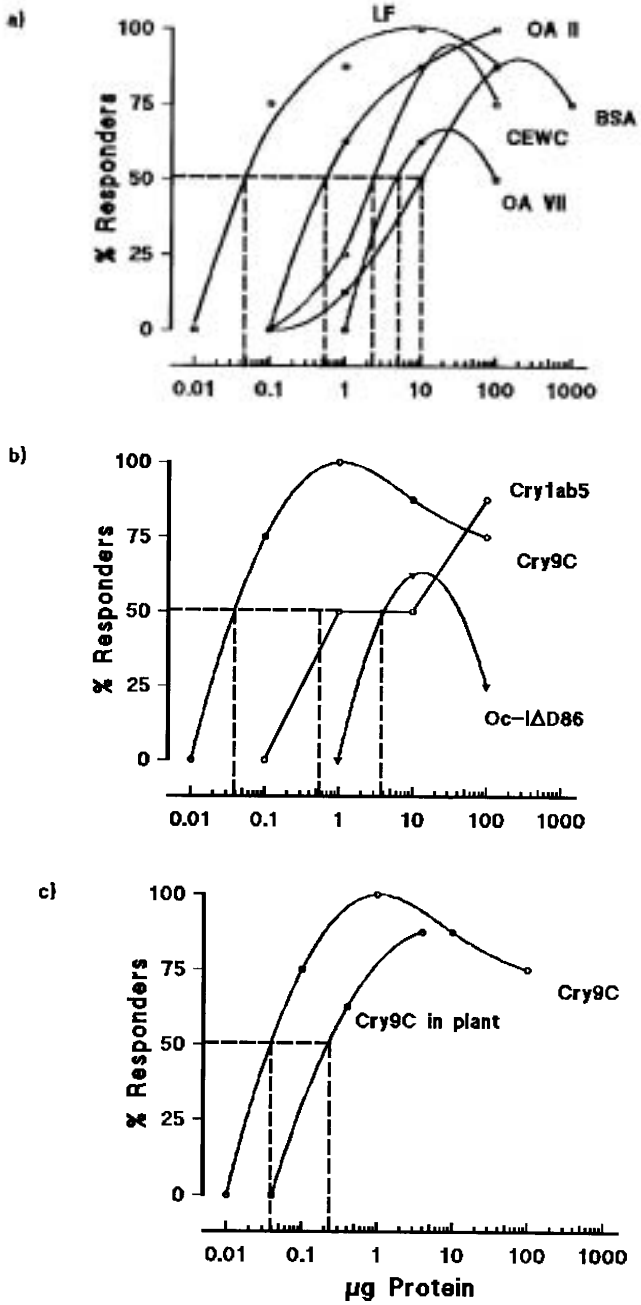

Figure 1: Dose response curves obtained following intraperitoneal administration of either reference dietary proteins (a), novel proteins (b) or novel protein when expressed in plant (c).

The development of sensitisation is a multi-stage process involving various elements of the immune system, hence the factors influencing the development of sensitisation are numerous. The type and magnitude of immune response to a protein is related to the amount of protein administered together with its inherent allergenic potential.

The amount of antigen administered is a critical factor in determining the development of an immune response. The amount of material available for examination is a critical factor in determining the experimental approach adopted. Assessment of the allergenic potential following intraperitoneal administration of the pure protein, for example, only requires milligram quantities of material to evaluate induction of sensitisation whereas studies using oral administration will require gram quantities of material [2]. The use of carrageenan at this stage in the development of methodologies has several advantages in that it provides diagnostic strength through decreasing variability in responses and the number of animals required. Also the number of treatments and/or dose levels of allergen required are reduced, which becomes a significant factor in the conduct of the assessment particularly related to oral sensitisation.

Although sensitisation following intraperitoneal exposure indicates potential allergenic properties, if the protein is degraded during digestion this property may not be retained following oral exposure. Nevertheless, the demonstration of inherent allergic potential of a protein following intraperitoneal administration represents a critical first step in the assessment process. It is important to show that the chosen animal model is capable of recognising a similar profile of allergens and epitopes as man [1,6] particularly for the novel proteins under investigation which is crucial with regard to interpretation of negative results following oral exposure. Studies examining protein stability to digestion have in the past employed commercially produced antibodies of the IgG class. Since the allergenic epitope may differ from the antigenic epitope against which the IgG was raised, in order to follow the fate of allergens during digestion and processing the antibodies need to be directed against the allergenic epitopes. Therefore this type of analysis should be directed toward the production of reaginic antibody, preferably of the IgE class as found in man. Sera raised by intraperitoneal administration can be utilised to track the fate of the IgE epitope during *in vitro* digestion. This approach is currently being investigated.

Plants contain a complex mixture of proteins which will be recognised as allergenic in mammalian species. Therefore, invariably there will be a background allergenic response in the animal model following exposure to plant proteins. It is generally acknowledged that the potential risks of GM-derived food crops should be related to those of traditional plant breeding, and in this context, to determine if the genetic modification has altered the allergenic characteristics of the crop. When assessing the allergenicity of novel proteins expressed by the plant the contribution of these accessory factors need to be considered. In addition to the introduced protein and newly expressed food proteins the food crop contain other constituents that influence the development of sensitisation through increasing gastrointestinal permeability or affecting immunomodulation. Bioactive materials in plants such as saponins that increase gastrointestinal permeability can increase the likelihood of sensitisation [2] or elicitation of allergic responses [7]. The levels of such factors produced by the plant may also be affected following genetic modification. Certainly reaginic responses were induced by Cry9C both in bacterial form and when expressed in plant following oral administration. However, as for the intraperitoneal studies, this observation needs to be addressed in relation to the responses of reference dietary proteins following oral administration within the model, and the influence of plant matrix on the reference protein response.

In conclusion these studies have shown that the BN rat can provide a suitable system for the investigation of potential allergens. Potential methodologies and experimental strategies have been identified. The model now requires validation against known protein benchmarks both in isolation and in the complete crop matrix. Once established, this type of analysis will contribute to the indicators currently adopted for determining potential for dietary allergenicity to man.

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Improvement of the safety and quality of refrigerated ready-to-eat foods using novel mild preservation techniques

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Partnership

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Objectives

The consumer demand for high quality foods which require a minimum amount of preparation led to the introduction of ready-to-eat, convenience foods preserved by mild methods only. An extensive range of foods qualifies as ready-to-eat, including raw vegetables, minimally processed (washed, trimmed, sliced) vegetables with or without dressings, and Sous Vide preparations (cooked vegetable and potato based dishes). Refrigeration is the main mild preservation techniques which these perishable food products rely on. Because of the difficulty of maintaining sufficiently low temperatures throughout the chain of production and processing to consumption, additional barriers are required to control the growth of spoilage and pathogenic microorganisms. This 4 year project, starting January 1993, develops these additional barriers such as bioconservation, modified atmosphere packaging (MAP) or coating (MAC), and coatings containing food-grade antimicrobial agents (active MAC). These barriers are optimised in combination with refrigeration on the basis of fundamental knowledge on microbiology, product physiology and preservation techniques in conjunction with the practical evaluation of safety and quality obtained with the (combinations of) mild preservation techniques.

Of particular concern for minimally processed, chilled foods are psychrotrophic pathogens such as *Listeria monocytogenes* and non-proteolytic *Clostridium botulinum*, which are able to grow at refrigeration temperatures The growth of such pathogens and of psychrotrophic spoilage bacteria is investigated here in relation to the food matrix (a_w, pH, preservatives, food components) and the storage environment, which both interact with their potential to survive or grow.

Project results

The project has shown that Minimally processing/Mild preservation is not a simple concept and that, in particular, MAP is not an "off-the-shelf" technology. To obtain a quality product, the concept needs to be carefully tailored to the specific physiological requirements of the product under the exact conditions prevailing (quality of processing applied, refrigeration, selection of MAP system, logistics, expected shelf-life, etc.). All results have been extensively disseminated towards both industry and scientific audiences.

Concerning safety, the project has brought about important new insight into the growth and survival of pathogens on Minimally Processed foods. It was found that a range of different food components supported the growth of certain cold-tolerant pathogens and that such effects could be very specific. The mechanisms underlying this effect were, for the first time, studied in much detail. The balans-interaction between pathogens and epiphytes on foods, as observed for *Listeria monocytogenes* on leafy salads has pinpointed the importance of the normal microflora in food safety and has identified consequences for Good Manufacturing Practices and disinfection treatments.

In Modified Atmosphere Packaging (MAP), many factors affect the keeping quality and shelf-life of MAP foods: product respiratory activity, head-space gas, head-space volume to product weight ratio, packaging material, storage temperature, processing, etc. Respiratory activity of the packaged product is of utmost importance with respect to quality. The respiratory activity depends on many different factors: storage temperature, processing (cutting, peeling, knife sharpness, dipping in chlorine), product (cultivar, origin, age, cultivation and storage histories), oxygen to carbon-dioxide ratio in head-space and absolute amount of oxygen (O_2). The former two have the biggest impact. Vegetables in prepared dishes are alive and need oxygen to maintain their quality. If O_2 is below a critical limit, anaerobic respiration is switched on and off-flavours are produced.

With Iceberg lettuce and carrots it was proven that very sharp blades only minimally affected respiration rates and microbiological counts. However, stationary knives caused a strong incline in these factors and, thus, reduced acceptability. Stationary blades probably cause localised bruising near cut surfaces resulting in more spoilage. A chlorine dip reduced microbial loads and increased acceptability scores in both cutting treatments. Undipped rotation cut lettuce scored better than dipped, stationary blade cut lettuce. Thus, a good cutting technology is of paramount importance.

Whereas it was commonly thought that microorganisms in general are sensitive to carbon dioxide, it was shown here that the cold-tolerant pathogens are not sensitive at all and really may pose a health hazard in MAP systems, unless additional precautions are taken. Such precautions could be the use of bacteriocin-producing lactic acid bacteria microorganisms (LABs) or their bacteriocins. Much effort was invested in finding LABs that actively produced suitable bacteriocins at chill temperatures under MAP conditions. No LAB with this trait was found. However, a bacteriocin producing *Enterococcus* was found and thoroughly characterized. It was found that application of the culture was less effective than application of the pure bacteriocin.

Chilling is an important preservative ‘hurdle’. Processing, transport, display and intermediate storage should all be at the same low temperature, e.g. 7°C or even better below, at least for produce that are not vulnerable to chilling injury. The use of as low as possible temperatures may well render other hurdles obsolete.

Changes in temperature should be avoided. Higher temperatures speed up spoilage. Fluctuating temperatures cause in-pack condensation, which will again stimulate spoilage.

Biocoatings, totally natural and edible, were developed for physical protection and to carry bacteriocins and other natural antimicrobials. This area is rapidly developing now and the participants in this project have moved much to the forefront of this development. The results can easily picked up by industries and taken further into their practice. The participating companies have experienced that improvements can be developed and implemented in a rather short time.

A new custom-made GC-system was tested and compared to commercially available non-GC systems of the quantification of low levels of oxygen. Inaccuracies in the different systems were identified and quantified. The methods for accurate measurement were developed and described. The methods described are useful for routine true oxygen measurement by industry or regulators, also those working outside the vegetable/fruit area. For example, precisely controlled low levels of oxygen can be vital in packaging of cured and some fresh meats.

The effects of a wide range of raw material processing and storage variables were evaluated in model products. Significant effects on quality were observed for all parameters. These findings are immediately applicable to industry. The effects of processing packaging and storage conditions on losses of ascorbic acid/conversion to dehydroascorbic acid were quantified. Guidelines to minimise the loss of this vitamin are applicable in industry. Packaging materials were selected for a range of commercial products that considerably improve product-package compatibility compared with current commercial practice.

Noticably, conditions can prevail that encourage growth of pathogens, the cold-tolerant *Listeria monocytogenes* especially in prepared salads, the non-proteolytic *Cl. botulinum* in REPFEDs/sous-vide type of preparations. By current standards, the presence of these pathogens may not be completely avoided in (raw) vegetables.

Many of the aspects of non-proteolytic *Cl. botulinum* were demonstrated here for the first time. The ability of sub-lethally damaged spores to grow in suboptimal conditions (NaCl, pH, organic acid, gaseous atmosphere) at refrigeration temperatures were characterised and the ability of many cooked vegetables to support growth of non-proteolytic *Cl. botulinum* was demonstrated for the first time. Particularly interesting was the discovery that vegetable juice can aid the recovery of heat damaged spores.

The project has developed knowledge and expertise on the design and application of edible coatings. Such coatings are 100% food-grade and form a physical protection on the product. We are now able to tailor gas permeability properties of films to specified band-width deemed optimal for MA-packaging of respiring products. Different types of commercial coatings have been as well tested in the project. With carrots subjected to coarse abrasion peeling, appearance scores normally decline rapidly due to dehydration and stress response reactions such as lignin synthesis. Use of the commercial coating 'Nature Seal' or a laboratory prepared pectin-calcium chloride coating advised by CIRAD.SAR considerably improved product appearance. Other coatings based on gluten (Opta Glaze) or oil coatings were not successful. More practical tests of performance still need to be undertaken on a broader scale to allow for a solid evaluation.

In effect, edible films and coatings certain for fruits and vegetables have been designed in this project. The knowledge gained in the process, should enable research and practice to fine-tune this technology for near-future application in minimally processed fruits and vegetables. Active edible coatings were found to be a promising concept, allowing (bio)preservation, MA-generation and physical protection to be combined in one process. The research performed on this topic has set the course to further develop "minimal packaging" or "invisible packaging" concepts.

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Exploitation

Based on the results from the project, food producing industry has been given targeted advise, e.g. via VALUE and FLAIR-FLOW Europe workshops. The project has not generated knowledge or technologies that are commercially interesting for patenting. Industrial application is very well possible in many respects, but involves improvements in safety and quality that are not due to facts or protocols that are, a priori, worth patenting or marketing. The results obtained are best exploited by wide diffusion to commercial and scientific parties interested and this has been undertaken (see below). The dissemination effectuated to date has been quite thorough. The detailed results from the project have been made available in many communications for (inter)national audiences, displaying both the scientific progress and the practical relevance for food processing industries many of which are SMEs. The routes followed were publications in scientific (112 times) or trade journals (15), appearances on conferences or workshops (91) and on specific EU-funded (40), often SME-targeted meetings (FLAIR-FLOW, VALUE, RETUER). In addition, Flair-Flow Europe one-pagers (3), Progress Highlights (10) and RETUER Handouts (1) were issued. A selection of papers published in the scientific literature is given on the next pages.

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Harmonization of safety criteria for minimally processed foods

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Abstract

The EU Concerted Action “Harmonization of safety criteria for minimally processed foods (FAIR CT96-1020)” has been started in September 1996. This Concerted Action brings together a significant number of representatives from food processing industries, from private and governmental research organisations and from legislative and consumer organisations.

Their objective is to provide a sound scientific base for the setting of standards and regulations relating to the safe production and distribution of minimally processed foods.

From the inventory of legislation and good manufacturing practice codes (31) can be concluded that there is no clear definition of 'minimally processed foods' and that the safety criteria are very different from country to country and from product group to product group. Traditional safety guidelines cannot be applied for new technologies or combined technologies.

1 “Minimally processed foods”: a broad definition

“Minimally processed foods” are foods that have been harvested, cleaned, trimmed, size-reduced, perhaps heated to inactivate enzymes and decrease microbiological loads, and reduced in temperature to prolong quality retention. Essentially, the least amount of processing required to permit an effective distribution under controlled conditions is employed to ensure that the food is as close to fresh as possible (Brody, 1997).

The traditional means to control microbial spoilage and safety hazards in foods, such as freezing, blanching, sterilisation, curing and use of preservatives have been replaced by new, innovative techniques such as mild heating, modified atmosphere and vacuum packaging and the employment of natural antimicrobial systems. Commonly, all products rely on refrigerated storage and distribution for their preservation, both from microbiological and quality retention standpoints. Within the project, minimally processed foods are defined as products that:

- undergo a mild preservation process: the final processing temperature falls between 0-100°C
- rely on refrigerated storage and distribution
- have a water activity higher than 0.85
- have a pH higher than 4.5

This definition is consistent with the definition of the European Chilled Food Federation but slightly different from the definition of the Codex Alimentarius Commission.

2 There is a fast growing market of minimally processed convenience foods

There is a strong, rapidly growing consumer demand for food products that are fresh-like, healthy and safe to eat with a high degree of convenience. In the period '91-'95 world wide sales increased with 40 % and for Europe the increase was 50 % (table 1). Recent figures from France indicate that the evolution to fresh-like products is still growing (table 2). The retail sector is really pushing the market of chilled products (table 3).

Worldwide sales are estimated at 40 billion US dollars in 2000, in 1995 the sales were estimated to be 12 billion, a yearly increase of more than 25 %.

But not only the definition of 'minimally processed foods' is not clear. Also many different definitions of chilled food can be found and also the distribution channels that are taken into account can be very misleading. But there is no doubt about the fact that the sales of minimally processed foods are rapidly increasing.

Table 1: Growth in chilled food sales (US$m) led by ready meals

	1991	**1995**	**% +/-**
North America	4440	6092	37.2
Japan	2557	3573	39.7
Western Europe	1574	2361	50.0
Australia	14	31	121.4
	8585	12057	40.4

Table 2: Product trends France 1997

Product	**Evolution**	**Sales MFF**
Fresh salads	+37 %	902
Fresh soups and sauces	+26 %	355
Fresh entrees	+21 %	1964
Dried prepared meals	+12 %	200
Fresh prepared meals	+11 %	1677
Fresh pastry	+10 %	997
Frozen prepared meals	-4 %	3031
Regional french meals	-8 %	1986
Exotic prepared meals	-8 %	527

Table 3: Retail is pushing the market

	Manufacturer's brand	Retailer's brand	Total
Frozen	347	232	579
Chilled	**481**	**1695**	**2176**
Ambient stable	1045	796	1841

3 Potential safety hazards associated with minimal processed foods

The major microbiological concerns associated with minimal processed foods centre around two types of microorganisms: psychrotrophic and mesophilic pathogens. Psychrotrophic microorganisms can grow at refrigeration temperatures, mesophilic pathogens can survive under refrigeration and grow during any temperature abuse of the food.

With IVth Gamma foods, a very important health hazard is the growth of psychrotrophic (low-temperature) pathogens such as *Listeria monocytogenes, Yersinia enterocolitica, Aeromonas hydrophila* and *Vibrio parahaemolyticus*. Some of these may proliferate at temperatures down to 3°C. Other pathogens, for instance *Salmonella typhimurium, Escherichia coli* O157:H7 and *Staphylococcus aureus* are mesophilic but may still come to grow due to temperature abuse, which in practise seems to be inevitable. With Vth Gamma products, which receive a pasteurization treatment, spore forming pathogens such as *Clostridium botulinum* and *Bacillus cereus* may survive and, in the absence of the competing natural microflora, may flourish when storage conditions are favourable. Among both types of sporeformers, psychrotrophic strains have been identified.

4 Actual status of safety criteria for minimally processed foods

4.1 Safety criteria in production and legislation are at variance throughout the European Union

In most countries, food business operators are required to comply with general and specific hygiene rules, and to develop procedures for food hygiene based on the principles of HACCP. Several national or international branch organizations have also developed codes of practice for the production of chilled foods with extended shelf life. A code of practice is rather advisory than prescriptive in form, and compliance with the code can only be recommended, not enforced.

During the first year of the project, an intensive search has been conducted on actual legislation, existing codes of practices and safety recommendations for the production and distribution of IVth and Vth Gamma products. All information has been summarised in a 46 page inventory report.

The inventory report indicates clearly that we are still far from reaching a set of harmonised criteria. For instance, a lot of differences in legislation can be found in required chilled storage and cooling requirements. This lead to remarkable differences in operation cost for the producers in different countries of the EU. In Denmark, for instance, cooked products should be cooled from 65 to 10°C in 3 hours, in France the cooling requirement demands a cooling from 60 to 10 °C in less than 2 hours (table 4). It is not obvious why these differences in national regulations have been introduced, but they do have an important impact on the type or level of processing applied for similar products.

The complexity of the products and the combination of different technologies make traditional simple safety concepts like 6-D or 7-D for pasteurised products not applicable. Also the choice of the reference microorganism and desired log reduction is not clear (table 5).

Further research is needed to establish pasteurization concepts in combination with the desired shelf life and actual chilled storage temperatures.

Another example are the criteria for the washing water for the cleaning of fresh vegetables, as recommended in different codes of practices (table 6). In most EU member states, the use of

hypochlorides for the desinfection of prepared vegetables is standard procedure, whereas a substantial number of memberstates specifically by law forbid the use of any such compound.

The allowed chlorine concentration is normally expressed as “free available chlorine” (in ppm or mg/l). The amount of “total chlorine” corresponds to the amount of chlorine added to the solution. A part of the chlorine combines with organic materials and approximately 80% of the added chlorine is effective (= free chlorine). Some codes of practice give also recommendations for the amount of residual chlorine after rinsing and dewatering.

As a result, there may be similar products on the market in different EU member states which differ in the level of safety. Both from the view of consumer health as well as of fair competition, uniform safety criteria should apply throughout the European Union.

Table 4: Legal time-temperature requirements for cooling and chilled storage in different EU countries (FAIR CT96-1020, Inventory Report).

Country	Chilled storage	Cooling after heating
Belgium	max. 7°C	immediately
Denmark	5°C MAP minced meat: 2°C	• from 65°C to 10°C in 180 min. • if sold within 3 hours: no requirements
Finland	meat based products: 6°C other chilled products: 8°C	to 10°C in 120 min., exceptions allowed
France	depends on stage of production, e.g. in retail, storage at ≤ 4°C	to 10°C in 120 min. (usually from 63°C to 10°C in less than 120 min.)
Italy	meat products: -1 - 7°C fish products: 0 - 4°C	for meat products to 10°C in 120 min.
Spain	0 - 3°C	not specified, specific for each product
Sweden	< 8°C	not specified
The Netherlands	max. 7°C	as quick as possible
UK	8°C	as quick as possible
Norway	-1 to 4°C retail: < 7°C	• from 60°C to 7°C in 4 hours (in general) • to 10°C in 120 min. (for meat as ingredient in ready-to-eat meals)

Table 5: Criteria for pasteurization process, storage temperature and shelf life, recommended in different national codes of practice (FAIR CT96-1020, Inventory Report).

Code of practice	**Pasteurization**	**Storage temperature**	**Shelf life**
European Chilled Food Federation, 1996	• 2 min. at 70°C Reference m.o.: *L. monocytogenes* • 10 min. at 90°C Reference m.o.: psychrotrophic (non-proteol.) *C. botulinum.*	≤ 3 °C retail: ≤ 4 °C	not stated
SYNAFAP, 1997 **France**	determined by the processor (after hazard analysis)	< 4°C	• determined by the processor • validation shelf life protocol
TNO Voeding, 1994 **The Netherlands**	• 10 min at 90°C • Reference m.o.: psychrotrophic (non-proteol.) *C. bot.* typeB D_{90}=10 min z=10°C if T>90°C or z=7°C if T<90°C	0 - 3°C retail: 0-5°C	• max. 42 days: • max. 3 weeks outside facility • max. 1 week in home fridge
Campden & Chorleywood Food Research Association, 1996 **United Kingdom**	• 2 min. at 70°C • 10 min. at 90°C • 10 min. at 90°C + hurdles	deep chill storage: < 3°C retail: ≤ 8°C	• short shelf life: < 10 days • long shelf life: > 10 days
National Food Processors Association, 1989 **USA**	• 5D reduction of *E. coli* O157:H7 in ground beef • 7D reduction of *Salmonella* in poultry	< 4.4°C (40 F) retail: < 5°C (41 F)	scientific evidence needed to support labelled shelf-life

Table 6: Criteria for the washing water to rinse fresh vegetables, recommended in different national codes of practice (FAIR CT96-1020, Inventory Report).

Country	Code of practice	Requirement for washing water
France	Centre Techniques Interprofessionel des Fruits et Legumes, 1996	• max. 4°C • 120 ppm free chlorine, provided products are rinsed efficiently with drinkable water • contact time: 2 min.
USA	National Association of Fresh Produce Processors, 1993	• 50 - 100 ppm of total chlorine • residual chlorine: 1 ppm
Canada	International Fresh-Cut Produce Association, 1996	• 100 - 150 ppm of total chlorine • 2 to 7 ppm of free chlorine (residual after contact) • pH = 6.0 to 7.0
United Kingdom	Campden & Chorleywood Food Research Association, 1992	Disinfection with chlorinated water
The Netherlands	Productschap Tuinbouw, 1996	No hypochorides allowed in washing water

4.2 Chilled foods distribution needs improvement

Minimally processed foods rely heavily on proper refrigeration during storage, distribution and retailing, since only growth control techniques are used (IVth Gamma products, shelf-life about six days) or mild preservation techniques are applied but storage is for rather long periods (Vth Gamma products, shelf-life up to six weeks).

Manufacturers recognise the potential for temperature abuse during distribution or storage of foods requiring refrigeration. The weak chill chain during distribution has been reported in several studies (Brody, 1997); and average temperatures in consumers' fridges up to 7-8°C may be considered as a general rule.

Product temperature should be maintained slightly above freezing (maximum 4°C) to guarantee an optimal safety for chilled foods. Any deviation from these optimal temperatures can cause serious effects on the growth of psychrotrophic bacteria in these foods. Microbial lag phases (during which their is no growth or a decline in microbial numbers) and generation times (duration between a formation of a daughter cell and its division into two new cells) increase as refrigeration temperatures decrease (table 7).

Table 7: Generation times or time until toxin formation by some psychrotrophic pathogens during growth in food. Adapted from Snyder (1996).

Pathogen	Temperature °C	Generation time (h)	Food
Listeria monocytogenes	0.0	110.0	corned beef
	3.0	37.6	roast beef
	5.0	43.0	raw cabbage
	5.0	44.0	cooked meat
	10.0	21.7	lettuce
	10.0	8.2	corned beef
Yersinia enterocolitica	0.0	67.4	imitation crab legs
	3.0	18.0	boiled shrimp
	7.0	10.3	cooked beef
	10.0	12.0	imitation crab legs
Pathogen	**Temperature °C**	**Time to toxin formation (h)**	**Food**
Clostridium botulinum Type E	3.3	964	Fish
	4.0	644	Fish
	4.4	1320	Crab meat
	5.0	426	Fish
	6.0	456	Beef stew
	7.0	243	Fish
	9.0	163	Fish
	10.0	138	Fish

5 Recommendations for harmonized criteria

The project is currently in the process of setting up recommendations for further rationale and harmonization of safety criteria. We believe that following directions should be taken into account to further investigate and standardise the control measures for IVth and Vth Gamma products:

5.1 Risk assessment

Risk assessment, the first part of risk analysis, is the scientific evaluation of known or potential adverse health effects, resulting from exposure to foodborne hazards. Risk assessment can be an extremely useful tool to quantify risks associated with minimally processed foods. More research and data are needed, however, to better quantify the overall risks associated with minimally processed foods. Also the occurrence of and risks associated with more or less new, foodborne pathogens (e.g. VTEC) should be considered.

5.2 Scientifically based control measures

With attention to Good Manufacturing Practices, sanitation, hygiene, product formulation, storage temperature, length of refrigerated storage, and microbial control measures, extended shelf life refrigerated foods can be produced of high quality and minimal risk for foodborne illness.

Because their is doubt about whether refrigeration alone is sufficient to assure the safety of chilled foods, further research should be conducted to the product formulation of products with additional hurdles: low pH, low water activity, added organic acids and protective cultures, modified atmosphere packaging etc.

5.3 Shelf life assessment and validation

Acceptable product shelf life (e.g. days, weeks) at specified temperature limits should be established and monitored to help manage food quality and safety. Microbial models to predict the growth of microorganisms during chilled storage, microbial challenge tests and other scientific validation studies should be further developed and validated in real food products. The level of temperature abuse in the cold chain to be taken into account for the risk assessment is a very sensible discussion.

5.4 New technologies

The potential of some “new”, non-thermal methods to extend the shelf life and improve the safety of minimally processed foods should be further investigated. Examples of these new technologies are ionizing radiation, pulsed electric field technology, pulsed high-intensity light, high hydrostatic pressure, …

5.5 Consumer information and education

Consumers should be aware of the potential risks associated with chilled foods (restricted shelf life, pasteurized products, need for refrigeration, …). Further attention should be paid to safety labelling of minimal processed foods and clear information towards the consumer.

6 Information dissemination of the HARMONY project

The Concerted Action will also strongly promote the outward direction flow of information from the project group to (federations of) food producers and consumers as well as to relevant governmental representatives. The project has been presented in 2 Flair Flow one-pagers. A Website dedicated to the HARMONY project has been developed, and can be reached from
http://www.harmony.alma.be.

A copy of the inventory report can be ordered from the co-ordinator or downloaded from the HARMONY Website as a Word-document:
http://www.harmony.alma.be/f_results/f_inventory/inventory.html

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The HACCP-Concept - Its impact on the food industry

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Abstract

Public awareness of food safety continues to be high and the provision of safe and high-quality food is uppermost in the minds of both producers and consumers. Hazard Analysis and Critical Control Point (HACCP) is the system of choice for identifying, evaluating and controlling hazards which are significant for food safety. The concept was developed for the food industry more than thirty years ago and its widespread application has accelerated in the past six years due to the introduction in European Directives of the requirement for hazard analysis, and customer requirements for their suppliers to have an effective food safety management programme.

FLAIR Concerted Action No. 7 brought together scientists and industrialists in two subgroups. One subgroup produced a reference document on the use of combined processes for food preservation. A second produced a simple guide which would promote a common application of HACCP across Europe. The principles of HACCP, as described by Codex, were explained together with advisory notes with special reference for small and medium sized enterprises.

Concerted action No 7

FLAIR Concerted Action No. 7 brought together scientists and industrialists in two subgroups. One subgroup aimed to gain a better insight and understanding of combined methods for food preservation, whilst the second subgroup aimed to promote safety in food production through the application of the HACCP system.

Combined processes subgroup

The ‘hurdle’ subgroup included 14 representatives from 11 countries: Belgium, Denmark, France, Germany, Ireland, Italy, The Netherlands, Slovenia, Spain, Sweden, and the United Kingdom. They assembled data on the combinations of preservation processes used for foods. Different ‘hurdles’ in a food often have a synergistic effect, so several ‘hurdles’ used simultaneously will result in safe and stable foods which have high sensory and nutritional properties. Many traditional foods may use ‘hurdle technology’ unintentionally, but the approach has found widespread application in food product development. The subgroup’s findings were published as a report (1) intended as a user friendly reference for the application of combined processes for food preservation in industrialised as well as developing countries.

The HACCP subgroup

Representatives on the HACCP subgroup came from 10 different countries: Belgium, Denmark, France, Germany, Ireland, Norway, Portugal, Spain, Sweden and the United Kingdom. Meetings were held in several locations: Zaragosa, Chipping Campden, Dublin, Leuven and Paris.

Development of the HACCP user guide

The HACCP subgroup aimed to produce a simple, practical guide to HACCP which would promote a common application across Europe. Initially the brief was to address both quality and safety aspects of food production, but it was agreed that as quality attributes tend to be specific to a company, the guide would focus on product safety assurance only.

The principles of HACCP are applicable to all sectors of the food and drink industry including small and medium sized enterprises (SMEs). Specific problems faced by SMEs (e.g. insufficient HACCP knowledge and expertise, insufficient technical expertise, insufficient technical resources) should not be a discouragement to applying the principles of HACCP. The guide produced by the subgroup aimed to provide a clear description of HACCP and its application to food safety assurance.

Following the completion of the guide, and its publication in 1993 (2), the text was translated by HACCP specialists from the countries represented in the subgroup and from The Netherlands and Finland. Of all the reports from FLAIR funded projects, the HACCP document has been the most widely requested (estimated to be in excess of 1800 enquiries with an estimated 5000 copies in circulation).

The development of the HACCP User Guide coincided with international developments on the principles of HACCP, the terminology used in HACCP systems, the practical application of the principles of HACCP, and draft European Directives on hygiene aspects of food production. Key influences on the approach to the development of the HACCP User Guide came from:

* National Advisory Committee on Microbiological Criteria for Foods (NACMCF), Washington (1992) (3)
* HACCP: A Practical Guide, CCFRA (1992) (4) (5)
* Joint FAO/WHO Codex Alimentarius Commission Food Hygiene Committee (1993) (6) (7).

With the endorsement by the Codex Alimentarius Commission in 1993, that the HACCP system is the most cost-effective approach to food safety, and the recognition of the role of Codex in matters relating to health and trade, the work of Codex, including their standards, guidelines and recommendations, are taken as the ‘benchmark’ for national requirements in food safety.

The HACCP system has become the internationally recognised and accepted method for food safety assurance. Whilst the original application of HACCP by the Pillsbury Company, NASA and the US Army Laboratories was for microbiological safety, the system is used to address both physical and chemical hazards which can have an effect on the production of safe food.

The HACCP system

The HACCP system is a systematic approach to the identification, assessment and control of hazards which are significant to health. The system is based on seven principles and aims to focus control, and resources, at the critical control points. The principles should be applied to all stages of an operation, including production, processing, manufacturing, preparation and use of the food (i.e. from primary producer to final consumer). The principles are:

1. Conduct a hazard analysis.
2. Determine the Critical Control Points (CCPs).
3. Establish Critical Limit(s).
4. Establish a system to monitor control of the CCP.
5. Establish the corrective action to be taken when monitoring indicates that a particular CCP is not under control.
6. Establish procedures for verification to confirm that the HACCP system is working effectively.
7. Establish documentation concerning all procedures and records appropriate to these principles and their application.

Guidelines for the Application of the HACCP System are described by Codex (7) and can be found with additional advisory notes and worked examples in several references (5) (8). The success of a food safety programme also depends on the adherence to Good Hygienic Practice and Good Manufacturing Practice.

Implementation of HACCP

The speed of development of HACCP systems has varied amongst the food industry in Europe, due to a variety of factors: for some companies food safety assurance was well managed but the system was not called HACCP; many food companies have a quality system which meets the requirements of the ISO 9000 standard; the US seafood rule requires all seafood to be produced under a HACCP system; US meat plants will have similar requirements in the future; the European Directive on the hygiene of foodstuffs does not have an explicit requirement for documentation and records; suppliers to large retailers, particularly suppliers of own-label products, have had to demonstrate that a HACCP system is in place as a requirement for trade; own checks at critical points in products covered by vertical directives (which amounts to HACCP).

Assessment of HACCP systems

Throughout the food industry, the system implemented to demonstrate food safety assurance has been scrutinised not only by the food businesses themselves, but also by customers and regulatory authorities. The early assessments found that whilst the concept of HACCP was understood, the principles were misapplied. One tendency was to include all aspects affecting the product in the HACCP system. This resulted not only in unmanageable systems which were difficult to keep up to date but also with most of the process steps being identified as “critical”. Clearly the misidentification of CCPs dilutes the effectiveness of HACCP in pinpointing the critical steps and enabling the resources of the business to be focused on the steps critical to food safety.

Food inspectors must assess the HACCP plan and confirm that it is operating effectively. The absence of the requirement for documentation and records in the horizontal hygiene directive has brought difficulties for the authorities in seeking compliance with the directive.

Food industry response

Guides have been produced in many areas of the food industry, to enable that sector to develop their HACCP systems more consistently. Technical expertise may be absent in smaller businesses, so guides play an important role in helping these companies to develop their HACCP system.

The documentation of the hazard analysis and resultant food safety system has been variable. The key references provide suitable suggestions (2), (5), (8).

Training of personnel involved in HACCP teams has varied, depending on the size and resources of the business. This has resulted in numerous training packages and training providers becoming available, some of which have been of doubtful quality. In the UK, a group of representatives from both the food industry and government were brought together and a training standard agreed. The "Certificate in HACCP Principles" is awarded to those who have followed an approved course and have successfully completed an examination. The examination tests the candidate's understanding of the terminology and their judgement in applying the principles of HACCP for a specific scope with a given food scenario. The training standard and examination is administered by the Royal Institute of Public Health and Hygiene. A syllabus is also under development by other nationally registered organisations who have established hygiene related training courses.

Future considerations

Food safety risk analysis associated with biological hazards is being addressed by government bodies at national and international levels. Risk analysis comprises three components: risk assessment, risk management and risk communication. The WTO Sanitary and Phytosanitary Agreement also encourages the wider use of systematic risk assessment in the management of food safety. The Codex Alimentarius Commission Food Hygiene Committee is currently developing a document (9) on the principles and guidelines for the conduct of microbiological risk assessment. The general principles are:

* Microbiological Risk Assessment must be soundly based upon science.
* There should be a functional separation between Risk Assessment and Risk Management.
* Microbiological Risk Assessment should be conducted according to a structured approach:

- hazard identification
- hazard characterisation
- exposure assessment
- risk characterisation.

The formalised approach to risk analysis in food microbiology is in its infancy and is likely to play a more important role in the determination of the level of consumer protection that a government considers necessary and achievable, i.e. food safety objectives. It is then the responsibility of food companies to define their policy and programmes to meet the food safety requirements.

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Session 2:

Food, Nutrition and Well Being

Effect of different dietary carbohydrates on colon function. Design of healthier foods

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Abstract

The overall objective of the project was to develop a scientific basis for recommending new dietary guidelines and for implementing the production of novel industrially-produced foods containing carbohydrates that have a protective effect on the colonic mucosa.

In particular the project addressed the following aims:

- To investigate the patterns of SCFA formed by fermentation of a variety of complex carbohydrates by the human gut microflora *in vitro*.
- To assess whether a shift in the composition of the diet from simple sugars (e.g. sucrose) to complex carbohydrates (e.g. starch) has the potential to block steps leading to neoplastic transformation in the colon.
- To investigate, using human volunteers, animal models and *in vitro* methods, the possible mechanisms (including faecal concentration of short chain fatty acids, long chain fatty acids, bile acids, calcium, bacterial species) underlying the protective effects of selected complex carbohydrates, so that more effective carbohydrate sources can be developed.

The work of the project fell under four main headings:

1. *In vitro* studies of carbohydrate fermentation by the human gut microflora
2. Animal studies designed to evaluate critically the potential protective role of complex carbohydrates, especially starches, in colon cancer
3. Dietary intervention studies in human volunteers to provide more definitive information on the protective effects of complex carbohydrates on colon damage in man.
4. Development and use of *in vitro* systems based on isolated colon cells in culture to investigate mechanisms involved and to identify gut luminal factors, particularly gut flora metabolites, with modulating activity towards cytotoxic and genotoxic damage in the gut.

1 Introduction

Epidemiological data suggest that diets rich in lipid and low in starch and fibre may increase the incidence of colon cancer (Cassidy et al 1994). It has been proposed that these correlations of dietary habit and cancer risk are a consequence of some dietary components acting as tumour promoters and others as protective factors. It has been demonstrated that diets deficient in starch, cellulose and calcium increase colon mucosa proliferation, considered to be a marker for increased risk of colon cancer (Caderni et al, 1991; Lipkin, 1988). Furthermore, rats given boluses of sucrose exhibit a burst of cell proliferation in the colon (Stamp *et al*, 1993). Such observations may have implications for cancer risk in man since most western diets incorporate relatively high amounts of sucrose and processed foods are often supplemented with sucrose and consumed on an empty stomach.

There is therefore, preliminary evidence that the type of carbohydrate in the diet, particularly whether it is a simple sugar or a complex carbohydrate, can have marked effects on the colonic mucosa function possibly influencing colon cancer. Dietary carbohydrates likely to have the greatest effect on the colon are those that are poorly digested in the small intestine and hence pass intact into the large bowel. There are three main types of such carbohydrates (Englyst *et al*, 1992):

1. *Non-starch polysaccharides (dietary fibre).* These encompass a wide range of polysaccharides including soluble and insoluble fibres (e.g. cellulose, hemicellulose, gums) and pectins and are largely derived from plant cell walls or algae.

2. *Resistant starch.* Most dietary starch is broken down by amylase in the small intestine, but some starches resist digestion as a consequence of their inaccessibility to amylase (e.g. starch in intact seeds and grains), chemical resistance to the enzyme (e.g. raw potato starch and amylomaize starches such as Hylon VII), or because of retrogradation of the amylose polymer caused by successive heating and cooling (e.g. heated and cooled potatoes, and the processed starch CrystaLean used in many of the present studies).

3. *Non-digestible oligosaccharides (NDO).* Some low molecular weight carbohydrates comprising 3 - 10 sugar moieties, such as stachyose raffinose, fructo-oligosaccharides and xylo-oligosaccharides, possess particular glycosidic linkages that are not susceptible to the hydrolytic enzymes in the small bowel, and so pass into the colon (Rumney and Rowland 1995). The xylo-oligosaccharide used in the present project is produced by from xylan by enzymic hydrolysis and is comprized of 2 - 5 xylose units.

It has been proposed that many of the effects of starch and non-starch polysaccharides and NDO on the colon are a consequence of the fermentative activities of the gut microflora. Fermentation of carbohydrates yields short chain fatty acids (SCFA), primarily acetic, propionic and butyric acids which may directly influence the colonic mucosa resulting in changes in cell proliferation rates, apoptosis (programmed cell death of damaged cells) and differentiation (Roediger, 1996; Cummings, 1997). In addition, carbohydrates may alter directly, or indirectly via SCFA formation, the physico-chemical conditions in the gut lumen, e.g. pH, and modify the composition of the microflora and the bacterial synthesis of carcinogens and promoters (Rowland, 1996). Since the various SCFA have very different metabolic fates (e.g. acetate is absorbed and reaches the liver and muscles where it is used as an energy source, whereas butyrate is a preferred energy source of colonocytes and induces cellular differentiation in colon cell lines), the extent of fermentation and pattern of SCFA is likely to be of crucial importance in determining the physiological effects of a particular carbohydrate.

2 Aims and objectives

The overall objective of the project was to develop a scientific basis for recommending new dietary guidelines and for implementing the production of novel industrially-produced foods containing carbohydrates that have a protective effect on the colonic mucosa.

In particular the project addressed the following aims:

- To investigate the patterns of SCFA formed by fermentation of a variety of complex carbohydrates by the human gut microflora *in vitro*.
- To assess whether a shift in the composition of the diet from simple sugars (e.g. sucrose) to complex carbohydrates (e.g. starch) has the potential to block steps leading to neoplastic transformation in the colon.
- To investigate, using human volunteers, animal models and *in vitro* methods, the possible mechanisms (including faecal concentration of short chain fatty acids, long chain fatty acids, bile acids, calcium, bacterial species) underlying the protective effects of selected complex carbohydrates, so that more effective carbohydrate sources can be developed.

3 Results

The work and results of the project can be grouped under four sub-headings:

1. *In vitro* studies of carbohydrate fermentation by the human gut microflora
2. Animal studies designed to evaluate critically the potential protective role of complex carbohydrates, especially starches, in colon cancer
3. Dietary intervention studies in human volunteers to provide more definitive information on the protective effects of complex carbohydrates on colon damage in man.
4. Use of animal models, human volunteers and *in vitro* systems based on isolated colon cells in culture, to investigate mechanisms involved and to identify gut luminal factors, particularly gut flora metabolites, with modulating activity towards cytotoxic and genotoxic damage in the gut.

3.1 *In vitro* studies of carbohydrate fermentation by the human gut microflora

The work on the project has shown that gut bacteria found mainly in the colon, ferment different carbohydrates to varying extents. For example starches resistant to breakdown in the upper gut were less readily metabolized by bacteria than digestible corn starch, although there were considerable differences between individual human volunteers in fermentation capacity. More importantly, the products formed varied depending on the type of carbohydrate, with non-starch polysaccharides such as soy fibre, or apple fibre being fermented mainly to acetate, while substantial amounts of butyrate are produced from starches.

3.2 Animal studies on colon functions related to cancer

The animal studies provided valuable information on the influence of carbohydrate type on various gut microfloras and gut mucosal changes related to carcinogenesis in the colon. Our results demonstrated that boluses of sucrose increased cell proliferation in the colon, but had an irregular influence on aberrant crypt foci (ACF) and did not increase the incidence of tumours in the colon.

ACF are early alterations of the colonic mucosa which appear after the administration of carcinogens to rodents

In rats colonized with a human gut microflora (HFA rats), both digestible and resistant starches (RS) modified gut physiology and gut microbial metabolism in a potentially beneficial manner and usually the effects were more pronounced in the RS-fed rats. The RS used was the retrograded, high amylose starch 'CrystaLean'. However, an increase in mucosal cell proliferation was observed in the rats fed RS.

CrystaLean starch-fed rats exhibited less carcinogen-induced DNA damage in the colon than those fed sucrose, digestible starch or soy fibre. Overall, the results suggested therefore that CrystaLean may suppress the initiation phase of carcinogenesis (genotoxicity) but increase cell proliferation.

Other studies conducted as part of the project indicated protective effects of starchy foods. For example, a long-term colon cancer bioassay in rats provided evidence that pasta reduced the incidence of colon tumours by comparison to sucrose-fed animals.

3.3 Dietary intervention studies in human volunteers

A dietary intervention study in human volunteers indicated that over a long term, a diet rich in starch reduced colon mucosa proliferation. The effect of a high intake of starch on proliferation was not dependent on the contemporary reduction of simple carbohydrates. The study also demonstrated that a short-term (three weeks) reduction in dietary intake of sucrose was ineffectual in reducing colon mucosa proliferation. There were however changes in the faecal microbial flora associated with the low sucrose diet, in particular an increase in lactobacilli and a decrease in the anaerobe/aerobe ratio. The study also identified *L. acidophilus* and *Bifidobacterium breve* as marker organisms characteristic of subjects on the low sucrose diet.

3.4 Investigations of mechanisms involved in protective effects of carbohydrates.

It seemed likely that the influence of carbohydrates, particularly starches and dietary fibres, on the colon mucosa proliferation and damage, was mediated by SCFA. This hypothesis was tested in this project in a series of *in vitro* experiments, studies in animal models and in patients with intestinal disease.

In vitro studies into the modulating effect of SCFA on induction of DNA damage in colon cells, demonstrated that n-butyrate and acetate reduced the level of damage, induced by H_2O_2. In contrast, propionate and i-butyrate had no protective effects. It should be noted that n-butyrate and acetate are utilised best as energy sources by the colon cells. It was also found that protection against DNA damage was apparent in cells exposed to mixtures of SCFA characteristic of starch fermentation, but not with SCFA mixtures typical of soy or apple fibre fermentation.

There was evidence from *in vitro* studies in human colon cell lines that n-butyrate could induce apoptosis in the cells. Apoptosis is a mechanism by which damaged cells are removed from a tissue and is considered to play a protective role in colon cancer by eliminating cells which might otherwise develop into tumours.

A further protective mechanism that seemed feasible from our studies was the induction in colon cells by SCFA of the protective enzyme glutathione transferanse (GST) which detoxifies foreign compounds.

Evidence that SCFA can modulate cell proliferation in the human colon came from studies in ulcerative colitis (UC) patients who have an elevated colon cancer risk. The high rate of proliferative activity in these patients was decreased after SCFA enemas. In contrast, familial polyposis (FAP) patients, who also exhibit increased cancer risk, seemed refractory to these treatments. Slow-release butyrate pellets, which could provide a more acceptable means for delivering SCFA via the oral route were tested in a rat model and found not to affect colonic proliferation and ACF formation and progression. It may still however be worth testing the pellets in UC patients, in which a beneficial effect of SCFA has been demonstrated.

Overall, the results do not support the hypothesis that sucrose is a risk factor in colon carcinogenesis. However, starchy foods, as opposed to sucrose, appear to be protective. The increase of the content of complex carbohydrates in the diet is, potentially, an effective and cheap dietary variation that may assist in the reduction of colon cancer risk. Caution needs to be exercised however in relation to resistant starch, which exhibited both beneficial and adverse effects on parameters associated with colon carcinogenesis.

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Quantitative assessment of the human gut flora using a panel of rRNA targeted hybridization probes

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Abstract

Maintaining a normal microflora in the gut is important in human health. Considerable effort has been expended in the past attempting to influence the composition and activity of the microbial flora of the gut by diet and the consumption of live bacteria (probiotics). However, these efforts have been seriously hampered due to the inadequacy of traditional (i.e. phenotypic) approaches to reliably detect and identify the microorganisms. The purpose of this study is to further develop, refine, evaluate and apply molecular genetic approaches for the qualitative and quantitative monitoring of the human intestinal microflora. The project includes: (1) The construction of a comparative phylogenetic framework of gut microorganisms based on 16S rRNA. (2) The development of a panel of probes targeting different levels within the taxonomic hierarchy. These methods are applied to investigate: (1) The spatial distribution of defined bacterial population groups in gnotobiotic rats. (2) The population structure in different age groups. (3) The effectiveness of prebiotics, probiotics and synbiotics in gut flora manipulation and management. (4) Competitive exclusion of pathogens by dietary supplementation. At the present stage of the project a phylogenetic framework of known gut bacteria has been almost completed. A collection of validated probes covering the described bacterial flora at the group level and in part at the species level is now available. Optimised protocols for RNA extraction from human faecal samples for quantitative dot blot hybridization or for in situ hybridization using fluorescently labelled oligonucleotides have been established. The successful development of a protocol for probe-dependent in situ detection of bacteria in thin sections of gut tissue is a breakthrough. A software programme affording fully automatic counting of in situ hybridized gut microflora has been worked out.

Introduction

It is generally accepted that diet plays an important role in the maintenance and improvement of human health as well as prevention of disease [1]. In this context, the intestinal flora is considered to be key in influencing human well-being. It is thought that the metabolic potential of the human colon rivals that of the liver. This can be attributed to the resident microbiota. Under normal homeostatic

conditions, the intestinal microflora is of central importance in preventing colonization by pathogens, and is also considered to have many beneficial local and systemic roles such as improved lactose tolerance, supply of short chain fatty acids as energy substrates for the host, anti-tumour properties, neutralisation of certain toxins, stimulation of the intestinal immune system, reduction of triglyceride levels and production of vitamins [2, 3, 4]. Considerable efforts have been made to influence the composition of the intestinal microflora in such a way that colonization of the gut by pathogenic bacteria and the formation of toxic compounds by bacterial activity is prevented, thus improving the health potential of the hindgut [5]. The European consumer is increasingly confronted with functional food products which are purported to have health promoting effects. A crucial aspect of these products are their effects on the colonic microflora. One strategy of microbial flora management is based on the consumption of beneficial live microorganisms, such as *Bifidobacterium* and *Lactobacillus* species, in certain dairy products (probiotics) [6]. The hope is that the exogenous addition reaches the colon in a viable form and exerts advantageous properties therein. Another strategy aims at stimulating growth of resident beneficial bacteria and the suppression of potentially harmful microorganisms by the application of particular food ingredients (prebiotics) [7]. These non-viable dietary components therefore support a specific type of fermentation. The identification of factors controlling/influencing the composition of the human intestinal flora and the effective implementation of microflora management (including prebiotics and probiotics) are seriously hampered by the inadequacy of current methodologies of assessing microflora composition. Currently used methods are laborious and based almost entirely on phenotypic approaches that are unreliable and certainly lack the resolving power necessary to analyse the complex microbiota of the human gut (known to include over 400 described species). Recent advances in the field of molecular biology are revolutionising the characterisation and identification of microorganisms. For example, molecular sequence analysis, particularly of rRNA, provides an immensely powerful tool for determining the genetic interrelationships of microorganisms, spanning great, as well as measuring close, evolutionary distances [8]. Furthermore, by utilising diagnostic sequences within the rRNA it is possible to design gene probes to facilitate precise identification [9, 10]. By using PCR-technologies, even non-culturable microorganisms are accessible [11]. We have therefore started to more fully determine the diversity of the gut flora, a hitherto neglected task in the probiotic and prebiotic approach. The purpose of the study is to develop, refine, validate and apply molecular genetic approaches for a vastly improved qualitative and quantitative monitoring of the human intestinal microbiota - an essential step forward for determining the validity of the functional food concept.

Results

To generate a phylogenetic framework of human gut microorganisms a systematic analysis of the 16S rRNAs has been undertaken. The sequencing effort has concentrated on numerically dominant taxa of the human intestinal tract. Reference strains belonging to the genera *Bacteroides, Eubacterium, Clostridium, Peptostreptococcus, Veillonella/Megasphaera*, and *Bifidobacterium* have been grown and subjected to 16S rRNA gene sequencing. Particular effort has been put into sequencing species of the genera *Bifidobacterium* and *Enterococcus* both of which are dominant taxa in the human gut. About 30 different species of these genera have been sequenced (Table 1).

Besides sequencing strains belonging to known species good progress has been made on the isolation of new gut flora diversity. Two approaches were used: 1. Over 200 strains have been isolated from enrichment studies using inulin and fructooligosaccharides as substrates both in batch

and continuous fermentations. Based on partial 16S rRNA sequencing of 150 and on total 16S rRNA sequencing of 30 of these isolates phylogenetic analyses indicate that 20 potentially new species have been isolated and deposited in a public culture collection (Table 2).

Table 1: List of reference species for which 16S rRNA gene is being sequenced

Bifidobacterium			Enterococcus
B. adolescentis	B. choerinum	B. longum	E. casseliflavus
B. angulatum	B. coryneforme	B. magnum	E. durans
B. animalis	B. cuniculi	B. minimum	E. faecium
B. asteroides	B. gallicum	B. pseudocatenulatum	E. hirae
B. bifidum	B. gallinarum	B. pullorum	E. malodoratus
B. boum	B. globosum	B. subtile	E. avium
B. breve	B. indicum	B. suis	E. solitarius
B. catenulatum	B .infantis		E. dispar
			E. saccharolyticus

Table 2: Faecal bacterial strains isolated with various combinations of selective media and prebiotic substrates

Substrate	Medium	No of isolates	Substrate	Medium	No of isolates
FOS	Azide	12	FOS	FOS indicator	22
FOS (batch)	Azide	11	FOS	Nutrient	5
FOS	Bacteroides	29	Inulin	Azide	1
FOS (batch)	Bacteroides	10	Inulin	Bacteroides	6
FOS	Clostridia	23	Inulin	Clostridia	3
FOS	Beeren's	29	Inulin	Beeren's	17
FOS (batch)	Beeren's	17	Inulin	Rogosa	7
FOS	Rogosa	8	Inulin	Wilkins Chalgren	14
FOS (batch)	Rogosa	5	Inulin	Inulin indicator	10
FOS	Wilkins Chalgren	2			

FOS = fructooligosaccharides

Since knowledge of the 16S rRNA sequence alone is not sufficient to know the role of this particular organism in the human gut, the phenotypic feature of the isolates such as growth substrates and catalytic potential are presently being studied. Further strains were isolated from human faecal samples because of interest in certain phenotypic features such as acetogenesis from hydrogen and carbon dioxide or the ability to use the quercetin-3-glucoside, a flavonoid relevant to nutrition [12]. Sequencing of the 16S rRNA of these isolate revealed the phylogenetic position of these isolates

which had not been possible based on their phenotypic features. 2. The second approach to describing new gut diversity has been based on the genotypic analysis of 16S rDNA obtained from the faecal flora of an adult person. Using an optimised technique for the isolation of DNA from faecal samples, 16S rDNA was amplified with PCR (10 and 25 cycles) by using primers that bind to the majority of bacterial species or to specific phylogenetic groups such as the bifidobacteria. The resulting amplicons have been cloned and more than 250 clones have been isolated, characterised and partially sequenced (1000 bases). Further rDNA clones were isolated by amplifying rDNA from a 70-years old person and from a newborn. Both universal and bifidobacteria specific primers were used for amplification by PCR. Table 3 gives an overview of the clones isolated and the primers used for amplification.

The preliminary comparative phylogenetic analyses indicate that most of the clones belong to one of the following phylogenetic groups: Bacteroides/Prevotella group, Clostridium coccoides subgroup and Clostridium leptum subgroup. Moreover, the presently available data indicate that 80% of the dominant flora has not yet been isolated and described. However, nothing is known about the phenotypical properties of these organisms. Therefore, cultural studies on the same faecal samples will begin in order to asses the cultural versus the non culturable components of the intestinal flora.

Table 3: 16S rDNA clones obtained by amplification with PCR of DNA isolated from human faecal samples

Sample origin (age)	PCR amplification target	Number of rDNA clones stored/sent	Sequencing status
Adult (38)	All bacteria except bifids[1]	512/512	in progress > 500 clones
	Bifido-specific[2]	100 & 50/100 & 50	30 & 12 done, No bifids
	Universal[3]	200/200	no started
Elderly (70)	All bacteria except bifids[1]	300/100	done 12
	Universal [3]	300/200	done 60, No bifids
Newborn	All bacteria except bifids[1]	300/100	done 100
(1,5 mo)	Universal[3]	100/100	done 36, No bifids

1. primer pair Bacteria 008F - Bacteria 1517R
2. primer pairs Bifids 008F - Bacteria 1517R, and Bifids 008F - Bifids 1412R
3. primer pair Bacteria 350F - Bacteria 1517R

Whereas the sequencing of the majority of known gut microorganisms has been almost completed the new diversity detected with the genotypical approach is overwhelming. More than one thousand 16S rDNA clones await to be sequenced. The new sequence information is used to improve the basis for probe design and validation. The data retrieved will improve our knowledge on the microbial diversity in the human gut.

Oligonucleotide probes labelled with radioisotopes or with fluorescent dyes specifically indicate the presence of a target organism in the gut ecosystem. The use of probes necessitates their design and validation. As a first step in probe design diagnostic signature sequence regions in the 16S rRNA that may serve as targets for oligonucleotide probes have been identified. Depending on the degree of

similarity such sequence regions have been used to detect bacteria at the various levels of phylogenetic hierarchy. Upon identification of potential 16S rRNA target regions, corresponding oligonucleotide probes were aligned with all available 16S rRNA sequences to determine the theoretical specificity of the probe as indicated by the number of nucleotide mismatches. Probes were selected based on their ability to perfectly match their target sequence in hybridization, allowing at least two mismatches with the rest of the sequences in the database. Both group-specific and species-specific probes were designed. The theoretical specificity of the probes as determined by sequence alignments was verified experimentally. For the experimental assessment of probe specificity a collection of 117 reference species has been selected. The presently available 45 validated probes cover the numerically dominant intestinal microorganisms at the group level and partly at the species level.

In order to obtain a realistic picture of the intestinal microflora composition it was necessary to optimise probe utilisation. In case of dot blot hybridization the extraction of faecal samples and the blotting procedure were optimised. Protocols for the fixation and permeabilisation of gram-positive and gram-negative bacterial cells in faecal samples were improved for unbiased cell counting by in situ hybridization. In order to facilitate the counting of fluorescently labelled cells, automatic techniques were introduced in the project. A software programme for fully automatic counting of fluorescently labelled cells was applied and the automated counting procedure was validated on faecal samples with validated oligonucleotide probes targeting all bacteria, bifidobacteria, bacteroides and the *Clostridium coccoides-Eubacterium rectale* cluster. The performance of the automated counting procedure is characterised by the parameters given in Table 4:

Table 4: Performance of automated counting procedure

Parameter	Value
Mean time needed to obtain a correctly focused image	40 sec.
Mean image analysis and storage time	10 sec.
Mean time needed for change of wells	5 sec.
Mean time needed for change of slides	5 sec.
Fraction of fatal out-of-focus incidents	0.01

The introduction of automated counting is of utmost importance for future applications of the probe methodology, because it allows huge numbers of samples to be analysed.

In contrast to the classical phenotypical enumeration methods, the use of oligonucleotide probes has the advantage of not depending on the culturability of the organisms. Since a number of significant studies in the past used classical enumeration methods it was most useful to compare these with the molecular methods developed in this project. We therefore compared dot-blot-hybridization or in-situ-hybridization with classical enumeration methods. The comparison of classical enumeration techniques with the molecular probing techniques developed in this project was done on human faecal samples using in situ hybridization and quantitative dot blot hybridization, respectively, in comparison to colony counts. Using selective conditions for the *Bacteroides* group, the genus *Bifidobacterium* and for *Escherichia coli*, human faecal samples from adults were analysed. This comparison revealed that the proportion of the colony-forming organisms belonging to these taxa relative to the total anaerobes was greater than the corresponding relative RNA measured by dot blot hybridization. This has been interpreted to mean that the underestimation of total anaerobes results in a systematic overestimation of the respective subgroup. The results

obtained by culture-based enumeration on the one hand and by in situ hybridization with fluorescent 16S rRNA hybridization probes targeted at all bacteria, bifidobacteria and the Bacteroides/Prevotella group on the other hand were compared by analysing faecal samples from 12 healthy female volunteers. The total number of bacteria (viable and non viable) was determined with the DNA-binding dye DAPI. Only 76% of the bacteria detectable with DAPI were also detected with probe EUB 338 that detects all metabolically active bacteria. The culturable fraction was 32% of the cells detected with DAPI. In situ hybridization indicated that the proportion of bifidobacteria and Bacteroides/Prevotella in relation to total metabolically active bacteria was 6% and 32%, respectively. This implies that a significant number of intestinal organisms belong to other genera or are still unknown. It is concluded that whole-cell in situ hybridization offers a more objective enumeration of intestinal organisms than classical cultivation techniques.

A protocol for in situ detection of bacteria in thin sections of gut tissue has been worked out and is presently applied to gnotobiotic rats to study the spatial organisation of defined bacterial population groups. For that purpose microbial communities were introduced into germfree rats as a model system. So far, in most of these experiments only a limited number of animals was used to get experience with respect to association conditions, sampling from different locations of the gastrointestinal tract and the embedding, cutting, fixation and hybridization of tissue specimens. To analyse the development of the bacterial community structure in infants faecal samples were taken over time from newborn children. Total RNA extraction has been initiated and samples will be analysed taking advantage of the optimised dot blot hybridization procedure mentioned above. In addition the longitudinal and the luminal versus mural distribution of microorganisms has been studied using both in situ hybridization and classical enumeration. The data are still being collated. In order to assess age-related changes in the composition of the gut microflora faecal samples have been collected from 30 adults and 12 elderly people so far. RNA extraction has recently been started. The samples will also be analysed with fluorescence in situ hybridization aided by image analysis and the temperature gradient gel electrophoresis technique.

The idea of probiotics implies that the supplemented organisms remain in the gut for time periods long enough to allow them to take part in the interactions of the gut microflora and to influence the microbial composition in a way beneficial to the host. One of the most important questions in this context concerns the survival of probiotics in the gastrointestinal tract. To determine the survival of probiotics in the presence of bacteria indigenous to the human intestinal tract the acid and bile tolerance of the probiotic strains selected for further studies have been tested. In parallel the survivability of the probiotic strain *Lactobacillus plantarum* 0407 in an anaerobic stirred and pH controlled batch culture fermentation has been investigated. Initial results of the latter study are presently collated and analysed. To study the survival and competitiveness of the commercial probiotic strains *Bifidobacterium bifidum* BB12 and *Lactobacillus plantarum* 0407 in an animal model system, gnotobiotic rats monoassociated with either one of these strains were subsequently associated with *Bacteroides vulgatus* which is an indigenous species in the human intestinal tract. Conversely, gnotobiotic rats monassociated with *Bacteroides* were subsequently associated with the probiotic strains. The colonisation potential of the bacteria was monitored by both in situ probing and culture-based enumeration. The results indicate that *Bacteroides* colonised the germfree rats immediately in high numbers no matter whether it was applied as the first organism or the second organism. In contrast, the number of associated probiotic bacteria depended on the sequence of colonisation. The colonisation potential of *Bifidobacterium bifidum* BB12 or *Lactobacillus plantarum*

0407 was significantly better in rats already monoassociated with *Bacteroides* than in germfree rats. The data also suggest that *Lactobacillus plantarum* 0407 suppressed the growth of *Bacteroides* to some extent. Owing to the finding that the probiotic strains' preferred sites of colonisation were the caecum and colon, potential probiotic effects are expected to occur in the caecum and colon of the animals. Experiments are under way to test the effect of prebiotics on the colonisation potential. For that purpose commercial preparations of oligofructose are under study.

Preliminary investigations have been initiated to identify prebiotics that may be used in conjunction with the probiotics to improve their survival and their in situ metabolic activity. The potential of lactulose on the survivability of the probiotic strain *Lactobacillus plantarum* 0407 was therefore investigated in stirred batch culture fermentation systems. The data are still being collected and evaluated. Although still at an early stage, preliminary experiments have already been initiated by on testing the ability of probiotics to exclude enteropathogens. However, results are at an early stage and still being collated.

The suitability of probiotics, prebiotics and synbiotics for use in fermented foods was investigated at the laboratory and pilot plant scale. In particular, the sensory properties of fermented milk containing a probiotic *Lactobacillus plantarum* strain added to the standard culture was investigated. The highest scores for appearance, texture, initial mouthfeel, creaminess and flavour were given by trained personnel to a product manufactured with both a *Streptococcus thermophilus* strain and the probiotic *Lactobacilllus plantarum* strain. This combination was therefore tested in factory trials. The product obtained in the production trial was excellent but the make-time of 16 hours was too long. Experiments testing the survival of *Lactobacillus reuteri* during storage of fermented milk products under various conditions indicated significant loss of viability.

Conclusions

A big step forward has been made in the effort to develop a phylogenetic framework of gut microorganisms. Both human intestinal reference organisms obtained from culture collections and strains newly isolated from human faecal samples were systematically cultivated and subjected to partial and total 16S rRNA sequencing. In addition, more than 1500 rDNAs have been cloned from faecal DNA of individuals from three different age groups and the 16S rRNA of more than 700 of these have been sequenced. Although the molecular inventory is not yet complete, it is already evident from preliminary phylogenetic analyses that a very high proportion of the human gut flora has to date eluded cultivation and remains undescribed. In spite of the high phylogenetic diversity at the species level it appears that the majority of this new diversity can be assigned to known phylogenetic groups at a higher level of hierarchy.

Oligonucleotide probes covering the dominant bacterial taxa of known gut microorganisms at various phylogenetic levels have been designed and mostly validated. The successful development of a protocol for probe-dependent in situ detection of bacteria in thin sections of gut tissue is of particular interest because this technique can also be applied to human tissues. To automate microscopic counting of fluorescently labelled bacteria a software programme affording fully automatic counting of in situ hybridized gut microflora has been worked out. The performance of the automated counting procedure was validated and it was found it is much more accurate than quantitative culturing. A comparison of classical enumeration techniques with quantitative dot blot hybridization revealed that the classical enumeration technique was biased due to the underestimation of total

anaerobes resulting in a systematic overestimation of the respective subgroup. The comparison of the culture-based enumeration methods and in situ hybridization with fluorescent 16S rRNA hybridization probes led to the conclusion that whole-cell in situ hybridization offers a more objective enumeration of intestinal organisms than classical cultivation techniques.

This offers the opportunity to investigate the effect of nutritional supplementation or any other factor causing disturbance of the global structure of the human colonic microbiota. Basic aspects such as the investigation of microbial model communities in space and time and the monitoring of age related changes in the human gut microflora or more applied aspects such as the testing of the effectiveness of probiotics, prebiotics or synbiotics on gut flora manipulation and management are in progress.

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The role of fat and CHO in the European diet on health

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Abstract

Objective: The European multicentre dietary intervention study CARMEN (Carbohydrate Ratio Management in European National diets) was intended to investigate the effects of dietary fat reduction of about 10 energy percent (en%) and a concomitant change in the ratio of simple to complex carbohydrates up to either 2/3 or 3/2 on long-term food intake, body weight, body composition and blood and urine chemistry in overweight volunteers.

Methods: 400 overweight to obese volunteers ($20 \leq$ age ≤ 55; $26 \leq$ BMI ≤ 35), were randomized in four groups. Two experimental groups had free access to fat-reduced products and foods high either in complex (CCHO) or in simple (SCHO) carbohydrates. A dietary control group (CD) received products with the usual composition. A second control group (CS) receiving no foods from the study centre was used to determine seasonal variations. For each diet intervention group up to 100 commercial food products were available in a shop system. Before, during and up to 6 months after the intervention period dietary intake, parameters of lipid and glucose metabolism and anthropometric data were measured.

Results: During the intervention period 70 % of fat intake and 66 % of CHO intake was covered from shop products. In the CD group the fat intake was 36.1 en%, CHO intake 46.2 % with a SCHO/CCHO ratio of 0.96. In the SCHO and CCHO groups the fat intake reduced by 10.5 en% and 7.9 en% ($p<0.001$), the CHO intake increased by 8.5 en% and 4.7 en% and the SCHO/CCHO ration changed to 1.28 and 0.58 ($p<0.001$), respectively. As a result of these dietary changes body weight reduced by 0.94 kg ($p<0.05$) in the SCHO and 1.81 kg ($p<0.001$) in the CCHO group.

Conclusions: *ad libitum* reduction of dietary fat produces a moderate but significant reduction of body weight, esp. body fat. The concomitant increase in either simple or complex CHO did not reveal additional significant differences.

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Introduction

Obesity is a major and growing health problem. It is associated with a higher mortality risk for diabetes, hypertension and hyperlipidemia, and is itself an independent risk factor for cardiovascular disease. Moreover, it is connected with the prevalence of malignancies (breast cancer, colonic cancer) and osteoarthritis. Excess food intake is generally believed to be the primary cause of body weight gain. However, there are indications that the fat/carbohydrate ratio (F/CHO) is related to energy intake, a lower ratio resulting in a lower intake. One reason could be that the conversion of dietary CHO to F in the body costs 23 % of the original energy content of CHO, while the cost of

deposition of dietary fatty acids as triglycerides is only 3 %. Moreover, individuals seem to differ in their capacity to increase fat oxidation when offered a high-fat, low-carbohydrate diet. In subjects predisposed to become obese, a high-fat diet may result in a higher storage of fat, which is supported by the observation that in these subjects the satiating effect of fat is smaller than that of equicaloric amounts of protein or carbohydrates. In a small number of intervention studies it has been shown that a lower fat intake reduces body weight and energy intake, with evidence that overweight-to-obese subjects achieve more weight loss than normal weight subjects (Astrup et al., 1997).

Recently this topic became subject of a clinical debate in the New England Journal of Medicine (Connor et al., 1997; Katan et al., 1997). The debate and correspondence focussed on the importance of low-fat diets in the treatment of obesity and possible consequences in relation to blood lipids and cardiovascular disease. Pro's and cons agreed on the urgent need for prospective long term studies on the effect of lowering fat content of the diet and the concomitant effects of increasing of the different types of carbohydrates.

The CARMEN (CArbohydrate Ratio Management in European National diets) study has been initiated to address this topic. Furthermore, it will elucidate the role of sugar as part of the CHO intake. CARMEN will contribute to the understanding of the role of food, and in particular the role of sugar, for health and well-being of the European consumer and may be instrumental in fighting overweight and obesity recognized as a disorder of growing importance; this is one of the major objectives of research in Area 3 of the Agriculture and Fisheries Programm.

The first objective is to investigate the effects of a dietary fat reduction of about 10 en% and a concomitant change in simple (SCHO) and complex (CCHO) carbohydrates of 5 en%, leading to a change in the ratio SCHO/CCHO up to about 2/3 or 3/2, on long term (6-18 months) food intake and body weight of overweight-to-obese volunteers (BMI 26-34; average 30) with a habitual fat and carbohydrate intake of 35-45 and 40-50 en% respectively.

The second objective is to investigate the acceptance of the proposed diets in various countries and the effects of the dietary changes on well-being, body composition, energy exchange, physical exercise, CHO- and F-metabolism, and on insulin response and resistance of participants.

Material and methods

The project was composed of a core, multi-centre, parallel comparison study in five European research centres (see list of authors). Furthermore, five centre-specific studies were designed to complement the core multi-centre study.

Multi-centre intervention study

Food shops and food delivery system

In order to reduce fat intake by 10 en% and to increase at the same time simple or complex carbohydrate intake, volunteers were provided with a selection of food products for a period of six months, by means of a newly designed and validated laboratory food shop system. In each research centre a small supermarket was installed with shop facilities to provide volunteers with a known and recorded choice of food items. A computer program was developed to record the food items taken by the volunteers by means of a barcode reading system. Furthermore, the program enabled the

control of selection of the appropriate food items taken by the subjects allocated to the different groups and to calculate, based on a cumulative energy-nutrient intake calculation program, the contribution of food items coming from the shop related to the total individual energy and nutrient intake.

Food selection

To select the experimental food items necessary to reach the dietary changes during the multi-centre intervention study, two lines of food products were made available to compose the experimental and control diets for the volunteers: a) a high-fat/reduced-fat product line and b) a high-SCHO/high-CHO product line. Selection of food products was based on manufacturer's food composition data. All food products were commercially available, labelled brand products and provided with a barcode. From both food product lines combinations of food products were composed assuring, from the products provided, a fat intake by the volunteers which was either comparable with the average fat intake in the country concerned (control diet (CD) group), or a lower fat intake with either a high-SCHO (SCHO-group), or a high-CCHO (CCHO-group) intake.

Contracts with food companies

For the settlements and contracts with the food companies or retailers to have the food products forwarded for free or as cheap as possible, contacts with the local industrial partners were made on a centre-individual basis. For a number of international operating companies (Kellogg's, Coca Cola, Unilever, Nestlé, Danone, Mars) the coordinating centre and industrial partner made the necessary negotiations with the various European headquarters to get green light for their cooperation. After approval on a European level for free delivery of food products (Kellogg's, Coca Cola, Mars) or for reduced rates (Unilever) the local representatives were identified. For further contacts the local shop manager took the lead.

Volunteers

Volunteers were recruited from the population in the neighbourhood of each research centre. After a preliminary enrolment, volunteers received complete information about the aims of the study, both verbally and in writing. Furthermore, volunteers were screened to check whether they met the defined inclusion criteria regarding age (40 subjects (20m; 20f) $\geq$ 20 and $<$35 yr; 40 subjects (20m; 20f) $\geq$35 and $\leq$55 yr; BMI $\geq$26 and $\leq$34 kg/m^2); health (medical questionnaire, medical examination, blood and urine chemistry); alcohol intake ($\leq$28 unit/wk); weight change between recruitment and start of the study ($\leq$5 kg); and intensive sporting activities ($\leq$7 h/wk). In each of the five research centres it was scheduled to enrol 80 subjects (40m; 40f) into the study. They were allocated to a control-season (CS) group (10m; 10f) or to an experimental group (30m; 30f), stratified by age, sex and BMI. The CS group received minimal attention during the project and served to determine and to correct for possible seasonal variations in body weight.

Run-in

The run-in period lasted five weeks and was incorporated into the project for three reasons: 1) to evaluate for each volunteer belonging to the experimental group the amount of food products to cover about 70 % of the habitual fat intake and 50 % of the habitual carbohydrate intake; 2) to perform baseline measurements and 3) to gain experience with the complete experimental set-up

and to accustom the volunteers to the experimental set-up. During the run-in period all volunteers belonging to the experimental group had free access via the shops in each research centre to food products with a for each country normal composition with respect to fat and carbohydrates. Volunteers were free to take as many products as they wanted. As a number of products (e.g. fresh fruit, fresh vegetables, fresh meat) was not provided, volunteers were allowed to buy products in ordinary supermarkets. In the 5th week of the run-in period, subjects filled out a 7-day weighed dietary record.

Intervention

After the run-in period was finished, subjects were allocated to three nutritional groups, stratified by age, sex, tertile of simple to complex carbohydrate intake and fat intake based on the 7-day weighed dietary record. Subjects in the low-fat/high-simple carbohydrate (SCHO) group were provided for six months with fat-reduced products and with a variety of products with a high content of simple carbohydrates. Subjects in the low-fat/high-complex carbohydrate (CCHO) group were provided for six months with the same variety of fat-reduced products as the SCHO group and with a variety of products with a high content of complex carbohydrates. Subjects in the control diet (CD) group were provided for six months with a variety of products with the usual composition.

Furthermore, measurements were performed on food intake, body weight, body composition, and blood and urine chemistry (table I).

Run-out

A run-out period of six month was incorporated into the project to investigate the changes in food intake after an intervention period of six months. Changes in the primary (body weight) and secondary parameters (body composition, blood lipids and hormones) were also studied.

Centre-specific studies

To complement the core multi-centre Study five centre-specific studies were designed according to:

- Energy- and CHO metabolism: Pattern of food intake
- Energy exchange, body composition, substrate oxidation and spontaneous physical activity
- Characterization of syndrome X
- Food intake validation
- Sensory aspects of experimental diets alteration in sensoric preferences and sensitivity

Results and preliminary conclusions

Since 316 subjects completed the intervention period, the drop-out rate of 21 % is acceptable, moreover taken the estimated drop out rate of 20 % for the power calculated at the start of the study into account.

The control on food intake by means of food distribution by the laboratory food shop was achieved successfully; 70,2 % of fat intake and 66,1 % of carbohydrate intake was reached while 70 % and 50 % was aimed for.

Table II shows the characteristics of subjects, who completed the intervention.

Table I: Time Schedule

		Blood sampling, anthropometric measurements	Urine chemis-try	3-day records	7-day records	Questionnaire on physical activity
Preliminary phase	4 weeks	X		X		X
Run-in	4 weeks	X	X		X	
Randomiza-tion	4 weeks					
Intervention	4 weeks	X	X			
	4 weeks			X		
	4 weeks	X	X	X		
	4 weeks	X				
	4 weeks			X		
	6 weeks	X	X		X	X
Follow up	after 3 months	X				
	after 6 months	X			X	X

Table II: Characteristics of subjects completing the intervention period and drop outs

	completers (n=316)	drop outs (n=50)
SCHO/CCHO/CD/CS (n)	76 / 83 / 77 / 80	16 / 14 / 18 /2
male / female (n)	155 / 161	20 / 30
age (years)	39 ± 9	36 ± 9 *
weight at screening (kg)	88.4 ± 12.3	91.2 ± 11.1
BMI at screening (kg/m²)	30.1 ± 2.6	31.0 ± 2.2 *
weight at start intervention (kg)	89.0 ± 12.4	92.6 ± 11.9
BMI at start intervention (kg/m²)	30.4 ± 2.7	31.4 ± 3.0 *

Data are means ± SD

SCHO: simple carbohydrate group; CCHO: complex carbohydrate group; CD: control diet group; CS: control season group; significantly different between completers and drop outs *: $p<0.05$.

In general, subjects who dropped out in the different groups are significantly younger and depending on the experimental group tend to be more obese. This is particularly the case in the CD group (BMI 32.8 vs 30.4). A possible explanation for this could be that the more obese subjects expected to loose more weight although it has been clearly indicated at the start of the experiment that losing weight was not the first objective of the study. From the 7-day weighed dietary record filled out during the run-in period, a baseline value for the energy and macronutrient intake was calculated (see table III). During the six months of the intervention period food intake was registered by means of a 3-day weighed dietary record in week 5, 10 and 18 and by means of a 7-day weighed dietary record in week 26, at the end of the intervention period. Based on these 16 recorded days of food intake, an average food intake was calculated. From the food purchased from the shop minus the items that were returned and disposed, an accurate average level of food intake coming from the shop during the full six months period could be calculated (see table IV).

Table III: Average food intake during the run-in period, based on a 7-day weighed dietary record

intervention group	SCHO (n=75)	CCHO (n=82)	CD (n=76)
energy intake (MJ/d)	11.0 ± 4.0	11.0 ± 3.3	11.2 ± 3.6
protein (g/d)	93.2 ± 28.2	92.1 ± 24.7	92.8 ± 29.6
protein (en%)	14.5 ± 2.5	14.3 ± 2.2	14.0 ± 2.1
fat (g/d)	105.6 ± 45.7	106.9 ± 41.0	107.0 ± 40.3
fat (en%)	35.4 ± 5.4	36.3 ± 6.0	35.3 ± 6.0
total CHO (en%)	47.4 ± 6.1	46.1 ± 6.5	47.8 ± 6.5
- SCHO (g/d)	154.1 ± 81.3	143.0 ± 61.3	154.2 ± 68.5
- SCHO (en%)	22.8 ± 6.0	21.6 ± 6.0	22.7 ± 5.7
- CCHO (g/d)	154.5 ± 55.9	155.4 ± 50.9	162.9 ± 58.2
- CCHO (en%)	24.1 ± 5.8	24.0 ± 5.7	24.7 ± 5.8
- SCHO/CCHO ratio	1.04 ± 0.49	0.98 ± 0.45	0.99 ± 0.44
alcohol (g/d)	10.5 ± 13.0	13.4 ± 16.4	11.3 ± 12.8
alcohol (en%)	2.7 ± 3.2	3.4 ± 4.0	2.9 ± 3.2

Data are means ± SD
SCHO: simple carbohydrate group; CCHO: complex carbohydrate group;
CD: control diet group

Comparison of the average food intake from the shop, the total food intake based on the dietary records and the predicted energy needs is shown in table V. Predicted energy needs are based on calculated basal metabolic rate of the subjects, physical activity level and the recorded energy intake at baseline. On average, about 53 % of the predicted energy needs was provided by food products from the shop. During the six months intervention period, about 65 % of the energy intake as recorded by the subjects was purchased from the shop. The contribution of food products from the shop to total fat intake was on average 70,4 % (aim: 70 %); for carbohydrate the contribution was on average 66,1 % (aim: 50 %).

Table IV: Average shop intake during the 6 months intervention period

intervention group	SCHO (n=76)	CCHO (n=83)	CD (n=77)
energy intake (MJ/d)	6.3 ± 2.1	6.1 ± 2.1	6.8 ± 2.1
protein (g/d)	57.5 ± 22.6¶	68.0 ± 27.0 **	55.2 ± 26.1
protein (en%)	15.7 ± 4.0 **¶¶¶	18.8 ± 3.9 ***	13.7 ± 4.3
fat (g/d)	42.0 ± 15.7 ***	47.1 ± 17.6 ***	74.7 ± 26.8
fat (en%)	25.7 ± 5.7 ***¶¶¶	30.3 ± 7.7 ***	41.7 ± 7.3
total CHO (en%)	58.7 ± 6.1 ***¶¶¶	50.9 ± 6.5 ***	44.7 ± 5.8
- SCHO (g/d)	138.1 ± 55.2 ***¶¶¶	54.0 ± 20.0 ***	93.0 ± 40.1
- SCHO (en%)	37.1 ± 6.7 ***¶¶¶	15.4 ± 3.8 ***	23.0 ± 6.1
- CCHO (g/d)	81.2 ± 37.0 ¶¶¶	131.9 ± 63.7 ***	87.0 ± 39.8
- CCHO (en%)	21.6 ± 6.1 ¶¶¶	35.6 ± 7.6 ***	21.7 ± 7.1
- SCHO/CCHO ratio	1.89 ± 0.73 ***¶¶¶	0.47 ± 0.2 ***	1.23 ± 0.62

Data are means ± SD
SCHO: simple carbohydrate group; CCHO: complex carbohydrate group;
D: control diet group;
Significantly different from CD group: $^{**}p<0.01$; $^{***}p<0.001$
Significantly different from CCHO group: $^{¶}p<0.05$; $^{¶¶¶}p<0.001$

Regarding the effects of the six months dietary-intervention study on the primary parameter (body weight) and the secondary parameters (body composition, blood lipids and hormones), statistical analyses are in progress. In brief, the main result can be summarized as follows:

In the CD group the average 6 months fat intake was 36.1 en%, the CHO intake 46.2 en%, and the SCHO/CCHO ratio 0.96. The fat intake was lowered by 10.5 en% in the SCHO group, by 7.9 en% in the CCHO group ($p<0.001$). The CHO intake increased by 8.5 en% and 4.7 en% in the SCHO and CCHO groups, respectively, and the SCHO/CCHO ratio changed to 1.28 and 0.58 ($p<0.001$).

Due to these dietary alterations the body weight reduced by 0.94 kg ($p<0.05$) and 1.81 kg ($p<0.001$) in the SCHO and CCHO groups, respectively. In the CS and CD groups the body weight increased by 0.82 kg and 0.18 (ns), respectively. Fat mass changed by -1.27 kg ($p<0.05$), -1.79 kg ($p<0.001$) and +0.63 kg (ns) in the SCHO, CCHO and CD groups, respectively. Total cholesterol dropped slightly and significant in the SCHO and CCHO groups. No significant changes were found for HDL-cholesterol, triglycerides, fasting insulin, and glucose.

From the data on food intake, from the measured purchases and returns from the food shop during the run-in and intervention period and from the observed metabolic and anthropometric data it can be concluded that the objectives of the study were successfully achieved. The results will contribute to a better understanding of the role of amount and type of carbohydrates in relation to the amount of fat in the diet. Furthermore, this inter-relationship between both macronutrients and body weight control and metabolism will be better understood. It is anticipated to be relevant for further dietary guidelines in Europe.

Table V

intervention group	SCHO (n=76)	CCHO (n=83)	CD (n=77)
calculated BMR (MJ/d)	7.5 ± 1.1	7.5 ± 1.1	7.5 ± 1.1
PAL	1.65 ± 0.09	1.64 ± 0.09	1.65 ± 0.09
BMR * PAL (MJ/d)	12.5 ± 2.3	12.4 ± 2.2	12.5 ± 2.3
7 d record EI (MJ/d)	11.0 ± 4.0	11.0 ± 3.3	11.2 ± 3.6
predicted energy needs (MJ/d)	12.0 ± 2.5	11.9 ± 2.1	12.1 ± 2.4
EI shop / predicted (%)	52.4 ± 14.1	50.9 ± 14.0 *	56.6 ± 13.7
EI shop / recorded (%)	63.6 ± 18.0	66.8 ± 17.1	67.5 ± 18.9
fat shop / recorded (%)	64.9 ± 22.1 **	69.4 ± 17.2	76.9 ± 21.6
total CCHO shop / recorded (%)	65.9 ± 19.3	67.2 ± 22.0	65.1 ± 20.6

Data are means ± SD
SCHO: simple carbohydrate group; CCHO: complex carbohydrate group;
CD: control diet group;
EI: energy intake
BMR: basal metabolic rate, calculated according to the WHO table
PAL: physical activity level, estimated from the Baecke questionnaire
Predicted energy needs: calculated as (BMR*PAL*2 + 7d record EI)/3
Significantly different from CD group: *p<0.05; **p<0.01

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Effect of complex polyphenols on the colon carcinogenesis process

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Abstract

We studied the effect of complex polyphenols and tannins (CPT) from red wine (WCP) or thearubigins (TR) and theaflavins (TFu) from black tea on experimental colon carcinogenesis in F344 rats. Cell proliferation, a parameter associated with colon cancer risk, was not varied by chronic administration (90 d) of WCP (14.3 and 57.2 mg/kg/d). The highest dose of WCP

induced a decrease of the number of cells in the colon crypts. This effect was not due to increased apoptosis. WCP (57.2 mg/kg) fed continuously before or after the administration of azoxymethane (AOM) did no vary the number or size of aberrant crypt foci (ACF), colon pre-neoplastic lesions. The number of nuclear aberrations (NA) in colon mucosa was studied after administration of 1,2-dimethylhydrazine (DMH) and 2-amino-3-methylimidazo (4,5-f)quinoline (IQ), which require extensive metabolic activation. The effect of DMH and IQ was not varied by pre-treatment with WCP, TFu or TR (40 mg/kg/d/x 10 d). Similarly, the levels of total, secondary and long chain fatty acids were not varied significantly in animals fed WCP for 90 d. In conclusion, CPT did not influence parameters related to colon carcinogenesis. Long-term colon carcinogenesis experiments with these compounds are in progress.

Abbreviations:

CPT: complex, polyphenols and tannins; ACF: aberrant crypt foci; AC: aberrant crypts; NA: nuclear aberrations; AOM: azoxymethane; DMH: 1,2-dimethylhydrazine; HF: high-fat diet; LI: labelling index; WCP: wine complex polyphenols; TFu: theafulvine; TR: thearubigin fraction. DMH: 1,2-dimethylhydrazine; IQ: 2-amino-3-methylimidazo (4,5-f)quinoline ; 4AB: 4-aminobiphenyl..

Introduction

Colon cancer is one of the main causes of cancer in the industrialised world. It is generally agreed that dietary habits play a leading role in the induction of this type of cancer (World Cancer Research Fund, 1997). In the last years a great deal of attention has been dedicated of the discovery of food components which might have the potential of modifying the development of colon cancer, affecting one or more phases of the carcinogenesis process.

Among these food components, complex polyphenols and tannins (CPT) from tea and wine have been considered with considerable interest, since epidemiological studies suggest that populations consuming a high amount of green teas (World Cancer Research Fund, 1997) have a lower incidence of colon cancer. Some experimental studies have also shown a protective effect of green tea on colon carcinogenesis (Yamane et al., 1991).

On this basis we studied the actions of CPT from red wine and black tea, using short-term assays which are currently used to assess the potential of chemopreventive substances of preventing colon cancer.

Dietary components can affect colon carcinogenesis in two different ways: 1) varying cell proliferation (Lipkin, 1988), a high cell proliferation being a factor of risk for carcinogenesis; 2) altering the initial genotoxic effect of colon carcinogens on the mucosal cells (effect on the initiation phase; 3) modifying the growth and progression of cells initiated by carcinogens (effect on the promotion phase).

We report here the effect of CPT on colon mucosa cell proliferation, on the occurrence of aberrant crypt foci (ACF), a putative pre-neoplastic lesion in the colon and on the number of nuclear aberrations (NA) induced by colon carcinogens. Variations of the last two parameters are descriptive of effects on the initiation phase. We also studied the effect on the promotion phase of carcinogenesis by monitoring the growth of ACF in animals chronically fed CPT. Since bile acids act as co-carcinogens and promoters at the level of the colon mucosa (Cohen et al., 1980), we also measured the variations of faecal bile acid profiles in animals fed CPT.

Methods

Preparations of CPT

Complex polyphenols from red wine (WCP) were prepared in the laboratory of Veronique Chenier (Montpellier, France) within the framework of the project. Cabernet Sauvignon of the 1994 vintage was used as a starting material. Ethanol, sugar, organic acids and low molecular weight phenols were eliminated by column chromatography and a concentrated polymeric fraction was obtained (WCP) to be additioned to the food fed ad libitum to the rats..

Tea polyphenols were obtained in the laboratory of Mike Clifford (Guilford, UK). We tested theafulvin (TFu) and crude thearubigin fraction (TR) isolated from brewed black tea.

Colon mucosa proliferation

F344 male rats were fed with a high fat-high sucrose-low calcium diet (HF diet) to mimic human diets associated with a high colorectal cancer risk. Rats were then treated s.c. with 2 injections (one week apart) of azoxymethane AOM (15 mg/kg) or saline. Control rats were fed the HF diet for 90 d whereas treated rats were fed the HF diet additioned with WCP (14.3 and 57.2 mg/kg). At the end of this period, colonic mucosa proliferative activity was assessed in mucosal biopsies obtained after sacrifice and measured as ^{3}H-thymidine incorporation *in vitro* and autoradiography.

Modification of chemically-induced colon carcinogenesis

A) Experiments on initiation

Fisher 344 male rats were fed the HF diet (controls, n=15) and treated rats (n=16) were fed the same diet additioned with WCP (57.2 mg/kg/d). After 10 d the animals of both groups were treated s.c. with a single dose of AOM (20 mg/kg). The number and dimensions of aberrant crypt foci (ACF) in both groups were evaluated 30 d after the administration of the carcinogen.

B) Experiments on promotion

Fisher 344 male rats were fed a HF diet for 7 d and were administered AOM (15 mg/kg) s.c. twice with one week interval. Control rats (n=14) were kept on this diet for 90 d whereas treated rats

(n=14) were fed the same diet additioned with WCP (14.3 or 57.2 mg/kg/d). The number and dimensions of ACF in both groups were evaluated sacrificing the animals 90 d after the administration of the carcinogen.

C) Induction of nuclear aberrations (NA) by colon carcinogens

F344 rats were treated by gavage with saline or with WCP for 10 d, at the end of this period they were given the indicated dose of carcinogen 24 h before sacrifice. All carcinogens were administered by gavage dissolved in water except 2-amino-3-methylimidazo (4,5-f)quinoline (IQ) which was administered in water and 40% ethanol to make it soluble. All controls in the IQ group were administered the same volume of ethanol:water. Aberrations were scored on colon mucosal crypt histological sections after colouring slides with Feulgen-fast green staining. NA were scored in coded samples by conventional microscopy.

Bile acid and long chain fatty acid analyses in fecal samples

We analysed by gas-chromatography mass spectrometry the faecal content of the rats treated with AOM and fed WCP for 90 d at two dose levels (14.3 and 57.2 mg/kg). We used the same animals of the promotion experiment on ACF described before.

Results

We first studied the effect of CPT on colon mucosa proliferation. For these experiments we used two levels of WCP, chosen to be in the range of a high human exposure for an average drinker of red wine. The results are shown in Table 1- 2. After 90 d of feeding, a period long enough for studying effects of dietary variations on the colon of rodents, WCP did not significantly modify the number of labelled cells/crypt, of the labelling index and of the distribution of labelled cells along the crypt, all parameters connected with a variation of colon cancer risk. We only observed a small, but significant reduction of the number of cells/crypt with the highest dose of WCP, an effect of unknown biological significance in terms of cancer risk.

Table 1: Mucosal proliferative activity in AOM- and saline-treated rats fed HF diet or HF diet additioned with CPW as indicated

Groups	No. of labelled cells/crypt	Labelling index (LI)	No. of cells/crypt
Controls (n=13)	6.02±0.73	7.81±0.97	39.39±0.85
WCP 14.3 mg/kg (n=8)	4.86±0.37	6.24±0.46	39.81±0.93
WPC 57.2 mg/kg (n=9)	5.93±0.55	8.32±0.89	36.67±0.87 *

Values are means ± S.E.;= p=0.05 as compared with controls and rats treated with WCP 14.3 mg/kg.*

The decrease of the number of cells in the crypt with the highest dose of WCP could be explained by an increase rate of apoptosis in the intestinal mucosa, a factor which has a protective meaning, leading damaged cells towards programmed cell death. However, as shown in Table 3, this parameter did not seem to be varied after feeding WCP for 90 d.

Table 2: Distribution of the proliferative activity along different parts of the crypt in AOM and saline-treated rats and in rats fed with CPW as indicated.

Groups	% LI in the lower part of the crypt	% LI in the mid part of the crypt	% LI in the upper part of the crypt
Controls (n=13)	76.04±2.28	23.49±2.37	0.45±0.32
WCP 14.3 mg/kg (n=8)	81.22±3.18	15.12±3.42*	1.02±0.51
WCP 57.2 mg/kg(n=9)	71.16±4.39	28.82±4.38	0.00±0.00

Values are means ± S.E.= p<0.05 as compared with rats treated with WCP 57.2 mg/kg*

We then started investigating the effect of WCP on carcinogen-induced colon cancer lesions such as ACF. The results showed that WCP did not influence the number of ACF or ACF dimension (number of AC/ ACF) or the number of "large ACF" (supposed to indicate a progression of ACF towards tumour formation) in the initiation phase, when given 10 d before the carcinogen (Table 4) or in the promotion phase, when given for 90 d after carcinogen administration (Table 5).

Table 3: Number of apoptotic cells/crypt in control rats and in rats fed with CPW as indicated.

Groups	apoptotic cells/ crypt
Controls (n=13)	0.25±0.05
WCP 14.3 mg/kg (n=14)	0.35±0.05
WCP 57.2 mg/kg(n=14)	0.25±0.05

Values are means ± S.E.

Table 4: Number and dimension of ACF in control rats and in animals fed WCP 10 d before the administration of AOM. Rats were sacrificed 30d after carcinogen administration. Data are means ± S.E.

	No. of ACF/colon	AC/ACF	Large# ACF/colon
controls	42.94±3.00	1.67±0.05	3.84±1.39
WCP- treated	46.94±4.96	1.77±0.09	1.17±0.27

 # A large ACF in these experiments was composed of at least 4 aberrant crypts.

Table 5. Number and dimension of ACF in control rats and in animals treated with WCP (14.3 and 57.2 mg/kg) for 90 d after the administration of AOM (30 mg/kg). There were 14 rats in each group. Data are means ± S.E.

	No. of ACF/colon	AC/ACF	Large# ACF
controls	97.89±9.44	2.87±0.11	0.89±0.26
WCP-treated (14.3 mg/kg)	90.42±8.15	2.81±0.07	0.68±0.25
WCP- treated (57.2 mg/kg)	91.07±10.11	2.70±0.16	0.68±0.28

 # A large ACF in these experiments was composed of at least 9 aberrant crypts.

On the basis of the results reported in Table 4, WCP did not seem to have an effect on the initiation phase of colon carcinogenesis by AOM. This conclusion might be considered rush due to possible inadequacy of the experimental model employed. In fact WCP may have had effects on the activation and deactivation of colon carcinogens which might not become evident using AOM, a compound that requires a single oxidation step to be transformed into the ultimate carcinogen. To resolve this problem we tested the following additional carcinogens which require a more extensive metabolic handling to exert their genotoxic effect:

1) 1,2-dimethylhydrazine (DMH), precursor of AOM and transformed into AOM by a further oxidation step catalysed by monoxigenases;
2) 2-amino-3-methylimidazo(4,5-f)quinoline(IQ), which requires oxidation by monoxigenases and is further acetylated or conjugated with sulphuric acid into direct genotoxic compounds;
3) 4-aminobiphenyl (4AB), an aromatic amine which requires hydroxylation and acetylation to become genotoxic.

The modulating effect of WCP on the initiation phase of carcinogenesis with the above carcinogens was studied with the “nuclear aberration”(NA) assay , in which the effect of carcinogens on the colon is measured by scoring the number of acute cell toxicity effects on the colon mucosa, evaluated by nuclear morphology, 24 h after the administration of a carcinogen. This test has been demonstrated to be good indicator of carcinogenic potency in the colon (McLellan and Bird, 1988). The results of this approach are shown in Table 6-8. Both DMH and IQ induced the expected increase of nuclear aberrations over controls. On the contrary 4-AB did not induce NA in our experiment, a result in disagreement with previous data (McLellan and Bird, 1988). Negative results with this compound have also been reported by other authors (Wargovich et al., 1983).

CPW administration was not able to vary NA frequency tested with DMH or IQ or 4-ABP(Table 6-8).

We then repeated the same type of experiments with rats treated with TFu. As shown in Table 9-10 TFu failed to modify the induction of NA by DMH or IQ . Similarly TR was not able to modify NA induced by DMH (Table 11).

Table 6: Nuclear aberrations (NA) in colon mucosa of control rats and in animals fed HF diet additioned with WCP (57.2 mg/kg) for 10 d before the administration of DMH. NA were scored 24 h after carcinogen administration and are expressed as mean number of NA/ crypt ± S.E.

Groups	NA/crypt
Controls (n=3)	0.26±0.08
Controls+ CPW (n=3)	0.34±0.03
Rats treated with DMH (n=14)	3.27±0.43**
Rats treated with DMH + WCP (57.2 mg/kg) (n=14)	3.91±0.65**

***= p<0.01 relative to the respective controls*

Table 7: NA in colon mucosa after administration of IQ (250 mg/kg) to control rats and rats fed HF diet additioned with WCP (57.2 mg/kg) for 10 d. NA were scored 24 h after carcinogen administration and are expressed as a mean number of nuclear aberrations (NA) crypt ± S.E.

Groups	NA/crypt
Controls (n=2)	0.275 ; 0.235
Controls + WCP (57.2mg/kg) (n=3)	0.291±0.07
Rats treated with IQ (n=13)	0.996±0.14*
Rats treated with IQ + WCP (57.2mg/kg) (n=15)	1.462±0.29*

**=p<0.05 relative to controls*

Table 8: NA in colon mucosa after administration of 4-AB (100 mg/kg) to control rats and to rats fed HF diet additioned with WCP (57.2 mg/kg) for 10 d. Nuclear aberrations were scored 24 h after carcinogen administration and are expressed as mean number of nuclear aberrations (NA)/crypt ± S.E.

Groups	NA/crypt
Controls (n=7)	0.36±0.08
Rats treated with 4-AB (100 mg/kg)(n=14)	0.24±0.04
Rats treated with 4-AB + WCP (57.2 mg/kg) (n=15)	0.25±0.02

Table 9: Nuclear aberrations (NA) in colon mucosa of control rats and in animals administered TFu (40 mg/kg) for 10 d before the administration of DMH (20 mg/kg). NA were scored 24 h after carcinogen administration and are expressed as mean No. of NA/ crypt ± S.E.

Groups	NA/crypt
Rats treated with DMH (n=9)	2.82±0.42
Rats treated with DMH + TFu (40 mg/kg) (n=10)	3.28±0.61

Table 10: Nuclear aberrations (NA) in colon mucosa of control rats and in animals administered TFu (40 mg/kg) for 10 d before the administration of IQ (250 mg/kg). NA were scored 24 h after carcinogen administration and are expressed as mean number of NA/ crypt ± S.E.

Groups	NA/crypt
Rats treated with IQ (n=10)	0.85±0.19
Rats treated with IQ + TFu (40 mg/kg) (n=6)	1.27±0.28

Table 11: Nuclear aberrations (NA) in colon mucosa of control rats and in animals administered TR (40 mg/kg) for 10 d before the administration of DMH (20 mg/kg). NA were scored 24 h after carcinogen administration and are expressed as mean number of NA/ crypt ± S.E.

Groups	NA/crypt
Rats treated with DMH (n=12)	4.34±0.59
Rats treated with DMH +TR (40 mg/kg) (n=12)	4.66±0.82

Finally, we studied the effect of CPT on the levels of fecal bile acids in the animals treated chronically with WCP. The results, shown in Table 12, do not show a variation of the total faecal bile acid profile, although there was a non-statistically significant reduction at the highest dose. The levels of total long chain fatty acids were reduced by WCP, but the difference was not significant statistically.

Table 12: Levels of fecal bile acids and long chain fatty acids in control rats and in rats treated with WCP for 90 d.. All animals had been administered AOM (30 mg/kg s.c.) 90 d before sacrifice. Values are means ± S.E.

Groups	Total faecal bile acids (mg/g)	Total secondary bile acids (mg/g)	Total long chain fatty acids (mg/g)
controls (n=11)	1.82±0.23	0.189±0.022	1.599±0.791
WCP 14.3 mg/kg (n=13)	1.84±0.29	0.170±0.021	0.710±0.08
WCP 57.2 mg/kg (n=10)	1.17±0.17	0.182±0.026	0.700±0.10

The levels of individual bile acids, shown in Table 13, demonstrate that beta and omega-muricholic acid was decreased at the highest dose of WCP, but hyodeoxycholic acid was increased. The biological significance of these events is hard to grasp. However, the total amount of the more toxic secondary bile acids, which are supposed to act as co-carcinogens and promoters at the level of the colon, was not varied (Table 12).

Table 13: Levels of individual bile acids (mg/g fresh feces) in control rats and rats treated with CPW for 90 d.

bile acid	Control diet	WCP 14.3 mg/kg	WCP 57.2 mg/kg
lithocholic acid (II)	0.025±0.003	0.032±0.006	0.031±0.008
deoxycholic acid (II)	0.154±0.019	0.137±0.020	0.151±0.026
chenodeoxicholic acid	0.020±0.006	0.006±0.002	0.006±0.003
alfa-muricholic acid	0.109±0.021	0.096±0.035	0.103±0.039
hyodeoxycholic acid	0.112±0.040	0.095±0.026	0.279±0.055**
beta-muricholic acid	0.541±0.087	0.344±0.090	0.247±0.048*
omega-muricholic acid	0.861±0.143	1.137±0.193	0.357±0.103**

***=p<0.01 as compared with controls and WCP 14.3 mg/kg. *=p=0.05 as compared with controls. (II) = secondary bile acid.*

Discussion

The administration of WCP with the diet, prior or after the administration of AOM, does not seem to vary a series of parameters correlated to colon carcinogenesis in the rat.

Of all proliferation parameters analysed (number of cells/crypt, labelling index, distribution of the proliferative activity along the crypt) we observed a significant decrease in the number of cells/crypt in the rats treated with the highest dose of WCP. The significance of variations of this parameter in terms of colon cancer risk is not clear. In fact, some authors consider a low number of cells/crypt to be a protective factor (Newmark et al., 1990), some others the reverse (Lipkin et al., 1996). It should be noticed that we observed a decrease in the % of labelled cells in the middle crypt compartment in the rats treated with the intermediate dose of WCP. All the other parameters were not varied compared to controls. This isolated and not dose-dependent difference is probably biologically irrelevant. WCP, therefore, do not influence cell proliferation in the colon.

Given the uncertainty in the interpretation of the variation of crypt height in these experiments, we controlled for the number of apoptotic cells/crypt in the existing histological specimens. In fact there is chance that an increased apoptosis might protect against cancer, eliminating mutated or damaged cells in the mucosa. However, such an effect was not observed in the rats fed WCP.

We also studied a series of parameters which can predict the effect of a dietary supplement on the initiation phase (induction of ACF or NA) or in the promotion phase of colon carcinogenesis (growth of ACF).

The presence of CPT from red wine (WCP) or tea (TFu and TR) in the diet did not seem to modify these processes, induced with a variety of carcinogens (AOM, DMH, 4-ABP and IQ).

The supplementation with WPC at the highest dose tended to decrease the total concentration of bile acids and of long chain fatty acids in the faeces. However, these differences were not statistically significant. Bile acids are co-carcinogens and promoters at the level of the colon. Secondary bile acids, which are supposed to be the most toxic compounds, were not varied by CPT administration.

In conclusion the data seem to indicate that CPT do vary a series of parameters related to colon carcinogenesis. However, since protective effect of green tea extracts and red wine solids have been described by some authors (Yamane et al., 1991; Clifford et al., 1996) we are in the process of performing a long-term carcinogenesis experiment with CPT from red wine and tea to reach a definitive conclusion.

Acknowledgements

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Possible health benefits of consuming probiotic bacteria

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Abstract

The work described in this review was undertaken for an EU funded Shared Cost project entitled Second Generation Functional Foods - Microbial Flora and Intestinal Function (AIR-CT92-0256). The objectives of this project were to improve understanding of probiotic actions on health and to develop an understanding of probiotic bacteria that would allow them to be effective at realistic levels of intake. A technique to modify the genome of bifidobacteria was developed. Bifidobacteria strains were investigated for their ability to produce potentially beneficial metabolites. Two strains produced metabolites that repressed the growth of potential intestinal pathogens .The potential of probiotics to reduce the risk of cardiovascular disease, and cancer were investigated. The microgel electrophoresis (Comet) assay, the aberrant crypt foci (ACF) assay and the intrasanguinous host-mediated assay for determining genotoxic damage were used as markers for cancer risk. *In vitro* studies showed that bacteria do not assimilate cholesterol and feeding a strain reported to assimilate cholesterol did not lower blood levels of cholesterol. Bacteria with bile sale hydrolase (BSH) activity were selected and characterised. Significant cholesterol lowering effects were found in pigs, but not in hamsters or rats. Reduced DNA damage (induced by 1,2 dimethylhydrazine), anti-genotoxic effects and reduced numbers of ACF were found in rats fed certain strains of bacteria.

Introduction

The work described in this review was undertaken for an EU funded Shared Cost project entitled Second Generation Functional Foods - Microbial Flora and Intestinal Function (AIR-CT92-0256). The principal scientists and the organisations participating in the project were: Dr LC Lievense and Dr JM Fletcher (coordinators) of Unilever Research,Vlaardingen, Dr R Leer and Dr I van Luijk of the TNO Nutrition and Food Research Institute, Prof WH Holzapfel, Prof B Pool-Zobel of the BFE-IHT Karlsruhe, Dr F Ahrens of the ISF Wahlstedt, Prof C Romond of the University of Lille, Prof Palenzona, Prof B Sgorbati, Prof D Matteuzzi and Dr M Rossi of the University of Bologna, Prof Verstraete and Dr I Smet of the University of Gent, Prof I Rowland and Dr C Rumney of the BIBRA International, Dr G MacFarlane and Dr A McBain of the MRC Dunn Clinical Nutrition Centre. In this large and multi-disciplinary project a wide variety of biochemical, microbiological, physiological and nutritional materials and methods were employed. Only the most important, or novel methods that have been specifically developed for the project are mentioned below. The project was funded for three years and in this time a great deal of work was carried out and a large amount of data obtained. In the scope of this review it is only possible to highlight some of the results and conclusions of the project.

Probiotic Food Products (PFPs) are foods that contain live micro-organisms. The probiotic concept has a long history dating back 100 years to Eli Metchnikov who first proposed that consuming live bacteria was beneficial to health and longevity. The starting point for the EU Shared Cost project was the realization that despite this long history and some commercial success, the scientific status of probiotics was not firmly established and there was not yet a biomedical consensus that consuming live bacteria is beneficial for any aspect of health. Furthermore, many of the studies

claiming benefits of probiotic consumption had only been performed with high intakes of live bacteria that are not attainable in normal food products.

The health claims associated with consumption of PFPs are many and diverse (for recent reviews see 1,2,3). The best documented claims of probiotic efficacy are those associated with gastrointestinal function, such as alleviation of lactose intolerance, treatment of irritable bowel syndrome and treatment or prevention of diarrhoea caused by pathogenic micro-organisms. The evidence in favour of probiotic consumption reducing the risk of cardiovascular disease (CVD), reducing geno-toxicity and the risk of cancer is considerably weaker. However, because these diseases represent the major health risks facing the consumer in developed nations the work of this project concentrated on exploring these potential benefits of PFP consumption.

In previous investigations of probiotics, beneficial effects have generally only been achieved with high intakes of bacterial strains and since existing PFPs are mainly restricted to dairy products with a low viable count of the probiotic bacteria, such an intake is often unrealistic. Therefore another objective of this research project was to develop the scientific and technological basis of a new generation of PFPs that would be effective at more realistic intakes. The research activities undertaken to reach this objective included: development of methods to identify and amplify the beneficial activity of probiotic strains, understanding the factors influencing colonisation capacity of potential probiotic strains and development of methods to enhance the growth and establishment of probiotic strains in the intestine.

Molecular biology and colonisation resistance capacity

The main focus of research within this topic was directed towards understanding how probiotic micro-organisms, or their metabolites, might influence bile acid metabolism and how functional modification of the microbial population of the gut might be achieved.

i) Bifidobacteria constitute a major part of the normal human intestinal flora. They compete with less desirable components of the intestinal flora like enterobacteria or clostridia. Moreover, several bifidobacteria have been found that produce conjugated bile salt hydrolase (BSH), an enzyme that might be involved in cholesterol lowering. Previously the molecular biology of *Bifidobacterium* spp. had not been developed because of the difficulties associated with the transformation of this genus. Moreover, specific vectors and regulons were not available. Consequently, an important scientific task of this subgroup was to develop the molecular methods to (over)express homologous or heterologous genes in *Bifidobacterium*.

ii) Conjugated bile salts are synthesised in the liver, transported to and stored in the gallbladder and secreted in the upper part of the small intestine. Through resorption by the small intestine and transportation by the blood, they again come into the liver, a process called the enterohepatic cycle of bile acids. The hydrolysis of conjugated bile acids, through the BSH activity of bifidobacteria or lactobacilli, may cause the withdrawal of bile salts from the enterohepatic cycle, thereby reducing serum cholesterol levels and investigation of this hypothesis was an important objective of the whole project. Within this sub-topic the research targets were analysis of BSH genes in several lactobacilli and amplification of the BSH activity of selected strains by genetic modification.

iii) The effectiveness of probiotic action is, amongst other factors, determined by the ability of the micro-organism to (sometimes transiently) colonise the intestine. It is anticipated that micro-organisms that have the ability to colonise, produce proteins or induce the host to produce proteins, that cause the adhesion to the intestine. Studies were undertaken to develop a model system for measurement of adhesion, with the objectives of exploring the relationship between aggregation of bacterial cells and colonisation ability and in addition to isolate proteins that might be involved in adhesion.

iv) It has been suggested that certain bifidobacteria produce compounds that actively repress the growth of clostridia and enterobacteria, while at the same time promoting the growth of endogenous bifidobacteria. The production, analysis and effectiveness of such compounds was studied. In addition the need for the presence of the bacterial cells for expression of this activity was studied.

Materials and methods

In general, standard microbiological, biochemical and molecular techniques were used. A variety of micro-organisms have been used in these studies, obtained from the following sources:

- Bifidobacteria and lactobacilli used for molecular and adhesion studies were obtained from the Type Culture Collection of the Istituto de Microbiologia Agraria (University of Bologna).

- Bifidobacteria used in the studies on the interference with the intestinal flora were obtained from the collection at the Laboratoire de Bactériologie de la Faculté de Pharmacie, Lille.

- *L. plantarum* 80 was described by Scheirlinck et al (4) and *L. plantarum* ATCC 8014 by Posno et al (5). *L. gasseri* NCK65 was a gift from T. Klaenhammer (Raleigh, NC, USA) and *L. animalis* 362 and 364 were isolated at Unilever Research Laboratorium Vlaardingen, during this project, from the gastro-intestinal tract of a hamster.

Results

a) The molecular biology of bifidobacteria:

A convenient and reproducible procedure was developed to genetically transform *Bifidobacterium* spp. in a co-operative study of the group at TNO- Rijswijk and the group at the Pharmaceutical Sciences department of the University of Bologna. The method, which is applicable to all *Bifidobacterium* spp. tested so far, was based on electroporation of bacteria made competent by preincubation of the bacteria in electroporation buffer for several hours at 40C. Transformation of *Bifidobacterium* could be achieved with pDG7, a vector based on a cryptic plasmid originating from *Bifidobacterium* (pMB1) and with plasmid vectors from *Corynebacterium*, but not with vectors carrying replicons from *Lactococcus* or *Lactobacillus*. Based on the pMB1 replicon a large family of plasmids was constructed, to be used as promoter screening vectors or expression vectors in *Bifidobacterium*. Also a small 2.8 kb plasmid pTRE3 was constructed that replicates stably in *Bifidobacterium* for at least 200 generations without selection pressure. Several promoters were cloned and shown to be active in *Bifidobacterium* and several antibiotic resistance genes could be expressed under their own promoters. Attempts to clone the cholesterol oxidase gene from *Streptomyces* in *Bifidobacterium* were unsuccessful, probably as a result of breakdown by a restriction/modification system that recognises DNA with a high GC content.

b) The molecular biology of BSH activity from Lactobacillus

Three genes encoding BSH (also known as conjugated bile salt hydrolase, cbh) activity from *L. plantarum* ATCC 8014 and *L. gasseri* NCK 65 were cloned. Expression vectors were constructed to determine expression of the genes in different *Lactobacillus* hosts. Whereas efficient expression was obtained in *L. plantarum* with vectors containing the three above-mentioned *cbh* genes, as well as with a vector containing the previously isolated *cbh* gene from *L. plantarum* 80, virtually no expression was observed in *L. casei*, a strain which in itself shows no BSH activity. In order to obtain insight into the relation between the sequence or structure of the proteins encoded by the *cbh* genes and enzyme specificity, the nucleotide sequences of the *cbh* genes cloned were determined. Comparison of the nucleotide sequences and deduced amino-acid sequences from *L. plantarum* 8014 with those reported for *L. plantarum* 80, showed that the two *cbh* genes code for almost identical enzymes, despite the fact that the activities and specific activities towards glyco- and tauro-bile salts were significantly different. The two *L. gasseri* genes encoded enzymes that were structurally different and showed different activity spectra. Results of mRNA measurements indicated that differences in enzyme activity levels can, to a large extent, be ascribed to differences in mRNA levels. The results also indicated that all *cbh* genes analysed are preferentially expressed during the stationary phase of growth.

c) Aggregation, adhesion and colonisation

On the basis of their aggregation ability, two different subpopulations of lactobacilli and bifidobacteria were found in a single strain of each species. By investigating the adhesion of the two subpopulations to *in vitro* cultured human cells, an inverse relationship between aggregation and adhesion was found. Two proteins were isolated that might be involved in the aggregation of bacterial cells, a aggregation promoting factor (APF) and a cell surface protein.

d) The influence of Bifidobacterium metabolites on the intestinal flora

Two *Bifidobacterium* strains were selected that produced compounds upon fermentation in milk, that were able to repress faecal clostridia in mice. Moreover the growth of *Bacteroides fragilis* was reduced and growth of endogenous bifidobacteria was enhanced. It was shown that viable cells were not required for this activity. The strains did not survive gastric transit and poorly colonized mice. The active compounds were sensitive to heating and partially sensitive to lyophilization and oxidation at room temperature. The addition of cysteine hydrochloride stabilised the compound. It was determined by ultrafiltration that the activity primarily was located in the 100-300 kDa fraction. There were indications that high molecular weight glyco-proteins were involved. Hydrolysation of the milk with a proteolytic enzyme before fermentation destroyed the activity. Investigations into the mode of action showed that antimicrobial actions were not involved. In a collaboration with the group of Prof. Verstraete (University of Gent), the effects of the *Bifidobacterium* fermented wheys on the microbial flora, independent of the host, were investigated in the SHIME reactor. However, the SHIME reactor did not exactly reproduce the intestinal flora of the human intestinal tract and therefore the results could not be directly compared with the effects obtained in *in vivo* studies using mice. Subsequent human trials showed that a positive modification of the human intestinal flora was obtained, similar to that observed in mice.

Discussion

The rather recalcitrant genus of *Bifidobacterium* was made accessible to molecular genetic techniques through the development of an effective transformation system and a variety of plasmids, based on a small cryptic *Bifidobacterium* plasmid. Through the cloning and molecular analysis of several BSH genes from lactobacilli, novel strains have been developed which may help investigations of the effects of BSH activity on the gastro-intestinal tract and on blood cholesterol levels. The results also allow the determination of the effects of BSH activity on resistance of intestinal bacteria towards bile salts. This type of information is of importance with regard to e.g. the selection of strains with probiotic properties. Finally, a better insight in the relationship between structure and function of BSH, as revealed in this study, will allow a better use of this type of enzyme as a specific amidase. It has been shown that the composition of the (human) microbial flora can positively be altered by the addition of a *Bifidobacterium* fermented whey, without the concomitant addition of the bacterial cells. The active compounds are heat and oxidation sensitive, but can be stabilised by the addition of cysteine hydrochloride. During the EU contract an industrial application was developed in the form of an infant formula. It has been shown that the formula induces accelerated maturation of the intestinal flora in prematures. New applications of *Bifidobacterium* fermentation metabolites might be expected in future.

Cholesterol-lowering activity

There have been several scientific publications claiming that consumption of fermented milk reduces the concentration of cholesterol in the blood of experimental animals and man (6,7,8). It has been proposed that the active cholesterol-lowering agent is the live bacteria (probiotic) contained in the fermented material. In the existing literature four plausible mechanisms by which probiotic bacteria might lower blood cholesterol have been proposed :

i) Inhibition of hepatic HMG-CoA-reductase by short chain fatty acids, produced by the activity of probiotic bacteria, leading to reduced *de novo* synthesis of cholesterol.

ii) An increased activity of bacterial BSH in the small intestine, causing a reduced efficiency of bileresorption and increased loss of bile acids and their derivatives in the faeces.

iii) Bacterial assimilation of cholesterol by probiotic bacteria, causing an increased excretion of dietary and endogenous cholesterol in the faeces.

iv) Oxidation of dietary and endogenous cholesterol by probiotic bacteria, resulting in formation of unabsorbable metabolites that are lost in the faeces.

At the outset of this project a review of recent scientific developments strongly indicated that inhibition of *de novo* cholesterol synthesis by short chain fatty acids was most unlikely to be an effect of probiotic feeding. Oxides of cholesterol are potentially toxic to the host animal and therefore possibility iv (above) was not pursued as a reasonable route to safe cholesterol lowering. The focus of experimental work within the project was the possibility of blood cholesterol lowering by the mechanisms of either cholesterol assimilation by probiotic bacteria or, bile salt hydrolysis by probiotic bacteria (ii and iii above).

Materials and methods

The phenomenon of apparent cholesterol "assimilation" by bacteria was studied *in vitro.* In addition, the effects on blood cholesterol levels and cholesterol excretion of feeding a bacteria previously reported to assimilate cholesterol (RP32, ATCC 43121), was investigated in pigs and hamsters (10^{11}cfu/day/animal).

The activity of BSH in bacteria and in digesta contents was assayed independently by a radiochemical and a HPLC based method. Strains of bacteria with low and high BSH activity were identified and isolated from the intestinal contents of pigs and hamsters. The expression of BSH activity and substrate specificities of this enzyme were characterised. The significance of BSH activity for survival and growth of bacteria in the intestinal microbial eco-system was studied using an *in vitro* model of the gastro-intestinal tract (SHIME; Simulation of the Human Intestinal Eco-system). The survival of genetically modified variants of the same strain (*L. plantarum 80*, prepared by TNO), expressing high or zero activities of BSH was also studied in this system.

The effects on blood cholesterol concentrations and faecal bile acid excretion of feeding bacteria (10^{10}-10^{12} cfu/animal/day) with high BSH activity was studied in pigs, mini-pigs, rats and hamsters. The *in vivo* survival of bacteria with BSH activity after feeding and their influence on intestinal BSH activity was assessed in post-mortem digesta samples and using ileal digesta taken from rats fitted with an ileostoma. The metabolism of bile acids after deconjugation was investigated with respect to identification and quantification of metabolic end products in the faeces.

Results

A series of *in vitro* experiments showed that cholesterol assimilation by bacteria does not occur. From these experiments it was concluded that apparent "assimilation" only happens when bacteria are allowed to grow in the presence of cholesterol suspended in conjugated bile acids. The activity of bacterial BSH deconjugates the bile which precipitates in the low pH caused by bacterial fermentation. Some of the suspended cholesterol co-precipitates with the bile and after centrifugation it only appears as though the bacteria have assimilated cholesterol. Nevertheless literature claims of cholesterol-lowering by feeding bacteria with apparent cholesterol assimilating activity were investigated. Serum cholesterol levels were determined in pigs and hamsters fed bacteria with apparent 'assimilation' activity (RP32, ATCC 43121). Groups of pigs and hamsters were fed 10^{11} cfu per day of live bacteria, but compared with control animals there was no effect on blood cholesterol levels, or excretion of cholesterol (and its microbial metabolites) or excretion of bile acids.

Most of the efforts of the project within the subtopic cholesterol-lowering were subsequently directed towards investigation of the BSH hypothesis (see ii above). The populations of intestinal LAB from hamsters, rats and pigs were screened for BSH activity and for further beneficial properties with respect to their survival in the gastrointestinal tract and their use as potential probiotic strains. During these studies it was discovered that the gastrointestinal flora of the hamster is abnormal compared to the human, with respect to a high population of bacteria and a high BSH activity in the stomach and upper small intestine. These features make the hamster a less suitable model to investigate any effects of potential probiotics. The rat was found to be more similar to the human. Strains of gut bacteria from rats, hamsters and pigs with high BSH activity were isolated and identified. The BSH activity of the isolated strains was characterised with regard to substrate specificity, optimum conditions for enzyme expression, optimum pH, and stability to freeze-thawing.

The microbial ecological significance of BSH activity was investigated using the *in vitro* SHIME system. Inoculation experiments showed that strains with BSH activity survived better than strains without BSH in the SHIME. However in cultures in the presence of bile salts without nutrients the presence of BSH activity was detrimental to survival. The possibility was studied that bile salts are toxic to bacteria because they enter the cell in a protonated form and cause intracellular acidification by release of the co-transported proton. Based on literature values for the interrelationships between bile salt-cholesterol metabolism and experimental data the potential positive effect of increasing intestinal BSH activity by probiotic feeding was calculated. This calculation showed the theoretical feasibility of cholesterol-lowering by feeding probiotic bacteria with BSH activity.

The effects on ileal digesta of feeding bacteria with BSH activity were investigated by using rats fitted with an ileostoma. It was shown after feeding bacteria with BSH activity that the population of the ileal digesta was markedly altered, although the total number of LAB changed only a little, and that the BSH activity was increased.

The effect on blood cholesterol concentration of feeding several different strains of bacteria with BSH activity were investigated in experiments on hamsters, rats, pigs and mini-pigs. In these experiments the animal diets were designed to have a similar composition as a typical western diet and cholesterol was added to ensure an initial hypercholesterolaemia. Live bacteria were fed at daily doses of 10^{10} to 10^{12} cfu per animal. In the experiments using hamsters and rats there were no effects on blood cholesterol and no effects on bile or cholesterol excretion. In the experiment that used mini-pigs there was a trend towards a cholesterol-lowering effect of approximately 5%. In the pig feeding experiment, blood cholesterol concentrations were reduced by 11-26% compared with a control group. The reduction in cholesterol levels was associated with an increased concentration of bile acids in the faeces. In all of these experiments there was no evidence of any adverse effects on growth or health of the host animals of feeding the bacteria. A significant reduction of the enzymes beta-glucuronidase and azoreductase was observed in the faeces of mini-pigs during the period of probiotic feeding.

The effects of feeding the oligosaccharides lactulose or inulin on blood cholesterol levels were studied in the rat and the hamster. These materials cannot be hydrolysed by mammalian enzymes, but they are broken down and utilised by the bacterial flora of the intestine. Several published studies have claimed that feeding these materials reduced blood cholesterol levels via changing the populations and metabolic activities of the gut flora. However, feeding lactulose or inulin at inclusion levels in the diet of 5% or 10% had only a temporary lowering effect on blood cholesterol in hamsters and no effect in rats. Feeding lactulose, but not inulin, caused an increase in the numbers of bifidobacteria in the caecum and ileum of hamsters. There was no effect on blood cholesterol levels of feeding LAB bacteria combined with oligosaccharides.

Discussion

The possibility that consumption of fermented milk products and probiotic bacteria might reduce blood cholesterol levels has been in the scientific literature for many years. The hypothesis of cholesterol lowering by assimilation was attractive because in principle it is a safe route, not involving modification of bile composition. In addition, two groups had published studies describing the success of this approach. However, the results obtained from our *in vitro* studies led to the conclusion that the theory of probiotic induced cholesterol-lowering by assimilation was incorrect.

During the course of the work described in this report a scientific publication appeared which reached the same negative conclusion (Klaver & van der Meer, Appl. Eviron. Microbiol. **55** 1120-1124, 1993). An effect on blood cholesterol levels in pigs after feeding a bacterial strain reported to reduce cholesterol by assimilation could not be confirmed.

After dismissing the assimilation hypothesis, the work of the consortium was mainly directed towards investigation of the BSH hypothesis of cholesterol-lowering. This hypothesis was based on two assumptions; firstly, if probiotic bacteria survive to reach the small intestine the BSH enzyme would be sufficiently active in the prevailing conditions to significantly increase the amount of deconjugated bile, secondly that deconjugated bile salts would be resorbed with significantly less efficiency than intact bile and the unabsorbed bile acids would be lost in the faeces It should be emphasised that to influence cholesterol levels any probiotic with BSH activity must be effective in the small intestine, which is the site of bile acid resorption. Effects on bile acid metabolism may occur during passage of digesta through the small intestine and therefore colonisation is not a prerequisite for a probiotic based on BSH activity.

The work of the project explored the assumptions behind the BSH hypothesis and sought to demonstrate a cholesterol-lowering effect. The results obtained have greatly increased our understanding of bacterial BSH in several important respects; the distribution of this activity within different bacteria has been determined, the activity of the isolated strains has characterised with regard to substrate specificity, optimum conditions for enzyme expression, optimum pH and stability to freeze-thawing. Direct investigation of the effect on blood cholesterol levels of potential probiotic strains with BSH activity has been undertaken in hamsters, rats, pigs and mini-pigs. A lowering effect on the concentration of blood cholesterol was only obtained in two studies, carried out in pigs and in mini-pigs.

The observation of cholesterol lowering in pigs fed bacteria with BSH activity and a concurrent increase in bile acid excretion is extremely important as offering the first positive evidence for the BSH hypothesis. Further studies are however needed:

i) To confirm the findings and to determine the dose-response relationship between the amount of fed bacteria and the size of cholesterol reduction. The number of bacteria fed per day (10^{11} cfu) was large in relation to a realistic intake for humans, but the magnitude of the cholesterol reduction was also large (10-29%) in relation to expected beneficial effects of reduced cholesterol levels.

ii) To determine the chemical identity of the increased bile acid output. Concerns have been expressed over the possible harmful effects of increased secondary bile formation as a result of elevating BSH activity in the intestine. Secondary bile acids may act as irritants and tumour promoters in the mucosa of the intestine. This potential problem needs to be investigated, particularly as in the pig experiment, described above, the lowering effect on blood cholesterol levels was maintained after the cessation of probiotic feeding. Therefore it is possible that colonisation of the intestine with the fed high BSH bacteria had occurred and effects on bile acid metabolism may persist after feeding of the bacteria is stopped.

iii) To show a benefit in a controlled human feeding study. To gain a consensus in the biomedical community for the effects of consumption of a probiotic it is clearly desirable to demonstrate these effects in human subjects.

Anti-genotoxic and anti-carcinogenic effects

Experimental studies *in vitro* and *in vivo* have suggested that consumption of lactic acid bacteria (LAB) may exert anti-carcinogenic effects, although the mechanisms involved and interspecies differences have not been adequately explored. The objectives of the project were therefore :

i) To identify strains of LAB (lactobacilli, streptococci and bifidobacteria) that could exert anti-genotoxic and anti-carcinogenic properties.

ii) To investigate the survival of potentially beneficial probiotic strains and their effects on other constituents of the colonic microbial eco-system.

iii) To investigate mechanisms involved in the anti-genotoxic and anti-carcinogenic effects of LAB.

A variety of mechanisms have been proposed for the anti-genotoxic and anti-carcinogenic action of probiotics, including adsorption and inactivation of carcinogens, inhibition of the metabolism and activation of dietary carcinogens and synthesis of anti-carcinogenic substances. These potential mechanisms have been explored in the course of the present project using *in vitro* and *in vivo* rapid methods (see below) and assays for specific enzymes associated with carcinogen metabolism. In addition to LAB strains, the effect of inulin, a substance which stimulates numbers of LAB in the colon has been studied. The effects of adding potentially beneficial probiotic strains and/or inulin on the populations and metabolic activity of other bacterial constituents was studied using *in vitro* models of the colonic microbial eco-system.

Materials and methods

Conventional cancer bioassays are too expensive and time-consuming to allow screening of LAB strains for anti-carcinogenic activity and do not facilitate studies of mechanisms of action. To circumvent these problems a variety of rapid methods were used to assess genetic damage and neoplastic changes in the colonic mucosa and in the liver namely:

a. The single cell microgel electrophoresis (Comet) assay, which measures DNA damage shortly after carcinogen exposure.

b. A short term assay for preneoplastic colon damage - the aberrant crypt foci (ACF) assay

c. The intrasanguinous host-mediated assay. This assay is an *in vivo* version of the Ames test and assesses genotoxic damage in the liver.

Sprague Dawley rats fed a semi-synthetic (SSA) diet based on casein and starch were used for most of the studies to ensure comparability of data. In the *in vivo* microgel electrophoresis (Comet) assay, rats were gavaged with one or four daily doses (10^{10} cell/kg body weight) of a culture of LAB (grown for 24 h in MRS medium, centrifuged and resuspended in saline). The carcinogens N-methyl-N-nitroso-nitroguanidine (MNNG, 7.5mg/kg body weight) and 1,2 dimethylhydrazine (DMH, 15mg/kg body weight) were given p.o. 8hr following the final LAB gavage and the rats were killed 16 h later. Colon cells were isolated by protease digestion, layered in agarose on slides and subjected to electrophoresis. After staining the DNA, the length of the fluorescent DNA ‘comet’ was assessed by image analysis.

For *in vitro* studies, colon cells were isolated from untreated rats and incubated for 30 min in the presence or absence of a genotoxin (MNNG) and potential anti-genotoxic factors or LAB cultures. ‘Comets’ were assessed as described above.

In the aberrant crypt foci (ACF) assay, rats were fed either SSA diet or a modification (CO25) with increased fat content (25% w/w). Rats were dosed with AOM i.p (2 x 25mg/kg) and then one week later were fed diets containing freeze-dried LAB, with or without inulin (5% w/w in diet). After 3 months, the rats were killed, colons removed and stained with methylene blue and ACF counted.

The activity of bacterial enzymes, b-glucuronidase and b-glucosidase in caecal contents from rats treated with LAB or inulin, and the conversion of the dietary carcinogen IQ to its genotoxic metabolite, 7-OHIQ, were assessed by established methods. The effect of LAB treatment on xenobiotic metabolising activities in the colonic mucosa was also determined.

Single and three-stage continuous *in vitro* culture models were used to study; a) the survival of potential probiotic bacteria, b) the impact on the populations of other constituents the microbial flora, c) potential genotoxic enzyme activities; glucuronidase, b-glucosidase, azoreductase, arylsulphatase and nitroreductase and d) the concentration of potentially carcinogenic metabolites.

Results

In the *in vivo* comet assays, protective effects against MNNG or DMH-induced DNA damage were *Lactobacillus acidophilus*, *L. gasseri, L. confusus, B. breve, B. longum.* When several strains of one species were compared in the DMH assay, only 1 of 4 strains of *S. thermophilus* and 1 of 3 strains of *L. bulgaricus ssp. delbrueckii* exhibited protective effects*.* The importance of viable cells was demonstrated by the lack of anti-genotoxic activity of heated *L. acidophilus.*

The influence of feeding *L. acidophilus*, (10^8/g diet) or dietary inulin (reported to stimulate LAB numbers in the gut), on ACF induction by azoxymethane (AOM) in rats fed either a low fat or high fat diet was investigated. Significant lowering effects on ACF numbers of the LAB and inulin treatments were seen, with the protective effects being more potent in the animals given the high fat diet. In a subsequent study. *B. longum* (10^9/g diet) was shown to have similar protective effects. The combined treatment with *B. longum* and inulin was additive in terms of the protective effect on AOM-induced ACF.

The host mediated assay was used to determine whether the ability of LAB to bind chemical carcinogens *in vitro* could modify their uptake and genotoxic effects in the liver. Simultaneous oral dosing of one of the cooked food carcinogens MeIQ, MeIQx, Trp-P-2 or the fungal toxin aflatoxin B1, with a dense cell suspension of *B. longum* or *L. acidophilus* did not decrease the concentration of the carcinogen in the liver, nor did LAB treatment affect the level of induced mutations in the liver.

Oral administration of *B. longum,* or *L. casei*, significantly inhibited the conversion by caecal bacteria of IQ to 7-OHIQ. The activity of the b-glucuronidase was also decreased by *B. longum* and /or inulin. In contrast, LAB treatment had only minor effects on colonic mucosal xenobiotic metabolising enzymes. The exception being a significant increase of cytochrome P450-reductase activity in the colon caused by treatment with L. acidophilus.

The severity of DNA damage (assessed by the Comet assay) induced by MNNG in isolated rat colon cells, was decreased by incubation with acetate (a product of LAB fermentation of carbohydrates) by the presence of a metabolically active culture of *L. acidophilus* or an acetone extract of the culture.

In vitro fermentation studies were carried out using two prospective probiotics: *Bifidobacterium longum* NCFB 2259 and *Lactobacillus acidophilus* 1237. Experiments were made using single and

multiple-stage continuous culture systems to investigate potential probiotic effects of these organisms on the large intestinal microbiota, with respect to alterations in the composition of the colonic ecosystem as well as reductions in bacterial synthesis of putative genotoxic enzymes and toxic products of dissimilatory amino acid metabolism. Neither the lactobacillus nor the bifidobacterium were able to establish in climax gut ecosystems and with the exceptions of a suppression of bacteriodes population by *B. longum* and stimulation of Gram positive cocci by *L. acidophilus*, the candidate probiotics had no major effects on the generic composition of intestinal bacteria growing in fermentation systems.

Short term modulations in bacterial metabolism induced by adding the prospective probiotics were observed. Concerning fermentation, *L. acidophilus*, increased amino acid deamination and lowered proteolysis while *B. longum* stimulated dissimilatory tyrosine metabolism in the gut microbiota, resulting in large increases in phenol production.

Experiments aimed at investigating probiotic effects on the synthesis of hydrolytic and reductive enzymes involved in the formation of genotoxic metabolites showed that depending on cultural conditions, beta-glucuronidase synthesis was reduced by both organisms, but that beta-glucosidase formation was invariably reduced. With *L. acidophilus* nitroreductase activities also increased. Thus depending on unknown ecological factors, addition of two toxigenic micro-organisms to intestinal microbiota cultured *in vitro* could result in what may be considered to be harmful manifestations.

Discussion

The results of the *in vivo* assays in the colon indicate that consumption of certain LAB can lead to potentially beneficial effects on early changes (genetic damage and aberrant crypt foci) in the colonic mucosa considered to be predictive of cancer. It is clear from the Comet assay data, that not all LAB exert protective effects and furthermore, that these effects are strain specific. The ACF studies provided further evidence that probiotics may exert cancer-preventive effects in the colon and indicated that dietary components such as inulin that increase LAB numbers in the colon, presumably as a consequence of their content of poorly-digestible oligosaccharides, may also be protective. The latter studies strongly indicated that a strategy of providing both LAB and oligosaccharides in the diet, could be much more effective, in terms of beneficial effects, than the two treatments given separately. It seems likely from the results of another *in vivo* assay performed, the host-mediated assay in the liver, that probiotics will be less effective in preventing DNA damage at sites outside the gut. These results also demonstrated that *in vitro* experiments showing that LAB bind carcinogens, have little biological significance *in vivo* .

The studies have provided insights into potential mechanisms of action of LAB against damage induced by carcinogens. The importance of viable organisms for the prevention of colonic DNA damage in vivo, and the lack of effect of non-growing LAB cultures on DNA damage in vitro suggest that products (probably short-lived) of metabolically active cells are the active anti-genotoxic agents. *In vitro* studies indicate that fermentation products such as acetate may be important. Another mechanism of action of probiotics may be by decreasing the production of genotoxic or carcinogenic derivatives of dietary carcinogens in the gut. It was also apparent that administration of LAB to rats resulted in a decrease in activity of a number of gut microflora enzymes, e.g. b-glucuronidase, which releases carcinogens from biliary conjugates secreted in to the gut. However *in vitro* experiments aimed at investigating probiotic effects on the synthesis of hydrolytic and reductive enzymes

involved in the formation of genotoxic metabolites showed that depending on cultural conditions, beta-glucuronidase synthesis was reduced by both organisms, but that beta-glucosidase formation was invariably reduced. With *L. acidophilus* nitroreductase activities also increased. Thus depending on unknown ecological factors, addition of two toxigenic micro-organisms to intestinal microbiota cultured *in vitro* could result in what may be considered to be harmful manifestations. There was little or no evidence to suggest that LAB consumption was associated with changes in activity of enzymes in the colonic mucosa.

In addition to providing evidence that LAB may have beneficial properties in terms of cancer prevention, the research programme has identified rapid techniques, the microgel electrophoresis and ACF assays, that may be useful as screening method to assess potentially beneficial probiotic candidates.

Overall conclusions

The work of the project was successful in advancing scientific knowledge in many areas of relevance for understanding the potential benefits of probiotic consumption. A variety of techniques were developed to understand and manipulate potential probiotic actions. In the two areas of probiotic interaction with health chosen for investigation, potentially important influences of probiotic feeding were demonstrated and considerable progress was made in understanding the mechanistic basis of these actions.

In the three years since the end of the project several further studies in animals and man on the effects of consuming PFPs have been published. It is disappointing that these studies have often consisted of isolated observations without a mechanistic explanation or rationale to understand the basis of observed effects. To progress the science underlying the probiotic concept and help move towards a consensus that probiotic consumption may significantly improve human health a great deal of further work needs to be undertaken.

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Demonstration of nutritional functionality of probiotic foods

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If functional foods are to make an increasing impact on the European market, it is necessary to ensure that the consumer receives clear messages about the products and that the health claims are validated. *The main goal of the project is to give rise to well documented probiotic foods.* For the industry marketing probiotic foods it is very important to be able to demonstrate the beneficial effects of these products. Large numbers of products already exist today. Their impact on human health needs better documentation, otherwise the confidence of the consumer in functional foods will disappear.

The project objectives are divided into four tasks: 1) to establish a scientifically based selection of probiotic bacterial strains currently available for functional foods 2) to demonstrate the beneficial value of probiotic products in human pilot testing both in children and in adults, applying molecular tools for identification of gastrointestinal flora 3) to demonstrate and meet the functional and technological requirements essential for the industrial production of probiotics as functional foods 4) to disseminate the knowledge and results to the extended audience consisting of the group of industrial users, authorities and consumer organisations.

The following achievements can be listed this far:

1) Scientific selection and safety criteria (*Newsletter 2)* of listed probiotic strains.

2) Well-planned clinical pilot protocols.

3) Clinical pilot testing on children with viable *Bifidobacterium lactis Bb-12* and *Lactobacillus GG* resulted in a significant improvement of eczema (atopic dermatitis).

4) Clinical pilot testing with healthy human volunteers showed that the immunomodulatory capacity of *Lactobacillus johnsonii -1* and *Lactobacillus salivarius 118* was not linked to an inflammatory type of immune response. The beneficial immune effects were demonstrated by showing mucosal IgA response in probiotic treated groups .

5) An antagonising effect of *Lactobacillus johnsonii* Lj-1 and *Lactobacillus salivarius* 118 on *Clostridium perfringens* was shown in clinical testing on healthy adults.

6) Novel methods for adhesion studies were compared and developed on cell lines, mucin assays and biopsies. Human intestinal mucus appears to be strain-specific and the age of the target group may be worthy of consideration when planning a schedule for probiotic therapy. Specific strain surface properties (aggregation) are important factors in adhesion and colonisation as demonstrated with *L. crispatus* M247 and the mutant strain MU5.

7) Full nucleotide sequences of the selected probiotics and methods to handle faecal samples and biopsies for molecular analysis, methods to measure survival and population dynamics in faecal samples and biopsies have been developed.

8) Protocols for fermentative properties of probdemo strains have been developed

9) Pilot scale and technical viability protocols for Probdemo strains have been established - the aim is to demonstrate that all the probiotic strains included in the project can be produced on a large scale with an acceptable yield and a good performance and viability.

10) Production of a series of demonstration workshops and newsletters and manuscripts.

Introduction

Functional foods incorporating probiotic bacteria with scientifically supported health claims have great potential for improving quality of life and already constitute a rapidly growing EU and export market. Europe has traditionally had a leading position the probiotic market. Considerable confusion and scepticism, however, exists on the part of consumers, consumer organisations, and the scientific community about the claims associated with probiotic products. This greatly hampers further exploitation of probiotic bacteria in functional foods and weakens the market position of European producers in the face of competition.

The main goal of the project is to give rise to well documented probiotic foods. For the industry marketing probiotic foods it is very important to be able to demonstrate the beneficial effects of these products. Large numbers of products already exist today. Their impact on human health needs better documentation, otherwise the confidence of the consumer in functional foods will disappear. To speed up the adaptation of probiotic food technology, and enhance the attractiveness of new probiotic foods, it is essential to demonstrate the up-to-date basis for marketable claims. This will be established by presenting the health and nutritional benefits of probiotic bacteria and foods, with special emphasis on intestinal integrity and immune modulation, exploiting validated methods for the selection of novel probiotic bacteria and foods, and disseminating the knowledge obtained to the extended audience consisting of both industries, authorities and consumers.

Objectives

The project objectives are divided into four tasks:

1) *to establish a scientifically based selection of probiotic bacterial strains currently available for functional foods.*
2) *to demonstrate the beneficial value of probiotic products in human pilot testing both in children and in adults, applying molecular tools for identification of gastrointestinal flora.*
3) *to demonstrate and meet the functional and technological requirements essential for the industrial production of probiotics as functional foods.*
4) *to disseminate the knowledge and results to the extended audience consisting of the group of industrial users, authorities and consumer organisations.*

Table 1: Demonstration project on probiotics - Probdemo CT96-1028 tasks

Nutritional Functionality of Probiotic Foods	
Task 1. *Selection and verification of probiotic strains*	
Task 2 *Clinical pilot testing on humans*	Task 3 *Technological properties of probiotic foods*
Subtask 2.1 *Clinical pilot testing on children*	*Subtask 3.1* *Probiotic properties*
Subtask 2.2 *Clinical pilot testing on adults and patients with GI-disorders*	*Subtask 3.2* *Fermentative properties of probiotic foods*
Subtask2.3 *Establishment of novel methods*	*Subtask 3.3* *Large scale production methods*
Task 4. *Dissemination of knowledge of probiotic products*	

The four objectives are approached in the following way:

1) The selected list of probiotic strains has been chosen for demonstration purposes according to scientific and safety criteria and research results.

2) The potential of probiotics will be shown in treatment of food allergy infants and preventive diarrheal treatment for small children as well as in healthy children. The effect of probiotics will be demonstrated in healthy adults and in adults with inflammatory bowel disease (IBD/ Crohn's disease).

3) Functional requirements will be established by demonstrating existing *in vitro* methods of probiotic strain properties and reflecting these to the clinical testings. *The main focus this far has been on demonstrating adhesion* in vitro *and also* in vivo *using human biopsies.* The demonstration of the technological criteria will also be carried out for probiotic products (storage stability), and pilot production of probiotic strains.

4) Dissemination of knowledge will be established by annual workshops (*Workshop* 1 was held on the *Safety of probiotics* in Helsinki, Finland 1996, *Workshop* 2 was held on *Novel probiotic research tools* in Cork, Ireland October 1997, Workshop 3 is held in Haikko, Finland October 1998). Two Newsletters has been published as well as a review manuscript on Safety of probiotics for *Int. J.Food Microbiology*.

State of progress

Selection of strains

Six actual probiotic strains were chosen for demonstration purposes : Lactobacillus johnsonii LJ-1, Lactobacillus paracasei F19, Lactobacillus GG (L. rhamnosus ATCC 53103), Lactobacillus salivarius LM2-118, Lactobacillus crispatus M247 (cog+) and Bifidobacterium animalis Bb-12 (Bifidobacterium lactis). In addition Lactococcus lactis MG1363, L. crispatus MU5 (cog-), and Streptococcus thermophilus will be used as reference material.

Clinical pilot testing

The clinical pilot trials are continuously in progress. The trials started by obtaining an approval for the pilot clinical testing from Ethical committees in respective countries (Finland, Ireland, Denmark, Sweden, Switzerland), thereafter patients fulfilling the set criteria (Salminen & Mattila-Sandholm, 1998) have been recruited and randomised to different treatment groups as indicated in the outlines.

Treatment with viable *Bifidobacterium lactis* Bb-12 and *Lactobacillus* GG has resulted in a significant improvement of eczema in children. Clinical pilot testing with healthy human volunteers have shown that the immunomodulatory capacity of *Lactobacillus johnsonii* -1 and *Lactobacillus salivarius* 118 was not linked to an inflammatory type of immune response. Mucosal IgA was demonstrated as well as anticlostridial effect.

Table 2: Clinical pilot testing status during the second year period. Selected faecal/biopsy samples will be distributed to molecular analysis of GI-flora as well as conventional microbiology, hematological and immunological analysis.

Clinical pilot testing	PROBDEMO Strain	Current status
Healthy children	*Lactobacillus paracasei (ARLA)*	in progress
Children with atopic eczema	*Lactobacillus GG (VALIO),* *Bifidobacterium lactis (Chr.HANSEN),* *Lactobacillus paracasei (ARLA)*	demonstrated effect demonstrated effect planning phase
Children - prevention of diarrhea	*Bifidobacterium lactis (Chr HANSEN)*	in progress
Healthy adults	*Lactobacillus johnsonii (NESTLE),* *Lactobacillus salivarius (UCC),* *Lactobacillus paracasei (ARLA)*	demonstrated effect demonstrated effect planning phase for the Elderly
Adults with IBD (irritable bowel syndrome)	*Lactobacillus johnsonii (NESTLE),* *Lactobacillus salivarius (UCC),* *Lactobacillus paracasei (ARLA)*	planning phase in progress planning phase

Molecular methods

In order to demonstrate the applicability of the molecular methods the following three probiotic strains have been selected for a detailed characterization including *Lactobacillus rhamnosus* GG, *Lactobacillus paracasei* F19 and *Bifidobacterium lactis* Bb-12. The obtained sequences allowed for the design of species-specific probes and primers based on the V1 region (GG & F19) or the V2/V3 and V6 region (Bb-12) of the 16S rRNA. These probes and primers have been validated in dot blot hybridization and PCR experiments using pure cultures and are now being used in the clinical testing experiments. A suitable protocol was developed for the *in situ* detection of probiotic cells in pure cultures. This protocol has been adapted to study the detection of individual bacterial cells in fecal and biopsy samples. A fluorescein labelled eubacterial probe has been used to detect bacterial cells in fecal samples. A fluorescein-/Cy 3-labelled *Lactobacillus* probe has been applied in Fluorescent In Situ Hybridization (FISH) experiments in biopsy samples. The TGGE analysis from clinical testing in children revealed that the diversity of the microflora increases in time in the early stages of life of these young children (< 1,5 years old). In eczema trials, bands resembling *L. rhamnosus* GG and *B. lactis* Bb12 can be recognized.

Strain properties

Adhesive properties of the PROBDEMO strains have been tested via various adhesion assays carried out by Nestle, Turku University and VTT. One of the demonstrative purposes has been to show the link between *in vitro* adhesion assays, mucin assays and adhesion on colonic biopsies. This has been demonstrated with *Lactobacillus cripatus* M247 and the mutant strain of *L. crispatus* (MU5), lacking the receptor for the secreted aggregation protein normally gluing the cells together. No MU5 colonies could be found or identified in the samples tested, whereas the M247 strain showed adhesive behaviour on both cell-lines, mucin assay and the biopsy samples. It was also demonstrated that the adherence to human intestinal mucus appears to be strain specific and the age of the target group may be worthy of consideration when planning a schedule for probiotic food therapy. Preliminary screening on the hydrophobicity of probiotics strains with regard to their adhesive properties was started.

A review report as well as a newsletter on the Safety issues of probiotics was prepared during 1998: Salminen, S., von Wight, A, Morelli, L., Marteau, P., Brassart, D., de Vos, W., Fonden, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S-E., Mattila-Sandholm, T. Demonstration of Safety of probiotics - a review. 1998. Int. J. Food Microbiol. In press. Furthermore individual actions on safety are ongoing with PROBDEMO strains; as an example the plasmid content and stability as well as translocation in a mice model has been checked on Lactobacillus paracasei ssp paracasei F19. Lactobacillus rhamnosus strains, as well as some other lactobacilli, are known to be resistant to vancomycin -to confirm the safe nature of the vancomycin resistance genes of Lactobacillus rhamnosus strain GG, it was shown that its van genes were distinct from the van genes of enterococci.

Establishment of an *in vitro* model to test the immunogenicity of probiotic strains with regard to induction of pro-inflammatory and regulatory cytokines was started. New markers of immunomodulation were shown to be a useful tool to characterize probiotic activity of specific LAB strains. Methods to measure mucosal antibody levels using flow cytometry have been developed. *Lactobacillus salivarius* 118 has shown positive responses in mucosal immune response (IgA). Serum measurements of IgA, IgM, IgG as well as cytokines IL-1•, Il-1ß, IFNγ, TNF•, IL-4, IL-2 and IL-6 in healthy individuals receiving probiotics have this far not demonstrated any significant changes compared to control groups. Furthermore no differences in the phagocytosis activity of monocytes receiving 118 has been shown. No changes in haematological parameters have been demonstrated.

Fermentative properties

Products have been produced with the different probiotic strains alone and together with supporter cultures to fasten the acidification process. The supporter cultures used has been either a *Streptococcus thermophilus* culture (St 20) or a yoghurt culture (YC 380). Kinetics of acid production, number of different lactic acid bacteria, survival of probiotic strains, texture of the product and sensoric properties have been tested. Possibilities to stimulate the growth and acid production from the probiotic organism by the addition of glucose, yeast extract and milk protein fractions has been tested. Preliminary results from the trials were reported on the second Workshop of the Probdemo-project held in Cork, Ireland in October 1997. Products with and without supporter culture were produced and demonstrated for the meeting of the Probdemo group in Siena, Italy, on April 1998.

Large scale production

Chr. Hansen have in collaboration with Valio Ltd. prepared two general procedures for the production of all the strains in the PROBDEMO project, one for production of lactobacilli and bifidobacteria and one for the production of the lactococci strain. *The aim is to demonstrate that all the probiotic strains included in the project can be produced on a large scale with an acceptable yield and a good performance and viability.* The stability of batches of frozen and freeze-dried cultures are being followed for one year for each strain. Most of the tests are within their 6-9 month period and variations have been demonstrated with the stability with regard to temperature sensitivity of both frozen and freeze-dried cultures.

Pilot scale production of Probdemo project strains as a starter powder has been carried out, based on standard procedures. No optimisation of the production process for an individual strains will be included in this project. As far nine months storage stability of the concentrated, freeze-dried powder has been demonstrated. It was interesting to see, that the *L. crispatus* mutant strain did not tolerate the production process of the starters properly, but those cells which survived in the production process, also survived during nine months storage time.

Dissemination of knowledge

The workshops and newsletters and other publications are considered to be of utmost importance for the dissemination of project results since the main purpose of the programme is to demonstrate the knowledge for the External Audience. The dissemination has been established by annual workshops (*Workshop* 1 was held on the *Safety of probiotics* in Helsinki, FI 1996, Workshop 2 was on *Novel probiotic research tools* in Cork, IE October 1997). Two Newsletters have been published (Newsletter 1, Newsletter 2) as well as a FLAIR-FLOW leaflet. Numerous inquiries are being constantly addressed to the coordinator - these derive from the workshops, FLAIR-FLOW dissemination as well as the high number of project presentations.

Achievements

The following achievements can be listed this far:

1) Scientific selection and safety criteria (*Newsletter 2)* of listed probiotic strains.

2) Well-planned clinical pilot protocols.

3) Clinical pilot testing on children with viable *Bifidobacterium lactis Bb-12* and *Lactobacillus GG* resulted in a significant improvement of eczema (atopic dermatitis).

4) Clinical pilot testing with healthy human volunteers showed that the immunomodulatory capacity of *Lactobacillus johnsonii -1* and *Lactobacillus salivarius 118* was not linked to an inflammatory type of immune response. The beneficial immune effects were demonstrated by showing mucosal IgA response in probiotic treated groups .

5) An antagonising effect of *Lactobacillus johnsonii* Lj-1 and *Lactobacillus salivarius* 118 on *Clostridium perfringens* was shown in clinical testing on healthy adults.

6) Novel methods for adhesion studies were compared and developed on cell lines, mucin assays and biopsies. Human intestinal mucus appears to be strain-specific and the age of the target group may be worthy of consideration when planning a schedule for probiotic therapy. Specific strain

surface properties (aggregation) are important factors in adhesion and colonisation as demonstrated with *L. crispatus* M247 and the mutant strain MU5.

7) Full nucleotide sequences of the selected probiotics and methods to handle faecal samples and biopsies for molecular analysis, methods to measure survival and population dynamics in faecal samples and biopsies have been developed.

8) Protocols for fermentative properties of probdemo strains have been developed.

9) Pilot scale and technical viability protocols for Probdemo strains have been established - the aim is to demonstrate that all the probiotic strains included in the project can be produced on a large scale with an acceptable yield and a good performance and viability.

10) Production of a series of demonstration workshops and newsletters and manuscripts.

Future actions

Pilot testing on children

Enrolment progress has been completed as planned and the detailed clinical parts of the study will be reported this year. Parameters for immune function assessment and intestinal microflora and metabolic activity assessment will be further refined for the future pilot clinical studies. A pre-pilot test on healthy children has started with *Lactobacillus paracasei* ssp *paracasei* F19. The test is performed to investigate the survival of the bacteria in the gut of the children and to demonstrate the tolerance of the children to the bacteria. A diarrheal prevention test is in progress with *B. lactis* on day-care centre children.

Pilot clinical testing on elderly

The impact of *Lactobacillus paracasei* ssp *paracasei* F19 on the well-being of elderly will be demonstrated. The test will be conducted together with the Karolinska Institute on healthy elderly people living in a home for elderly in Stockholm. The impact on well-being, the function of the GI-tract and microbial flora in the gut will be studied. Healthy elderly persons having a diminished acid secretion in the stomach due to colonisation by *H. pylori* will be included in the study.

Gastrointestinal disorders

To facilitate the human studies, probiotic feeding trials have been conducted in IL-10 knock out mice in order to study the effects of probiotic consumption (*L. salivarius* 118) 16 weeks on modulating gut flora and onset of IBD. Future actions will focus on probiotic preparations and Crohn's disease patients for 6 week feeding period as well as probiotics for Crohn's disease in remission. This far the ethics approval committee has been granted for the trials and patient recruitments will commence in the near future.

Series of experimental trials on colonoscopy patients consuming whey drinks fermented with further strains from the PROBDEMO group except *Lb. crispatus* strains M247 and MU5 will progress. Another line for future research is the adhesion of probiotic strains to the healthy and diseased part of colon with partial ulcerative colitis. The outcome from these trials helps in planning of actual clinical experiments on the effects of probiotic strains on the symptomology of inflammatory bowel disease.

Molecular methods

The project proposes to demonstrate the applicability of novel molecular methods to determine the fate and activity of probiotic bacteria in the human gastrointestinal (GI) tract after their administration via food products. By using molecular techniques, culturing of the ingested cells is not a prerequisite and cells which become non culturable during their passage and residence in the human GI tract can still be detected. The application of molecular techniques will complement the conventional plating approaches and in many cases will be faster and more reliable. Moreover, the activity of probiotic cells in the GI-tract can be determined as well as the expression of specific genes by hybridization of specific molecular probes to rRNA or mRNA.

Technological properties

Establishment of an *in vitro* model to test the immunogenicity of probiotic strains with respect to the induction of proinflammatory and regulatory cytokines will be established - different probiotic strains will be tested for their ability to induce a specific cytokine pattern.

Future demonstration on technological viability will progress. The evaluation of fermentative properties of PROBDEMO strains will continue. Workshop 3 in Haikko, October 1-2, 1998 will provide an exhibition panel for products with different properties. Demonstration of the influence of large scale production on adhesion properties of selected number of probiotic strains will start. It is important to find out if the probiotic manufacture and propagation in fermented milk has an influence on the probiotic properties of the strain.

Dissemination of knowledge

Workshop 3 will be held in Haikko, Finland Oct 1st to Oct 2rd, 1998 entitled *Functional Food Research in Europe*. Also a pressforum and panel statements of the following issues will be held: 1) Probiotic safety, 2) Regulation and legal aspects, 3) Consumer aspects, 4) Clinical use of probiotics, 5) Marketing images and visions, 6) *In vitro* methods, 7) Animal models, 8) Selection criteria, 9) Immunological effects of probiotics on healthy consumers. The workshop includes also all the FAIR projects on functional foods as presented in their state-of -art of today. *Workshop* 4 - 5 for 1999-2000 are in the stage of planning (the aim is to combine the end-seminar with STARLAB).

List of publications

First reporting period:

Alander, M., Mattila-Sandholm, T. (eds). 1996, Selection and Safety Criteria of Probiotics. 1st Workshop, Demonstration of the Nutritional Functionality of Probiotic Foods, FAIR CT96-1028.

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Increased consumption of fruits and vegetables within the EU: Potential health benefits

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Abstract

A Core Human Study was carried out in Ireland, Northern Ireland, Spain, France and Holland. Response of volunteers to increased intake of carotenoid components of fruits and vegetables was determined using a wide range of biochemical and physiological measurements. Particular attention was paid to whether the antioxidant capability of •-carotene, lutein and lycopene, demonstrated in vitro, was apparent in relation to increased oxidation resistance of low density lipoprotein (LDL) ex vivo. Comparison of baseline oxidative and antioxidant status, and levels of consumption of vegetables and fruits and, hence, carotenoids, between volunteers from different countries was an important element of this research.

Complementary Studies were also performed at each centre. These included: protective effects of carotenoid-rich foods against LDL and DNA oxidative damage; carotenoid bioavailability; barriers to increased vegetable consumption; serum carotenoid profiles and carotenoid content of fruits and vegetables most frequently consumed in the EU.

Carotenoid supplementation did not influence the oxidation resistance of LDL. However, increased consumption of carotenoid-rich fruits and vegetables was associated with increased LDL oxidation resistance, and higher plasma concentration of total and specific carotenoids (pre-supplementation) was associated with lower levels of DNA damage. These, and other results, are outlined in more detail below.

Background

We have long known that low intake of essential nutrients creates predisposition to deficiency disorders. However, we are only beginning to understand that many previously unappreciated components of our diet may prove important in promoting wellbeing and preventing a range of

chronic diseases. Within the last decade, as research into the relationship between diet and health has proliferated, so too has the body of data from epidemiological and experimental studies that indicate vegetables and fruits contain substances which appear to reduce the risk of cancer and cardiovascular disease.

Fruits and vegetables are rich in antioxidant compounds and it is proposed that antioxidants such as vitamins E and C and pro- and nonprovitamin A carotenoids, reduce the risk of chronic diseases by protecting against free-radical mediated damage. Low density lipoprotein (LDL) is particularly susceptible to free radical attack.

In early atherosclerotic lesions, cholesterol-laden macrophages are found accumulated in the sub-endothelial space of the artery. As the disease progresses most of these cells die, generating fatty streaks that may ultimately develop into arterial plaque. LDL which has become oxidatively modified has properties that could explain the formation of these lipid laden macrophages. The hypothesis is that the chemotactic properties of oxidised LDL (oLDL) within arterial tissue results in accumulation of blood monocytes which penetrate the artery wall and differentiate into macrophages. If the oxidative damage to LDL is sufficient to damage the protein moiety, the macrophage take up oLDL via the scavenger receptors. Uptake by scavenger receptors is unregulated, unlike tightly controlled LDL receptor uptake, thus large numbers of lipid-laden macrophage accumulate forming a fatty streak.

Based on studies demonstrating the atherogenicity of oLDL, it is possible that fatty streak formation can be prevented, or reduced, by antioxidants that are capable of protecting LDL against oxidation. The most abundant natural antioxidant in LDL is α-tocopherol but other substances with antioxidant activity have been identified: for example, the carotenoids β-carotene, lycopene, cryptoxanthin, zeaxanthin and lutein. Of the 600 or so carotenoids found in nature, about 40 are regularly consumed by humans, largely in the form of coloured fruits and vegetables and, of these 40 carotenoids, those mentioned above are the most easily identifiable in human blood. Although much research has focused on the biological properties of β-carotene, this carotenoid may not be particularly effective at high oxygen tension such as found in blood and lungs. There are indications that lycopene is a more efficient quencher of single oxygen and, that lutein is more efficient in quenching lipid peroxyl radicals. Despite these findings the role of the nonprovitamin A carotenoids in preventing oxidative modification of biomolecules has not been adequately determined. In this project we sought to determine whether supplementation of the diet with β-carotene, lycopene or lutein, alone or in combination with vitamin E, increased the oxidation resistance of LDL.

There are also suggestions that antioxidants in fruits and vegetables may reduce risk of certain cancers. Carcinogenesis is a complicated multi-stage process which is assumed to proceed via a three-stage model. Initiation seems related to DNA alteration. Thus a major protection against cancer initiation may reside in maintaining low rates of damage with efficient repair. Initiation is followed by promotion and the final stage is the development of a pre-malignant into a malignant lesion. In view of the importance of DNA damage to carcinogenesis, agents capable of reacting with, and chemically modifying, DNA are potential carcinogens. For example, hydroxyl radical attack generates a whole series of modified bases and a multiplicity of other products. As yet, specific types of hydroxyl radical damage to DNA that participate in carcinogenesis have not been identified but it may be that faulty repair of free radical induced lesions is responsible. There is little doubt that oxidant stress can cause DNA damage. Conceivably, therefore, unless the stress is sufficient to kill

the cell, oxidants could be involved in initiation, promotion or progression of cancer. This potentially cancer-inducing oxidative damage might be prevented, or limited, by dietary antioxidants.

Several authors have reviewed the extensive literature suggesting a protective role for higher vegetable and fruit consumption in cancer incidence and the epidemiological evidence for a negative association between the consumption of these foods and reduced risk is impressive. There are also convincing associations between low plasma concentrations of β-carotene and higher cancer risk. However, any observed protective effect of a particular diet is usually ascribed to a nutrient(s) in which the researcher has a particular interest, but for many of the studies performed it is not possible to be confident that a particular factor is the effective agent. In this project we set out to determine the range of protective effects of specific carotenoids against DNA damage.

Despite the fascination of researchers for studying cell and tissue response to synthetic carotenoids, or those isolated from the food matrix, we are well aware that consumers and the food industry are seeking information on foods which will contribute to a healthier diet. Thus, in this project we have considered response to isolated carotenoids and foods and diets rich in those carotenoids; contributed to a more extensive database on the carotenoid composition of fruits and vegetables, assessed relative carotenoid intakes and quantified plasma concentrations in similar adult population groups from five regions within the EU, performed a preliminary examination of barriers to increased vegetable consumption in seven regions within the EU, and provided information on carotenoid bioavailability and interactions.

Specific objectives and results

* to determine whether increased intake of the carotenoids P-carotene, lutein and lycopene increases the oxidation resistance of low density lipoprotein;

Apparently healthy, male and female adult volunteers with normal biochemical profile, not taking any regular medication or supplements, having a stable lifestyle, and consuming diets typical of the region were recruited from 5 regions: Coleraine (Northern Ireland), Grenoble (France), Zeist (Netherlands), Cork (Ireland) and Madrid (Spain); approximately 80 volunteers per centre. A wide range of biochemical measurements were made on blood and urine samples. Volunteers were supplemented with α-tocopherol for 4 weeks (100 mg/d); followed by supplementation with palm oil carotenoids (predominantly β- & α-carotenoid), lutein or lycopene for a further 12 weeks (approx. 15 mg carotenoid/d), followed by a combined supplement of the single carotenoid (palm oil, lutein or lycopene) +α-tocopherol for a further 4 weeks (approx. 15 mg carotenoid and 100 mg vitamin E/d). The study was placebo controlled. See Fig. 1 and Table 1 for study design and analyses performed, respectively.

Supplementation with α-tocopherol resulted in an increase in α-tocopherol serum levels, while producing a strong decrease in g-tocopherol. Supplementation with lutein elevated serum lutein and zeaxanthin (approximately 5 and 2-fold, respectively). Supplementation with lycopene, also containing β-carotene, phytoene, phytofluene, Ecarotene, resulted in a 2-fold increase in lycopene and, on average, a 30-50 % increase in β-carotene levels. Supplementation with palm fruit β-carotene, also containing α- carotene, resulted in a 5-fold and 14-fold increase in β-carotene and α-carotene, respectively.

Supplementation (weeks)

0* 4* 16* 20*

1. [--- vit. E 100 mg---][palm oil carotenes 15 mg/d][vit. E + palm oil carotenes]

2. [--- vit. E 100 mg---][-----lutein 15 mg/d ----------][------ vit. E + lutein ----------]

3. [----vit. E 100 mg---][----lycopene 15 mg/d -------][------ vit. E + lycopene -----]

4. [--- vit. E 100 mg---][--------placebo ----------][-------vit. E + placebo ------]

* Blood & urine samples were taken at weeks 0, 4, 16 & 20 for analysis as described in Table 1.

Volunteers underwent initial biochemical & lifestyle screening before random allocation to one of four groups. Treatment groups contained similar numbers of male and female volunteers (n = 20 per group, 10 male & 10 female; 80 per country for 5 countries; 25 to 45 years of age; non-smokers; normal biochemical profile; stable lifestyle). Volunteers received supplements of α-tocopherol, and/or palm oil carotenes or lutein or lycopene as described above.

Figure 1: Core Study Protocol (5 countries; FR, GB, IE, NL & ES)

Table 1: Analyses

Water-soluble antioxidants;	Markers of protein damage;
ascorbic acid (plasma)	thiols (plasma & whole blood)
uric acid (plasma)	
Lipid-soluble antioxidants;	Lipid damage;
carotenoids (serum, LDL, HDL)	fatty acids (LDL)
retinol (serum, LDL, HDL)	aldehydes (LDL)
tocopherol (serum, LDL, HDL)	malondialdehyde (LDL, urine)
	Cu-stimulated oxidation (LDL)
Antioxidant enzymes cofactors;	DNA damage & repair (lymphocytes);
superoxide dismutase (RBC)	
glutathione peroxidase (RBC)	strand breaks
Zn (plasma)	enzyme sensitive sites
Cu (plasma)	8-hydroxy deoxyguanosine
Se (plasma)	

Despite these increases in serum concentrations, there was no evidence that supplementation of the diet, with any of the carotenoids or vitamin E, increased LDL oxidation resistance in an ex vivo test (Cu-stimulated oxidation).

* the influence of increased carotenoid intake on biomarkers of oxidative status;

Carotenoid supplementation had no consistent effect on biomarkers of oxidative status plasma vitamin C, vitamin E, copper, selenium or zinc; glutathione; protein oxidation products; lipid oxidation products; antioxidant enzyme activity; and DNA damage.

* whether there is a synergistic effect between carotenoids and vitamin E;

There was no significant effect on LDL oxidisability and other biomarkers of oxidative and antioxidant status when carotenoids were provided in conjunction with vitamin E.

* differences in carotenoid intake & status in adults from different countries;

The French had a significantly higher intake of total carotenoids than other participating centres. Northern Ireland, Ireland and The Netherlands all had similar total dietary carotenoid intakes with the Spanish having a lower intake. The French had the highest intake of both β-carotene and lycopene with the Spanish having the lowest intake of all carotenoids except lutein (+zeaxanthin) and β-cryptoxanthin.

Carrots were the main source of β-carotene in the diets of the volunteers in all countries except Spain where spinach was of slightly greater importance. Carrots also contributed between 60 % (Spain) and 90 % (Ireland) of the α-carotene in diet. There was a greater diversity of vegetables contributing to the α-carotene dietary intake in the Spanish diet, however, the intake of α-carotene in the Spanish sample was significantly lower than that in the other European countries.

The main sources of lutein in Northern Ireland and Ireland were peas, broccoli and eggs, respectively. Spinach was the main source of lutein in the other three countries where it contributed 34 % of lutein in the Spanish diet, 30 % in the Dutch diet and 31 % in the French diet. Other foods which contributed to lutein intake varied between countries: for example, in the Spanish and French diet other main sources of lutein were lettuce and eggs, whilst broccoli and peas were good sources in the Dutch diet.

Fresh tomatoes were the main source of lycopene. Tinned tomatoes, pizza, tomato soup and tomato puree were other contributors to intake. In Spain tomato puree was the only other main source of lycopene (apart from fresh tomatoes), contributing 42 % of intake. For the other four participating countries, tinned tomatoes contributed between 9 % in the Dutch diet and 23 % in the Irish diet. Pizza contributed approximately 16 % of the lycopene intake in Northern Ireland, France, The Netherlands and Ireland, and tomato soup contributed 10 % in the French diet and 29 % in the Dutch, Northern Irish and Irish.

Oranges, orange juice, and tangerines were the main sources of β-cryptoxanthin at all centres; contributing between 86 and 100 % of this carotenoid.

Examination of relationships between calculated dietary intake of carotenoids and plasma concentrations indicated that β-cryptoxanthin and α-carotenoid gave the strongest association between diet and blood values. The higher carotenoid intake by the French volunteers was associated with a higher serum total carotenoid concentration. Mean serum lutein concentration was highest in France; serum β-cryptoxanthin was highest in Spain; serum trans-lycopene was highest in

Irish males and lowest in Spanish males (females were similar at all centres and total lycopene was similar in both sexes between centres): serum β- and α-carotene concentration was highest in French volunteers.

* differences in antioxidant and oxidative status more generally,

There were no significant differences between plasma vitamin C concentrations between centres. Plasma copper concentration was lowest in Spain and plasma zinc concentration was highest in Northern Ireland. There were no significant differences in plasma selenium between centres.

Plasma thiol concentration tended to be lowest in Spain. There were no significant differences in fasting whole-blood total-glutathione between centres. However, fasting whole-blood reduced-glutathione concentration was highest in France and Spain.

There were no consistent differences in mean glutathione peroxidase activity, and no differences at all in mean superoxide dismutase activity, between centres.

Urinary malondialdehyde was lowest in the French volunteers, however, MDA tended to be higher in their LDL, whist other aldehydic products tended to be lower.

* the influence of carotenoid status on biomarkers of DNA damage;

Examination of lymphocytes obtained from the Spanish part of the Core Study indicated that there was no evidence for any protection against DNA damage in these cells resulting from the carotenoid or vitamin E supplementation regimen. However, significant correlation's were seen between specific sites of DNA damage and total carotenoids, lutein, β-carotene and lycopene, and the correlation's tended to be stronger at week 0 of the Core Study (baseline) than week at 16 (after supplementation). There were no significant correlation's between serum vitamin C, vitamin E or β-cryptoxanthin concentrations and the level of DNA damage.

Because of practical difficulties, samples of lymphocytes from all but the Spanish centre had to be pooled for estimation of DNA damage. Analysis of these pooled samples indicated that before supplementation the volunteers from different centres were equivalent. However, there was a striking difference between Irish male and female values, with men showing twice as much damage in lymphocyte DNA compared to women, who had similar levels to those seen in other countries.

Further Complementary Studies, demonstrated that rapid recovery of lymphocytic DNA from oxidative damage correlated with an elevated plasma β-carotene concentration.

* whether increased intake of carotenoid rich foods increased the oxidation resistance of low density lipoprotein in smokers and non-smokers;

Increased intake of carotenoid-rich fruits and vegetables (equivalent to 20-30 mg carotenoids per day for 2 weeks) increased the oxidation resistance of LDL.

* factors influencing the absorption and metabolism of β-carotene;

Analysis of triglyceride-rich fractions, isolated from plasma following the consumption of carotenoid-rich isolates or foods, demonstrated that lutein, if present at a relatively high dosage as compared to β-carotene, can interfere with β-carotene absorption, but not cleavage. There is also evidence that high intakes of β-carotene may reduce lutein absorption. Carotenoid absorption from food sources is generally lower than from isolates. The greatest difference was observed between absorption from isolated β-carotene vs. β-carotene absorption from a meal of carrots.

* barriers to increased vegetable consumption;

A questionnaire was administered to Core study volunteers, plus similar volunteers from two further countries (Italy and Germany). While 75 % of the respondents believe people should eat at least two pieces of fruit per day, only 55 % of the respondents ate the recommended quantity. 38 % ate only one piece of fruit per day, 8 % of the respondents ate less, or did not eat fruit at all. Fruit consumption was lowest in the Netherlands and France, and highest in Spain and Italy.

Most respondents (78 %) believed people should eat three or more portions of vegetables per day. Only 62 % report actually eating three or more portions daily. The quantity of vegetable consumption appeared highest in Italy. The frequency of buying and using (fresh) vegetables was highest in the Netherlands, where 33 % of the respondents bought fresh vegetables almost every day (5 % in the other countries).

Half of the respondents bought and consumed prepared vegetables at least once a week. Both frozen and canned vegetables were used weekly in 46 % of the households, and jarred vegetables used weekly in 21 % of the households. Most people used the different types of prepared vegetables within a few months after buying. Respondents gave a comparative evaluation of fresh, frozen, canned and vegetables in jars, based on their perception of various aspects. The evaluations in different countries were fairly similar. Fresh vegetables were considered to have the highest nutritional value, the best taste and the best appearance before and after cooking. Canned vegetables scored lowest on nutritional value, taste and appearance, but were considered most advantageous on storage, preparation, availability and price. Frozen vegetables were perceived to have a better nutritional value, taste and appearance than canned and jarred vegetables.

There was almost 100 % agreement in all countries on the nutritional value of fruit and vegetables. Everybody seemed to know that fruit and vegetables are good sources of vitamins. On the barrier side we found the following factors which should be taken into consideration in order to trigger increased fruit and vegetable consumption:

Family acceptance: A notable part of the respondents showed positive intentions towards eating more fruit and vegetables, but many found it difficult to include more in their diet. The most important factor was preferences of children and partners. In all countries the majority of respondents with children expressed a wish that their children would like vegetables more. In addition, one third of both men and women wished that their partner would like vegetables more. Respondents would try new vegetables if they thought others in the family would like them. Taste is obviously a major issue when considering ways to increase vegetable consumption and solutions have to be sought for in either introducing new ways of preparation or new vegetables.

Storage: Of the respondents, 44 % said they would eat more fresh vegetables if they were easier to store, and 27 % of the respondents (particularly in Germany and France) said they would eat more frozen vegetables if they had more room in the freezer.

Concern with contamination: Pesticides evoked the greatest concern in all countries. Concern with contamination during transport and in the shops was considerable in France and Spain.

* an improved database for the carotenoid composition of vegetables and fruits;

Additional analysis of food has been undertaken and new information on the effects of cooking procedures produced.

* improved dietary information;

These results demonstrate that supplementation with several carotenoids did not appear to influence a range of biomarkers of oxidative status. A response was observed to carotenoid-rich foods. These results re-focus attention on the importance of eating a healthy diet, containing higher amounts of fruits and vegetables.

* To promote collaboration and technology exchange between EU scientists and between scientists and the European food industry,

This project would not have been possible without effective collaboration, and technology exchange, between the European scientists and food industries involved.

* To harmonise protocols and methods to facilitate EU-wide comparison.

A 'batch analysis' approach (each centre being responsible for specific analyses) was used in this project. This eliminated interlaboratory variation in analytical results, and allowed comparison of values between population groups.

Conclusion

This project has demonstrated the potential health benefits of increased consumption of vegetables and fruits; significant relationships between plasma concentrations of specific carotenoids (but not other major antioxidant vitamins) and reduced DNA oxidative damage and significant reduction in LDL susceptibility to oxidation ex vivo. In as much as different patterns of carotenoid and fruit and vegetable intake, and different plasma carotenoid profiles, were observed in two regions (France and Spain) having lower chronic disease rates, benefit cannot be ascribed to any particular carotenoid; although it was notable that the consumption of lutein-rich vegetables was highest in these two regions. The lower total consumption of carotenoids, and lower plasma total carotenoid concentration, in Spanish volunteers has been observed in other recent Pan-European studies.

When interpreting the results of this project it is important to recognise that each group of volunteers was not necessarily representative of the overall population of their respective countries. It is known that the dietary habits in the South of France varies from the North, and that the profile of food intake in Catalonia is different from the overall Spanish diet. The use of the biomarkers of oxidative stress, used in this study, are also relatively new and their validity is still in question. The results gained as part of this project will contribute to the debate on the usefulness of such biomarkers.

The association between high levels of fruit and vegetable and lower risk of chronic disease is extremely strong, stronger than for calories, fat or fibre. It is highly likely therefore that there are multiple reasons for this association of which the carotenoid content of the diet may only be one small part. If emphasis is placed on achieving tissue concentrations of carotenoids seen in those populations with lower risk of chronic disease via increase fruit and vegetable intake, this will also have the effect of increasing intake of all potentially beneficial compounds present in these foods.

These results re-focus attention on the importance of eating a healthy diet, containing higher amounts of fruits and vegetables. They provide support for recommendations to increase fruit and vegetable intake, and for those seeking to implement such recommendations. However, issues of family acceptance and taste evaluation need to be tackled in order to develop strategies for increasing vegetable consumption in the EU.

The results obtained for volunteers from different countries within the EU in this project would not have been possible without the use of the common methodology and centralised analysis supported by funds from the Commission of the European Communities, Agriculture and Fisheries specific RTD programme.

It is obviously not possible in this short abstract to describe, or discuss, in detail the methods used and results obtained in both Core and Complementary activities of this project. Papers are appearing in the literature and current publications are listed at the end of this abstract. For additional information please contact Sue Southon.

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Session 3:

Meat

(special FLAIR-FLOW contribution)

Predicting beef quality at the early post-mortem period

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Abstract

One of the greatest challenges to the meat industry, in the present time, is the ability to predict and guarantee the quality of their final product. Many biochemical factors and physical measurements demonstrate powerful predictive ability in the early *post-mortem* period. Some of these have previously been recorded while others involve both assessment of novel meat components and the application of new and existing technological methods. Proteolytic degradation of myofibrillar proteins is being investigated and several components which may contain predictive information of meat quality are currently being employed in the development of immunoassays. It is hoped that this will lead to the production of a rapid detection kit for use in the factory. The ability NIR spectra to differentiate between tough and tender meat has been demonstrated. NMR has been effective in revealing a redistribution of water over the ageing process. Ultrasound, autofluoresence spectroscopy and image analysis are being investigated for prediction of both compositional and textural features of meat. Rigometry and early *post-mortem* measurements of pH, conductivity, impedance, Warner Bratzler shear force and colour are also under investigation as for their predictive potential. Many of these measurements show much promise as early *post-mortem* indicators of meat quality. Analysis is continuing to determine the practical usefulness of these indicators in an industrial situation.

Biochemical markers

Proteases

The biochemical mechanisms involved in meat tenderisation during meat ageing have been extensively researched (Taylor, 1995 & Koohmaraie, 1996). Proteolysis of key myofibrillar proteins and associated collagenous or skeletal proteins has been suggested to be the cause of meat tenderisation. Collagenous proteins form the support structure within and between myofibrils, between myofibrils and the sarcolemma, and link muscle cells to basal lamina (Taylor, 1995). Takahashi (1996), however, has proposed a non-enzymatic mechanism of meat tenderness. Two major systems are recognised, by many, for their role in meat tenderness development and in meat flavour onset. These are the cathepsin and calpain systems. A third protease system which consists of the aminopeptidases is involved in meat flavour onset. These proteases are sensitive to pH, temperature, ionic strength and specific ions. Despite their importance, both systems are very complex and difficult to measure as they exist in close association with powerful inhibitors. These protease systems and their inhibitors have been monitored to gain a more in-depth knowledge of the relationship between them and both technological and sensory attributes of meat. While the usefulness of some of these protease systems as indicators of quality have been questioned measurements of their inhibitors may hold more information. Calpastatin activity at 24h *postmortem* has been correlated with Warner Bratzler shear force values (Whipple *et al*., 1990 & Shackleford *et al*., 1991). Zamora *et al*., (1996) recently concluded that calpastatin activity at 1hr *postmortem* gave the best correlations of all the elements of the calpain system with toughness, i.e. tougher meat had higher levels of activity of calpastatin.

Protease activity has been applied to pork meat quality as well as that of beef. The large variability in pork meat quality has been characterised by pH, drip loss and colour to give rise to five pork quality classes. These classes have been assigned the following names pale, soft, exudative (PSE), red, soft, exudative (RSE), pale, firm, exudative (PFE), red, firm non-exudative (RFN) and dry, firm, dark (DFD) (Chizzolini *et al*., 1996 & Garrido and Honikel, 1996). Muscle aminopeptidases and cathepsins have been shown to be effective in distinguishing between these quality classes (Table 1).

Table 1: Use of aminopeptidase and cathepsin proteolysis in the identification of pork quality classes.

QUALITY CLASS	PROTEOLYTIC ACTIVITY
DFD	Evidences of low breakdown process
RSE	Evidence of high breakdown process.
RFN	Evidence of intermediate breakdown process

RSE - red, soft, exudative: RFN - red, firm non-exudative: DFD - dry, firm, dark.

Results have shown that the activities of both cathepsins B and B+L were significantly higher in PFE than in RSE and RFN samples. Both aminopeptidase B and alanyl aminopeptidase exhibit higher rates of activity in both DFD and RFN than in RSE and PSE classes. Further assessment of these systems in both pork and beef will determine their practical usefulness in the assessment of meat quality.

Proteolytic fragments

Electrophoretic techniques such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and capillary zone electrophoresis (CZE) have been employed to identify proteins and proteolytic fragments which vary during the ageing of meat. SDS-PAGE can separate proteins on the basis of their molecular weight while CZE separates compounds on a charge to mass ratio. As muscle proteins are broken down the disappearance of the parent compound can be traced as can the appearance of the proteolytic fragments. The amino acid composition of these fragments can be determined by sequence analysis and computerised data basis used to confirm their origin (Tsitsiloni *et al*., 1996; Troy *et al*., 1997; Casserly *et al*, 1997 & Mullen *et al*., 1998a). Both the parent compounds and fragments will be quantified and correlated with quality attributes to determine the usefulness of their dynamics in predicting meat quality. SDS-PAGE analysis has revealed that myofibrillar proteins such as troponin T, desmin and nebulin are degraded while proteolytic fragments such as the 30kDa and 110kDa components increase over the ageing process. To date one of the principle degradative changes detected on examination of SDS-PAGE of myofibrils is the appearance of a 30kDa component which is also positively correlated with tenderness (Olson *et al*., 1977 & Utterhaegan *et al*., 1992). Sequence analysis of this component has shown high homology with troponin T (Table 2), (Tsitsilonis *et al*., 1996).

Troponin T is located close to the Z-line in the myofibrillar structure and may impart stability to this region which is progressively degraded during meat ageing (Hendrick *et al*., 1975). The 110kDa fragment, has revealed high homology with C-protein (Table 2), (Tsitsilonis *et al*., 1996 & Casserly *et al*., 1997), which serves to act as a clamp to hold and stabilise bundles of myosin molecules together in the thick filament (Bailey, 1982). The degradation of C-protein could partly result in loss of myofibrillar structural integrity such as the disappearance of the transversal alignment of the Z-disc (Taylor *et al*., 1995). Other fragments of interest, which exhibit changes over the ageing

process, are currently being investigated by this technique. Proteins such as nebulin, desmin and titin are also being assessed. Degradation of nebulin, for example, over the ageing process, could trigger alterations in the myofibril which could influence the tenderness of the meat (Taylor *et al*., 1995).

Table 2: Comparison of sequenced primary structure segments of bovine proteins with known proteins. Fragment represent myofibrillar fragments obtained using SDS-PAGE.

Molecular weight myofibrillar fragment	Identified sequence	Identified parent compound	Homology
30kDa	**EVHEPEEKPR-PRLLAAPKIP-EGEKVDFDDIQ**	**Troponin-T** (52-68, rabbit troponin T, fast skeletal muscle isoforms)	70%
110 kDa	**EQPEVDVWEL-LKNAKPSEYE-KIAFQYG**	**C-Protein** (203-229, human, myosin binding protein,slow-type muscle)	88%

Methods are currently being developed, using CZE, to analyse the soluble components of beef over the ageing process. One such method has lead to the isolation and analysis of some specific peptides, which have revealed good correlations with the pork quality classes. Profiles of these soluble components have been obtained using another method incorporating an acid phosphate buffer. This method is currently being standardised to allow for identification and quantification of these fractions, which can be subsequently correlated with meat quality.

HPLC analysis provides a rapid and sensitive tool for the isolation and identification of many compounds. As a result of HPLC analysis of sarcoplasmic components of bovine LD three components, which increase over the ageing process, have been identified (Figure 1).

These components have been assessed by mass spectrometry and amino acid sequencing to reveal their identity (Table 3). One of these fragments (1283.5Da) revealed an 87.5% homology with glyceraldehyde-3-phosphate dehydrogenase. Comparison of the 1722.9Da component with the known troponin T structures revealed an average identity of 50% confirming results obtained by other authors, (Nakai, *et al*., 1995). A higher molecular weight fraction, 5572Da demonstrated 100% homology with creatine kinase. Creatine kinase (43kDa) is a myofibrillar protein which is localised at the M-line and appears to be bound to M-protein as a 1:1 complex (Turner *et al*., 1973).

Free amino acids and natural dipeptides from porcine *M.longissimus dorsi* have been isolated and quantified by HPLC analysis. Significant differences were observed in the content of some amino acids and the level of carnosine and anserine between quality classes.

The content of free and bound amino acids and peptides were monitored in soluble extracts from bovine LD (Mullen *et al.* 1998b). Samples were assayed at 1hr, 1d, 3d, and 14d *post-mortem* using an amino acid analyser. Significant increases were observed in these parameters over the ageing process (Figure 2). Samples have also been extracted at 6h, 24h and 48h *post-mortem* in order to determine the relationship between these peptides and amino acids and meat tenderness and flavour.

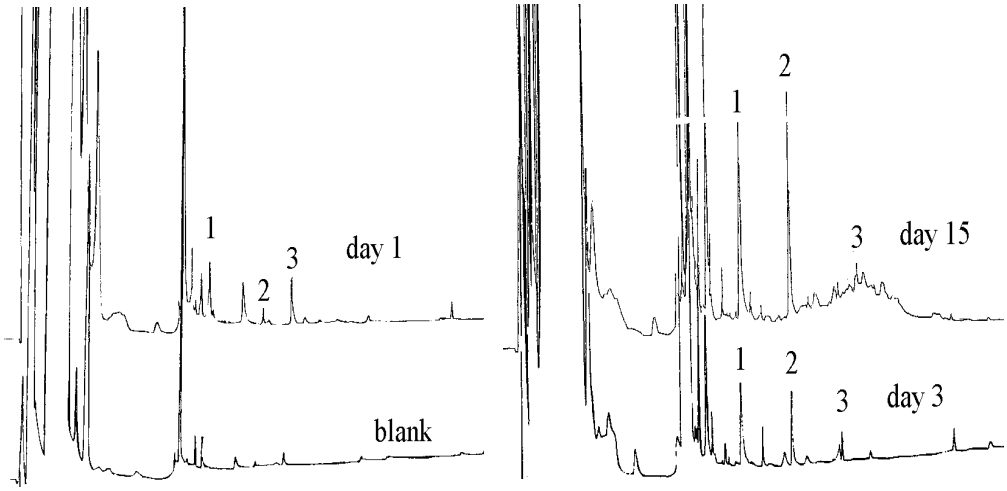

Figure 1: HPLC profiles of soluble components extracted from bovine *M.longissimus dorsi*, during ageing for 15 days. Peaks labelled 1-3 were collected for further identification.

Table 3: Comparison of sequenced primary structure segments of bovine proteins with known proteins. Fragments represent soluble fragments isolated using HPLC analysis

Molecular weight soluble fragment	Identified Sequence	Parent Protein	% homology
1283.5 Da (Fraction 1)	KVVKQAS-EGPLK	Glyceraldehyde-3-phosphate dehydrogenase	
		(258-265) rabbit, mouse, pig	87.5%
		(260-267) human	87.5%
1735 Da (Fraction 2)	APPPPAEVPEV-HEEVH	Troponin T	
		(29-42) quail and chicken	56.6%
		(39-54) rat	37.5%
5570 Da (Fraction 3)	DPIIQDRHGGF-KPTKKHKTDL-NHENLKGGDD-LDPNYVLS	Creatine Kinase	
		(4-42) rat muscle	100%
		(90-128) rat skeletal muscle, rabbit M chain	100%
		(90-128) human M chain, chicken muscle, T.calfornica, T.marmorata, trout, G.gallus, dog brain, rabbit brain, mouse.	97%

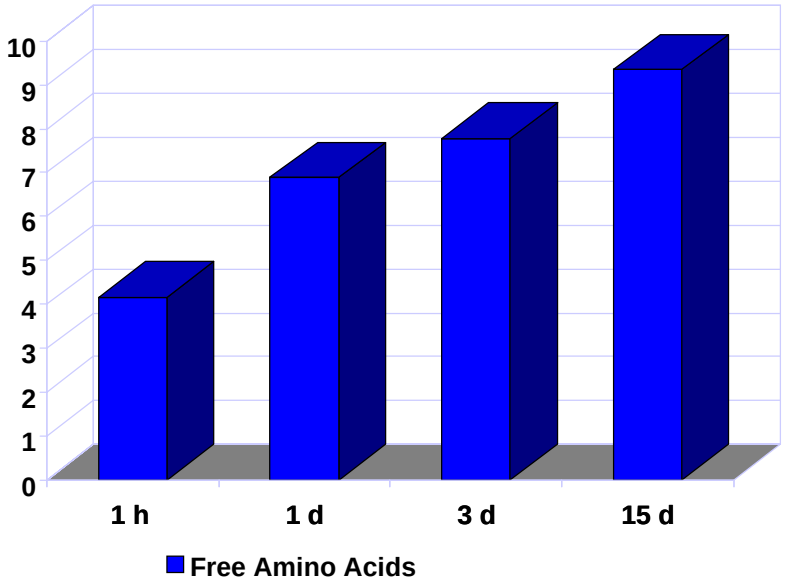

Figure 2: Total free amino acids in beef *M. longissimus dorsi* during ageing (n=3). Average values obtained at 1h,1d, 3d & 15d *post-mortem* are presented. Units are μmol/ml.

Connective tissue

Electrophoretic analysis, combined with ion-exchange chromatography, enzyme analysis and mRNA studies have provided a more comprehensive insight to the proteoglycan composition of muscles. Of the proteoglycan constituents measured between two different muscles, sulphate proteoglycans were more predominant in tough rather than tender muscle. Studies on the expression of decorin mRNA between different animals demonstrated a potential in predicting toughness in muscle. The total expression of decorin was higher in *M. semitendinosis* than in *M. psoas major* the (Table 4).

However, when this was monitored in relation to the collagen expression (decorin/collagen) the opposite was observed. Decorin is known to interact with collagen fibrils. It influences fibrillogenesis (formation of fibrous collagen) and inhibits collagen fibre diameter. As the decorin/collagen ratio is lowest in the *semitendinosis* muscle, it would be expected that the collagen fibre diameter would also be lowest in this muscle. Electron microscopic studies of the collagen fibre diameter confirms this (Table 5).

Table 4: Expression of decorin and collagen in bovine *M. semitendinosis* and *M. psoas major*

	ST/PM	**n**
Decorin (mRNA)	1.6 ± 0.2	4
Decorin/ Collagen I (mRNA)	0.6 ± 0.2	1
Decorin (protein)	1.4 ± 0.2	6
Decorin / OH-proline	0.7 ± 0.2	6

SM= Semitendinosus PM= Psoas major: All values are given as mean ± SE. n = Number of animals.

Table 5: Measurements of collagen fibre diameter, using electron microscopic studies, and Warner Bratzler shear force (Instron) in bovine *M. semitendinosis* and *M. psoas major*

	ANIMAL					
	1		2		3	
	ST	**PM**	**ST**	**PM**	**ST**	**PM**
Collagen fibre diameter (nm)	49.8 ± 7	23.2 ± 3	81.2 ± 9	43.9 ± 5	70.5 ± 8	37.4 ± 11
Instron (N)	76.4 ± 16	32.5 ± 2	102.4 ± 20	43.2 ± 5	67.7 ± 9	33.7 ± 3

All values are given as mean ± stdev. ST= Semitendinosus, PM= Psoas Major.

Development of ELISA test kits

The application of immunoassay technology to the analysis of non-clinical samples in the Food and Agricultural sectors is growing steadily (Rittenburg *et al*, 1992). It is a powerful analytical tool that depends on the interaction between an antibody and the antigen being measured. It can provide a rapid, economical, highly sensitive and specific analysis and is relatively simple to perform.

This analytical method has been used in the detection of food contaminants such as antibiotics, pesticides and microbiological organisms (Rittenburg, 1990). More recently the potential of this method for investigating endogenous food components such as vitamins, enzymes and structural proteins has been realised (Finglas *et al*, 1990; Doumit *et al*, 1996). Immunoassays are also be adapted to meet the increasing needs of the meat industry to be able to control and predict the quality of the final product.

Proteolytic fragments of structural proteins, in meat, which appear on SDS-PAGE over the ageing period and may be associated with meat tenderness, have been identified and isolated. Portions of their amino acid sequence have been read (see Table 2) and computerised databases used to confirm their origin (Tsitsilonis *et al*., 1996; Troy *et al*., 1997; Casserly *et al*, 1997 & Mullen *et al*. 1998a). The amino acid sequences have also been used to prepare quantities of equivalent synthetic peptides. These will be used as immunogens to prepare antibodies for development of a specific immunoassay. Antibodies are currently being produced and enzyme linked immunoassays (ELISAs) set up for the 30kDa and 110kDa SDS-PAGE bands. Soluble peptides that appear during ageing have also been identified (see Figure 1.), isolated and their amino acid sequences analysed (see Table 3). The amino acid sequence of these three peptides are also being used, in the same way, for the development of an immunoassay. Studies are being set up to evaluate the measurement of these peptides as predictors of meat tenderness by comparison with other physical methods and sensory analysis.

The calpastatin/calpain proteolytic system which has been implicated in the tenderisation process has also been targeted for the development of a rapid immunoassay test. Commercially available human calpastatin peptides and synthetically prepared bovine peptide sequences of calpastatin have been used in the production of calpastatin-specific antibodies for immunoassay development. Samples of calpastatin extracted from raw meat samples have been prepared and are being used to test the specificity of these antibodies. Western blotting methods will be used to check the cross-reactivity of the antibodies also.

Physical markers

The ability of NIR spectra to differentiate between tough and tender steaks is outlined in Figure 3. Promising correlations have been observed between NIR and both Warner Bratzler and sensory attributes measurements (Hilrdum *et al.*, 1994 & 1995). These have been obtained under controlled situations and the significance of these results needs further assessment. Investigations are continuing into the practical usefulness of this methodology as a predictive tool in meat quality.

The mechanical properties of meat have been characterised by two sono-elastography parameters - the propagation velocity and the attenuation co-efficient of the mechanical wave. Results indicate that sono-elastography can be used to follow rigor mortis onset and ageing in meat. Work is currently underway to analyse the usefulness of this technique in characterising the composition (lipids, collagen, water) as well as the mechanical properties of meat.

NMR has proven to be a powerful technique for the study of transformations in muscle tissue and has successfully detected a redistribution of water over the ageing process. Work is ongoing to record more data and evaluate them with chemometrics.

Initial analysis of images of meat obtained under both UV and visible light has correlated well with textural features while autofluoresence spectroscopy is being assessed for fat, water and connective tissue determination.

The influence of different chilling regimes on muscle shortening and rigor development has been assessed using the Rigotech® rigormeter (Figures 4 & 5).

Early *post-mortem* measurements of conductivity and impedance have been acquired and assessed σfor their predictive ability of both pork meat quality and cook yields of processed hams. Measurements of early *post-mortem* pH, colour and Warner Bratzler shear force are also being investigated as potential indicators of ultimate meat quality.

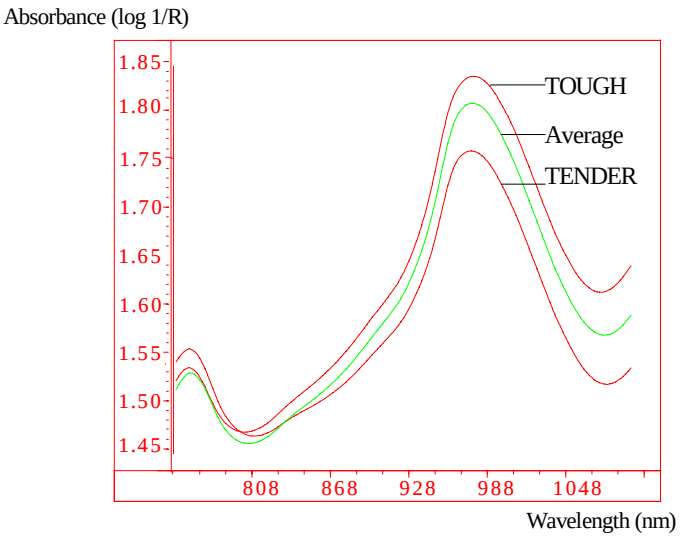

Figure 3: Mean spectra from bovine M. *longissimus dorsi* (n=24), together with spectra of the toughest and most tender samples in the set according to sensory analysis.

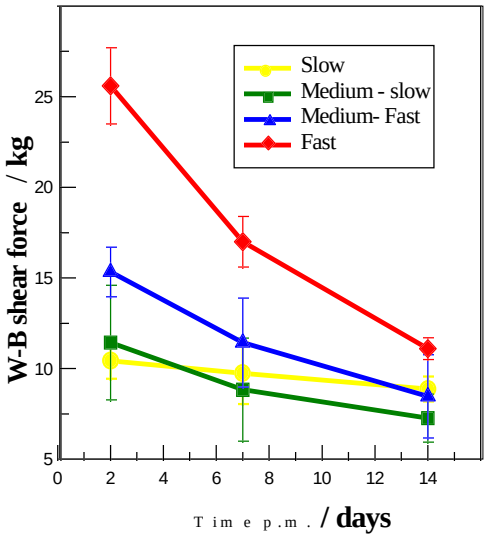

Figure 4: Relationship between Warner Bratzler and time *post-mortem* using four chilling regimes.

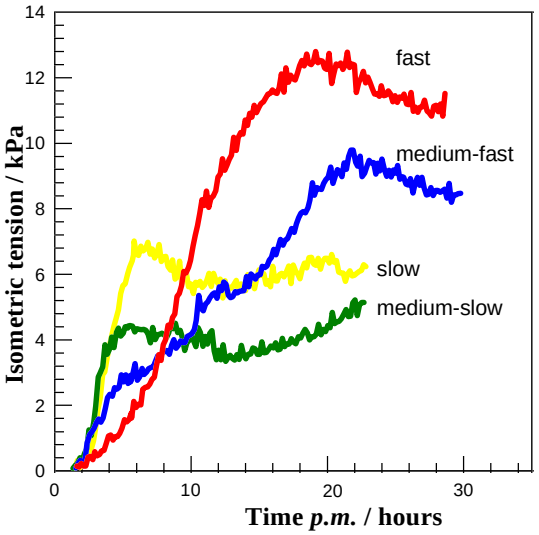

Figure 5: Relationship between Isometric tension (measured using Rigotech® (Rheologia Instruments AB, Sweden) and time *post-mortem* using 4 chilling regimes.

Conclusion

Certain challenges are faced now by the meat industry. In order to maintain a viable and competitive industry of economic growth and improved public perception these challenges need to be addressed. Providing a product of consistent quality is one of the most crucial responsibilities of the meat industry. To accomplish this goal early *post-mortem* indicators of the ultimate quality of the product are required. Many of the biochemical components of muscle outlined in this paper are potential candidates for quality indicators. Similarly, the results of the physical measurements of meat are also suggestive of their powerful potential as early *post-mortem* indicators of quality. Analysis is continuing to determine the practical usefulness of these indicators in an industrial situation and it is hopeful that rapid, sensitive, accurate indicators of meat quality will be available to the meat industry.

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Safe poultry meat production including HACCP application in the poultry industry

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Abstract

Poultry meat is by far the most popular animal food product. There are no religious restrictions concerning the consumption of poultry meat and quality, availability in an enormous amount of different ready to eat products and price are other important attributes to make poultry meat the world's favourite meat. Nevertheless the poultry industry is facing threats. These threats relate to hygiene, the presence of potentially pathogenic micro-organisms as Salmonella and Campylobacter, and the lack of confidence consumer's show after publicity on outbreaks of food-borne disease or other scandals involving poultry or other products of animal origin.

Government and industry have introduced quality control and quality assurance systems, the latter including HACCP (Hazard Analysis Critical Control Point) systems. This paper deals with aspects of the production of safe poultry meat and discusses methods to prevent poultry products from becoming contaminated with pathogenic micro-organisms. The principles of HACCP will be outlined and some examples are given.

Introduction

Hygiene during poultry production and processing relates to the presence or absence of potentially pathogenic micro-organisms (Salmonella, Campylobacter, *Escherichia coli* and *Staphylococcus aureus*) as well as the presence of spoilage organisms (Pseudomonas and Brochotrix). In both cases their presence may result in considerable economic losses to society and industry, by causing human food-borne disease or by spoilage of the products if handled and stored under improper conditions.

Consumers demand for a larger variety of products with a shorter preparation time and therefore a revolutionary process of mechanisation and automation took place in the poultry industry. In modern poultry processing nowadays slaughter capacities of more than 6000 birds per hour on one line are normal practice. Hygiene during production, transport, slaughter and processing therefore has become even more important than in earlier years.

Not only the costs society has to pay for cases of human food-borne diseases, but also the reassurance of consumers and restoration of consumer confidence in poultry (food) products, have led to more stringent hygiene measures and quality control schemes in the whole production chain.

Publicity with regard to Salmonella outbreaks in poultry and reports on Salmonella and Campylobacter infections in live birds and contamination of their products have made consumers very critical.

There are several literature surveys from the USA, The Netherlands, Sweden, United Kingdom on contamination of poultry products with potentially pathogenic micro-organisms. The data show that

products at retail level can be found contaminated with Salmonella (all serotypes including *enteritidis* and *typhimurium*), *Campylobacter jejuni* and *Listeria monocytogenes* and that *Escherichia coli* O157H7 only could be isolated in a very low percentage of samples. These are important organisms, which have the opportunity to increase in live birds, probably as a consequence of present intensive husbandry practices, without causing any sign of disease. It can be stated, that with regard to the relation between contamination of live birds and the contamination of the final product, the number of bacteria present differ per pathogen: Campylobacter 10^7 colony forming units per gram, *Escherichia coli* (not O157H7) 10^5 per gram and Listeria and Salmonella 10^3 per gram, which means that the consequences of the slaughter process in relation to contamination also differs per organism. Due to stress conditions during catching, transport and slaughter and breakage of intestines during slaughter and evisceration, the organisms may cause contamination of the processed bird and by cross-contamination also the whole flock.

Table 1 summarises literature data on prevalence of potentially pathogenic micro-organisms in poultry (Bryan & Doyle, 1995; Mulder, 1997).

Table 1: Prevalence of pathogenic micro-organisms on poultry meat

Micro-organism	Prevalence (%)
Campylobacter jejuni	0-100
Clostridium perfringens	63
Clostridium botulinum	0.3
Escherichia coli $O_{157}H_7$	1.5
Listeria monocytogenes	5 (>100 cells)
Salmonellae	0-100
Staphylococcus aureus	29
Yersinia enterocolitica	8 (non-pathogenic)

Prevention of contamination

The poultry production chain can be described in terms of the Hazard Analysis Critical Control Point concept, pointing at the critical points in several phases of production. The stages in the application of the HACCP concept are shortly described in Table 2. Full descriptions of the HACCP system can be found in Codex Alimentarius (1969) or ICSMF (1998). As a result of an EU-AIR project a summary on quality and safety assurance systems has been published by Hinton et al, 1997.

The implementation of quality assurance systems, including HACCP, can only effectively be done when the production is already performed under codes for good hygienic practices (GHP) or good manufacturing practices (GMP).

The application of HACCP in the live poultry production is questionable. Recent however an examples was published regarding a hazard analysis plan for exempt poultry production in turkeys (Anon, 1998). Several examples exist in the processing phase, from transport to scalding, evisceration, chilling and cutting.

Table 2: Stages in the application of HACCP

Identification of hazards	Micro-organisms, toxins, residues etc
Risk analysis	Ranking risks according to their severity and frequency
Identification and classification of CCP*	Where control must be exercised, the degree of control (CCP1 or CCP2)
Selection of control options	Effectiveness
Setting of critical limits	A criterion, which must be met
Selection of monitoring options	Utility, reliability, accuracy
Exercise control	Implement quality assurance

CCP1 = a location, practice, process or procedure where control is possible in order to prevent hazardous situations, whereas at CCP2 possible hazards are minimised but not totally controlled.

Government and industry in the western world have agreed on reduction of pathogens in products of animal origin and specifically in poultry meat products. These pathogen reduction plans include the implementation of plant specific HACCP plans, standard operation procedures (SOP's)for sanitation and testing programmes for the major pathogens. In The Netherlands and other European countries the emphasis is on Salmonella and Campylobacter in the live birds, with the final aim to lower the Salmonella contamination rate of the processed birds below 10% in the next 2.5 years. For Campylobacter this percentage is 15%. Several measures (examples are logistic hatching and logistic slaughtering) in the whole production chain accompany the testing programme.

During the slaughtering and evisceration process the requirements as pointed out in the European Union Directives have to be followed. In this directive there are no requirements with respect to visual carcass contamination. Visual observation of processed birds is one of the means of monitoring efficiency of plucking, evisceration and washing procedures. It is obvious that in case dirt is still visible after washing the carcasses there is no need for additional microbiological examination.

In the USA the USDA/FSIS Pathogen Reduction Programme also includes plant specific HACCP plans, SOP's and testing for pathogens, including critical limits, monitoring plans and corrective actions. Testing for pathogens is on Salmonella (tests conducted by FSIS) and *Escherichia coli* (tests conducted by the plant). This plan, better known as Megareg[1], also includes the so-called zero-tolerance with respect to faecal contamination. Faecal contamination can be controlled by use of feed withdrawal strategies and by the optimal management of transport and holding conditions before slaughter. As visual faecal contamination does not predict presence of Salmonella and / or *Escherichia coli*, but makes their presence likely, processors cannot rely only on the efficiency of the slaughtering process, but should concentrate on measures during the live bird phase as well.

[1] Megareg was announced July 25, 1996 and came into force Jan 26, 1998. The main aim is to reduce the contamination with pathogens and that the industry should take their responsibility to control the poultry production process. For large establishments (500 or more employees) the programme started Jan 26, 1998 and will be fully implemented in all establishments by Jan 25, 2000

This zero-tolerance rule will influence the evisceration technology and carcass washing performances as at present applied. Contamination due to intestinal breakage will be controlled more and more, so from that point of view the visual faecal contamination will be decreased and products will stand the microbiological tests. Probably more important in this respect will be the contamination caused by micro-organisms present in the crop and the way croppers operate.

HACCP is important, however quality control and quality assurance programmes should be in force after the implementation of all methods and technologies, which make the production of safe poultry products possible. Not all methods and technologies available are already implemented. Partly this has to do with the profitability of the poultry industry, which prevents large investments without reduction in costs for workers.

Table 3 summarises those methods and technologies, which could be implemented, as they have already shown under semi-practical conditions their effectiveness.

Table 3: Technologies and methods to be implemented for a safe poultry meat production (Mulder et al., 1993)

Area	Technology/method
Hatchery	a. Dipping of eggs b. No re-use of transport trays or through cleaning and disinfection c. Cleaning and disinfection of containers
Genetics	a. Production of resistant chicks
Husbandry / Management	a. New litter systems b. Application of competitive exclusion micro-flora's c. Specific pathogen-free housing
Feed	a. Pelleting and expanding techniques b. Organic acids c. Probiotics
Processing	a. Flock monitoring/logistic slaughtering b. Cleaning in place systems c. Crate washing equipment d. Combined scalding and plucking e. Cleaning and scalding in multistage scalders f. New evisceration technology
End Product	a. Lactates/Lactic acid b. Inorganic phosphates c. Ionising radiation

If by implementation of these technologies and methods no 100% absence of pathogens can be guaranteed, and additional quality assurance plans also can not improve the situation, an end product treatment should be considered. This treatment to guarantee microbiologically safe products to be applied after washing the carcasses. Although not world wide accepted, as their use is often considered as hiding bad hygienic situations, with the new Pathogen Reduction Plans in force all over the world, these treatments could give the extra safety guarantee to ready-to-cook poultry products.

Therefore the efficacy of existing and new treatments (chemical, physico-chemical and physical treatments are available) for carcass should be studied under present hygiene and quality assurance schemes.

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Decontamination of meat, meat products and other foods using steam condensation and organic acids

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Summary

Substantial rises in the occurrence of food poisoning outbreaks and commercial requirements to extend the safe, high quality shelf-life of food, has focused attention on decontamination systems. A comprehensive survey of literature on decontamination indicated that steam condensation and organic acids had the best potential of the physical treatments and chemical treatments respectively.

Experimental studies are underway at the University of Bristol to investigate the potential of steam condensation at, above and below atmospheric pressure as a decontamination system for red and white meat, meat products, vegetables, herbs and fruit. The synergistic effect of adding organic acid to sub atmospheric steam is also being evaluated. Comparison studies are also being made using hot air, water immersion, infra-red and ozone.

Reductions of over 2 $\log_{10}$ CFU cm^{-2} have been achieved in at, above and below atmospheric pressure pilot plants. Optimum systems and time treatment combinations are very product specific. For example, pressurised steam is particularly effective in decontaminating peppers. However, soft fruit are far more amenable to treatment in atmospheric steam.

The bacterial reductions achieved using the heat treatments are consistently less than would be predicted from the measured temperature-time cycles that are produced in the pilot plants.

Introduction

All handling operations, especially those that involve cutting, dicing, shredding, grating and mincing, are likely to contaminate the food being processed (1). The interior of most raw products from animal or vegetable origin and cooked products have very low, if not zero levels, of initial bacterial contamination. Bacterial contamination, pathogenic and spoilage, is present on the surface of raw products and the surfaces of materials coming into contact with both raw and processed products. It will be transferred to any new surface created during the handling operation, thus reducing both the safety and storage life of the food being processed. Investigations on the attachment of bacteria to surfaces indicate that they are most vulnerable immediately after contact and before they become firmly attached.

Significantly reducing the number of pathogenic and spoilage organisms on the surface of food will substantially reduce food poisoning and extend the shelf life of food. However, the process should not change the sensory properties, especially the appearance of the food.

Table 1 shows some of the treatments that have been used in decontamination studies and the range of bacterial reductions reported.

The treatments used rely on either chemical or physical methods with the physical mainly based on the application of heat. Bacteria are very sensitive to heat and far less heat is required to kill bacteria

on the surface of a food than to change the food's appearance. The problem is how to rapidly raise and lower the temperature at the surface of the food so fast that little heat penetrates into the product.

Thermal destruction of bacteria using condensing steam is the most promising of the physical methods. Reductions of up to 6 $\log_{10}$ CFU cm^{-2} have been reported in investigations without irreversible changes to the surface of food. Steam based beef carcass decontamination units are in industrial use in the USA. Investigations have therefore been carried out at Langford using condensation of pressurised steam, followed by vacuum cooling, to obtain the rapid heating and cooling cycle required. However, any system using pressure vessels is likely to be expensive to install and will probably have to be operated in a batch mode. A system using steam at atmospheric pressure is simpler to construct and automate so experiments have also been carried out using atmospheric steam. Heat sensitive food are unlikely to survive treatments at temperatures approaching 100°C. Therefore, in parallel, studies have been carried out using steam under vacuum. High rates of heat transfer can still be achieved but surfaces can be maintained at set temperatures between 50 and 100°C.

Table 1: Typical bacterial reductions ($\log_{10}$ CFU cm^{-2}) achieved by various decontamination treatments

Treatment	Range reductions	Treatment	Range reductions
Physical treatments		Chemical treatments	
'Cold' water	< 0.5 to < 1.4	Organic acids	1.2 to 3.5
'Hot' water	< 0.5 to > 3	Chlorine	< 2
Steam	3 to 6	Trisodium phosphate	2 to 2.5
Ultraviolet	2 to 3	Hydrogen peroxide	2 to 3
Visible light	0.5 to 1.5	Ozone	0.3 to 3
Microwave	~2		

The application of organic acids offers the best potential of the chemical treatments. Reductions of up to 3.5 $\log_{10}$ CFU cm^{-2} in bacterial numbers have been achieved in laboratory trials. Organic acids have the advantage of being a natural component of many foods and are not thought of as a chemical additive by many consumers. The synergistic effect of adding organic acid to sub atmospheric steam is also being evaluated.

In a number of cases the reductions achieved using steam and organic acids have been compared with results from parallel studies using hot air, infra-red and immersion heating systems or chemical treatments using ozone.

Experimental studies

A similar approach has been adopted in all the decontamination investigations.

A performance specification was initially drawn up for the pilot plant required i.e. pressurised, atmospheric or sub-atmospheric. The pilot plant has then been designed, constructed, commissioned and its performance tested against the specification. In most cases the pilot plant has

then required modification to improve its performance. In the case of the pressurised steam system the pilot plant is still being modified to increase the rate of heating.

A series of trials was then carried out with different foods in each of the three pilot plants to determine the conditions that would cause irreversible changes to the appearance of the food. Previous studies (2) had shown that the appearances of food could recover after a period in chilled storage. Changes in surface appearance were therefore assessed immediately after chilling and after approximately 24 h in chilled storage. Initially changes to food was assessed using instrumental colour measurements and subjectively by a small panel. However, no consistent correlation could be obtained between the subjective and instrumental measurements. Since the instrumental measurements sometimes failed to identify unacceptable changes in visual appearance it was abandoned in favour of the subjective approach.

From the visual trials, sets of time-temperature conditions were identified in the different pilot plants that would produce no, slight or marked changes in appearance of the food of interest. In some products, i.e. some herbs and leafy salads, no acceptable thermal conditions that were likely to result in bacterial destruction without unacceptable appearance/quality changes were identified.

Time-temperature combinations that were considered to have the best potential to produce substantial bacterial reductions were then selected from the appearance sets. Trials were then carried out using these conditions on samples of inoculated or naturally contaminated food and the resulting bacterial reductions determined. Some initial work indicated that the use of vacuum and pressure could result in movement of surface bacteria into the food. In these circumstances surface swabbing techniques could overestimate the reductions achieved by the treatment. To evaluate this effect incision (homogenisation) and swabbing techniques were used in parallel in some experiments.

Pressurised steam

The pressurised steam decontamination rig consists of a steel process vessel, steam generator and vacuum pump protected by a condenser chamber (Figure 1).

The working section of the process vessel is approximately 1 m long by 0.6 m diameter. Steam from the boiler (Fulton 48 kW EFS) is admitted through a 19 mm pipeline into the top centre of the 140 l process vessel. The vacuum pump (Edwards “One-Stage 80”) is connected via a 51 mm pipe and used to draw a vacuum for evaporative cooling. An ice filled condenser chamber fitted in-line between the process vessel and the vacuum pump protects the pump from steam ingress and damage. A further connection to the process vessel permits venting to atmosphere. The connections to steam, vacuum and vent are all controlled by pneumatically assisted solenoid valves. Instrumentation includes six T-type thermocouples reading temperatures within the vessel (product, wall and steam temperatures), and a pressure transducer with digital readout. All data was logged by a PC via a Datascan 7320 transducer interface (Measurement Systems Ltd) and Labtech Notebook software.

Figure 2 shows the temperature and pressure changes in a pressurised steam decontamination cycle. After the food is loaded the vacuum plant is used to draw a vacuum and remove non-condensable gasses from within the vessel. Steam is then introduced into the vessel where it condenses on and rapidly increases the surface temperature of the food. After the surface has reached the required temperature it is rapidly vacuum cooled. Initially this is achieved by venting the vessel to atmosphere. When the vessel reaches atmospheric pressure the vent is closed and the vacuum pump activated to continue cooling.

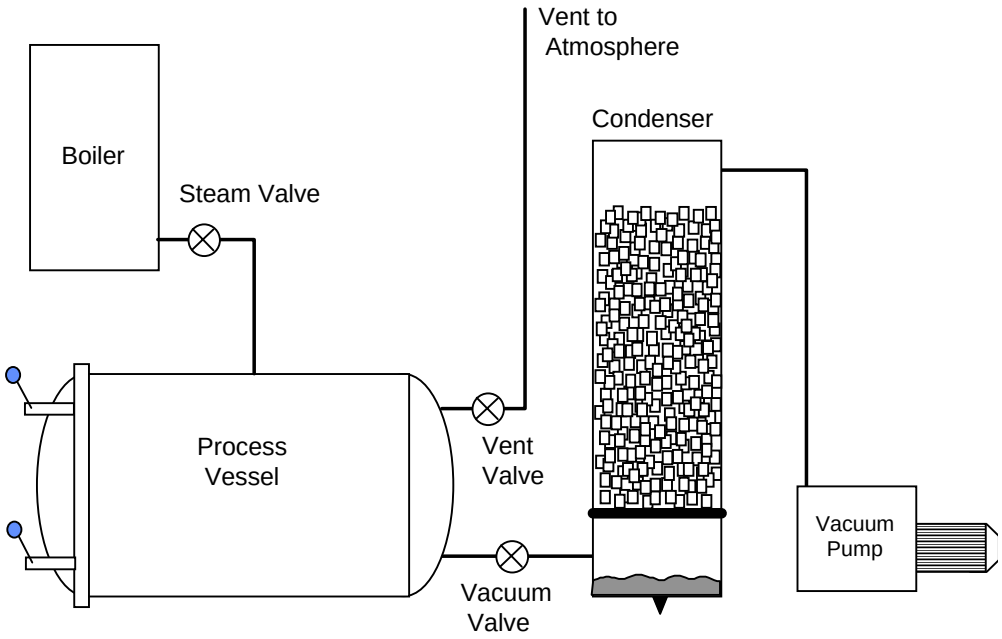

Figure 1: Schematic (not to scale) of pressurised steam decontamination pilot plant.

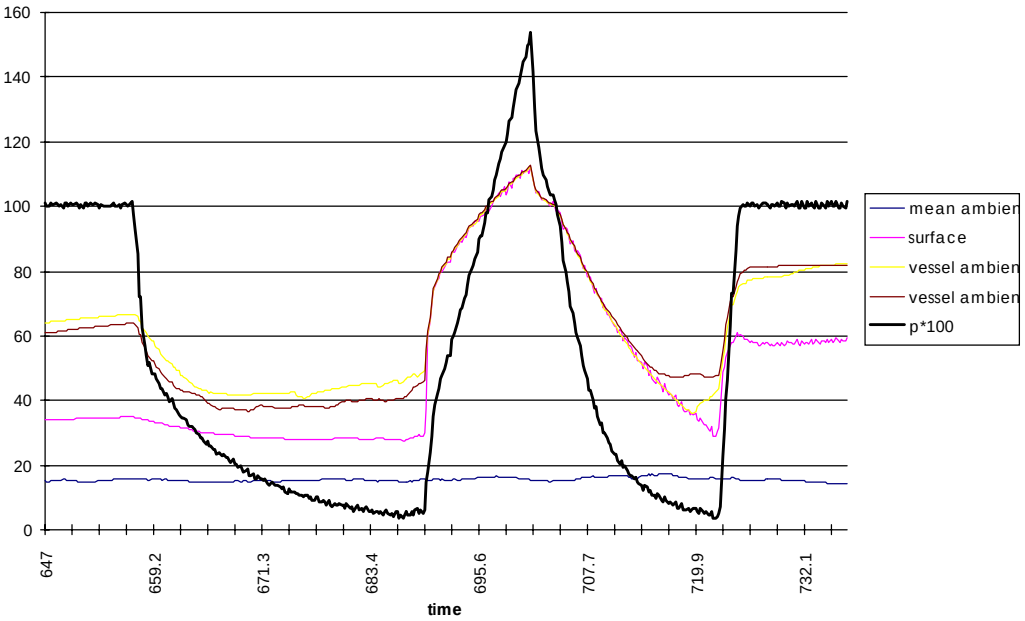

Figure 2: Example of decontamination cycle in pressurised steam pilot plant.

The pilot plant has been used to investigate the decontamination of peppers, soft fruit, red meat primals and consumer cuts.

Atmospheric steam

The atmospheric steam pilot plant has a much simpler design and mode of operation than the pressurised steam system. The working section of the plastic process vessel is approximately 0.6 m high by 0.6 m diameter. Steam from the boiler (Fulton 48 kW EFS) is continuously fed into the top of the vessel. As the steam fills the vessel it displaces any air and since it is lighter than air it remains in the vessel with a slight spillage from the open bottom of the vessel. The rate of spillage is adjusted with the steam valve. A pneumatic lifting device raises the product to be decontaminated into the vessel, holds it there for a set exposure time and then lowers it out of the steam. The product can then be cooled by immersion in iced water.

Investigations have been carried out on the decontamination of red meat cuts, whole poultry and poultry portions, soft fruits, peppers and herbs. A second processing vessel, approximately 2 m high by 0.6 m internal diameter has been constructed and used to investigate the decontamination of freshly slaughtered lamb carcasses.

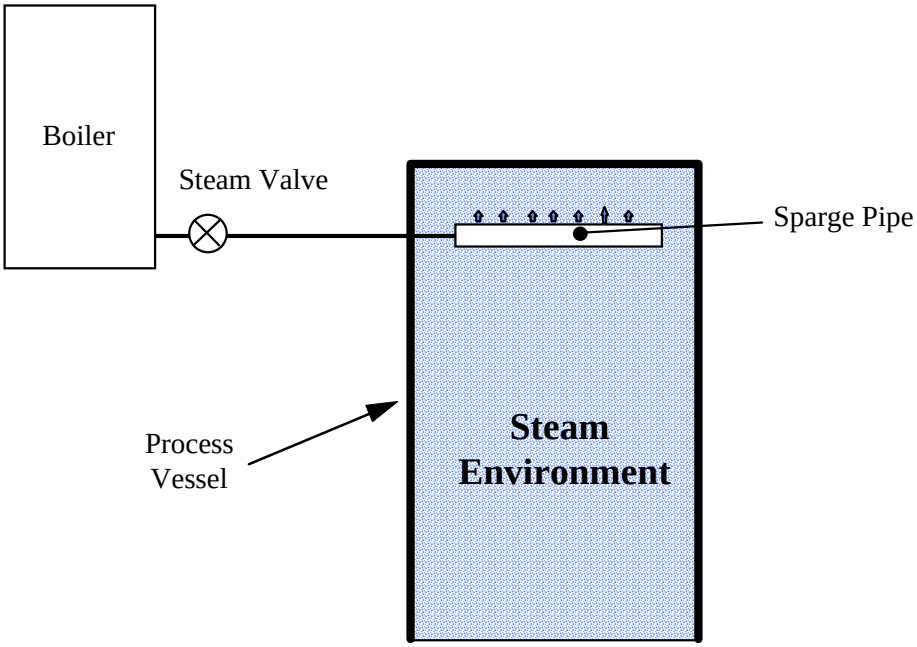

Figure 3: Schematic (not to scale) of atmospheric steam decontamination plant.

Sub atmospheric steam and organic acids

Four identical sub atmospheric steam pilot plants (Figure 4) were designed and constructed at the University of Bristol, Langford site. The pilot plants were then supplied to microbiology groups of the partners in the project, MATFOST (Norway), University of Vienna (Austria), National Food Centre (Ireland) and the University of Bristol (UK).

In operation the sample of the product to be decontaminated is suspended from a rack. The process vessel (approximately 0.45 m high by 0.3 m diameter is placed over the rack. The vessel is then

evacuated to remove non-condensable gasses. Steam is then introduced and the desired temperature maintained by controlling the vacuum pressure within the vessel. After the desired treatment time has been reached the steam is shut off and vacuum cooling applied.

Additional operations were carried out when the synergistic effect of adding organic acid to sub atmospheric steam was evaluated. A set amount of organic acid at the desired concentration was sprayed onto the surface of the product. In different trials the acid was sprayed before or after steam treatments and different residence times were also employed.

Investigations on the decontamination of beef (lean and fat), poultry (skin on and skin off), paté, pig skin, peppers, apples and lettuce have been carried out in the sub atmospheric steam pilot plant.

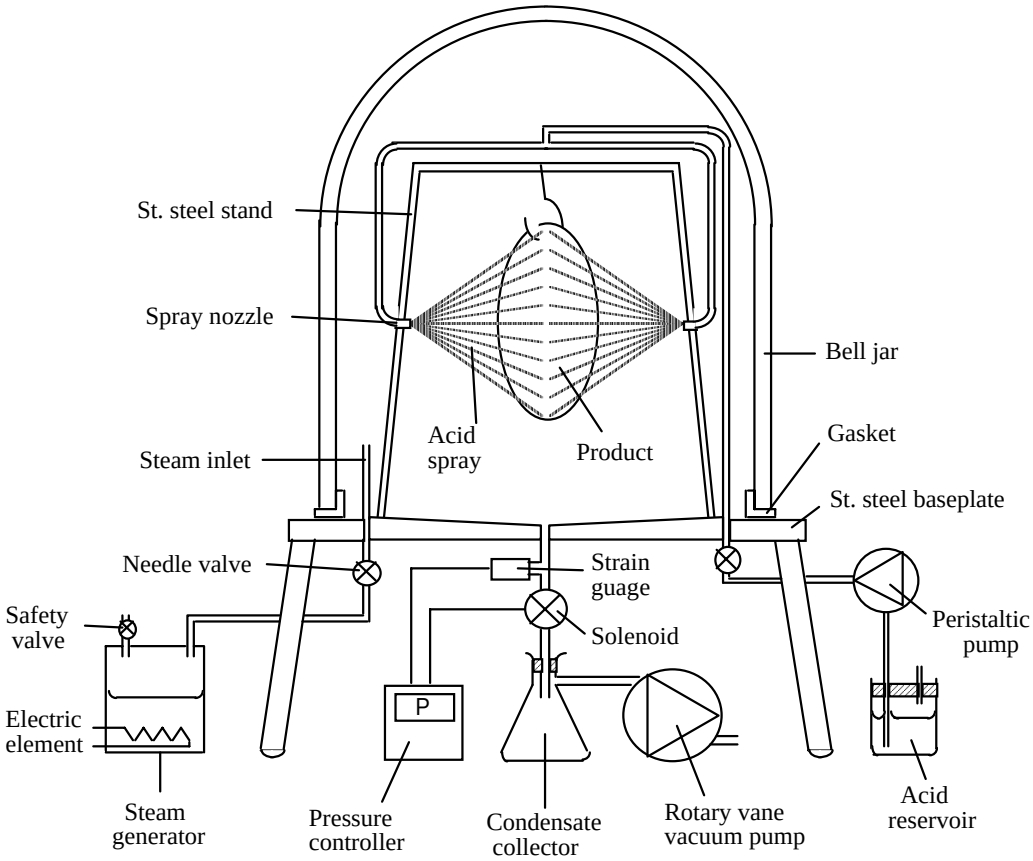

Figure 4: Schematic (not to scale) of sub atmospheric steam pilot plant.

Results

Pressurised steam

Initial studies on decontaminating beef showed no systematic difference between reductions produced on fat or lean surfaces (Table 2). On the surface of fat the reductions were not influenced by steam temperature while those on lean significantly increased with increase in steam temperature (Table 2).

Table 2: Bacterial reductions in pressurised steam system on fat and lean surfaces of beef samples.

Steam temperature	Reduction (log_{10} CFU cm^{-2})	
(°C)	Fat	Lean
100	2.59	1.23
120	2.41	2.53
135	2.15	3.44

Further studies have demonstrated an increase in bacterial reductions with increase in steam temperature (Table 3). The studies also show a marked difference in the ability of the outer or cut surfaces of beef muscle to withstand heat treatments without changes in its appearance (Table 3).

Table 3: Appearance changes and bacterial reductions in pressurised steam system at different steam temperatures.

Steam temperature	Appearance change		Reduction
(°C)	Cut muscle	Outer surface	(log_{10} CFU cm^{-2})
100	Slight	Negligible	2.25
120	Marked	Slight	3.26
135	Marked	Marked	4.08

Initial studies on the decontamination of green peppers compared steam under pressure with other heat treatments and ozone. Steam treatments resulted in higher bacterial reductions from both inoculated and naturally contaminated surfaces (Figure 5) than any of the other treatments.

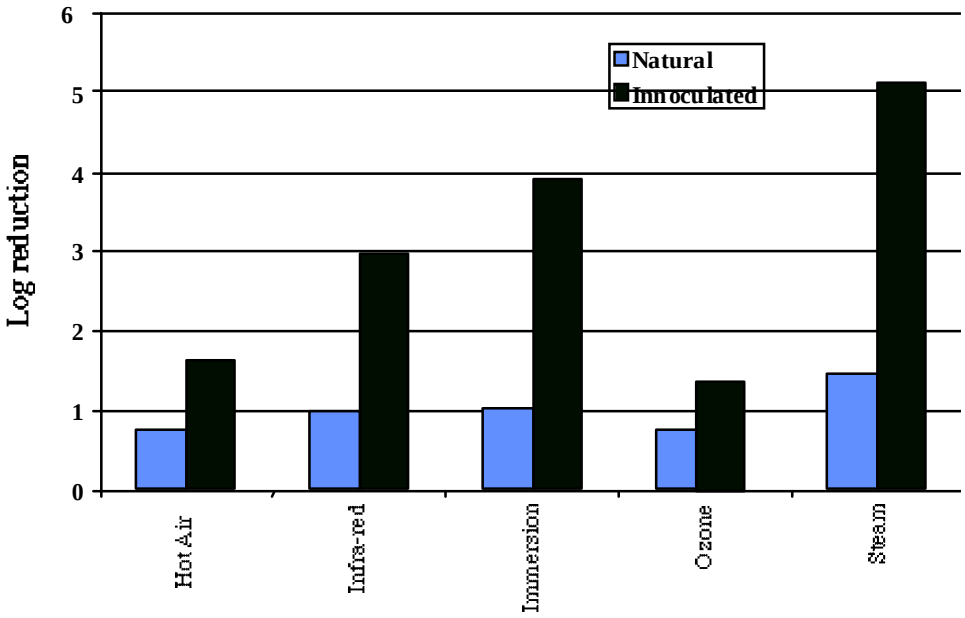

Figure 5: Bacterial reductions (log_{10} CFU cm^{-2}) on peppers achieved in different systems.

Further studies have used steam and hot air to produce heating and cooling cycles (Figure 6) that should result in, very large (>7 $\log_{10}$ CFU cm^{-2}) and similar, reductions to bacteria on the surface of peppers. The peppers were inoculated with 5 to 6 $\log_{10}$ cm^{-2} *Escherichia coli* O80 and bacterial reductions measured using surface swabbing and homogenised sample techniques. Reductions measured by swabbing were similar >3.88 and >3.51 $\log_{10}$ CFU cm^{-2} for steam and hot air respectively. Homogenised samples from steam treated peppers showed a similar reduction >3.74 $\log_{10}$ CFU cm^{-2}. However, only 1 $\log_{10}$ CFU cm^{-2} reductions were measured using the homogenising technique on hot air heated peppers.

Chilled soft fruit, raspberries and blackberries, could not be successfully decontaminated in the atmospheric steam decontamination plant. In preliminary trials all the pressure and temperature-time treatment combinations destroyed the structure of the berries.

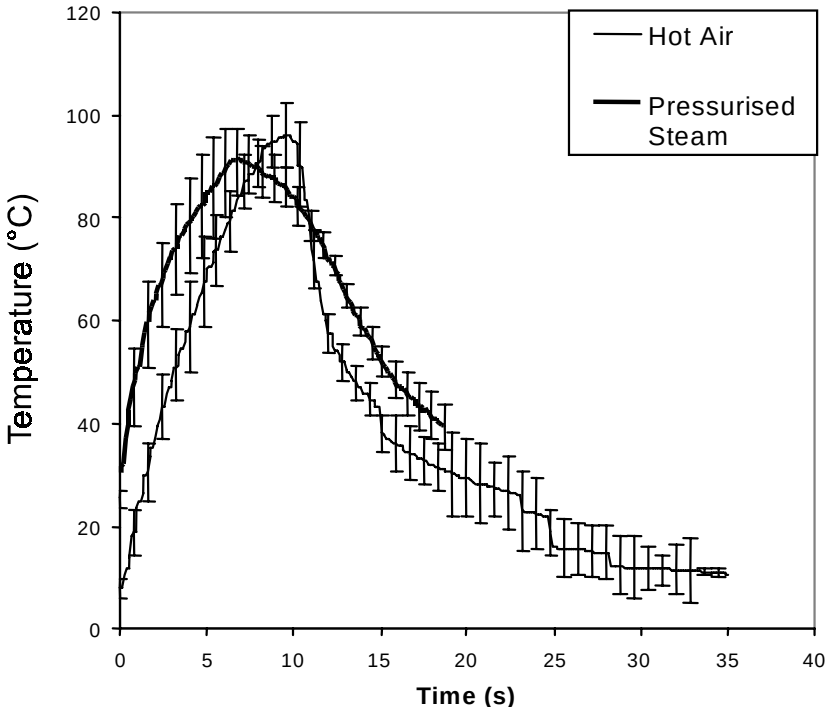

Figure 6: Temperatures immediately under surface of peppers during heating and cooling in pressurised steam and hot air (error bands ± 1 standard deviation).

Atmospheric steam

Initial studies on decontaminating beef showed no systematic difference between reductions produced on fat or lean surfaces (Table 4). A lower reduction in bacterial numbers was measured when the exposure time was increased from 6 to 8 s (Table 4).

Further studies have demonstrated a general trend for increase in bacterial reductions with increase in exposure time up to 6 s (Table 5). The studies showed, similarly to the pressurised steam studies, a marked difference in the ability of the outer or cut surfaces of beef muscle to withstand heat treatments without changes in its appearance (Table 5).

Table 4: Bacterial reductions in atmospheric steam system on fat and lean surfaces of beef samples.

	Reduction (log_{10} CFU cm^{-2})	
Exposure time (s)	Fat	Lean
6	2.62	3.32
8	1.54	1.66

Table 5: Appearance changes and bacterial reductions in atmospheric steam system at different exposure times.

	Appearance change		Reduction
Exposure time	Cut muscle	Outer surface	(log_{10} CFU cm^{-2})
2 s	Slight	Negligible	0.19
4 s	Some	Negligible	1.09
6 s	Marked	Slight	2.97
8 s	Marked	Some	1.91

Chilled soft fruit, raspberries and blackberries, were very successfully decontaminated in the atmospheric steam decontamination plant. Initial contamination levels of typically 10^3 to 10^5 g^{-1} were consistently reduced to below the level of detection of 20 g^{-1}.

Sub atmospheric steam and organic acids

Preliminary results on the skin of chicken breast samples (Figure 7) show that, as expected, there is a strong trend for the magnitude of bacteria reduction to increase with both steam temperature and exposure time. Reductions of approaching 2 log_{10} CFU cm^{-2} have been measured after a 10 s exposure to steam at 65°C. After 40 s exposure at temperatures of 80 or 85°C reductions approach 4 log_{10} CFU cm^{-2}.

The initial indications are that using organic acid sprays in conjunction to the condensing steam can produce an additional 2 log_{10} CFU cm^{-2} reduction in bacterial numbers. After spraying with acid, holding for 6 s and then a 40 s exposure to steam at between 70 and 75°C reductions were typically 5 log_{10} CFU cm^{-2}.

Overall there appears to be a trend for the degree of bacterial reduction to reach a plateau as the condensing temperature approaches 70°C.

Discussion and conclusions

Rapid progress has been made in the investigation of the decontamination of foods using condensing steam at different pressures. With some specific products, i.e. green peppers and some soft fruit, combinations of processing conditions and times have already been identified that may result in a significant increase in safety and shelf life without quality problems. In these cases we now need to investigate the sensitivity of the reductions to small changes in the decontamination procedure. We are then in a position to look into the engineering problems involved with the scaling up and handling of products at industrial scale.

There is a strong trend with both red and poultry meat for the highest bacterial reductions to be achieved on freshly slaughtered carcasses. More severe temperature-time treatments can also be applied to uncut surfaces that cut surfaces without causing irreversible quality changes.

Consistent throughout these investigations is a trend for the bacterial reductions to be substantially less than would be predicted from the temperature-time treatments. Fundamental studies are required to:

1. develop reliable and accurate methods of surface temperature measurement,
2. obtain reliable data on bacterial death kinetics in real foods.

Much of the published data on the relationship between temperature and microbial destruction has been obtained in pure cultures at low temperatures. Extrapolations have been made to higher temperatures and these data applied to real food. Our work indicates that this may substantially underestimated survival.

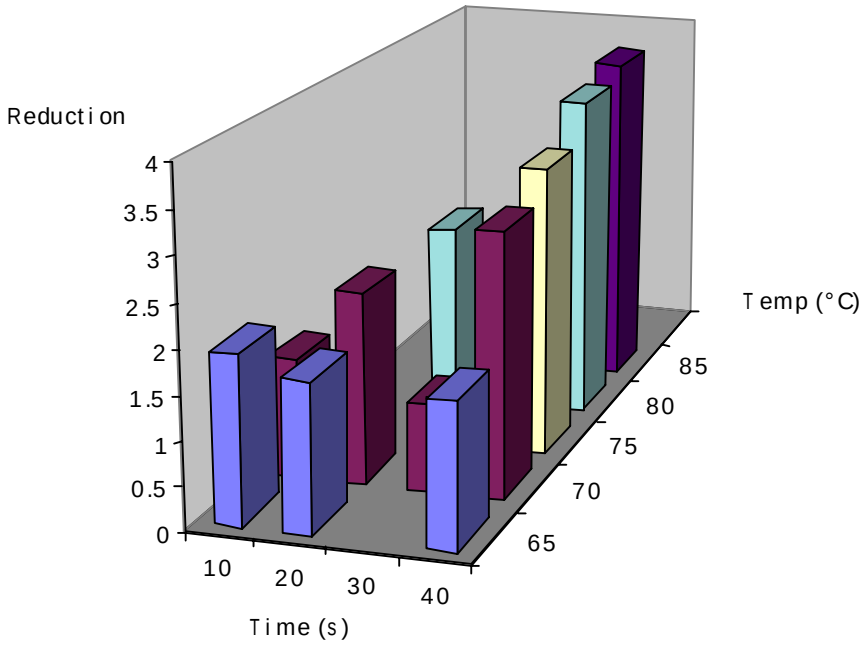

Figure 7: Bacterial reductions (log_{10} CFU cm^{-2}) on skin of chicken pieces produced by different steam temperatures and exposure times.

Acknowledgements

The authors would like to thank the European Commission under the FAIR programme for funding work on the decontamination of meat using modified steam and organic acids. The thirty month project involves partners from five countries (United Kingdom, Ireland, Belgium, Austria and Norway). The British Ministry of Agriculture Fisheries and Food (MAFF) are providing funding for a complimentary programme looking at different food products and a separate project on the decontamination of red meat using pressurised steam. Work on herbs, vegetables, fruit, poultry and lamb has been carried out as part of the MAFF funded Fellowship's research programme.

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Dry-Sausages ripening improvement

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Introduction

This project is aimed to define methods and tools for the improvement of the drying and ripening techniques of meat products (dry-sausages); special attention is given to the study of traditional products for which the preparation procedures are still based on empirical knowledge.

Many plants have been built without completely knowing the phenomena of mass transfer and the variations of temperature in the different parts of the sausage during drying; the study of mass transfer and the system of air pulsing is of great interest for this project and especially for the dry-sausages prepared in the south of Europe (France, Italy and Spain) in which the preparation technique is based mainly on the lowering of the water activity (a_w) values of the products rather than on the decreasing of the pH: the time of ripening for these products is quite long and the final quality depends essentially on temperature and mass transfer phenomena at the interface air-product.

The results of this study will help to acquire new knowledge and will enable the development of companies, especially small and medium enterprises, which mainly prepare traditional dry-sausages.

Objektives of the project

In order to achieve the above mentioned final result some intermediate objectives are planned to be reached:

- the description of water and solutes diffusion into the sausages as a function of the drying and ripening conditions;
- the definition of the chemical, microbial and sensory characteristics of traditional sausages produced in the countries participating in the project;
- the specification of the air conditions (speed, distribution, temperature, relative humidity) for obtaining an optimum fermentation and drying of the product and a controlled microbial growth on its surface;
- the development of a computer database concerning the physical-chemical of these meat products during ripening and the air characteristics in a ripening chamber as a function of the operating conditions.

To face these tasks an important intermediate goal of the project is the design, setting up and operation of two pilot-scale ripening chambers to be utilised in the study of the drying conditions by monitoring the product behaviour and the plant operating parameters.

The final output of this project is expected to consist in a full picture of the possibilities of improving the quality of typical sausage products by properly controlling the conditions operated by the ripening units; this desirable result would benefit the meat processing industry, the ripening plants manufacturers and obviously the dry-sausage products consumers.

Results obtained so far

Since the project beginning some important preliminary research tasks were completed concerning the description of water and solutes diffusion into the sausages as a function of the drying and ripening conditions and the definition of the chemical, microbial and sensory characteristics of traditional sausages produced in the countries participating in the project; some relevant results are briefly reported in this paper.

Water sorption properties of sausage meats

This study was conducted on two different kinds of Italian salami ("Crespone-Milano" and "Turista Buonpiemonte") two of Spanish sausages ("Salchichon Cular Cosido" and "Salchichon Casero") and two of French ones ("Varzi" and "Menage 250g") on purpose processed by the meat industries participating in the Project.

The water sorption isotherms were determined at two temperatures (10°C and 20°C) on the sausages taken both at the end of the drying stage (i.e. at the end of the first week of processing) and at the end of the ripening; on the whole 24 water sorption isotherms were built. Oswin's mathematical model was used for the description of the water sorption curves:

$$X_e = k \times \left(\frac{aw}{1 - aw} \right)^n$$

where: Xe is the mass ratio (water/dry matter) at equilibrium, a_w is the water activity, k and n are the Oswin parameters.

The following table lists the values of the Oswin parameters obtained for the different sausages:

Oswin parameters of the sausages

	Process time:	End of drying		End of ripening	
Sausage Identification	Temperature °C	K	n	K	N
Turista Buonpiemonte	20	6.9651	0.944	7.9982	0.934
Turista Buonpiemonte	10	7.5842	0.857	9.3529	0.811
Crespone Milano	20	6.5588	0.872	8.2012	0.854
Crespone Milano	10	6.3273	0.973	9.1508	0.887
Salchichon Cular Cosido	20	9.2140	0.808	7.9840	0.807
Salchichon Cular Cosido	10	10.504	0.744	9.0630	0.814
Salchichon Casero	20	8.1826	0.869	7.2154	1.027
Salchichon Casero	10	107185	0.639	10.361	0.811
Varzi	20	10.1749	0.844	13.551	0.717
Varzi	10	13.6078	0.511	14.3716	0.825
Ménage	20	9.1905	1.007	11.838	0.834
Ménage	10	13.9917	0.697	13.9814	0.714

Drying pattern in sausages under factory or lab plant conditions

Aim of this study was to provide useful information about some relevant parameters describing the drying of sausages prepared at factory level and, at lab plant level, to give an estimate of physical variables of prime importance in the drying processes: the diffusion coefficient of water, the thermal behaviour of the products being dried, the surface water exchange coefficient.

3 lots per type/partner of the aforementioned sausages were elaborated by the participating meat industries; the ripening of these products was monitored at plant level by recording the operating parameters (process times, air temperature and relative humidity, air speed, weight loss of the product) and by simple chemical determinations as pH, water and salt content, water activity, in two concentric fractions of the sausages taken at three processing times (fresh sausage, end of the first drying stage, end product).

The determination of the diffusion coefficient of water in the sausages was done by using the sample weights recorded in the experiment for the determination of the sorption isotherms.

The determination of the surface exchange coefficient: was carried out by means of lab scale thermostatic ovens in which the ripening of the products has been simulated at different room conditions. On these products the weight loss kinetics as well as the evolution of salt and water content in concentric fractions were investigated.

The following table lists the calculated values of the effective diffusion coefficient (m^2/s) of water in the sausage samples used in the determination of the sorption curves.

Effective diffusion coefficients of water in the sausages

RH	"Crespone Milano" END OF DRYING		"Crespone Milano" END OF RIPENING		"Turista Buonpiemonte" END OF DRYING		"Turista Buonpiemonte" END OF RIPENING	
	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C
90.3	6.00E-13	5.12E-13	8.29E-13	6.23E-13	1.71E-12	1.02E-12	3.30E-13	6.37E-13
84.3	3.21E-13	7.78E-12	4.74E-13	7.85E-13	7.97E-13	1.33E-12	4.96E-13	9.33E-13
75.3	6.82E-13	1.53E-12	6.11E-13	1.12E-12	8.96E-13	1.53E-12	6.80E-13	8.92E-13
70.8	6.70E-13	1.61E-12	8.66E-13	1.30E-12	9.66E-13	1.36E-12	6.57E-13	8.19E-13
57.7	1.22E-12	2.46E-12	1.65E-12	2.22E-12	1.43E-12	1.71E-12	1.39E-12	1.33E-12
52.9	1.61E-12	2.33E-12	1.46E-12	3.41E-12	2.16E-12	1.72E-12	1.57E-12	1.36E-12
RH	"Salchichon Cosido" END OF DRYING		"Salchichon Cosido" END OF RIPENING		"Salchichon Casero" END OF DRYING		"Salchichon Casero" END OF RIPENING	
	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C
90.3	3.02E-12	2.14E-12	3.68E-12	1.20E-12	3.97E-13	8.06E-13	-	1.19E-12
84.3	1.39E-12	1.57E-12	7.29E-13	5.83E-13	8.54E-13	1.43E-12	5.40E-13	1.38E-12
75.3	1.30E-12	1.27E-12	2.70E-13	3.36E-13	2.28E-12	2.83E-12	3.78E-12	3.10E-12
70.8	8.36E-13	6.51E-13	4.15E-13	3.39E-13	2.22E-12	3.98E-12	4.26E-12	3.51E-12
57.7	5.31E-13	1.54E-12	8.28E-13	1.09E-12	3.55E-12	4.55E-12	4.08E-12	5.10E-12
52.9	1.59E-12	4.87E-13	8.28E-13	5.19E-13	3.83E-12	5.37E-12	4.51E-12	6.10E-12
RH	"Varzi" END OF DRYING		"Varzi" END OF RIPENING		"Menage 250g" END OF DRYING		"Menage 250g" END OF RIPENING	
	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C
90.3	2.68E-13	7.37E-13	-	2.97E-13	4.55E-13	8.31E-13	9.62E-13	8.29E-13
84.3	5.12E-13	1.14E-12	4.86E-13	1.36E-12	6.45E-13	1.18E-12	3.61E-13	1.22E-12
75.3	1.88E-12	2.18E-12	1.50E-12	3.17E-12	1.51E-12	2.57E-12	1.87E-12	4.01E-12
70.8	1.73E-12	2.52E-12	1.37E-12	2.95E-12	2.07E-12	3.24E-12	1.94E-12	4.43E-12
57.7	2.03E-12	2.94E-12	1.95E-12	2.83E-12	2.78E-12	4.25E-12	1.45E-12	2.51E-12
52.9	1.79E-12	3.39E-12	1.90E-12	3.22E-12	2.98E-12	4.54E-12	1.64E-12	2.11E-12

The surface exchange coefficient of water in the sausages was found to be highly variable ranging between E-6 and E-9 (m/s) for the six different sausages and at the different experimental conditions (i.e. air temperature, relative humidity and speed).

The differences found between the various types of sausages are obviously due to the different characteristics of the products: size (external diameter), casing type (natural vs. artificial), grinding size of the meat.

The variability of the data within the same type of product depends on the value of the air temperature, relative humidity and speed of the various tests and is directly connected to the drying process of the sausages; this variability could also depend on the lot to lot differences.

The variability of the behaviour of the products reflects, even at this very small lab-scale, the real conditions in the industrial plants and this confirms the needs of a larger pilot-plant (which will be used in the future activities of this project) to adequately investigate the drying process of this meat product in order to obtain results transferable to the industrial scale.

To understand the above referred point it is important to imagine the dimensions and the capacity of the mentioned plants: lab-scale 10-15 Kg of fresh product, pilot-plant 200-300 Kg, industrial chambers 2000 to 6000 Kg.

Also the thermal properties were taken into account and mathematical models describing the thermal conductivity, the specific heat and the heat transfer coefficient of the sausages were specified and will be validated by using the recorded temperature data in the future activities of the project.

Growth of moulds under dry-sausages processing conditions.

The growth of various kinds of moulds of technological interest in sausage ripening was evaluated in a model system at different temperature and equilibrium relative humidity; the competition between starter cultures and other moulds was also investigated following the same experimental design.

Object of this study were mould strains of interest in the ripening of dry-sausages (*P. nalgionvensis, P. gladioli, P. candidum)* and some strains which can be encountered in some cases and which are able to produce toxins (*P. verrucosum, P. solitum, P. chrysogenum, A. ochraceus)*. The growth and competition of these strains was investigated, on agar gel culture media, at different temperature and relative humidity levels falling within the range of the values usually adopted for these variables in the course of sausages drying and ripening. In particular, ten experimental points have been studied: two replicates at the “centre” point (T=15°C, RH=85%), four “square” points (T=10 and 20°C, RH=80 and 90%) and four “star” points (T=15 and 22°C, RH=78 and 92%).

This study was completed by a competition test among the starter mould cultures and the undesirable strains; this test followed the above referred experimental design.

Culture strains tested.

Mould strains	Origin
1- *Penicillium candidum* M(PcM)	commercial starter
2- *Penicillium candidum* P (PcP)	commercial starter
3- *Penicillium gladioli* P (PgP)	commercial starter
4- *Penicillium gladioli* B (PgB)	starter isolated from salami
5- *Penicillium nalgiovense*(Pn)	commercial starter
6- *Penicillium solitum* (Ps)	undesirable strain isolated from salami
7- *Penicillium chrysogenum* (Pch)	undesirable strain isolated from salami
8- *Penicillium verrucosum* (Pv)	undesirable strain isolated from salami
9- *Aspergillus ochraceus* (Ao)	undesirable strain isolated from salami

Experimental design

First part of the study (growth conditions of the strains)

Independent variables: temperature (range 10 to 20°C), relative humidity (range 80% to 90%).
Dependent variables: lag phase duration, growth rate.

Second part of the study (evaluation of the interactions among the strains)

The strains selected for this test were:

- 3 starter strains: Pn, PcP and PgP, since they are the ones most frequently used and/or studied

- 4 undesirable strains: Ps, Pv, Pch and Ao, since they are often found in products during ripening, giving them an unpleasant appearance. In addition, Pv, Ao and Pch are able to produce toxins.

To better evaluate the results obtained in this study and to make them suitable for industrial application, it is useful to examine, one by one, the three successive stages of salami production, taking into consideration the wide range of technological parameters (RH and T) prevalent in industry.

-*Heating*. This stage lasts only few hours and does not play a very significant role in surface fungal growth, which occurs later; on the other hand, this stage is particularly important for starting the correct fermentation process produced by the bacteria (lactobacillus and micrococcus).in the meat

-*Drying stage*. The presence of surface moulds begins to show at this stage, which lasts for 5-7 days. Operating temperatures in this stage range from 15-20 °C, while the range of surface a_w values is extremely wide (0.80-0.95).

Given that all the strains tested develop under these conditions, on the basis of optimal parameters of temperature and water activity, the ones with the greatest growth probability in this stage are: PgP, PgB, Ps, Pv and Pch since their optimum a_w, ranging from 0.88 to 0.91, corresponds to that most frequently found in the products, but also Ao and PcM can be found. Under these conditions, these seven strains present a minimum lag time ranging from 1-5 days and a growth rate of 1-2.2 mm/day.

The surface of the product can be invaded by any of these strains which, at the end of the seventh day, could have a colony diameter, for example, of 4 mm for Pch, or even 13.2 mm for Ao.

These results already indicate the necessity for precise measures such as inoculating the product with a starter mould, since all the "undesirable" moulds can grow on it.

-*Ripening*. The final stage is carried out in a temperature range of 10-15 °C, while the surface a_w is in the same range as that indicated for drying; temperature is thus the only discriminating parameter between the two stages of the process and for the predominant development of the strains considered in the present study.

On the basis of optimum T values for these strains, only those relative to Pn and PcP are included in the ripening conditions.

The lag time and growth rate values for each mould were also calculated by keeping for each strain its own optimum a_w value and considering T=15 °C:

Lag time and growth rate values for the mould strains at RH=optimum and T=15°C

	Lag time (days)	**Growth rate (mm/day)**
Pn (starter)	4	2
PcP (starter)	5	1.2
PcM (starter)	4	1.6
PgP (starter)	5	0.7
PgB (starter)	5	1
Ps (undesirable)	5	1.3
Pv (undesirable)	6	1
Pch (undesirable)	3	1
Ao (undesirable)	14	1.1

From the table, it is clear that the starter strain most likely to predominate against the "undesirable" species is Pn, which shows the highest growth rate under the temperatures indicated. Although Pch showed a lower lag time (3 days) compared with Pn (4 days), its growth rate was in fact half that of Pn and it therefore revealed a lower invasive capacity.

Similar considerations allow the evaluation of other starter and non-starter strains using the same criteria. The overall results of prevalence are therefore:

Pn > PcM > Pch > Ps > PcP > PgB > Pv > PgP > Ao.

When the results obtained are compared, it can be seen that the relative humidity (RH) is more significant than temperature for all strains, except Ao; this explains why their latency time values, calculated on their optimum a_w value, are not so different from the optimal ones. Ao is the only exception, being much more susceptible to temperature than to relative humidity changes, as is shown by its long lag time at 15°C (14 days), in contrast with that relating to its optimal conditions (1 day).

After completing competition tests, it was observed that Pn can be subject to competition from Ao during drying (T•15°C), whereas during ripening (T•15°C) it is the dominant strain, when compared with Pch, Ps and Pv, however, Pn does not predominate in the case of comparable initial contamination.

PgP during the drying stage is inhibited by Ao and Ps; during the ripening stage, it dominates over Ao and, as in the case of PcP, does not dominate over Ps, Pch and Pv.

Ao can predominate during the drying stage; if it is present in high concentrations, it can completely invade the product even during the ripening stage.

PcP during the drying stage is inhibited by Ao, Pch and Ps; during the ripening stage, it dominates only over Ao. It was not observed to be competitive with Ps, Pch and Pv.

The 3 starters which underwent the second test can in effect be found to coexist with Pch, Ps and Pv on the product if they were present initially at comparable levels; in fact, considering the duration of the ripening period, the parameters studied (lag time and growth rate) for each of these strains become less significant.

In the light of these considerations, the employment at high inoculation levels of starter moulds on salami is essential to prevent the development of undesirable species.

Only on this basis can the scale of results obtained from the first test, relating to the individual starters under examination, be of practical validity and use.

The present study has revealed the possibility of reproducing the actual conditions for an industrial ripening process with a model system which is easy to set up.

The interest of the proposed method lies in finding out the optimal conditions of growth both of starter mould strains and of "undesirable" ones in the production of ripened cured salami, leading to the providing of guidelines for industrial application.

The most logical follow-up to this work would be the putting into practice in an industrial context of the suggestions arising from it, a study of the "technological" aspects of the most common starter strains, and an accurate analysis of undesirable moulds.

Chemical, microbial and sensory characteristics of dry-sausages

Samples from the above referred 3 lots per type/partner of sausages elaborated by the participating meat industries, for a total of 18 lots, were also analysed in order to investigate the chemical and microbial transformations occurring in sausages in the course of drying and ripening and at the same time to give a definition of the chemical, microbial and sensory characteristics of traditional sausages produced in the countries participating in the project.

Future activities of the project

Two pilot-scale ripening chambers have been assembled expressly for this project and are now operating (preliminary tests) at two participants sites; these plants will be used for validating the results of the studies about the drying properties of the sausages and to manufacture three lots per type of the sausages object of this research with the aim of reproducing their main characteristics (investigated in the first part of the project, as above referred) under strictly controlled operating conditions. This will give useful information about the possibilities of improving the quality of typical sausage products by properly controlling the conditions operated by the ripening units.

Expected influence of the project results on the meat industrie

The final results of this project are expected to have influence on different levels of the meat products chain:

the food machinery manufacturers, in this case the meat machinery and ripening units industry should be interested in the application of the results in the design of their products, especially in the control of the air conditions in the drying and ripening chambers to obtain an optimum drying of the products;

the meat processing industry, the sausage makers could achieve a better understanding of same processes underlying the fermented sausages production and at the same time improve the quality of their produce by properly controlling the fermentation and ripening processes;

the sausage products consumers could find better and safer products as to chemical, microbial and sensory properties.

Conclusion

The results obtained so far in this project (drying properties of sausages, growth of moulds under dry-sausages processing conditions and chemical, microbial and sensory characteristics of dry-sausages) will be used in the future work, the end of the project is planned for February 2000, to validate the operating parameters of the drying and ripening processes performed, under strictly controlled conditions, in the above referred pilot scale chambers.

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Feed supplementation in pigs and the quality of raw meat products

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Summary

Monogastric animals like pigs and poultry enhance the degree of unsaturation in their fats by feeding highly unsaturated oils. Higher unsaturation of fats enhances the degree of oxidation of fats which leads to rancidity. Also softer fats are observed. Vitamin E in the diet counteracts the oxidation. This paper shows that up to 6 % rapeseed-, sunflower- or olive oil can be used for fresh pork and cooked hams for a limited time of storage without negative effects. Long frozen storage leads despite vitamin E in the feed to off-flavours. Raw pork meat products (raw hams and salami type products) which are produced and stored rather long at ambient temperatures in the presence of oxygen exhibit soft fats with an unpleasant appearance and off-flavours at the end of the usual time of shelflife. The supplementation of the feed with vitamin E retards the process of oxidation but it cannot prevent it.

Introduction

Within the framework of the EU shared cost project AIR 2 - CT94 - 1577, acronym “Dietox“, a group of 13 partners worked on “Dietary treatment and oxidative stability of muscle and meat products“. Mainly pigs but also chicken and turkey and rabbits were fed with various concentrations of unsaturated fats (rapeseed [see table 1], sunflower, olive oil and acorns). Additionally tocopherol acetate and/or carotene up to 200 mg/kg feed were supplemented as an antioxidant. Copper salts up to 175 mg/kg feed were used as prooxidants. This paper considers only the feeding trials with pigs.

Fatty tissue of pigs and intramuscular lipids even without the supplementation of unsaturated oils in their feed are composed of about 60 % unsaturated fatty acids. A higher degree of unsaturation in feed will enhance the unsaturation of lipids in the carcasse. Fats, however, get softer with an increase of unsaturated fatty acids and become more susceptible to oxidation. The simultaneous addition of vitamin E as an antioxidative vitamin retards the development of oxidation and rancidity. Copper salts added to pig feed as a growth promoter should enhance in tissues the velocity of oxidation.

The changes of vitamin E in tissues during storage and processing are so far unclear.

The quality of fresh meat, fresh meat products (cooked sausages and hams) and shelf stable raw meat products like raw ham and salami-type sausages were investigated. Chemical analyses were done on vitamin E content of meat and meat products. Also the oxidation of fatty acids and the composition of fats were studied.

With regard to physical characteristics, water holding capacity, colour were investigated. In most cases the samples were also sensorically evaluated during and after chilled or frozen storage. Due to vast amount of data this paper deals solely with processed meat products. Especially the ones with a long shelflife like raw hams and salami type sausages due to the long influence of oxygen on these products will be the focus of this paper.

Material and methods

Several partners of the project used different dietary fat supplements in the feed of pigs. Also the concentrations varied from 2 to 6 % (table 2). Vitamin E was administered with the feed between no addition and 200 mg/kg. The feed supplements were given from a live weight of 25 - 30 kg until the day of slaughter (100 kg plus, live weight). An example of the feed composition is presented in table 3.

Generally the main ingredients were wheat, barley, maize and soy beans with an addition of a mineral and vitamin mix. The isoenergy of all feeds was either achieved by restricted feeding (see table 3) or by exchange of wheat, barley and maize against the oil with feed given ad libitum.

The vitamin E was supplemented as oxidation-protected •-tocopherol acetate which was hydrolyzed in the body to •-tocopherol. Plant oils as seen in table 4 contain a natural content of tocopherol with e.g. on supplementing the feed with 6 % rapeseed oil added 20 to 50 mg tocopherols to the diet.

An experimental set up by one partner is given in table 5. The addition of copper salts was considered to be used as an prooxidant. But in all experiments we could not detect an important influence of the copper on the quality of meat and meat products. Neither did we find an increase of copper concentration in muscle nor in fatty tissue.

Raw sausages were prepared solely from the meat and fat of the pigs in the trials. The proximate composition and other characteristics are given in table 6 and 7 for matured salame Milano and Coppa. The fresh and matured composition of the raw sausages salami-type in Germany are seen in table 8.

Results and discussion

Fresh tissue

Fig. 1 shows that there is with the isoenergetic feed supply given ad libitum no influence neither on weight gain by the supplementation of vitamin E nor with 2 % rapeseed oil. Also no change in feed consumption nor in feed efficiency was observed by the feeding regimes. The fatty acid composition of the fat in the carcass is shown in fig. 2 backfat exhibits an increase in monounsaturated fatty acids (mainly oleic acid) but also in dienic and polyenic fatty acid by 6 % rapeseed oil. The saturated fatty acids are reduced from 45 % in control to 32 % in the pigs which were fed with rapeseed oil. The concentration of vitamin E is enhanced by 50 - 100 % (see fig. 3 in backfat) by 100 mg vitamin E/kg feed and further on with 200 mg vitamin E/kg feed. 2 % rapeseed oil enhances the vitamin E from 5.8 mg vitamin E/kg tissue to 6.2 mg/kg tissue only slightly.

During chilled storage for 15 days the vitamin E content is reduced by 15 to 30 % (fig. 3). Frozen storage for 27 weeks in vacuum keeps the vitamin E concentration unchanged. Addition of vitamin E also reduces the fatty acid oxidation products measured as TBARS values (fig. 4). The main effect is observed between no addition and 100 mg vitamin E/kg feed. The addition of 6 % rapeseed oil leads to higher TBARS values in samples which were with supplemented vitamin E.

The sensoric evaluation of grilled pork chops after 3 months of frozen storage showed a clear advantage of controls without rapeseed oil but also acceptable flavour noted of the samples with rapeseed oil with little influence of vitamin E supplemented to the feed. After 35 weeks of frozen storage a clear preference for the control samples is presented in fig. 5. Pork shops with 2 % rapeseed oil without vitamin E in the feed gets the lowest degree of positive marks. Vitamin E improves the values.

In summary, fresh meat can be produced with a content of unsaturated oils up to 6 % and even stored frozen for several months with an acceptable flavour. After 35 weeks of frozen storage a strong negative influence on flavour is observed. The addition of vitamin E protects the product even after 9 months of frozen storage.

Meat Products

The vitamin E content of cooked ham (fig. 6) remains constant during preparation and storage nearly at the same level as in fresh muscle tissue. The sensoric evaluation of the meat which was obtained from pigs with 2 % soybean oil showed there is no significant influence of vitamin E addition (fig. 7). The flavour was only slightly influenced by vitamin E. Like with fresh meat cooked hams can be produced without sensorical problems with at least 2 % soybean oil. Vitamin E addition does not exert a positive effect in this product.

Raw hams of German type and raw sausages (Salami type products) are shelfstable products and are stored at ambient temperatures for many months in the presence or absence of oxygen. A higher degree of unsaturation in the fat may cause the development of rancidity.

Our own experiments showed that there is a reduced weight loss in hams produced from meat with contained enhanced amounts of vitamin E (fig. 8). The reason is most probable the enhanced stability of membranes in this intact muscle product. During fermentation by the drying process the vitamin E concentration remained quite high the lean muscle and the adipose tissue of the fat lager (fig. 9). With 2 % rapeseed oil it was somewhat lower than in controls.

In the sensory evaluation, however, the 2 % rapeseed oil lead to a number of deductions (e.g. soft fat) and flavour (fig. 10). The vitamin E content improved the values somehow but not to the level of the control.

In this 9 weeks old products the negative influence of rapeseed oil is clearly seen.

With German raw sausages (fig. 11) which were stored for 27 weeks there is an enhancement of vitamin E content in the first 25 days (fermentation period) due to the loss of water (weight). A further storage up to 27 weeks usually reduces the vitamin E content. It existed a clear influence of vitamin E in the diet of the pigs.

The oxidation of fats increases with time. The TBARS-value, measuring one oxidation product the malondialdehyde, is enhanced early during fermentation (fig. 12). Consequently the sensorical evaluation of all raw sausages were not satisfactory after 27 weeks of storage.

The Italian colleagues found higher values of vitamin E (table 9) in salame Milano. They used sunflower oil. The TBARS value, however, were in the similar range (table 10) as we reported in fig. 12.

In the sensorical evaluation they observed like us a significant decrease in firmness, a slightly paler colour and a reduced colour homogenity on a cut surface. All other sensorical characteristics including greasiness (slight increase with sunflower oil) aged taste (reduced with oil) and global acceptance (slight decrease) were not significantly different (table 11). The results with Coppa agree very well with the data obtained for salame Milano (table 12). The reason for this different result in comparison to our data may be explained with a higher vitamin E content, the use of nitrate instead of nitrite in our sausages and the acceptance of slightly rancid flavour in salami in Italy in contrast to Germany.

In conclusion: Long aged raw meat products tend to have softer fats and develop a more rancid flavour than products without supplemented unsaturated oil in the diet. The rancidity develops in these products during the long shelf like at ambient temperature in the presence of oxygen.

Table 1: Composition of rapeseed oil

C16 : 0	palmitic acid	3 - 6 %
C18 : 1	oleic acid	52 - 66 %
C18 : 2	linolic acid	17 - 25 %
C18 : 3	linolenic acid	8 - 11 %
C20 : 1	eicosaenic acid	1,5 - 3.5 %
(SFA)	saturated fatty acids	< 10 %
(MUFA)	mono unsaturated fatty acids	ca. 60 %
(PUFA)	poly unsaturated fatty acids	ca. 30 %

Table 2: Use of feed supplements for pigs by partners in the Dietox project

partner	1 & 2	3	4 & 5	6	
dietary fat	rapeseed oil	rapeseed oil	sunflower oil	olive oil/sunflower oil	acorns
% in feed	6	2 and 6	6	3 6	
vitamin E supplement in feed (mg/kg)	0 / 100 / 200	0 / 100 / 200	0 / 100 / 200	0 / 100	

Table 3: Sample of feed composition for pigs (*Bossi, Chizzolini (1996*))

38 %	wheat
38 %	maize
21 %	soybean
2 %	vitamin and mineral mix
0 and 6 %	sunflower oil
0, 100 and 200 mg/kg feed	tocopherol acetate
0, 35 and 175 mg/kg feed	copper salt

The energy consumption was kept isoenergetic by feeding 90 % of the feed with sunflower oil in comparison to control

Table 4: Concentration of tocopherols in oils and animal tissues (mg/kg)

	sum Tocopherols
maize(corn)oil	460 - 1200
rapeseed oil	580 - 800
soybean oil	320 - 1500
sunflower oil	380 - 800
lean pork	2 - 5
pork back fat	10 - 14
lean beef	3 - 6
lean chicken	4 - 6
milk	0.2 - 1
butter	10 - 33
egg	5 - 11

Table 5: Example of an experimental design (*Bossi, Chizzolini, 1996)*

diet	feed supplements
1	Control
2	+ 6 % oil 0 Vit. E 35 ppm Cu
3	" 100 ppm " "
4	" 200 ppm " "
5	" 0 " 175 " Cu
6	" 100 ppm " " " "
7	" 200 ppm " " " "

Table 6: Proximate composition, NaCl, nitrite concentrations and pH of matured *salame Milano* (mean of all diets) (*Chizzolini et al. 1996*)

Moisture	%	37
Protein	%	28
Fat	%	29
Ash	%	5.5
NaCl	%	4.2
Nitrite	mg/kg	ca. 4.5
pH		5.45

Table 7: Proximate composition, NaCl, nitrite concentrations and pH of matured *coppa* (mean of all diets) (*Chizzolini et al. 1996*)

Moisture	%	44
Protein	%	28
Fat	%	22
Ash	%	6.9
NaCl	%	5.8
Nitrite	mg/kg	9 – 14
pH		6.2

Table 8: Proximate composition, NaCl, nitrite concentrations and pH of fresh and matured german salami-type sausage (mean of all diets)

		fresh	*matured*
Moisture	%	57	39
Protein	%	16	23
Fat	%	23	32
Ash	%	3.8	5.4
NaCl	%	2.8	4.0
Nitrite	mg/kg	100	10 - 20
pH		5.8	

Table 9: Vitamin E content (mg/kg) of matured *salame Milano* (mean ± standard deviation) *(Chizzolini et al. 1996*)

diet	*salame Milano*
1	3.98 ± 0.29
2	8.22 ± 0.88
3	10.31 ± 0.31
4	10.83 ± 0.85
5	7.31 ± 0.45
6	9.16 ± 1.42
7	12.09 ± 0.60

diet 1: no addition; diet 2 to 7:with 6 % sunflower oil; diet 2 and 5: no vitamin E; diet 3 and 6: 100 mg vitamin E/kg feed; diet 4 and 7: 200 mg vitamin E/kg feed; diet 2 to 4: 35 mg Cu/kg feed; diet 5 to 7: 175 mg Cu/kg feed

Table 10: TBARS (mg MDA/kg) of matured *salame Milano* for one slaughter session (*Chizzolini et al. 1996*)

Diet	Session 2
1	0.40 ± 0.12
2	0.28 ± 0.04
3	0.29 ± 0.09
4	0.18 ± 0.03
5	0.17 ± 0.04
6	0.14 ± 0.04
7	0.23 ± 0.09

diets: see table 9

Table 11: Sensory evaluation[a)] scores of *salame Milano* (mean ± standard deviation) (*= significant difference P < 0.05 between diet 1 and diets 2 - 7 together) (*Chizzolini et al. 1996)*

	diet 1[b)]	diet 2	diet 3	diet 4	diet 5	diet 6	diet 7	diet 1/ diets 2-7
firmness	4.60 ± 0.49	3.93 ± 0.74	3.55 ± 0.74	3.57 ± 0.82	3.42 ± 0.90	3.67 ± 0.77	3.08 ± 0.69	*
colour-intens.	3.60 ± 0.66	3.12 ± 0.71	2.76 ± 0.67	3.04 ± 0.64	2.97 ± 0.71	3.07 ± 0.62	2.87 ± 0.62	*
colour-homog.	3.16 ± 0.86	2.78 ± 0.86	2.68 ± 0.79	2.76 ± 0.79	2.79 ± 0.81	2.75 ± 0.75	2.63 ± 0.82	*
greasiness	2.22 ± 0.83	2.45 ± 0.81	2.75 ± 0.87	2.75 ± 0.76	2.67 ± 0.94	2.61 ± 0.82	2.89 ± 1.96	
aged taste	3.24 ± 0.78	3.05 ± 0.83	3.05 ± 0.78	2.99 ± 0.81	2.97 ± 0.86	3.07 ± 0.79	2.89 ± 0.86	
global accept	2.82 ± 0.80	2.34 ± 0.84	2.48 ± 0.86	2.56 ± 0.86	2.46 ± 0.79	2.47 ± 0.68	2.45 ± 0.85	

[a)] 4 point scale, values from 1 to 4 increasing (e.g. 1 soft; 4 firm); [b)] diets: see table

Table 12: Sensory evaluation[a)] scores of C*oppa* (mean ± standard deviation) (*= significant difference P 0.05 % between diet 1 and diets 2 - 7 together) (*Chizzolini et al. 1996)*

	diet 1[b)]	diet 2	diet 3	diet 4	diet 5	diet 6	diet 7	diet 1/ diets 2-7
firmness	3.51 ± 0.69	3.27 ± 0.77	3.32 ± 0.74	2.99 ± 0.79	3.05 ± 0.74	3.12 ± 0.78	2.77 ± 0.79	*
colour-intens.	3.64 ± 0.63	3.42 ± 0.60	3.34 ± 0.77	3.59 ± 0.76	3.33 ± 0.65	3.44 ± 0.62	3.01 ± 0.63	*
colour-homog.	2.92 ± 1.00	2.70 ± 1.01	2.45 ± 0.88	2.70 ± 0.92	2.34 ± 0.89	2.66 ± 0.99	2.68 ± 0.78	*
greasiness	1.88 ± 0.73	2.11 ± 0.88	2.14 ± 0.87	2.27 ± 0.87	2.37 ± 1.01	2.19 ± 0.97	2.38 ± 0.95	
aged taste	2.92 ± 0.76	3.01 ± 0.72	2.89 ± 0.74	2.93 ± 0.73	2.93 ± 0.71	2.97 ± 0.78	2.81 ± 0.74	
global accept	2.71 ± 0.92	2.55 ± 0.85	2.42 ± 0.98	2.44 ± 0.88	2.47 ± 0.85	2.59 ± 0.96	2.68 ± 0.70	

[a)] see table 11; [b)] diets: see table 9

Effects on blood lipids in healthy humans from more unsaturated pork meat

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Abstract

The effect of an increased content of unsaturated fatty acids of pork fat on blood lipids was evaluated in an intervention study with strict dietary control in 12 male subjects. Diets with meat and fat from pigs fed either a basal feed with 2% fat or the same basal feed with the addition of 6% rapeseed oil (with and without additional vitamin E) were prepared and served in periods of 3 weeks each in a randomized cross-over design. Blood samples were taken at start and end of each dietary period. The intervention diets based on pigs fed rapeseed oil had a lower content of saturated fatty acids and a higher content of polyunsaturated fatty acids while the relative content of monounsaturated fatty acids was virtually identical. Blood total cholesterol concentration was significantly lower (approx. 4%) when diets prepared of meat and fat from pigs fed rapeseed oil were taken for three weeks. The changes in blood cholesterol concentration were slightly larger than expected from the differences in fatty acid composition. Addition of vitamin E to pig feed had only marginal effects on vitamin E content of the diets. In conclusion, realistic changes in feeding strategies of pigs can improve the nutritional value of pork meat and fat.

Introduction

Epidemiological observations already in the 60ies suggested that diet composition and especially dietary fatty acid composition was a key factor in relation to risk for coronary heart disease (CHD)[1]. Low prevalence of CHD in populations with a low intake of saturated fatty acids such as the Mediterrian countries, was observed, while total fat intake was not associated with risk for CHD. Similar conclusions were recently drawn from a prospective study of American women [2]. The biological explanation to the relation between fatty acid intake and CHD is their effect on blood cholesterol concentration. High blood cholesterol concentration is an established risk factor for development of CHD.

A large number of experimental studies have evaluated the impact of changes in dietary fatty acid composition on blood cholesterol concentrations and algorithms predictive of the effect of dietary fat changes for cholesterol changes have been developed [3-7]. These evaluations consistently show that an increased intake of saturated fatty acids of a chain-length of 12 to 16 will increase plasma total cholesterol and low density lipoprotein (LDL) (the most atherogenic lipoprotein) cholesterol concentrations. The saturated fatty acid stearic acid (C:18) and the monounsaturated fatty acids are neutral or show a slight decreasing effect and the polyunsaturated n-6 fatty acids lower blood cholesterol concentration. Differences between the fatty acids have also been documented regarding their effects on high density lipoprotein (HDL). A low concentration of HDL-cholesterol is associated with an increased risk for CHD. Saturated fatty acids increase HDL cholesterol to some

extent, monounsaturates are neutral, while high intakes of polyunsaturates tend to lower HDL cholesterol. The long chained polyunsaturated fatty acids of the n-3 family, found mainly in marine oils and in plant products, have other health beneficial effects. They lower plasma triacylglycerol concentrations and show positive effects on events related to blood coagulation and on immune responses. Based on present knowledge the official recommendations in many countries is a reduction of the intake of cholesterol raising saturated fatty acids to less than 10 % of energy intake, an intake of polyunsaturates not exceeding 10% of energy intake and the remaining fat in the form of monounsaturated fatty acids.

In most industrialized countries meat products account for a substantial part of total fat and saturated fat intake, and the epidemiological results have sometimes been interpreted as a need for a reduction of meat intake. Meat and meat products are, however, quantitatively important sources of essential nutrients such as vitamins and minerals and a decreased meat intake could have negative impact on the overall nutritional value of the diet. Thus there is a need to explore to what extent meat fat composition can be improved. The potential for improvement of fatty acid composition from a human nutritional point of view is especially relevant for pig meat and fat, where the feed fat content and type directly influence especially back fat composition [8-10]. The aim of the present study was to explore the effects of realistic changes in pig feed composition on the nutritional value of pork fat.

Materials and methods

Female pigs (Danish Landrace x Danish Yorkshire) were randomly assigned to i) basal feed (barley, wheat, soybean, 2% fat), ii) basal feed + 6% rape seed oil and iii) basal feed + 6% rapeseed oil + 200 mg vitamin E . The animals were given *ad libitum* access to feed and water from 25 to 100 kg live weight (slaughter). After slaughter meat and fat were used to prepare human diets where the pork fat contributed with 90% (80 g/10MJ) of the total fat content. To assure a constant and homogenous content of fat and fatty acids most of the meat was blended and incorporate in minced meat dishes, sausages, patés etc. An example of the daily menu is given in table 1. Duplicate portions of the diets were taken and analyzed for fatty acid composition and vitamin E content.

The three diets were evaluated in an intervention study with 12 young men. All food was prepared and served or delivered from the department and each diet was served for 3 weeks in randomized order with wash-out periods of minimum 3 weeks. Blood samples were taken at start and end of each dietary period and analysed for blood lipid and vitamin E concentration.

Table 1: Example of one day's menu

	Gram per 10 MJ		Gram per 10 MJ
Breakfast		**Lunch**	
Whole-wheat bread*	100	Rye bread	60
Sausage*	30	Paté*	50
Marmalade	20	Sausage*	20
Orange juice	250	Salad	100
		Fromage frais	30
Snacks		Pear	150
Whole-wheat bread*	90	Apple juice	200
Paté*	40		
Chocolate cake*	50		
Apple	150	**Dinner**	
Sweets	20	Meat sauce*	300
Apple juice	200	Pasta	60

*food items containing pork fat and/or pork meat

Results

Addition of rapeseed oil, rich in the unsaturated fatty acids, oleic acid, linoleic acid and α-linolenic acid, markedly increased the concentration of those fatty acids in backfat of the pigs (Lauridsen C. et al, personal communication). As the back fat contributed with the major part of the total pork fat of the human intervention diet these differences were reflected in the differences in the fatty acid composition of the human diets (figure 1). A lower content of saturated fatty acids (SFA) and a higher content of polyunsaturates (PUFA) was found in the diets prepared from pigs fed the rapeseed oil. Another important difference was the higher content of α-linolenic acid (C18:3) (4% versus 1.4%). The content of monounsaturates (MUFA) was virtually identical in the diets.

Intake of the diets with fat from pigs fed rapeseed oil, (B) and (C), resulted in a significantly lower total cholesterol concentrations compared to the diet with fat from pigs fed the basal diet (table 2) [11]. On average there was a difference of 4%. No significant differences were observed for LDL- and HDL- cholesterol concentrations. Compared to cholesterol concentrations at start of the dietary intervention period, i.e. reflecting habitual diet composition, there was a lowering of cholesterol concentrations of 5-8% in all dietary periods.

The increased vitamin E intake of the pigs fed feed iii) resulted in only slightly higher vitamin E content of the human diets prepared of the meat and fat: 0.7, 0.9 and 1.2 mg/10 MJ, for diet A, B and C, respectively. This low intake of vitamin E gave a reduction of plasma vitamin E concentration of the participants in all three dietary periods. However, the reduction was most pronounced in the group receiving diet B, while no difference was observed in plasma vitamin E concentrations at end of diet period A and C (table 2).

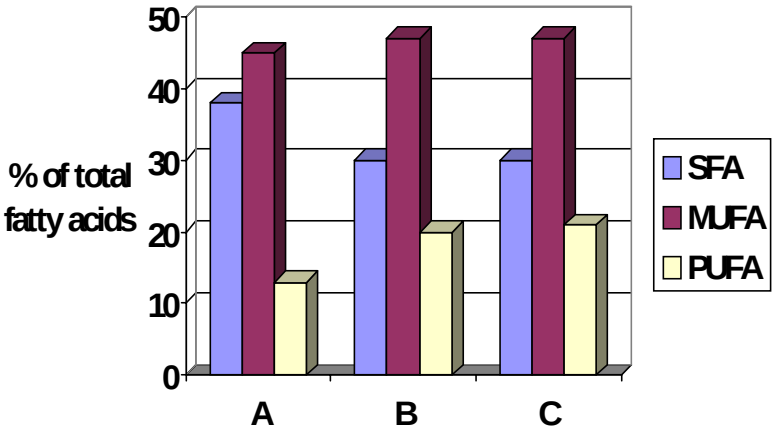

Figure 1: Fatty acid composition of the intervention diets

Table 2: Plasma total cholesterol and α-tocopherol concentration at end of the three dietary intervention periods (mean±SD)

	Diet A	Diet B	Diet C
Total cholesterol, mmol/l	3.62±0.58	3.47±0.57	3.44±0.49
α-tocopherol, μmol/l	18.2±3.5	16.9±3.8	17.8±3.1

Discussion

This study shows that it is possible with realistic changes in feeding regimes to change the fatty acid composition of pig fat in a way that has putative beneficial effects on blood cholesterol concentration in human subjects concerning a pork based diet. Based on meta-analyses of results from pharmacological and dietary interventions it has been estimated that a reduction of total cholesterol concentrations of 1% will result in a risk reduction for CHD of 2% [12]. Consequently, the observed difference of 4% could , if extrapolated to populations, have a significant impact on the prevalence of CHD. It is also likely that these changes of fatty acid composition would have more pronounced effects in subjects with higher serum cholesterol concentrations, than in this study group of young men. The differences in fatty acid composition between diets were smaller than anticipated when planning the study, basing calculations on published data for pig fat composition. This may be due to the relatively unsaturated nature of the basic diet. On the other hand the observed effects on total cholesterol were slightly larger than could be predicted from the analyzed fatty acid composition. As these predictions are mainly based on studies with vegetable fats it is possible that the pattern of fatty acids in animal fat affects blood lipids differently. The results also suggest that even the diet with fat from the pigs fed the basal diet had a better fatty acid composition with regard to blood lipids than the subjects = habitual diets. Presumably this could be attributed to the exclusion of dairy fats in the test diets.

The fatty acid composition of diets made from the rapeseed oil fed pigs matches the recommended fatty acid composition with less than 10% of energy from saturated fatty acid. In addition, the content of α-linolenic acid corresponds to the recommended minimum intake of n-3 fatty acids of 1% of energy and the content of linoleic acid is also sufficient to cover the need for essential fatty acids. The only potential draw back of a diet based on pig fat is the low content of vitamin E as in most animal fats. The present recommended intake of vitamin E is 6-8 mg/day, while the total intake in this study was only 1–1.5 mg/day. In addition the observed plasma vitamin E concentrations suggest that the higher content of polyunsaturates in diet B and C increased the body demand for vitamin E. This illustrates the importance of a balance between polyunsaturates and antioxidants. While it may be unrealistic costly to increase the vitamin E content of pork fat to correspond to the recommended intake, it may be worthwhile to consider addition of vitamin E to processed foods prepared from pork with a higher unsaturated fat content.

Conclusions

Moderate and realistic changes in fat content and fatty acid composition of pig feed had significant and potentially beneficial effects on the nutritional value of pork fat and meat. A human diet, in which a major part of total fat content was derived from pigs fed 6% rapeseed oil, had a composition virtually identical to present dietary fat recommendations. A relatively high content of vitamin E in pig feed had only limited impact on the vitamin E content of the pork fat, but may protect processed pork products against oxidation during storage and thus improve sensoric characteristics and shelf-life of such products.

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Dietary treatment and oxidative stability of muscles and meat products. Nutritive value, sensory quality and safety

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Abstract

Poultry and pig meat were found to have lower fractions of saturated and higher fractions of unsaturated fatty acids in the lipids, when the production animals were raised on feed supplemented with olive oil, high-oleic sunflower-oil or high-oleic rapeseed-oil under realistic production conditions. Meat products made from pigs, raised on feed supplemented with 6% oil, combined with the meat and lard in a normal diet was shown to have positive health effect in a dietary intervention study in human subjects. For any product certain percussions in relation to protection against rancidity and formation of toxic oxidation products need, however, to be considered. Such protection has, for a number of traditional European pork and poultry products, been shown to be possible through addition of vitamin E to the feed. Protection against oxidative processes was found most important for processed and pre-cooked meats, while use of copper salt as growth promoters for the animals had little if any effect on oxidative processes in the meat. The higher degree of unsaturation gave softer fat resulting in technological problems for production of certain type of hams and sausages, but not when the oil supplementation to the feed was reduces to 2%. By considering the entire production chain, it has thus been possible to establish directions for improving the nutritive value of meat products without accepting decreases in shelf-life, toxicological safety or overall wholesomeness.

Introduction: A strategy for improving the nutritive value of pork and poultry

A change in the fatty acid profile of the human diet towards a higher degree of unsaturation is expected to have positive health effects. High mono-unsaturated lipids as those dominant in the Mediterranean diet are thus known to have positive effects on blood parameters, and substitution of saturated fatty acids with oleic acid have been found to lower the cholesterol level. Since the fatty acid distribution, in particular in the adipose lipids, in monogastric animals like the pig is highly dependent on the fatty acid distribution in the feed, especially for high fat feeds, it should in principle be possible to improve the nutritive value of meat and fat by a change toward more unsaturation in the animal feed, provided that lipid oxidation in the feed, in the live animal and in the meat products can be controlled (1).

For meat production, such changes towards higher mono-unsaturation in meat and meat products entail a number of problems related to development of rancidity during storage and related to the functional properties of the fat. Such problems should be dealt with on a scientific basis prior to endorsing any recommendations of changes in the animal feed. The higher degree of mono-unsaturation will, for the feed available for modern farming, result in a concomitant increase in poly-unsaturated fat in the meat, meat products and backfat. Meat and fat with increased levels of mono-unsaturated and especially poly-unsaturated lipids are more exposed to oxidation leading to rancidity, discoloration and increasing levels of toxic lipid oxidation products and cholesterol

oxidation products. As for the functional properties of the fat, increased softness due to increased unsaturation may cause technological problems during processing and also change product characteristics in undesirable direction.

Traditional farming provides inspiration for solving the problems related to oxidation, as pigs like the Iberian pig and poultry raised under extensive conditions from fresh plant material like acorn and grass receive a high dietary supplement of natural antioxidants providing protection against oxidation of the unsaturated lipids and of cholesterol in muscle tissue and backfat. These antioxidants are the homologue tocopherols and tocotrienols (vitamin E) of which only α-tocopherol is retained in significant amount in the body, and a large variety of carotenoids and plant polyphenols. α-tocopherol, which is commercial available as a feed additive in form of a stable acetate ester, is generally considered to be the most important of these antioxidants, while the role of carotenoids and polyphenol are less clearly understood (2). During the last few decades an increasing number of investigations have explored the beneficial effects of supplementation of α-tocopherol acetate to feed for chicken (3), turkeys (4), pigs (5), cattle (6), veal (7) and farmed fish (8). In general, very positive effects have been observed for colour stability and decreased tendency for development of rancidity, and dietary α-tocopherol may accordingly also be expected to yield at least some protection to meats from animals raised on feed with added vegetable oil.

Farming depends on a high utilization of the feed and high performance is often ensured by the use of growth promoters. In Europe non-hormonal growth promoters like copper salts are preferred to growth hormones. However, copper is an oxidation catalyst and addition of copper to feed carries the risk of promoting oxidation in the live animal and in the meat after slaughter, especially when the feed contains a higher fraction of unsaturated lipid (9). When proposing investigations of the feasibility of changing the fatty acid profile in a more healthy direction for poultry meat and pork, a possible negative effect of copper as growth promoters had accordingly also to be taken into account combined with a detailed study of α-tocopherol as protector against oxidation. These three factors were accordingly considered in the DIET-OX project in the period 1995-1997, a project, which had the over-all goal of improving the nutritive value of pig and poultry meat under realistic production conditions without accepting decreases in shelf-life, toxicological safety or over-all wholesomeness.

DIET-OX: from feed to food and beyond

In the planning of the investigations the following three factors were taken into account when compounding the feed: (i) vegetable oil with a high fraction of the mono-unsaturated oleic acid was added to the feed in order to improve the nutritive value, (ii) the natural antioxidant α-tocopherol was added as the more stable acetate ester in order to protect the more unsaturated meat and fat, and (iii) copper(II) sulfate was added as a growth promoter in order to mimic real production conditions where copper addition may carry the risk of unintended oxidation. The basis of the DIET-OX project is the feeding plan presented in Table 1, which consists of 10 different combinations of addition of vegetable oil, vitamin E and copper to a basal feed based on barley. The oil source was different in different countries and an example of other variations was addition of β-carotene to chicken feed.

For the pigs and poultry raised on the feeds of Table 1 or slight variations hereof under realistic production conditions, the project covered the effects of changes in animals fed on (i) the performance, (ii) on physiological parameters in the live animal, (iii) on the oxidative stability of meat

and meat products, and (iv) on the improvement in nutritive value as studied in dietary intervention studies in human subjects. Besides this integration along the production chain from feed to food and beyond, the European dimension was also covered, as traditional products from different European regions were included with the goal of changing the fatty acid profile of meat produced in the Northern part of Europe toward a more Mediterranean profile. The partners and their individual tasks are listed in Table 2, from which the different meat products investigated also are evident.

Table 1: Feeding regime. The basis of the DIET-OX project[1]

1.	Basal diet based on barley[2]
2.	Basal diet plus 6% rape-seed oil[2]
3.	Basal diet plus 6% rape-seed oil plus 100 mg Vit. E
4.	Basal diet plus 6% rape-seed oil plus 200 mg Vit. E[2]
5.	Basal diet plus 6% rape-seed oil plus 35 mg Cu
6.	Basal diet plus 6% rape-seed oil plus 35 mg Cu plus 100 mg Vit. E
7.	Basal diet plus 6% rape-seed oil plus 35 mg Cu plus 200 mg Vit. E
8.	Basal diet plus 6% rape-seed oil plus 175 mg Cu
9.	Basal diet plus 6% rape-seed oil plus 175 mg Cu plus 100 mg Vit. E
10.	Basal diet plus 6% rape-seed oil plus 175 mg Cu plus 200 mg Vit. E

[1] This feeding plan was used by the Danish and German pig line with high oleic rape-seed oil. In a second feeding trial with the German pig line only 2% rape-seed oil was added. For the Italian pig line sunflower oil was used, see Table 2.
[2] Meat and lard from pigs raised on these three feeds were used in the controlled randomised single-blind dietary intervention study in healthy, human subjects.

Table 2: The DIET-OX partnership 1995-97.

Department of Dairy and Food Science
Royal Veterinary and Agricultural University, Rolighedsvej 30, 1958 Frederiksberg, Denmark:
Study of the effect of diet and its antioxidant/ pro-oxidant balance and lipid unsaturation on the oxidative stability of pig meat and meat products produced from pigs from the Danish pig line.

Research Department of Human Nutrition
Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg, Denmark:
Evaluation of the nutritional value of pork and pork products from the Danish pigline, intervention studies with healthy human volunteers.

Department of Product Quality
National Institute of Animal Science, Research Centre Foulum, P.O. Box 39, DK-8830 Tjele, Denmark:
Breeding of pigs from the Danish pig line. Oxidative status of live pig with emphasis on skeletal muscle function and raw meat quality.

Institut für Chemie und Physik
Bundesanstalt für Fleischforschung, E.C. Baumann Strasse 20, D-95326 Kulmbach, Germany:
Effect of vitamin E in relation to a changed fatty acid profile in pork and pork products like ham and raw sausages – technological aspects.

Istituto di Scienza e Tecnologia degli Alimenti
Universita' degli studi di Parma, Via del Taglio 8, 43100 Parma, Italy:

Effect of diet on oxidative changes in the whole range of pork eaten in Italy, including Parma ham, Salami and Coppa.

Istituto di Allevamenti Zootechnici (DIPROVAL)
Universita' di Bologna, Villa Levi, Coviolo, 42100 Reggio Emilia, Italy:

Effect of feed composition on growth rate and health for pigs from the Italian pig line.

Departemento Produccion Animal
Facultad de Veterinaria
Universidad Complutense de Madrid, Avda puerta de Hierro s/n, 28040 Madrid, Spain:

Effect of dietary natural antioxidants on the oxidative stability of dry meat products produced from heavy Iberian pigs.

Department of Food Technology
National Food Biotechnology Centre, University College Cork, Ireland:

Effect of diet on the oxidative stability of chicken muscle foods including interaction between anti-oxidative nutrients and copper as growth promoter.

Station de Reserches sur la Viande
Institut National de la Recherche Agronomique (INRA) Theix, 63122 St Genés Champanelle, France:

Effect of dietary fat, antioxidants and iron on the oxidative stability, the colour stability, flavour and consumer acceptability of turkey meat.

Department of Animal Nutrition
Institut de Recerca I Technologia Agroalimentaries (IRTA)
Centre De Mas Bove, Apartat 415, 43280 Reus, Spain:

Oxidative stability of fresh and cooked chicken meat from chicken raised on feed containing different fats, pro- and anti-oxidants, including the less investigated carotenoids.

Korn-og Foderstof Kompagniet
Groendalsvej 1, DK-8260 Viby J, Denmark:

Subcontractor supplying feed and expert knowledge.

Hoffmann-La Roche Ltd.,VFET – Animal Nutrition
4002 Basel, Switzerland:

Subcontractor supplying anti-oxidants and expert knowledge.

Danish Meat Research Institute
Maglegaardsvej 2, DK-4000 Roskilde, Denmark:

Evaluation of meat quality and production of products from meat from pigs from the Danish pig line.

Nutrition Laboratory
Department of Clinical Veterinary Medicine, University of Cambridge 307, Huntingdon Road, Cambridge CB3 OJQ, England:

Effect of dietary lipid n-6: n-3 ratio on cardiac function of pigs.

Live animals

The physiological parameters of the production animals raised on the supplemented feed are indicative of the well-being of the animals during growth. No indication of any negative effect on the animals was, however, found. Pigs raised on the oil-supplemented feed generally showed a shift from glycogen metabolism towards lipid metabolism as also indicated by a significant increase of free fatty acids in plasma (10). Copper supplementation only increased the copper level in the liver but not in the muscles of the pig, and increased copper level in the liver was interestingly enough found to be paralleled by an increase in liver α-tocopherol. For chicken, removal of both copper and iron supplementation decreased growth and feed efficiency, while removal of one of these essential metals had only marginal effect on feed efficiency (11). Copper was found to improve growth rate and increased feed intake for pigs given ad libitum feed but not to increase feed utilization. For heavy pigs as those used for production of Parma ham, no positive effect on performance was detected with a copper addition higher than that permitted by current EU rules for heavy pigs. This seems to be in contrast to the positive effect of high copper additions for lighter pig, confirming that the EU-rules for copper supplementation, in addition to the beneficial effect for the environment of low copper supplementation, do not give poorer performance, as far as the heavy pig is concerned (12). For chicken, copper was found not to affect the oxidative stability of breast or thigh muscle microsomes and copper had no significant effect on fatty acid profiles (13). When the antioxidant and oxidative status of the pigs was evaluated in terms of blood and liver analysis, no indication of oxidative stress or vitamin E deficiency symptoms was seen.

Raw meat

Addition of 6% high-oleic vegetable oil to pig feed was found to change the fatty acid profile towards more unsaturation in backfat from 41- 45 % to 30-32% to a lesser degree in the muscle lipid and hardly in the phospholipids (10). The sensory quality of meat and meat products is strongly affected by oxidation processes and the central part of the project dealt with the study of oxidative deterioration of the meat during processing and storage. For the Danish pig line, supplementation of the feed with 6% rapeseed oil did not influence colour stability and lipid oxidation of fresh or pre-frozen chill stored pork chops negatively (14). Some negative effects were, however, noted for the German pig line (15). For the Italian pig line, a remarkable result was, that oil addition without supplementation with α-tocopherol increased the uptake of α-tocopherol from the basic feed resulting in an increase in 100% of vitamin E in the meat. Cholesterol, which especially in highly processed meat is becoming oxidized forming toxic cholesterol oxides, was found to be protected by α-tocopherol and also meat pigment oxidation was found to be slower (16).

For the Iberian pig line, a relationship between the concentration of α-tocopherol in the meat and susceptibility to lipid oxidation was clearly demonstrated. Also the fat source was found to affect the susceptibility of the resulting meat to lipid oxidation, and animals fed a diet enriched with animal fat (tallow or lard) were found less susceptible to lipid oxidation than other fat sources (rapeseed oil or sunflower oil) (17). The influence of increased lipid unsaturation and of addition of copper salts as growth promoters to the feed was also studied for meat and meat products from turkey and chicken together with the protective effect of especially α-tocopherol but also of ascorbic acid and β-carotene as antioxidants. As for chicken, replacement of tallow in the feed with olive oil clearly gave a change in the meat lipid toward more monounsaturation, but olive oil as feed additive did not adversely affect the oxidative stability and had no effect on drip loss. α-tocopherol, but not ascorbic

acid, added to the feed improved the oxidative stability of raw meat during frozen storage, although removal of supplemental copper for two weeks prior to slaughter together with supplementation with α-tocopherol reduced lipid oxidation in thigh meat (11). A clear positive vitamin E effect was in general found on the oxidative stability of poultry meat. For frozen turkey meat, lipid oxidation was also affected by dietary fat (rape-seed ~ soy > tallow) and specific muscle effects were noted (*Sartorius* > *Pectoralis*) (18). In chicken, β-carotene had a pro-oxidative effect under certain conditions, while an anti-oxidative effect was demonstrated for other conditions (19). In a storage experiment with chilled chicken meat from birds fed different combinations of β-cartotene and vitamin E, vitamin E appeared to be oxidized in the presence of β-carotene and it seems like β-carotene acts as an antioxidant only when tissue vitamin E is high, findings which certainly deserves further attention.

Meat products

A number of important processed pork and poultry products from different European regions were investigated in details. Including 6% rape-seed oil in the feed resulted in increased lipid oxidation in processed pork as studied for the traditional Danish dinner sausages and pre-cooked meat patties. Increased muscle vitamin E obtained through dietary supplementation yielded protection against lipid oxidation in such products, although 200 mg α-tocopherol/kg feed was necessary for full protection (14). Chopping of the meat and heat treatment induces oxidative stress and the positive effect was most evident for minced and pre-cooked meat and for pork chops stored in high oxygen atmosphere in order to increase colour stability. For meat products with longer shelf life such as raw sausages and hams produced from the German pigs together with frozen meat, vitamin E supplementation was found to exhibit positive effects on the intrinsic quality characteristics as evidenced by less lipid oxidation, while colour stability or drip loss was not affected.

For typical Italian products like Parma ham, salame and coppa, the increased unsaturation gave technological problems and would also result in problems in relation to official branding. Supplementation with 6% high-oleic sunflower oil decreased the level of saturated fatty acids in hams, coppa and salame produced from these animals by 15% and increased the level of mono-unsaturated fatty acids by 10% and poly-unsaturated by 15% (20). Such changes were detected also by an increasing iodine number, which were above the official limit for Parma hams. The softness of the fat resulted in smearing during production of salame and coppa with further negative effects on quality attributes like colour and firmness. Similar problems were encountered for production of raw Salami type sausages, an important German meat product, from pigs raised on feed supplemented with 6% rapeseed oil. Again smearing during batter production was seen, followed by insufficient drying of the sausages (21).

It became, however, possible within the framework of the project to raise additional pigs belonging to the German pig line, this time on a feed supplemented with 2% rapeseed oil. This level of oil supplementation was estimated from the practical experience obtained during sausage making with meat from the pigs raised on feed with 6% oil supplementation. This “iterative” approach was successful, as sausages were produced from meat from pigs raised on the feed supplemented with 2% oil without problems and the overall product quality was at least as good as for the reference sausages. The same conclusion was reached for hams, where 2% rapeseed supplementation also was concluded to be the maximum. This example illustrates nicely the compromises necessary

during the process of optimizing nutritive value of foods without decreasing the sensory quality and also the importance of investigating each product.

Supplementation with α-tocopherol acetate to the feed was for the ham produced from animals belonging to the Iberian pig line found to provide some but not full protection of the increased unsaturation induced in the lipid by the use of plant oil rather than animal fat (22). For the extensively reared animals, indication was found, by the study of membranal stability, of positive effects of other antioxidants than α-tocopherol. In future studies, this observation deserves further attention as plant materials contains a wide variety of polyphenols and carotenoids, which may find use as feed additives also for animals not raised extensively.

While oxidative damage in biological material traditionally is followed by changes in the lipids, future investigation probably will have more focus on the proteins as markers of oxidative stress. Proteins respond faster especially to activated oxygen species and pro-oxidants in the aqueous phase of a living system and protein oxidation was explored as a marker for damage to turkey meat (23). Meat from turkeys fed soy oil were very sensitive to oxidation, whereas meat from turkeys fed tallow were found more resistant to oxidative stress and the sensory properties of the meat reflected the fat source. Also protein oxidation was found more pronounced in the meat from the soy-oil fed turkeys. Carnosine, a muscle peptide, was found to be an efficient antioxidant when added to minced chicken thigh muscle both raw and cooked. This part of the investigation was more explorative than focused on practical use, but it should be noted that a combination of feed supplementation with α-tocopherol and addition of carnosine was found to yield the best protection of chicken thigh meat against rancidity and against cholesterol oxidation (24).

Meat and human health

The overall conclusion from the project seems clear, and the results from the project formulated as recommendations to farmers, to the meat industry and to the European public should lead to changes in farming practices. For several pig lines, for turkeys and chicken dietary modifications have been shown to be a valuable tool in changing the fatty acid profile of the meat and for pigs, especially of the backfat in a more healthy direction. In one case, for the Danish pig line, meat, lard and meat products were used to perform a controlled randomised single-blind dietary intervention study in human subjects in order to obtain a direct evaluation of the effect of three of the treatments of Table 1 of pork and lard on risk markers for coronary heart disease, and to measure the content and availability of vitamin E and selenium in the diet.

The conclusion from the intervention study was that it is possible to improve the nutritional value of pig fat by changing the feeding regime for the pig. Inclusion of 6% rape-seed oil in the pig feed resulted in a fatty acid composition of the fat, giving a 4% lower total cholesterol concentration in young, male subjects, corresponding to a decreased risk of getting a cardiac heart attack of 8%, when a diet based on meat and lard from these pigs for three weeks replaced a similar diet based on meat and lard from non-supplemented pigs (25). Notably, this conclusion is not based on speculations and assumptions, but on a real dietary intervention study with young healthy human subjects, and the study may constitute the first example, where all steps in the meat production are combined with a dietary intervention study. Positive health effects may even be larger for other population groups with lifestyles resulting in for example overweight or obesity.

The dietary intervention study further showed that the selenium available through pig meat is higher than normally assumed and that meat products from pigs raised even on the non-supplemented feed, at least for the investigated Danish pig line, has a more healthy lipid profile than expected. The message now to be disseminated to the European consumer is that pork (and poultry meat) is a valuable source of important nutrients and that the lipid profile can be and should be changed in order to approach that of certain vegetable oil normally associated with a healthy image.

Another important conclusion from the project in relation to nutritive value of meat, is that chicken meat is a very good vehicle for vitamin E, since absorption of vitamin E from feed is superior for chicken when compared especially to turkey but also to pigs. This finding should also be considered in relation to another conclusion from the dietary intervention study concerning vitamin E. The vitamin E blood level in the human subjects after the intervention period with the more unsaturated meat and lard showed a decrease, indicating that vitamin E supplementation from other sources should increase to meet the increased oxidative stress induced by the more unsaturated lipids (25). Chicken meat from birds raised on a supplemented feed may be such a source, together with certain plant based foods.

Conclusions and recommendations

In the DIET-OX project, pigs and poultry have been raised on feeds with or without supplementation of high-oleic vegetable oil, copper as a growth promoter and α-tocopherol as an antioxidant under realistic production conditions. The projects covered the effects of changes in the animal feed on: (i) the performance, (ii) physiological parameters in the live animal, (iii) the oxidative stability of meat and meat products, and (iv) the nutritive value as studied in dietary intervention studies in human subjects. On the basis of the results it is recommended: (i) that pigs are raised on feeds including up to 6% high-oleic vegetable oil and supplemented with α-tocopherol. The level of added oil and α-tocopherol (100 ppm or 200 ppm) should depend on the breed and on the type of meat products to be produced, and an oil addition of 2% may be the maximum for certain meat products; (ii) that more systematic research is initiated to provide more detailed guidelines for the use of unsaturated fats in animal feeding in order to allow production of meats with even lower concentration of saturated fatty acids, and antioxidant other than vitamin E is included in such investigations; (iii) that the economical aspects of increasing the level of unsaturated fat in pig feed and supplementation with α-tocopherol is being elucidated and that profitability for the European farmer of producing the nutritive improved meat products is ensured; and (iv) that current rules of addition of copper to pigs feed and chicken feed are not changed.

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Solving the problems of texture and flavour in low fat meat products

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Abstract

This project was undertaken to provide a better understanding of the underlying causes of the inferior quality of low fat meat products. By so doing a basis for the development of low fat meat products of acceptable quality would be laid. The project was wide ranging and multidisciplinary, with each of the five partners concentrating on certain tasks using local traditional products as models for the basic types of product, comminuted, emulsion cured and dried fermented. This paper reports on the results of the tasks undertaken by The National Food Centre, which were mainly concerned with flavour studies, the effect of processing factors on low fat beefburger quality and the effects of a range of fat substitutes and blends on the quality of beefburgers and frankfurters.

Introduction

Traditional processed meat products are relatively high in fat, particularly saturated fats, which have been associated with coronary heart disease. There is therefore a demand for meat products with a reduced fat content. Since fat plays an important role in the flavour, texture, succulence and satiety value of these products, reducing the fat content results in less acceptable products. Attempts by industry to replace fat with substitutes such as carrageenan have not been very successful.

Despite the importance of fat to meat products, the scientific basis for its functionality and its interactions with other ingredients, including fat substitutes is not well known. The objective of the project was to increase this knowledge base in order to provide a sound scientific basis for the development of low fat meat products of acceptable quality. It was the intention to provide knowledge to facilitate the meat industry to develop low fat meat products, rather than to develop new products *pre se.* By developing a better scientific understanding of the role of fat and its interaction with other ingredients and with processing factors the industry will be better placed to develop low fat meat products of acceptable quality and exploit their potential.

A consortium of five partners in three countries was brought together to carry out a project to address these problems. The consortium included one industrial partner involved in meat processing and the production of milk fractions as food ingredients. Five goals were identified in order to achieve the objective: (a) To relate molecular transformations and associations which occur in meat products on reducing fat levels to quality aspects; (b) To identify the essential flavour components and interactions in reduced fat products; (c) To characterise the ultrastructural and rheological properties of low fat meat products; (d) To evaluate and optimise different processing technologies in the manufacture of low fat meat products; (e) To assess limitations on the reduction of fat in products in relation to quality, safety and consumer acceptability. Each of these was divided into specific tasks with one or more partners assigned to each.

The tasks of The National Food Centre, which are reported here, were mainly concerned with goals (b), (d) and (e).

Flavour studies on low and standard fat meat products

These studies were undertaken because flavour problems are associated with low fat meat products, yet little is known about the underlying causes other than the fact that most of the flavour compounds are fat soluble. Furthermore, even less is known about how the various fat substitutes interact with the other ingredients in the release of flavour volatiles.

The flavour volatiles of several types of emulsified meat products with different levels of fat (5-30%) were studied. From the several hundred volatiles, a few of those giving the highest peaks were identified. Many of these have not been previously reported, but some of those found in frankfurters have been reported as present in cooked beef and pork.

There were essentially no differences between low fat and standard fat products in the occurrence of compounds found in the headspace. More importantly, however, fat content had a large effect on the quantities of volatiles released, with low fat samples having larger quantities than standard products. Nose space analyses indicated that the volatiles are released more rapidly from low fat formulations, which may explain much of the difference in flavour acceptability between low fat and standard fat products. These differences between low and standard fat frankfurters were not apparent, however, when smoke and spices were excluded from the formulation as most of the major volatiles (terpenes and phenols) derive from these ingredients.

The effect of fat replacers on the flavour volatile profiles of beefburgers also revealed some interesting results. Low fat beefburgers with oat fibre added released similar quantities of volatiles to high fat burgers. Hence, oat fibre seems to be able to trap the flavour volatiles and delay their release, thus overcoming the problem referred to above. Oat fibre also delayed the release of some, but not all, of the flavour volatiles from low fat frankfurters, whereas tapioca starch and whey protein did not. The effect of oat fibre would seem, therefore, to be dependent on the class of compound. Maltodextrin was found to be a useful binder for low fat salami and mortadella with some potential for flavour improvement.

Sensory profiling studies indicated that the low fat frankfurters gave a greater ‘overall flavour intensity and were in good general agreement with the instrumental measurements. Time-intensity studies reflected the profiling results in that the peak intensity of flavour was highest in the low (5%) fat frankfurter and lowest in the standard (30%) fat samples for each of the 5 panellists used. Thus, a decrease in fat from 30% to 5% increased the perception of flavour of some of the flavour attributes of frankfurters. The primary role of fat in this product would appear to be to trap these volatiles and release them gradually during mastication.

Effect of various processing factors on the texture and eating quality of low fat beefburgers

These studies were undertaken to see if the functionality of the fat present in formulations could be optimised by modifying the processing. It would then follow that the amount of fat in the formulation could be reduced without affecting eating quality. Reduced fat versions of meat products could then be offered without the use of non-meat ingredients.

Work on beefburgers using an all meat formulation showed that panellists preferred high fat burgers but the differences were not as large as might have been expected given the large range in fat

content (7-23%). Also, the data showed that in a low fat (8%) formulation water should not be added at above 8% to avoid excessive cooking losses.

An experiment on the effect of grind size showed that a medium grind size (5mm versus 2 or 10 mm) gave the best texture as burgers made with a 10mm grind size were too coarse while those made with a 2mm grind size were too soft. Also the use of different types of carcass fat (brisket, clod and suet) was studied. The type of fat used in a low fat (8%) formulation had little effect on the texture or sensory attributes and even when this was repeated in a high fat (23%) formulation none of the fat types significantly improved palatability over the others.

The effect of cooking method (grilling, frying, deep fat frying, girdling and oven roasting) on palatability and texture was studied in another experiment. Panellists preferred burgers that were cooked on a griddle with deep fat fried burgers being the least acceptable. This is the most common form of cooking used by fast food outlets. Panel results were broadly supported by textural analysis.

Effect of a range of fat substitutes and blends on the texture and eating quality of low fat beefburgers

Many ingredients have been investigated for use as fat substitutes in low fat meat products and a large number are available commercially. No comprehensive study has been undertaken to compare their performance in terms of eating quality either singly or in combination. These studies were carried out to identify the most promising ingredients and blends.

Seventeen commercially available functional ingredients were assessed in low-fat beef burger formulations. There were differences in cook yields between the different low-fat meats. The burgers containing alginate/calcium lactate had the highest cook yield at 71.30% and had good textural qualities, but scored low in overall flavour. Most additives tested increased the WHC by comparison with the full-fat (23%) control, which had the lowest WHC (26.15%). Sensory analysis showed that there was a trend for higher fat beef burgers to have increased tenderness, however, no significant differences were found between the low- and full-fat controls. Beef burgers containing pectin, micro-crystalline cellulose, oat fibre and carrageenan scored high in flavour and overall quality. In contrast the burger formulated with blood protein had poor overall quality and flavour attributes. Burgers containing tapioca starch, modified food starch derived from waxy maize and oat fibre significantly reduced Instron shear values.

Correlations between sensory and instrumental texture traits are shown in Table 1. It is interesting to note that the correlation between sensory tenderness and Kramer Shear measurements was as high as that between Kramer Shear and the other instrumental texture measurement, that is Warner Bratzler. The complexity of the textural assessment of this type of product is highlighted by the fact that the correlation between tenderness and overall texture was only 0.5, considerably lower than between sensory tenderness and instrumental texture measurements. These findings were confirmed in the other experiments.

Optimising the composition of ingredients has a major effect on the properties of low-fat products. A further experiment was carried out to assess the effects of added tapioca starch (ranging from 0 to 30 g/kg), oat fibre (ranging from 0 to 20g/kg) and whey protein (ranging from 0 to 20 g/kg) on the physical and organoleptic characteristics of low-fat beef burgers. Response surface methodology (RSM) was used to study the simultaneous effects of these three ingredients. Significant regression

models were ascertained for cooking properties (yield and WHC), the sensory attributes of tenderness and juiciness and all mechanical texture attributes. The level of tapioca starch (TS) influenced the models to the greatest extent. Its effect on cooking properties was highly significant, positive and linear. The presence of TS had a large negative linear effect on Warner-Bratzler and Kramer shear forces. For each significant regression model, TS exerted a linear effect and in some cases a quadratic effect. Both oat fibre and whey protein had a limited influence. There was no evidence for interactions among the variables. The results indicate that as TS is increased in low-fat beef burgers a succulent and tender low-fat product is obtained.

Tapioca starch, carrageenan, oat fibre, pectin, whey protein and a commercial mixture of carrageenan and locust bean gum were assessed for their ability to mimic fat characteristics in cooked low-fat (10%) beef burgers. Thirteen different blends of the ingredients were formulated in order to examine their effects on quality parameters of low-fat beef burgers. The beef burgers were tested for cook yield, water-holding capacity (WHC), retention of shape, sensory and mechanical texture analysis. Most blends significantly ($P<0.05$) increased both cook yield and WHC, in particular blends containing tapioca starch, oat fibre, whey protein and the carrageenan/locust bean gum mixture. These blends substantially reduced both Warner-Bratzler and Kramer shear values. Sensory analysis showed that beef burgers containing tapioca starch, oat fibre and whey protein were acceptable in terms of flavour and texture. The low-fat control was found to be the toughest and driest of the beef burgers examined. This study shows that blends of these ingredients can be used to offset the poor quality associated with low-fat beef burgers.

Table 1: Correlation coefficients between sensory and instron measurements

	T	M/J	OT	OA	W\B
M/J	.765***				
OT	.506*	.474*			
OA	.228	.091	.743***		
W\B	-.718***	-.583**	-.279	-.161	
Kramer	-.775***	-693***	-.252	-.052	.774***

T: Tenderness; M/J.: Moistness/Juiciness; OT: Overall Texture; OA.: Overall Acceptability; W\B: Warner-Bratzler Shear Force; Kramer: Kramer Shear Force; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; No superscript: Non significant

Effects of fat substitutes and smoking method on the quality of frankfurters of different fat contents

Replacing fat with lean to make low fat frankfurters results in too chewy a texture in addition to being uneconomical. Water is the cheapest substitute for fat, but unless some form of binder is used cook losses will be unacceptably high. These studies on the effects of various non-meat ingredients on the quality of frankfurters with different fat contents were undertaken because of the lack of information on their performance in this type of product. The effects of the two main smoking methods on the quality of frankfurters have been documented, but their interaction with fat content has not, so this was addressed in a further study.

The effects of fat level (5 and 12%), tapioca starch and whey protein on the hydration/binding properties, colour, textural and sensory characteristics of frankfurters were investigated. The formulations are shown in Table 2. Decreasing the fat content increased cook loss and decreased emulsion stability and product lightness. Fat reduction increased smoke, spice and salt intensities and increased overall flavour intensity and juiciness. Texture Profile Analysis (TPA) indicated that fat reduction

Table 2: Formulation for frankfurters with varying fat levels with and without tapioca starch and whey protein. Weights are presented for 12 Kg batches.

	Lean Pork	Lean Beef	Fat	Ice	H_2O	Na Asc[1]	Salt	PO_4^{2}	Smoke	Spice Mix
5\0*	3.25	3.51	0.49	0.74	3.74	0.006	0.18	0.03	0.006	0.06
5\1	3.25	3.51	0.49	0.69	3.43	0.006	0.18	0.03	0.006	0.06
5\2	3.25	3.51	0.49	0.69	3.43	0.006	0.18	0.03	0.006	0.06
12\0	3.17	3.42	1.53	1.20	2.34	0.006	0.18	0.03	0.006	0.06
12\1	3.17	3.42	1.53	1.08	2.16	0.006	0.18	0.03	0.006	0.06
12\2	3.17	3.42	1.53	1.08	2.16	0.006	0.18	0.03	0.006	0.06

[1]Na Asc = Sodium Ascorbate; [2] PO_4 = Phosphate. *The first number refers to the targeted fat level and the second to the ingredient. 0 = No added ingredient; 1 = Tapioca starch; 2 = Whey protein.

decreased gumminess and cohesiveness but the other parameters measured were unaffected. Addition of tapioca starch or whey protein reduced cook loss and increased emulsion stability. Whey protein did not effect the sensory characteristics but tapioca starch increased overall flavour intensity. Both ingredients increased hardness, adhesiveness, gumminess and chewiness as measured by TPA. Two-factor interactions between fat level and ingredient were observed for several parameters.

The effects of fat level (5, 12 and 30%), carrageenan and oat fibre on the hydration/binding properties, colour and flavour characteristics of frankfurters were investigated. Decreasing the fat content from 30% to 5% significantly increased cook loss and decreased water holding capacity and emulsion stability. Reduced-fat products were also darker and more red compared to the 30% fat controls. Addition of carrageenan or oat fibre reduced cook loss and increased both water holding capacity

and emulsion stability. Sensory evaluation (see Table 3) indicated that decreasing fat from 30% to 5% increased the intensity of smokiness, spiciness and saltiness and reduced the overall acceptability of the flavour. Carrageenan or oat fibre did not alter the colour of the frankfurters and neither ingredient had a significant effect on the flavour characteristics assessed.

The results of these two studies demonstrate that tapioca starch, whey protein, carrageenan or oat fibre can partially offset some of the changes which occur in low-fat frankfurters when added water replaces fat and protein level is constant.

Table 3: Influence of fat, carrageenan and oat fibre on smoke, spice, salt and other flavour intensities and on mouthfeel and overall acceptability of flavour

	Smokiness	Spiciness	Saltiness	Other flavour	MF	O/A
A:Fat Level						
5	4.2 [a]	4.4 [a]	3.0 [a]	2.2	3.2	3.5 [a]
12	3.6 [b]	3.7 [b]	2.8 [a]	1.9	3.1	4.0 [b]
30	3.4 [b]	3.3 [c]	2.5 [b]	2.1	3.1	4.0 [b]
SL	0.0003	0.0	0.0109	NS	NS	0.0088
B:Ingredient						
0	3.9	3.8	2.9	2.1	3.3	3.8
1	3.6	3.8	2.7	2.0	3.0	3.7
2	3.7	3.9	2.7	2.1	3.0	3.9
SL	NS	NS	NS	NS	NS	NS

0 = No added ingredient; 1 = Carrageenan; 2 = Oat Fibre. NS = Not significant. SL = Significance level. MF = Mouthfeel/oiliness. O/A = Overall acceptability of flavour. Different letters in the same column (within each main effect) indicate significant differences ($P<0.05$).

The interactive effects of fat content (5, 12 and 30% fat) and smoke method (solid aerosol or commercial smoke flavouring) on the texture, flavour, colour and cook losses of frankfurters were investigated. Cook losses from frankfurters smoked with solid-aerosol were higher than from products with an added smoke flavouring. Products smoked with solid-aerosol were more coloured than those with an added flavouring. The effects of the smoke method on colour and cook loss were dependent on the fat content and vice versa. A two-factor interaction between fat content and smoke method was observed for three Texture Profile Analysis parameters (hardness, adhesiveness and gumminess). No interactive effects were observed for any of the sensory characteristics. Frankfurters smoked with solid-aerosol were judged to be more smoky, more spicy, more salty, had better texture and were more acceptable than those with an added smoke flavouring. The results indicate that interactive effects are important considerations during the development of reduced-fat frankfurters and/or when changing the smoke process.

Conclusions

- Low fat and standard fat meat products had the same flavour volatiles but for low fat samples these were released in larger quantities.
- Flavour volatiles are released more rapidly from low fat formulations, which may explain much of the difference in flavour acceptability between low fat and standard fat products.
- Smoke and spices are responsible for most of the major volatiles (terpenes and phenols) in meat products such as frankfurters. Differences in flavour release due to fat content are not apparent in formulations lacking smoke and spices.
- Low fat beefburgers with oat fibre added released similar quantities of volatiles to high fat burgers. Hence, oat fibre seems to be able to trap the flavour volatiles and delay their release, thus overcoming the problem referred to above.

- Oat fibre also delayed the release of some, but not all, of the flavour volatiles from low fat frankfurters, whereas tapioca starch and whey protein did not.
- Panellists preferred high (23%) fat burgers to those with only 7% fat but the differences were not large
- Without the use of binders, the practical limit for added water in a low fat (8%) formulation is around 8% due to excessive cooking losses.
- In the preparation of low fat beefburgers, a medium grind size (5mm versus 2 or 10 mm) gave the best texture as burgers made with a 10mm grind size were too coarse while those made with a 2mm grind size were too soft.
- The type of fat (brisket, clod and suet) used in either a standard (23%) or low fat (8%) beefburger formulation had little effect on the texture or sensory attributes.
- Cooking method affected the palatability of low fat beefburgers. Panellists preferred burgers that were cooked on a griddle with deep fat fried burgers being the least acceptable.
- Increasing levels of tapioca starch in low-fat beef burgers improved succulence and tenderness.
- Blends of tapioca starch, oat fibre and whey protein can be used to offset the poor quality associated with low-fat beef burgers.
- Beefburgers containing pectin, micro-crystalline cellulose, oat fibre or carrageenan scored high in flavour and overall quality, but blood protein adversely affected overall quality and flavour attributes.
- Beefburgers containing tapioca starch, modified food starch derived from waxy maize and oat fibre significantly reduced Instron shear values.
- Reducing the fat content of frankfurters reduces the colour, improves their hydration/binding properties, increases the flavour and adversely affects their texture.
- The addition of tapioca starch, whey protein, carrageenan or oat fibre can partially offset some of the changes that occur in low-fat frankfurters when fat is replaced with water. They improve emulsion stability, cook losses and texture but their effects on flavour are minimal.
- Frankfurters smoked with solid-aerosol have better texture, are more acceptable but have higher cook losses than those with an added smoke flavouring. Due to interaction effects, changes in the fat content may alter the quality of the frankfurters depending on the type of smoke process used. Awareness of such interactions may be critical in the development of reduced fat frankfurters and/or if using a new smoke process.
- The relatively poor correlations between textural measurements and sensory scores of tenderness in emulsion type sausages were confirmed.
- Texture Profile Analysis gave more promising results but most of the textural parameters had non-linear relationships with fat level mainly due to the effect of differing protein contents. It is therefore important to hold protein content constant when comparing products with different fat levels.

Quality policy and consumer behavior towards fresh meat: Results of a European cross country study

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The study has been carried out with the financial support from the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific RTD programme, CT 95-0046, „Quality Policy and Consumer Behaviour". It does not necessarily reflect its views and in no way anticipates the Commission's future policy in this area. This manuscript presents only some of the results. The complete study can be downloaded from http://www.uni-hohenheim.de/~apo420b. The contribution of Kristina Glitsch and the other members of the project is acknowledged.

Abstract

The EU food quality policy is based on the principles of mutual recognition, subsidarity and consumer protection. Vertical harmonization does not play an important role any more. The measures favoured by regulators in the food sector are standards and labels. While the standards approach is pursued mainly in the area of food safety, the labelling approach is employed to avoid consumer deception. So far, a diversity of labels exist, but none of them has itself established successfully on the market. Consumers regard the place of purchase as the most important cue for meat quality when shopping. The country of origin is used by consumers both, as an important cue for food quality and food safety. The introduction of the labelling approach in the meat sector may result in a discrimination against foreign suppliers, if the importance of country of origin as a quality cue is further strengthened.

Background of EU food quality policy

Economic integration and interdependence among EU-Member States has increased sharply since the beginning of the eighties. While EU-Member States had already developed and implemented their own national food policies, according to the agreements reached by different national political interest groups, an common food policy was, and still has to be developed. The present approach chosen by EU food policy is based on three principles. Two of these principles emerged in the 80's: the principle of mutual recognition and the principle of subsidarity. The later principle displaced the horizontal harmonization approach, as pursued by EU food policy till the beginning of the 80's. The principle of consumer protection is gaining impetus since the beginning of the 90's. The BSE crisis resulted in additional efforts to improve food safety within the EU.

The principle of mutual recognition emerged from the Cassis de Dijon ruling of the European Court. The arguments and measures proposed by the Court ruling were eagerly picked up by the Commission (Mattera, p.74f). Vertical harmonization was no longer regarded as the sine qua non principle for the harmonization of trade between Member State. Subsidarity became the important principle in the enlarging European Community. The deception of consumers as a possible consequence of mutual recognition is discussed already in the Cassis de Dijon case. Ten years later, in 1989 the European Community's determination to implement consumer policy in Europe motivated the Commission to create the Consumer Policy Service (CPS). As a consequence of the BSE crisis, the CPS became the DGXXIV on 1 April 1997 with the denomination: Directorate-General for Consumer Policy and Consumer Health Protection.

The Court ruling settling the Cassis de Dijon case mentioned already the labelling approach as the means to protect consumer from deception. It is regarded as one of the available measures with less trade distorting effects than mandatory national standards. The labelling approach was picked up by the Commission rather eagerly. The Directorate General VI paved the way for the introduction of the Procted Designation of Origin (PDO), the Protected Geographical Indication (PGI), and the Certificates of Specific Character on EU level. These are examples of the labelling approach pursued in EU quality policy.

The mandatory or voluntary standard approach is chosen mainly for safety issues. Examples are HACCP and ISO 9000.

The labelling approach for food quality and the standards approach for food safety are the main instruments chosen by European Union food policy. While the safety aspect has been discussed in detail in the follow up of the BSE crisis (Club de Bruxelles, 1997) a discussion of the quality aspects is considered by the author to be missing.

Events marking the development of present approach pursued by EU food policy towards food quality include the Cassis de Dijon case in 1979, the presentation of a specific communication on Community food law to the Council and the European Parliament in 1985 by the Commission in the wake of the major White Paper on the completion of the internal market, the New Approach to technical harmonization and standards as laid down in 1985, and the Council Regulations on the protection of geographical indications and designations of origin together with the Council Regulations on certificates of specific character in 1992.

The approach to food policy in the future as proposed by the Commission, is presented in the European Commission Green Paper "The general principles of food law in the European Union" and in the Commission Communication "Consumer health and food safety", both in April 1997. Both papers together present a good outline of EU quality policy and are intended to serve as a basis for the public discussion.

In this paper the focus is on fresh meat. Here two regulations might have important implications for quality policy. The introduction (by regulation) of a bovine animal identification and registration system offers the possibility to make claims not only on the animal category (just beef or bull, heifer, cow etc.) but on all the other product and process quality characteristics. This identification system offers the opportunity for the fresh meat supply chain to introduce labels with cues signalling product or process quality. Quality cues have to be established on the market and in particular in the mind of the consumer for becoming a kind of brand. This is taken care of and further encouraged with the introduction of the beef labelling system, which is till 31.12.1999 voluntary and may become mandatory in 2000.

The beef labelling system seems to offer the opportunity to restore consumer confidence and increase beef consumption. But, hardly realized by policy makers, the beef labelling regulation may introduce barriers to trade between Member States, if the country of origin becomes an important cue on the label. As the following results clearly show, country of origin is already one of the main cues used by the consumer for food quality and this extends to food safety to some extent. The labelling regulation could strengthen the importance of country of origin for producers and result in trade distorting effects.

Quality in the shop

In the following some selected results of the research are presented. The analysis is based on the distinction between quality in the shop, eating quality and safety. Consumers have available some cues to judge the quality in the shop. The place of purchase (butcher or supermarket) might signal quality. As a result of the BSE crisis, retailers communicate the country of origin of the meat to consumers. Labels and the price are further cues available for the consumer when buying meat. Intrinsic cues are: colour, leanness, and marbling. These cues serve as signals for quality and can be experienced by the consumer when buying the meat. The eating quality itself is experienced in the process of consumption by the consumer. Eating quality characteristics are: tenderness, flavour juiciness, free of gristle, texture, colour, smell and leanness. Food safety is another issue. Food safety is a credence quality attribute, which is neither learned by visual inspection in the shop nor by eating the meat. Possible cues for meat safety are: freshness, way of production (e.g. organic), origin, price, producer. Other safety cues could be communicated by labels.

The results presented are the outcome of a survey and are intended to shed some light on the questions, what do consumer associate with the different cues for quality and how important are the quality cues? The data was collected in the Spring of 1997 through telephone surveys of 500 households in each country. The interviews were conducted by MRC (Market Research Centre), a commercial telephone survey organisation, using random-digit dialling procedures. Individuals responsible for household food shopping were the subjects of this sample. The questionnaire was translated (and independently retranslated) into each of the respective languages. The quality cues presented in the questionnaire are the outcome of three focus group sessions conducted in each of the countries investigated.

Table 1 shows that among meat characteristics which can be used for assessing meat quality in the shop, 'price' was distinctly considered to be the least helpful quality indicator in all countries, except in the United Kingdom. For beef and pork, the 'place of purchase' was one of the most important quality cues in all countries except in Sweden and the United Kingdom. In Sweden meat is sold by supermarkets. Butchers do not play a significant role. In the United Kingdom, supermarkets have established themselves in the premium quality segment. In all other countries butchers are regarded as delivering superior quality compared to supermarkets. The place of purchase is judged by consumers to be less important for chicken than for pork of beef. Among the intrinsic factors, 'colour' was the most important for all types of meat. 'Marbling' seems to be the most problematic quality indicator, since many respondents, particularly in the Spanish sample, were unsure and did not know how helpful they considered 'marbling' for assessing beef and pork quality.

The results clearly demonstrate, that consumers regard the place of purchase as the main quality cue when buying meat. Colour and to a lesser extent country of origin serve as additional cues. Fewer differences between the countries were obvious in the case of eating quality characteristics, as table 2 presents. In general, 'flavour' is one of the most important factors, while 'leanness', 'texture' and 'free of gristle' belong in many cases to the least important eating quality characteristics. Altogether, most of the factors, except 'leanness' and 'free of gristle' were rated very highly in each of the countries.

Table 1: Significant differences in the helpfulness of 'quality in the shop' characteristics - results of a t-Test

	Germany	Ireland	Italy	Spain	Sweden	United Kingdom
BEEF						
1st rank	Origin, place	Colour, place, leanness, origin	colour, place	place, colour	colour, origin, label	colour, leanness
2nd rank	Leanness, colour	Marbling, label	origin	leanness, origin, marbling, label	marbling	place, marbling, label
3rd rank	Marbling, label	Price	Marbling, label, leanness	Price	leanness	price, origin
4th rank	Price		price		place, price	
PORK						
1st rank	Place,	colour, leanness, place	Colour, place	colour, place	colour, origin	colour, leanness
2nd rank	Origin, colour, leanness	Origin	Origin, marbling, label, leanness	leanness, marbling, label, origin	label	place
3rd rank	Label, marbling	Label, marbling	Price	Price	marbling	marbling, label, price
4th rank	Price	Price			leanness	origin
5th rank					place	
6th rank					price	
CHICKEN						
1st rank	Place, origin, leanness, colour, label	Colour	Colour	colour, place	origin	colour
2nd rank	Price	Leanness, place	Place, origin	Leanness	label	leanness
3rd rank		Origin, label	Leanness, label	label, origin	colour	place, label
4th rank		Price	Price	Price	price, leanness, place	price
5th rank						origin

Source: Becker, Benner and Glitsch, 1998

Table 2: Significant differences in the importance of 'eating quality ' characteristics - results of a t-Test

	Germany	Ireland	Italy	Spain	Sweden	United Kingdom
BEEF						
1st rank	Tenderness, juiciness, flavour, smell	Tenderness, flavour	flavour	Flavour, tenderness, juiciness	flavour	flavour, tenderness, gristle, texture, colour, juiciness, smell, leanness
2nd rank	Colour, gristle, texture	Colour, leanness, juiciness, texture, gristle, smell	tenderness, juiciness, smell, colour, texture	Colour, smell	tenderness	
3rd rank	Leanness		leanness, gristle	Texture	juiciness, smell, texture, gristle	
4th rank				Leanness	colour	
5th rank				Gristle	leanness	
PORK						
1st rank	flavour, tenderness, juiciness, smell	Flavour, tenderness	flavour	Smell, flavour, tenderness, juiciness, colour	flavour	flavour, tenderness, gristle, smell, texture, colour, leanness, juiciness
2nd rank	colour, texture, gristle, leanness	Leanness, texture, colour, smell, gristle, juiciness	tenderness, texture, smell, juiciness, colour	Texture	tenderness, juiciness, smell, texture	
3rd rank			leanness, gristle	Leanness	colour, gristle	
4th rank				Gristle	leanness	

Source: Becker, Benner and Glitsch, 1998

Table 3: Significant differences in the helpfulness of 'safety' characteristics - results of a t-Test

	Germany	Ireland	Italy	Spain	Sweden	United Kingdom
BEEF						
1st rank	origin, freshness	Freshness	feed	freshness	freshness	freshness
2nd rank	feed	Origin	freshness	feed, organic, origin	origin	label, feed
3rd rank	organic, producer, label	Feed, organic, label	origin, label organic producer	label	label	origin, organic
4th rank	price	Producer	price	producer, price	feed	price
5th rank		Price			organic	producer
6th rank					price, producer	
PORK						
1st rank	freshness	Freshness	feed, freshness	freshness	freshness	freshness
2nd rank	feed, origin, organic, label, producer	Origin, label, feed, organic	organic, label, origin, producer	feed, organic	origin	label, organic, feed
3rd rank	price	Producer	price	origin, label	label	price, origin
4th rank		Price		producer, price	feed	producer
5th rank					organic	
6th rank					price, producer	
CHICKEN						
1st rank	freshness	Freshness	feed, freshness	freshness	freshness	freshness
2nd rank	free range	free range, origin	free range	free range, feed	origin	free range, label, feed
3rd rank	feed	Label, producer, feed	label, origin, producer	label, origin	label	price, origin
4th rank	origin	Price	price	producer, price	feed	producer
5th rank	label				free range	
6th rank	producer				producer	
7th rank	price				price	

Source: Becker, Benner and Glitsch, 1998

It is still widely disputed whether or not food safety is regarded as belonging to the category of food quality in the consumers perception process. As is demonstrated in table3 the same cues are used by consumers to evaluate meat quality and meat safety.

If we take the 'country of origin' as both a quality and a safety factor, we find that the ratings of the respondents in this survey are highly correlated. The Spearmann correlation coefficient is +0.62 for beef, +0.68 for pork and +0.60 for chicken, which may point to a strong interdependence between the quality and safety of meat. The same applies to 'price' as a quality cue, on the one hand, and as a safety factor on the other. Thus, it is not yet clear, if quality perception and safety perception are two different processes.

Concerning consumers' evaluation of the given safety indicators, it becomes obvious that 'freshness' plays a major role in assessing the safety of beef, pork and chicken. Therefore, it would be advisable for producers and retailers to communicate 'freshness' to the consumers.

A special feature of Italian consumers is that they perceive 'feed' to be a very helpful safety indicator for all of the three meats. 'Free range' is a characteristic of chicken which was perceived as being relatively important by consumers of all countries, with the exception of Sweden.

Generally, the 'country of origin' is most important for beef, in particular for German consumers, and least important for chicken. For all of the meats, 'price' is considered to be the least important factor for assessing the safety of meat.

Table 4: Most trusted sources of information about meat

	Germany	Ireland	Italy	Spain	Sweden	United Kingdom
1	Independent re-tailers/butchers	Butchers in the supermarket	Independent re-tailers/butchers	Independent re-tailers/butchers	Independent re-tailers/butchers	Butchers in the supermarket
2	Butchers in the supermarket	Independent re-tailers/butchers	Butchers in the supermarket	Butchers in the supermarket	Butchers in the supermarket	Independent re-tailers/butchers
3	Consumer Groups	Own opinion	Department of Health	Department of Health	Newspapers	Own opinion
4	Magazines	Reports	Friends	Consumer Groups	Own opinion	Newspapers
5	Reports	Farmer representatives	Consumer Groups	Own opinion	Friends	Government
6	Friends	Newspapers	Reports	Government	Food safety board	Labelling

Source: Becker, Benner and Glitsch, 1998

For establishing consumer confidence in the quality and safety of meat, it is important to know the extend consumer give trust in receiving information on meat quality. In a spontaneous question it was asked: "When looking for information of the safety of meat who or what do you most trust?" The answers were encoded by the interviewers.

Table 5: Information sources most trusted (frequencies in % of all answers)

	Germany	Ireland	Italy	Spain	Sweden	United Kingdom
Indep. Retailers/Butchers	37.5	9.8	28.3	25.6	10.7	9.3
None/Don't know	26.1	26.6	14.7	28.5	31.1	35.8
Butcher in the Supermarket	6.8	36.7	28.2	15.1	10.2	23.1
Consumer Groups	6.6	1.7	3.7	4.7	3.5	1.9
Magazines	3.8	0.3	2.5	1.5	2.1	0.7
Reports	3.7	2.8	3.6	1.8	2.2	1.5
Friends	3.5	1.9	3.8	1.7	4.0	1.7
Mother/Other Family Member	1.7	1.1	1.5	2.1	1.4	0.9
Other	1.5	1.0	0.2	0.2	3.5	1.8
Farmer Representatives	1.4	2.4	0.5	0.1	0.7	2.0
The Food Safety Board	1.4	1.0	0.2	1.3	3.5	1.4
Own Opinion	1.4	4.6	0.3	4.0	4.6	6.0
Food Writers	1.0	0.3	0.7	0.3	1.2	1.0
Radio Reports	0.9	1.6	0.0	0.9	2.5	0.9
Dep. of Health	0.8	1.2	6.0	5.6	0.4	0.9
Newspapers	0.6	2.3	2.7	0.8	7.6	2.9
Labelling	0.5	0.9	0.4	0.4	2.6	2.0
Television	0.3	0.2	0.4	0.2	2.4	1.2
Dep. of Agriculture	0.2	1.3	0.2	1.7	1.5	0.7
Meat Industry Organisations	0.2	0.6	0.1	0.0	0.7	0.4
Government	0.1	1.1	0.2	2.8	0.2	2.4
Meat companies	0.1	0.3	1.1	0.3	2.7	1.0
Local doctor/other medical	0.0	0.4	0.6	0.4	0.5	0.4

Source: Becker, Benner and Glitsch, 1998

The results are presented in table 4 and 5. Consumers in each of the countries consider public institutions to be reliable sources of information only to a very small extent. This particularly applies to the Department of Agriculture and to the Government in general. The Department of Health is somewhat of importance to the Italian and Spanish consumers.

Butchers emerged very significantly as the source consumers trusted most, even in Sweden and the United Kingdom where butchers do not play such an important role as in the other countries.

Consequences for the possibility of success or of failure of the labelling approach in the fresh meat sector.

Independent retailers and butchers are regarded as the most reliable source of information on meat quality. Quality is communicated in the shop by the selling personal to the consumer. As long as labelling efforts does not take on board the interest of the butchers and retailers, the success seems to be questionable. As the results clearly demonstrate, consumers regard the place of purchase as a very important quality cue when shopping for meat. Retailers and butchers are aware of it. Supermarkets use the shop in shop system to sell meat. Butchers claim, that they personally stand up for the quality. A label or brand will diminish the relevance of butchers and thus butchers will be reluctant to introduce other labels than their own. The detailed study of national quality policies (not covered here, but available on the internet) has demonstrated that efforts to establish labels in the meat sector are countless, but only retailer labels, in particular in the United Kingdom, gained any importance on the market. It seems very questionable whether the labelling regulation will be accepted by the market.

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Session 4:

Technological Methods to Improve Food Quality

High pressure processing of foods

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Published reports of the projects AIR-CT92-0296 and FAIR-CT96-1113 have been used as data source. Therefore we want to give special thanks to Prof. D. Knorr (Berlin University of Technology) and to Prof. B. Brooker (Institute of Food Research, Reading).

Abstract

High pressure (HP) technology offers major benefits to the food industry as it does not impair characteristics such as taste, flavour, colour or vitamin content. It is therefore an extremely interesting alternative to preserve foods instead of chemical, thermal or even radiation techniques. Currently, the FAIR-programme is supporting two projects on this emerging technology. The first project (FAIR-CT96-1113) is investigating the use of HP to improve the processing of liquid foods and derived products whilst retaining desirable properties, such as natural taste, colour and nutritional status. Research mainly concerns increasing digestibility, improving production of cheese and fermented milk products, optimising processing of fruit juices and designing high performance high pressure processing plants. The second project (FAIR-CT96-1175) aims to develop a scientific food engineering basis for design, evaluation and optimisation of combined high pressure/temperature processes for industrial application. Kinetic-based methodologies are being developed through detailed kinetic studies on safety and quality aspects of foods, including enzymes, microorganisms, food structure and sensorial/nutritional quality.

Research trends in food preservation and processing

Heating is pre-eminently the method mostly used in the processing and preservation of foods. Food products are treated at high temperature for pre-set time to render them microbiologically safe while maintaining an acceptable quality level. Nevertheless, conventional thermal processing often produces a number of undesirable changes in foods such as loss of colour, flavour and functionality. Since there is currently a growing consumer demand for fresh-like high-quality products with an extended shelf life, which are minimally processed and additive free, the major challenge for the food industry is to respond to these consumer demands. Several research projects concerning improvement of existing thermal preservation methods, assessment and optimisation of thermal processes and development of mild preservation methods have been or are being supported by the European Commission (Table 1).

In addition to these “traditional” preservation methods, research on new non-thermal preservation methods, such as the use of high hydrostatic pressure and high electric field pulses, has been stimulated (Table 2). In Europe, the first project dealing with this emerging technology was launched in 1992 under the AIR-programme while currently two new projects on high pressure processing are being supported in the FAIR-programme. Research results have shown that high hydrostatic pressure could qualify as an alternative to conventional thermal processing as it results in foods with improved flavour, colour and nutrient retention, less damage to raw materials and better texture. Some pressure preserved food products are already introduced on the market, mainly fruit-based products like juices and jams, yoghurts, avocado paste.

Table 1: Overview of European research projects in the area of thermal processing, thermal process assessment and minimal processing of foods.

Project number	Title of the project
AIR0125	Improvement of safety and quality of refrigerated ready-to-eat food using mild preservation techniques
FAIR3159	Research on factors allowing a risk assessment of pathogenic spore-forming bacteria in cooked chilled foods containing vegetables
AIR1519	Microbial safety and quality of foods processed by sous-vide as a method of commercial catering
AIR1326	Modified atmosphere systems in varying temperature regimes
FAIR1104	Novel high oxygen and noble gas modified atmosphere packaging (MAP) for extending the quality shelf life of fresh prepared produce
FAIR3155	On-line optical measurements as a basis for control during mild heat processing of vegetables
AIR0278	The (bio)chemistry and archestructure of fruit and vegetable tissue as quality predictors for optimising storage and processing requirements
AGRF0031	Study of the characteristics of raw materials for the manufacture of ready-to-use fruits
AIR1017	A new approach to heat distribution and heat penetration studies for thermal processing of foods in modern batch-type overpressure rotary retorts
AGRF0018	Development of an improved technology for in-pack thermal processing of particulated foods
AIR0746	The development of new time temperature integrators (product history indication) for the quantification of thermal in terms of food safety and quality
AGRF0033	Continuous microwave and Joule effect heat treatments of particulate fruit products which enhance quality while maintaining safety
AIR1284	Microwave sterilisation – Process validation, nutritional wholesomeness, product quality and the consumer interaction
FAIR1192	Optimal control of microwave combination ovens for the food heating

Table 2: European research projects in the area of new non-thermal preservation technologies.

Project number	Title of the project
AIR0296	High hydrostatic pressure treatment: its impact on spoilage micro-organisms, biopolymer activity, functionality and nutrient composition of food systems
FAIR1113	High pressure treatment of liquid foods and derived products
FAIR1175	Combined high pressure thermal treatment of foods: a kinetic approach to food safety and quality
FAIR3044	High electric field pulses: food safety, quality and critical process parameters

High hydrostatic pressure treatment: its impact on spoilage organisms, biopolymer activity, functionality and nutrient composition of food systems (AIR-CT92-0296)

General objectives

The general objective of the project is to investigate whether high pressure could qualify as an alternative to conventional thermal processing, in order to respond to the consumer demands for minimally processed, freshlike products that are of highest quality and safety and possess extended storage stability. There is still considerable lack of knowledge regarding basic information on the effects of pressure on food systems as well as a need for equipment and process development. The project will respond to these issues using the following approaches:

a) establishment of a sound scientific basis to improve microbial and toxicological safety of foodstuffs through the development of food processing methods based on high hydrostatic pressure treatment.

b) development of high pressure processes for the treatment of foodstuffs with the aim of maintaining and enhancing intrinsic food quality

c) extension of existing high pressure knowledge and its application, with the aim of developing novel non-thermal food preservation techniques and a means to modify functional and molecular properties of foods and food components.

The consortium of this project is presented in table 3.

Table 3: Consortium of the project AIR-CT92-0692

Co-ordinator:
Berlin University of Technology, Department of Food Technology, Prof. D. Knorr
Partners:
Université de Montpellier II, Unité de Biochemie et Technologie Alimentaires, Prof. J.-C. Cheftel
Rijksuniversiteit Gent, Laboratory of Food Technology, Chemistry and Microbiology, Prof. A. Huygebaert
Katholieke Universiteit Leuven, Laboratory of Chemical and Biological Dynamics, Prof. K. Heremans and Laboratory of Food Technology, Prof. M. Hendrickx
University of Reading, Department of Chemistry, dr. N. Isaacs
Unilever Research Laboratories Vlaardingen, dr. J. Smelt
GEC ALSTHOM ACB, dr. J.E. Lebas
FMC Europe Corp., dr. B. Mertens
CPC Europe Consumer Foods Ltd., dr. R. Stute
Universität Heidelberg, Physical Chemistry Unit, Prof. H. Ludwig
Universidad Autonoma de Barcelona, Food Technology Unit, Prof. B. Guamis Lopez
Institut Francais des Boissons de la Brasserie Maltiere, dr. P. Boivin

Description of the work

Consistent with the objectives, five research tasks are included in the project. In task 1 it is endeavoured to develop a standard pressure regime. Task 2 focuses on data collection, strategy development and establishment of models regarding pressure inactivation of micro-organisms. Furthermore, the effect of pressure on the structure-function relationship of isolated food components and model food systems is investigated in task 3. Task 4 concerns all activities related to pressure effects on the microscopic structure and macroscopic appearance of model foods and real food systems. In a final stage, the potentials of the application of high pressure for food processing are evaluated and research on the development of new pressurising units is established.

Achievements of the project

With respect to task 1 it was seen that due to variations in size and geometry of the pressure vessels used by the different partners, a general reference procedure was not obtainable. Nevertheless the objective of this task was met because recommendations were developed for time-pressure, time-temperature and pressure-temperature profile monitoring for individual pressure treatments and for the establishment of standardised operating procedures within each laboratory.

In task 2, inactivation of a wide range of vegetative micro-organisms was evaluated by examining the effects of pressure, temperature and time on the organisms. Simultaneously, the impact of immersion media and certain baroprotectants (e.g. glucose, sucrose, sodium chloride) was evaluated. Inactivation of spores on the other hand was achieved by a combination of pressure induced germination of spores and subsequent pressure inactivation (500-900 MPa) at elevated temperatures (35-50°C). Furthermore, studies on the mechanism of pressure inactivation were accomplished, including electron microscopy evaluation of morphological changes and examination of the membrane based ATPase which is considered as an important target in pressure inactivation.

The emphasis in task 3 was on quality aspects of foods including pressure induced protein gels, inactivation characteristics of food enzymes and pressure effects on the Maillard reaction.

From rheological studies it could be concluded that heat induced gels contain a higher density of long term bondings while high pressure induced gel networks are composed of weaker, short term bondings. Furthermore data obtained for β-lactoglobulin suggest that pressure induces aggregation through the formation of intermolecular S-S bonds.

Enzyme inactivation studies on polyphenoloxidase revealed significant differences in barosensitivity within various fruit and vegetable species. Furthermore the combined effect of pressure (300-750 MPa) and temperature (30-100°C) were studied using *Bacillus subtilis* α-amylase as an enzymatic model system.

The study of the effect of pressure on the Maillard reaction revealed the initial interaction of the sugar and the amino acid to be accelerated by application of pressure while the degradation of the amadori products is retarded.

In task 4 microscopic structure and macroscopic appearance of model food and real food systems were investigated. These studies included the following aspects: (i) structural analysis of protein gels and liquid dairy cream; (ii) microbial and enzymatic analysis of pressure treated goat milk; (iii) enzymatic browning of fruits and vegetables; (iv) sensory evaluation of apple juices; (v) texture analysis of pressure treated fruits and vegetables.

Finally in task 5 work has been carried out on utilising the benefits of high pressure technology for process development including high pressure blanching, whether or not in connection with subsequent freezing, drying or frying. Additional work on high pressure shift freezing and pressure thawing has been initiated. Besides, development of a semi-continuous unit (4.0 L, 400 MPa, 20-80°C) has been completed and tested in over 2000 cycles. Additionally, cleaning abilities and sanitary aspects have been examined. This unit has also been tested with real food systems (e.g. milk) by partners within the project. Finally economical analysis of high pressure processes as well as feasibility studies on industrial pressure systems for food in packages were conducted. Automated handling systems were designed and a comparison of estimated operation costs for food in bulk and food in containers was made.

High pressure processing of liquid foods and derived products (FAIR-CT96-1113)

General objectives

The principal aim of this research project is to develop the use of high pressure as a food processing and preservation technique, especially for liquid foods. It is believed that this unit operation could increase the competitiveness of EU-produced foods by improving their quality, extending their shelf life and offering the potential to devise new food products with novel taste and texture profiles.

In addition to devising protocols to improve the functional properties of milk and dairy products and the quality of fruit and vegetable juices, the objectives include nutritional assessment of pressure treated liquid foods and consumer attitudes to both high pressure processing *per se* and the quality of high pressure treated foods. Finally the objectives in the engineering of processing plants consist of designing and testing new components for an advanced high pressure equipment together with important aspects of cleaning and hygienic operation.

The consortium of this project is presented in table 4.

Table 4: Consortium of the project FAIR-CT96-1113

Co-ordinator:
Institute of Food Research, Reading, dr. B. Brooker
Partners:
Rijksuniversiteit Gent, Department of Food Technology and Nutrition, Prof. A. Huygebaert
National Dairy Products Research Centre, Cork, dr. T. Beresford
GEC ALSTHOM ACB, dr. J. Bignon
Queens University of Belfast, dr. D. Johnston
Campus Universitario de Bellatera, Barcelona, dr. B. Guamis
Institute of Chemistry and Biology, Karlsruhe, dr. P. Butz
Centre de Recherche Pernod Ricard, dr. J.-P. Savina
Institut National de la Recherche Agronomique, dr. A. Baron
St. Ivel Ltd., UK, dr. B. Gupta
Danone S.A. Barcelona, dr. A. Montserrat

Description of the work

For dairy products it will be investigated in which way proteins of bovine and caprine milk are affected by high pressure and what the effects are on the dairy products made from this milk. Since high pressure is also known to affect the activity of enzymes and the viability of bacteria, pressure treatment of cheese curd will be examined with respect to the possible acceleration of ripening and of salt penetration into the brined cheese. Besides, biochemical changes taking place in the pressure treated curd are focused on, allowing to identify the basis for pressure-induced changes in quality. In the area of fruit and vegetables juices, the work concerns the optimisation of protocols for high pressure treatment of juices, consistent with microbiological stability and stability of flavour and turbidity. In both the dairy and the juice area, it will be investigated whether pressure-treated foods lose some of their nutritionally important components or generate undesirable compounds that adversely affect organoleptic properties or are toxic.

Whatever advantages high pressure may bring to food processing and preservation, the final question will always be how acceptable pressure-processed foods will be. Therefore, consumer workshops and opinion polls will assess consumer attitudes to high pressure processing in France, Germany and Great Britain. Besides, trained panels of sensory analysts will compare freshly squeezed juice with pressure- and heat-treated juice.

Finally, the commercial viability and exploitability of high pressure processing depends on the availability of high pressure plants that have been designed specifically to address problems associated with certain foods. In this context, a study will be performed in order to improve design and performance of a semi-continuous bulk liquid plant. This includes the design of high pressure valves that can accommodate liquids containing particles, such as fruit pulp as well as general considerations about cleaning and hygienic control.

State of progress

The study on the effects of high pressure on milk has shown that it induces structural changes in casein micelles, leading to their partial or complete disruption, depending on the pressure used, and to denaturation of some of the whey proteins. Association between some whey proteins and casein submicelles, when coagulated by acidification or by rennet, can lead to gels with novel rheological and textural properties. With respect to the studies of cheese, optimal pressures that increase proteolysis (maturation) compared with controls have been identified and will be used in the next stage of the project. Work on the use of high pressure to accelerate brining of cheese has demonstrated more rapid penetration of salt when the cheese is pressurised and studies on the accompanying changes in biochemistry have started. The potential for the use of this approach in the accelerated ripening of cheeses is greater than was realised at the start of the project.

Protocols for the extraction of juice from apples and oranges and their pressurisation have been completed and tests on plastic packages for leakiness and organoleptic properties have identified a suitable material and closure for future use. Conditions for the inactivation of pectin methyl esterase (PME) in orange juice have been identified, although work on apple juice is continuing, and isolation of organisms from orange juice for future microbiological work has been completed. Hereby a major obstacle has been overcome in the path of extended shelf-life/quality of these products.

Work on the quality and safety of high pressure treated foods has shown that, within the range of pressures likely to be used commercially, no adverse affects of pressure are found. The stability of

milk lipids and naturally occurring antimutagenic compounds in fruits and vegetables are not affected by pressures up to 600 MPa, and the generation of off-flavours by enzymes released due to tissue damage during high pressure treatment occurs only in negligible quantities.

The design, construction and testing of a new high pressure valve that can accommodate liquids containing particles without leakage has been completed and has been found to give an excellent performance during the course of more than 1600 pressure cycles. This plant has provided treated milk and juice for a number of partners in the project. Although minor problems for hygienic control still exist, a major problem for the processing of fruit juices, soups and other particulate liquids has been overcome.

Combined high pressure thermal treatment of foods: a kinetic approach to food safety and quality

General objectives

The general objective of the project is to develop a scientific food engineering basis for design, evaluation and optimisation of combined high pressure/temperature (HP/T) processes, which will enable the industrial application of this novel preservation technology in a justified way. Indeed, most authors believe that the most safe and economically feasible use of high pressure in food preservation will be in combination processes, especially with moderate temperature elevation. This project will therefore focus on combined HP/T processes, in the range of 100 to 1000 MPa and 30 to 100°C. The project rationale is to take a non-product oriented approach and to deal with the common hurdles limiting the industrial introduction of any HP/T-preserved product: (a) safety, legislative aspects, (b) optimal quality and consumer acceptability and (c) performant technology. To solve these issues, kinetic-based methodologies to determine the effect of high pressure on food safety and quality related aspects are indispensable.

The consortium of this project is shown in table 5.

Table 5: Consortium of the project FAIR-CT96-1175

Co-ordinator:
Katholieke Universiteit Leuven, Laboratory of Food Technology, Prof. M. Hendrickx
Partners:
Katholieke Universiteit Leuven, Laboratory of Chemical and Biological Dynamics, Prof. K. Heremans
Unilever Research Laboratorium Vlaardingen, dr. J. Smelt
Technical Research Center of Finland, Food Technology, dr. K. Autio
Bundesforschungsanstallt für Ernährung, Institut für Chemie und Biologie, Dir. u. Prof. B. Tauscher
Ruprecht-Karls-Universität Heidelberg, Institut für Pharmazeutische Technologie und Biopharmazie, Prof. H. Ludwig
Technische Universität Berlin, Institut für Lebensmitteltechnologie und Gärungstechnologie, Prof. D. Knorr
National Technical Univesity of Athens, Laboratory of Food Chemistry and Technology, dr. P. Taoukis
Engineered Pressure Systems International NV, Belgium, ir. P. Colman and ir. F. Gorrebeeck
ABB Pressure Systems AB, Sweden, Mr. J. Hjelmqwist and Mr. J. Westerlund
Nestec Ltd., Nestlé research Center, dr. S. Crelier

Description of the work

The objective will be reached through implementation of eight research tasks. Firstly detailed kinetic studies will be performed on different safety and quality aspects. In task 1, inactivation kinetics of food quality related enzymes will be investigated. The enzymes studied will be the following: polyphenoloxidase (PPO), which causes enzymatic browning of several fruits and vegetables; lipoxygenase (LIP), which induces the production of off-flavours in vegetables, mainly in *leguminosae*; pectinmethyl esterase (PME) and polygalacturonase (PG), playing an important role in cloud loss in fruit and vegetable juices and in texture of fruits and vegetables in general; cysteine sulfoxide lyase (CSL), which is responsible for off-flavours in the *Brassica* family; myrosinase (MYR), which is important for the characteristic flavour and antimutagenic properties of *Brassica* vegetables. In task 2, the kinetics of microbial inactivation will be studied because this information is indispensable for establishing whether a process has a sufficient impact to ensure safety. This task will include research on non-sporeforming pathogenic bacteria, sporeforming bacteria and fungi. Task 3 will focus on the effects of high pressure and temperature on food structure and texture. Changes in the sol-like or gel-like nature, viscosity, gelification and coagulation, which may be desirable or undesirable, will be kinetically characterised. Both protein/polysaccharide sol/gel/coagulate systems and starch sol/gel systems will be studied. Finally in task 4, the effect of high pressure and temperature on nutritional and sensorial quality of foods will be investigated. As to nutritional quality, especially vitamins can severely suffer. On the sensorial side, the treated product usually looses its fresh colour and flavour, and there might be off-flavours formed. In this context, kinetic studies to determine the influence of pressure-temperature processes on vitamins (vitamin C, A and K), colour (chlorophyll) and flavour (isothiocyanates) will be performed. In each task an increasing order of complexity in the composition of the medium (from simple model systems over complex model systems to real food products) will be considered.

The data gathered in the previous tasks will be used as input for the development of a concept and terminology to evaluate HP/T process impact quantitatively, since this terminology shall be covering the issues of safety as well as of quality (task 5). In task 6, insight will be gained in the performances of state of the art high pressure equipment, in terms of process uniformity and repeatability and specific problems that can be improved in future design and upscaling will be identified. In a first stage, lab equipment of the different partners will be examined while in a next state, the pilot scale installations from the different constructors involved in the project. Task 7 will focus on the long-term evaluation of safety and quality of HP/T-processed products, which is needed when these products will be marketed. Hence this task will include a study of the long-term post-pressurisation behaviour of relevant safety and quality aspects, including resuscitation of sublethally injured micro-organisms, outgrowth of superdormant spores, regeneration of enzyme activity, changes in textural properties, generation of off-flavours, and propagation of HP/T-initiated reactions including breakdown of vitamins or pigments. Finally optimisation studies are indispensable, especially because pressure treatments will be combined with moderate temperature elevation. In contrast to pressure where detrimental effects to nutritional and sensorial quality of foods are limited, heat treatments are known to affect heat labile nutritional, sensorial and structural aspects to a large extent. Maximising quality retention within the constraints of the legislative requirements (inactivation of pathogenic bacteria as well as spoilage microorganisms and enzymes) will be subject of these optimisation studies (task 8).

State of progress

Until now, mainly kinetic studies on pressure-temperature induced changes in food safety and quality aspects have been focused on. The food quality related enzymes studied so far include polyphenoloxidase (PPO), lipoxygenase (LIP), myrosinase (MYR), pectinmethyl esterase (PME), cysteine sulfoxide lyase (CSL) and polygalacturonase (PG). For PPO, LIP and PME pressure-temperature domains in which inactivation occurs were demarcated and kinetic data about inactivation were gathered, allowing to construct pressure-temperature phase diagrams. For CSL, PG and MYR, on the other hand, mainly thermal inactivation has been investigated. The food safety determining micro-organisms investigated in detail are (i) a pressure-temperature resistant strain of *Listeria monocytogenes*, (ii) the sporeformers *Bacillus subtilis* and *stearothermophilus* and (iii) the moulds *Eurotium repens* and *Penicillium expansum*. While inactivation kinetics have been focused on for the vegetative micro-organisms and the moulds, germination kinetics and following inactivation have been examined for the sporeformers. As to food structure, the effect of combined pressure and temperature on starch sol/gel systems (potato and waxy maize) has been investigated with respect to viscosity, rigidity and gelation properties. Besides, the effects of pressure in the range of 0.1 to 800 MPa on model systems of casein, whey proteins and pectin (binary, tertiary and quaternary) have been investigated using light microscopy, electron microscopy, rheology and FTIR. Finally kinetic data sets have been gathered on pressure-temperature induced changes in sensorial and nutritional quality aspects such as vitamin C, A and K3, chlorophyll, isothiocyanates and hydroxy-methylfurfural. Furthermore, attempts have been initiated to develop mathematical models describing the kinetics of these P/T-induced changes, which can be considered as the preliminary work required for the development of a concept and terminology to assess the impact of high pressure-temperature processes.

Dissemination of the results

Dissemination of the results of the three EU-projects discussed above is and will be further accomplished by Flair Flow activities, presentations at international conferences, meetings and work shops, networks (e.g. European High Pressure Research Group, French Club Hautes Pressions, Hautes Pressions Technologies, United States of America High Pressure Consortium…), inter-project activities and publications.

Conclusions

Based on the above mentioned, it can be concluded that both consumers and industry will benefit from the high pressure research. This research will result in an increased choice of products, high quality beverages, minimally processed foods, free from preservatives, and improved safety and quality. Industry will be provided with predictive models for this processing technology, giving them the opportunity for developing new products and intermediates. Optimal design of high pressure processing equipment for liquid and solid foods will allow industry to satisfy consumer needs by the efficient production of more ‘natural’ foods.

Integrated membrane processes

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Abstract

The most important aspect for a cheese process to be applicable on industrial scale is good quality of the produced cheese, because consumers have high expectations regarding texture and taste. However, it is a widely recognised fact that some cheese processes applied nowadays – including the membrane-mediated processes – have quality problems. In this project a prototype for a new membrane mediated bioreactor process for the production of soft cheese will be constructed. In comparison to other existing membrane mediated cheese processes a higher quality standard and a more flexible process will be demonstrated.

Introduction

The production of natural food and non-food products by biocatalytic means is a hot topic in technological research. Enzymatic processing is characterized by high reaction specificity at a low temperature, which makes it environment-friendly. A drawback of enzymatic reactions is that they are often characterized by equilibrium constants that are not favourable for complete conversion to the desired product. To overcome this restriction, hence increase yield, reactions are typically done in systems where products are continuously removed by integrated separation steps. An example is the esterification of fatty acids and alcohols where the excess water is removed by distillation. Obviously, also other separation principles like extraction and crystallization can be combined with enzyme technology.

Membrane technology is a relatively new separation technology that can offer specific advantages when it is combined with enzyme technology. Especially the ability to separate very efficiently at low (enzyme reaction) temperature is very appealing to enzyme technologists. However, membranes offer another important possibility that make enzyme technology more flexible. Using enzymes in a membrane reactor, or in a circulating loop, prolongs the enzyme life considerably thus permitting a higher turnover per enzyme molecule. Such systems make it also more economic to use more expensive enzymes with special properties. In previous years ATO-DLO has executed various project on membrane reactors implementation: including, chiral resolutions, apple juice processing and enzymatic oxidation. In this EU-sponsored demonstration project “MEMCHEEP” started in 1997 and discussed today, we aim for a combination of a membrane reactor and several other unit operations to realize a (semi-) continuous cheese production process.

Current state of knowledge

The most important aspect for a cheese process to be applicable on industrial scale is good quality of the produced cheese, because consumers have high expectations regarding texture and taste. However, it is a widely recognised fact that some cheese processes applied nowadays – including the membrane-mediated processes – have quality problems.

In this project a prototype for a new membrane mediated bioreactor process for the production of soft cheese will be constructed. In comparison to other existing membrane mediated cheese processes a higher quality standard and a more flexible process will be demonstrated.

Enzymes with high proteolytic activity are immobilised onto a membrane surface for milk clotting. Therefore, the clotting enzymes will not leak into the final product. The destabilised milk, leaving the Enzyme Membrane Reactor, is coagulated in a heat exchanger by simple heating. Eventually, the curd is continuously concentrated. Through this technique soft cheeses can be produced with a dry weight up to 40%.

Demonstration objectives

The main objective of this project is to demonstrate the industrial viability of a membrane mediated bioreactor process for continuous and controlled rennetting of milk. In a second processing unit the milk is coagulated and continuously concentrated to soft cheese. With this technology the control over the different steps of the cheese production process will be improved, hence yielding improved cheese quality and reducing waste.

Quantitative objectives are a pilot production of 75 kilograms of consistent cheese per day and the extrapolation of the technical and economic results to industrial levels.

Work content

In the first stage of the project a prototype will be constructed. The membrane bioreactor performance will be optimised in terms of conversion degree, productivity, stability and cheese quality.

The cheeses produced with the prototype will be analysed and the data compared to a wide range of specifications in order to establish appropriate process parameters and to characterise the product.

Special attention will be given to membrane fouling to demonstrate a reliable continuous process. Furthermore, cleaning procedures will be established to maintain high enzymatic activity in the enzyme membrane reactor between sterilisation procedures.

After installation of the pilot plants the process parameters will be evaluated and optimised for pilot scale operation. Prototype cheeses will be produced on site and compared to conventionally manufactured products by end-users.

At the end of the project the results will be used for an economic and technical evaluation to confirm the viability of the process on plant scale versus existing process technologies.

Role of partners

The technology will first be implemented in a prototype at ATO-DLO, which will optimise the process conditions. In collaboration with the University of Calabria, ATO-DLO will assume responsibility for an optimal enzyme immobilisation. Moreover, the University of Calabria will study fouling reduction and enzyme deactivation.

Separem will develop a membrane module applicable for an enzyme membrane reactor and will construct two pilot plants to be located at the two end-users premises. The two end-users will test the prototypes and characterise the final products. They be instructed by ATO-DLO and U-Calabria and will assist in the optimisation of the pilot plants.

Exploitation plans and Target groups for the Extended Audience

ATO-DLO is active in a variety of areas and as a result has strong contacts with cheese producers, processing industries and scientists to foster technology exploitation. Separem's industrial strategy is to gain greatest leverage from its distinctive professional competence and state of the art technologies. The University of Calabria is involved in a number of research projects concerning membrane reactor systems and it has also contacts with a large number of Mediterranean dairies. Both MEVGAL and BBRD intend to stay ahead of the competition in the market of process technology.

To inform the market of the advantages of the new technology, the following target groups have been identified and will play the role of the Extended Audience:

- dairy companies interested in new process technologies and in producing new cheeses;
- engineering companies able to implement the technology (not only in dairy but also in fruit juice processing);
- universities and research centres focused on technologies to improve the quality of dairy products and optimisation of process technologies;
- companies concerned with food authenticity and hygiene.

Consumer groups and food regulatory agencies will be addressed in the phase of product characterisation (e.g. sensory panels)

The Extended Audience will be informed about the project by organised meetings (as this one) and symposia, as well as by regular project abstract and a final report. Targeted demonstration sessions will be organised at end-users' premises during the late phases of the project.

Why a demonstration phase now?

The practical use of immobilised enzymes in food industry has been proven on laboratory scale, particularly with membranes as carriers. However, the technology is still not accepted by the industry and should be proven by demonstrating its use on pilot scale.

Project Nr : FAIR CT-97-3148

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See also: Examples of demonstration projects in the life science programmes volume 2. Wakolbinger, W. et al (eds), EUR 17784 EN, DG XII, Brussels, (1998)

The preservation of quality and safety in frozen foods throughout the distribution chain

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Abstract

Frozen foods continue to make up an increasing proportion of the European diet. Demographic changes, towards increased numbers of single person households and families with both adults in paid employment, drive this growth. Despite the focus on frozen foods as convenience foods it should be remembered that frozen products often:

- Have a higher content of volatile nutrients
- Enjoy a relatively good safety record
- Have sensory characteristics preferred by the consumer.

A great deal of commercial and academic research has been invested in improving the quality of frozen foods. Nevertheless the perception of frozen foods as a convenience food remains. Much of the advantage in producing high quality frozen products is lost if they are poorly treated during distribution. Since the manufacturer expects the convenience image to often result in poor handling, he is loath to make the original investment in quality. This leads to a reinforcing circle of low quality products.

This paper describes a European concerted action aimed at tackling this problem. Our starting point is to understand the weak links in the chain and to propose best practice by the manufacturer, distributor, retailer and consumer to provide the highest quality and safety in frozen food products.

Introduction

Frozen foods continue to make up an increasingly large proportion of the European diet. Demographic changes towards increased numbers of single person households and families with both adults in paid employment continue to drive this growth.

Freezing as a preservation technique is too often perceived as a method to produce cheap and convenient products. However, despite the focus on the convenience factor, it should be remembered that high quality frozen products often:

- Have a higher content of volatile nutrients than those stored at chill temperatures;
- Enjoy a relatively good safety record;
- Have sensory characteristics preferred by the consumer to the unfrozen equivalent.

A great deal of research at a national level has been invested in improving the quality of frozen foods. Technical innovation has focused on techniques that we believe are increasingly producing new high-quality frozen foods with a good safety record.

Frozen foods provide a convenient and safe storage method for both the retailer and the consumer. However, common misunderstandings in the handling of frozen food may lead to deterioration in their textural and nutritional value.

The concerted action tackles the problems of the cold chain in four major initiatives. We are providing information for manufacturers, distributors, retailers and consumers. Our goal is to aid in the dissemination and understanding of existing best practice across the frozen sector. This should help lead to a virtuous circle as depicted in figure 1.

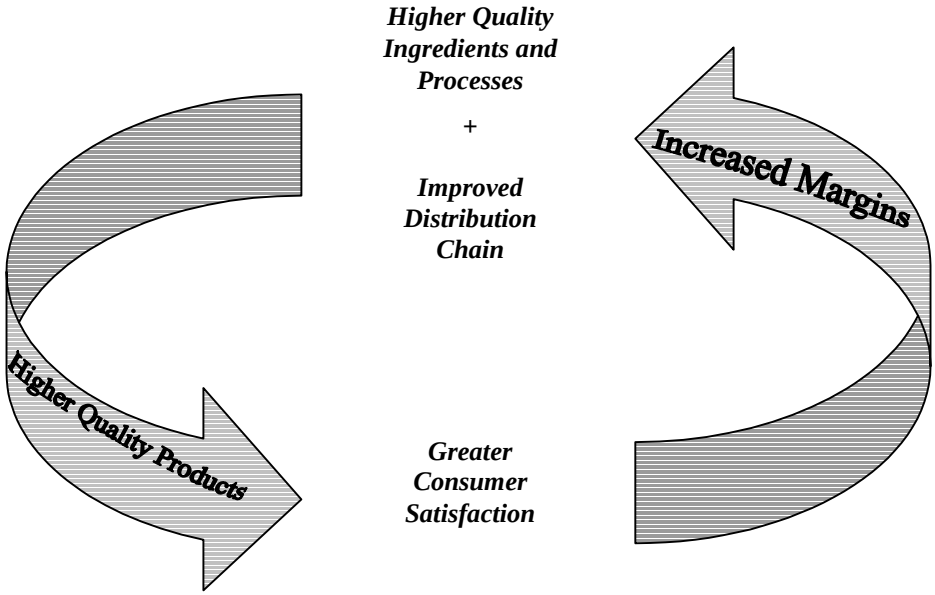

Figure 1: Describing a virtuous circle resulting from a more effective distribution chain for frozen foods.

More in-depth information is available to workers in the Industry through our book "Managing Frozen Foods" to be published by Woodhead in autumn 1999.

We are researching new techniques and processes, which we believe, will improve the quality, safety and nutritional value of the frozen products that are available to consumers across the European Union.

Research and development

Our work is focused in four areas. Scientific papers presented at plenary meetings 1 to 5 of the group are available by contacting Chris Kennedy. These areas are:

- Physical and chemical factors affecting the stability of frozen foods during storage;
- Microbiological effects of freezing and thawing foods;
- Temperature control during distribution;
- Exploration of the factors which may stabilise foods during distribution.

Physical and chemical factors affecting the stability of frozen foods during storage.

This group is studying a wide range of factors, which affect the stability of frozen foods. These include the choice of raw materials, nutrient reactions, protein changes and kinetics below the glass transition. Techniques employed by the partners include Magnetic Resonance Imaging, mathematical modelling as well as many standard laboratory techniques.

Although frozen storage normally greatly reduces reaction rates deterioration processes will continue slowly. Choice of input material has a strong effect on products, this has lead to the development of quality scales for many food types including fish (Nielsen, 1996).

Studies of orange juice have shown that freezing may be beneficial to orange juice quality (Silva, 1996). Pectinesterase (PE) the enzyme responsible for clarification and gelation in citrus juices has been shown to be partially deactivated under certain frozen conditions. This may offer a partial alternative to deactivation by heat which, results in more pronounced changes in the sensory properties of juices.

MRI was used, for example to study changes in frozen thawed trout, (Nott et al., 1997). This study showed that the freezing and thawing process resulted in significant increase in Magnetisation Transfer when the fish was frozen slowly. Similar changes were seen in longitudinal relaxation times T_1 and T_1^{sat}. The largest change in these parameters was in the initial freeze thaw cycle, as opposed to smaller changes on subsequent multiple freezing. This suggests that MRI may provide a useful tool for authenticating fresh fish products.

L. Boegh Soerensen (1997) of the Danish Food Control Laboratory is co-ordinating a study of the effects of temperature abuse on the sensory and physical properties of a wide range of products. Each of the laboratories involved has obtained samples of their chosen product direct from the manufacture and is subjecting them a regime of cold storage and temperature excursions typical of movement through the cold chain.

Samples are subjected to three periods of temperature abuse. The results of the project will be collated and presented at our September 1998 meeting prior to publication.

Microbiological effects of freezing and thawing foods

Specific interests of this group have focused on the effects of cold shock on the survivability and pathogenicity of psychrotrophic micro-organisms, the detection of stressed micro-organisms recovered from frozen thawed foods and the implementation of HACCP in frozen food production and distribution all the way to the home. Partners also continue to work on a range of foods and relevant pathogens.

George Nychas and Kyriaki Lambropoulou from the Agricultural University of Athens have carried out a long term study (Lambropoulou & Nychas, 1998) of the effect of frozen storage temperature, thawing rate and culturing method upon the quantification of *Salmonella enteritidis* and *Listeria monocytogenes* from raw fish. Their study concludes that *Salmonella enteritidis* is largely undamaged during frozen storage at temperatures down to -80°C for up to 7 months. *Listeria monocytogenes* showed a reduction of 2 logs during the first 15 days of storage at -80°C, however subsequent storage at this temperature and storage at higher sub zero temperatures resulted in little loss of viability.

Kevin Kerr (1996, 1997) from the Leeds General Infirmary has reviewed recent research on the bacterial Cold Shock Response (CSR) in food borne pathogens and assessed the implications of this bacterial response for the safety of frozen foods. The main conclusions drawn are that exposure of microorganisms to an environmental stress (chilling, freezing, acidity, heat) which results in sub-lethal damage has the effect of hardening the organism to subsequent stresses. This acquired protection may apply not just to the specific stress that the organism received but other forms of stress. For example the CSR caused by freezing can render the bacteria more resistant to the effects of heat. This has important implication for the use of hurdle technology approach in minimally processed products. Another significant concern regarding the cold shock response is that exposure to low temperatures may have a direct effect upon the virulence of pathogens. The need for more research on the CSR and its effect upon food borne pathogens was highlighted.

Temperature control during distribution

The group is concerned with improved devices and techniques for monitoring temperature distribution. It is also strongly involved in the development of new retail display cabinets that can overcome the conflict between high visibility, easy access displays and the requirements of good temperature control.

Maurizio Minossi from AEA srl, Italy (1996, 1998) described devices they have developed for temperature monitoring throughout the distribution chain. These include those suitable for wireless temperature monitoring within a cold store or retail site and those that can transmit data nationally or even internationally via mobile communication networks. Applications for the latter would include temperature monitoring of refrigerated vehicles. Data can either obtained either when requested or continuously.

The problems associated with the ozone depleting properties of CFC's and HCFC's are leading to their phasing out as refrigerant gases. This is seen as an opportunity to design and develop innovative solutions to display cabinet refrigeration which do not involve the use of halogenated hydrocarbons: these can be grouped into three categories:

- Direct systems with new thermodynamic cycles (e.g. air cycle Gigiel (1996) or with natural refrigerants;
- Indirect systems with single phase secondary refrigerants;
- Indirect systems with phase changing secondary refrigerants.

Giovanni Cortella & Fabio Polonara from the universities of Udine and Ancona in Italy (1997) have described the advantages and disadvantages of indirect expansion systems employing ammonia and ammonia/carbon dioxide. These include:

- Two-stage ammonia cycle with a single phase secondary refrigerant such as aqueous ethylene glycol or potassium acetate solutions;
- Two-stage ammonia cycles with carbon dioxide as the secondary refrigerant;
- One or Two-stage ammonia cycle cascaded to a carbon dioxide refrigeration cycle.

Exploration of the factors which may stabilise foods during distribution

A second project (Torreggiani *et al.,* 1998) is being co-ordinated by Danila Torreggiani of IVTPA in Milan. Partners are measuring the effects of different polymers on the low temperature properties of strawberry juice. Our understanding of how these polymers effect stability is poor. The group is able to use its expertise to determine a wide range of physico-chemical properties of the juices and a greater understanding of their effects may lead to better control of formulation and stability. The objective was to investigate how added carbohydrates affect the colour stability of frozen strawberry juices above the maximally freeze concentrated glass transition temperature T_g'. Differential Scanning Calorimetry (DSC) Dynamic Mechanical Thermal Analysis (DMTA) and dc Conductivity measurements were used to determine Tg'. It was found that there was no clear relationship between the anthocyanin loss and the amplitude of the difference between the storage temperature and the glass transition temperature of the freeze-concentrated phase of strawberry juices.

The use of Dewatering Impregnation Soaking (DIS) when coupled with Immersion Chilling and Freezing (ICF) is being investigated by Tiphaine Lucas and Anne Lucie Raoult-Wack from Cemagref BP and Cirad SAR BP, France. These treatments allow:

- efficient freezing of fruit and vegetables in sugar and salt solutions;
- manipulation of water content
- impregnation of foods with solutes

The use of solute impregnation and/or dewatering may be desirable for cryo-protective, nutritional, economic or product formulation reasons. Much of the current methodology in DIS and ICF is empirical, whereas the key to the successful application of these techniques is an understanding of heat and mass transport during the immersion processes. To this end, computer models of these systems are being developed (Lucas and Raoult-Wack, 1998).

Outputs

More detailed scientific information on the work of the partners can be obtained for the proceedings of our plenary meetings. There are five volumes of these so far. Guides to the basic do's and don'ts of frozen food production (Archer & Kennedy, 1998) and of frozen distribution (Fuller, 1998) have also been prepared and 2 further guides are in preparation, one dealing with microbiological analysis of frozen foods, the other a consumer fact sheet for distribution via retailers. These best practice guides are available from Chris Kennedy and via the Flair-Flow Network. A technical handbook on managing frozen foods (Kennedy, 1999) will be published by Woodhead Publishing in the Autumn of 1999.

Acknowledgements

We acknowledge the huge contribution that has been made to the success of this project by each of the 40 partners and contributors involved. We are grateful to the European Commission for funding this action through research contract CT96-1180.

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Protective cultures improve the efficacy of nisin

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Abstract

Nisin is a natural food preservative commercially used in products such as processed cheeses, dairy desserts and canned vegetables. It is member of the group of bacteriocin-like peptides called lantibiotics and is active against a broad range of Gram-positive bacteria and can even inhibit the outgrowth of spores of bacilli and clostridia. The practical application of nisin is still limited because of its low stability and activity at high pH and its limited efficacy in certain food matrices. A problem that might arise from the wide-spread use of nisin is the emergence of nisin-resistance in *Listeria monocytogenes* and other target bacteria. However by combining nisin with other biopreservatives or mild preservation techniques, possible restriction in the use of nisin could be overcome. One approach could be the use of nisin in combination with other bacteriocins differing in mode of action. The bactericidal activity of nisin against *Listeria monocytogenes* Scott A and a strain of *Bacillus cereus* and *Lactobacillus plantarum* was enhanced when it was used in combination with non-lantibiotic bacteriocins indicating a synergistic interaction between nisin and other bacteriocin molecules. Bacteriocin-producing protective cultures were shown to inhibit the emergence of nisin-resistant populations of *Listeria monocytogenes* at both 10° and 30°C. Efficiency of the protective culture was affected by the inoculum level and the type of strain used.

Introduction

Nisin is a classical example for a natural preservative commercially used in many countries for a long time. It is a member of a group of potent antibacterial substances called bacteriocins and is produced by certain strains of *Lactococcus lactis* subsp. *lactis*. These organisms can frequently be isolated from various foods including milk, meats and vegetable products and are generally recognized as safe (GRAS).

Nisin is a 34 amino acid polypeptide and because of the presence of some unusual amino acids such as lanthionine and β-methyllanthionine it belongs to a class of antimicrobial compounds called lantibiotics. It shows antibacterial activity against many Gram-positive bacteria including food-borne pathogens such as *Staphylococcus aureus* and *Listeria monocytogenes* and spoilage organisms such as *Clostridium butyricum*. As nisin is effective in preventing the outgrowth of spores of *Clostridium botulinum* and other *Clostridium* species it can be used as an adjunct to reduce the heating requirement of certain thermally processed foods. Nisin is permitted as a food additive in about 50 countries particularly for the inhibition of *Clostridium* ssp. in processed cheese and canned foods (1, 2) and has been successfully employed in other dairy products, cereal puddings and fermented beverages.

On the other hand, several restrictions to the efficacy of nisin limit its range of practical application. Nisin cannot be used to control Gram-negative bacteria, molds and yeasts as it is not active against these organisms. *Salmonella* and other Gram-negative food-borne pathogens, however, may be sensitized to nisin by processes destabilizing the outer cell membrane acting as a permeability barrier (3). Another limitation for the application of nisin in foods is its reduced biological activity,

stability and solubility at an elevated pH. It shows only limited activity in low-acid foods (pH above 4.5). Nisin molecules may also bind or absorb to certain food components such as protein or fat particles resulting in inactivation of the bacteriocin. For instance, nisin was found to be much less active against *Listeria monocytogenes* in milk with an increased fat content as compared to skim milk (4). Another problem which may limit the efficacy of nisin is the relatively frequent occurrence of spontaneous nisin-resistant mutants of *L. monocytogenes* and other target bacteria. A strategy to overcome those limitations may be the combined use of nisin with other biopreservatives or treatments improving the effectiveness of nisin, an approach referred to as combined processes (hurdle technology). For instance, it may be used in combination with other bacteriocins exerting a different mode of action. Nisin may also be applied together with an appropriate starter or protective culture showing antagonistic activity against the target organism. Many strains of lactic acid bacteria producing bacteriocins active against *Listeria monocytogenes* and other pathogens have been extensively studied and their potential to inhibit *Listeria* has also been tested in meat and other types of foods (5, 6, 7).

Studies on the effectiveness of those strategies were performed within the project „Development and practical implementation of novel combinations of nisin with other biopreservatives and mild processes that expand the range of application of the bacteriocin in assuring food safety and quality“ (FAIR PL95-1148) funded by the EC.

Enhancement of the bactericidal effect of nisin against selected target bacteria by combination with other bacteriocins

Six different bacteriocins (sakacin A from *Lactobacillus* sake Lb 706, enterocin B from *Enterococcus faecium* BFE 900, piscicolin 61 from *Carnobacterium piscicola* LV61, divergicin 750 from *Carnobacterium divergens* 750, carnocin 54 from *Leuconostoc carnosum* LA54, pediocin PA-1 from *Pediococcus acidilactici* PAC-1.0) were selected for studies aimed to demonstrate a synergistic effect with nisin against 3 target bacteria. All of them were non-lantibiotic compounds with a structure typical for the pediocin-like bacteriocins of class II (8). In these experiments, nisin was added at a concentration of 100 IU per ml to a culture of *L. monocytogenes* Scott A, *Bacillus cereus* F462690 or *Lactobacillus plantarum* ATCC 8014 and the number of survivors were determined after 3 min and 2 h. Nisin reduced viable counts of *L. monocytogenes* Scott A and *B. cereus* by about 3 and 4 log cycles respectively within 2 h and *Lact. plantarum* viable counts decreased by about 2 log cycles. A stronger reduction of viable counts of all 3 target organisms was observed when nisin was used in combination with partially purified preparations of sakacin A, enterocin B, piscicolin 61, divergicin 750 , pediocin PA-1 or carnocin 54. For instance, the combination of nisin with enterocin B resulted in a 6.0 log reduction of *B. cereus* whereas nisin alone reduced the *Bacillus* viable counts only by 4.3 log units (Figure 1).

It is obviously a synergistic and not simply an additive effect as the non-nisin bacteriocins were used at a level below the minimum inhibitory concentration (MIC) that means in quantities not affecting the viability of the target bacteria (Fig.1). It should also be mentioned that addition of a nisin-like bacteriocin from a *Lactococcus lactis* did not result in an enhanced bactericidal effect of nisin.

The synergistic interaction between the nisin molecules and the non-lantibiotic bacteriocin was dependant on pH. The enhanced bactericidal activity of nisin in combination with a pediocin-like bacteriocin was observed at pH 7.5 only. At pH 4.5, nisin was less effective in reducing viable counts of *L. monocytogenes* and *Lact. plantarum* and no synergistic effect between nisin and the bacteriocins sakacin A, enterocin B and pediocin PA-1 was observed .

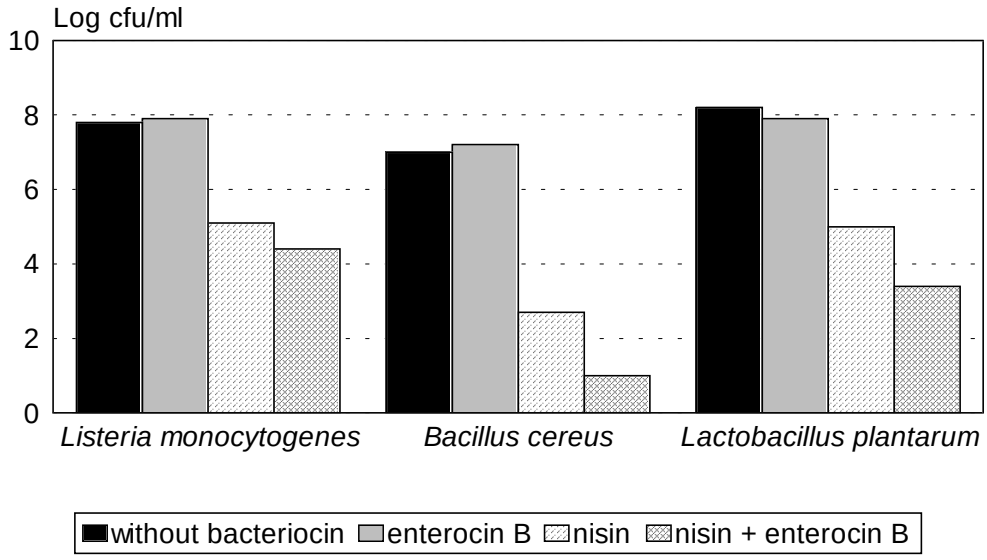


Figure 1: Reduction in viable counts of target bacteria by nisin (100 IU/ml) and enterocin B (128 AU/ml) in Standard I Nutrient broth, pH 7.5

Use of protective cultures to inhibit the emergence of nisin-resistant mutants of *Listeria monocytogenes*

Although nisin is effective in reducing viable counts of *L. monocytogenes* Scott A, nisin concentrations of 100 or 500 IU per ml do not kill all listerial cells. The population obviously contains some bacteria with an elevated resistance to nisin which can survive and are able to grow in the presence of nisin. This could be demonstrated in some experiments in which a culture of *L. monocytogenes* Scott A was exposed to nisin at concentrations between 10 and 500 IU per ml. With all nisin concentrations the initial decrease in viable numbers was followed by re-growth of survivors to nisin (Fig.2). Even at the highest nisin concentration of 500 IU per ml, *Listeria* numbers did not remain at a low level but rapidly increased again to high cell densities at 30°C. When the sensitivity of some surviving organisms to nisin was tested, a two- to fourfold higher tolerance to nisin was observed.

Several authors also reported on the occurrence of spontaneous nisin-resistant mutants of *L. monocytogenes* when *Listeria* cultures were exposed to nisin (9, 10). Up-to-now there are no reports on the frequency of nisin-resistant bacteria in foods. However, it cannot be excluded that the wide-spread application of nisin in the food industry results in the development of nisin-resistance in *Listeria* and other pathogens. The emergence of nisin-resistant bacteria may be prevented by a multiple-hurdle preservation strategy. The application of nisin in combination with a starter or protective culture showing antilisterial activity may minimize the risk of emergence of resistant *L. monocytogenes* populations in foods.

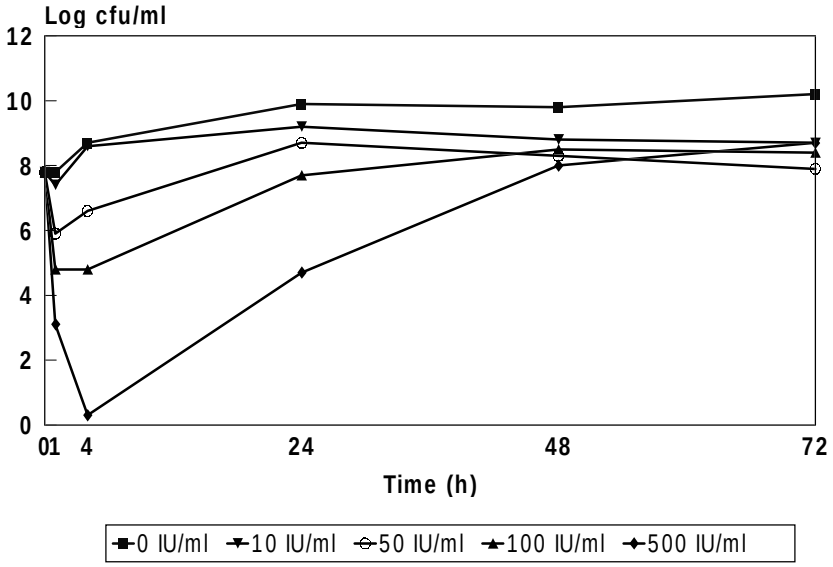


Figure 2: Bactericidal effect of various nisin concentrations on *Listeria monocytogenes* Scott A at 30°C

Three different bacteriocinogenic lactic acid bacteria which might be used as protective cultures in certain foods were tested for their potential to prevent re-growth of *L. monocytogenes* Scott A after exposure to nisin. *Lactobacillus sake* Lb 706 (11), *Enterococcus faecium* BFE 900 (12) and *Pediococcus acidilactici* PAC 1.0 (13) were chosen for the experiments.

Nisin-resistant mutants of these protective cultures were needed as they had to grow in the presence of nisin to exert their effect against the survivors. Exposure of the 3 strains to increasing concentrations of nisin resulted in mutants tolerating high amounts of nisin. These nisin-resistant mutants were added at a certain inoculum level to a culture of *L. monocytogenes* Scott A exposed to 100 IU of nisin per ml and bacterial viable counts were determined after different time intervals. Figure 3 shows the results obtained at 30°C.

All 3 bacteriocin producing strains showed an inhibitory effect on the growth of the listerial cells that had survived the bactericidal action of nisin. Differences in the efficiency, however, were observed between the protective cultures. *Lact. sake* Lb 706-1a was more effective in suppressing listerial growth than the other 2 lactic acid bacteria. With *Lact. sake* added, *Listeria* viable counts decreased continuously reaching numbers below 10 per ml after 48 h whereas *Ent. faecium* BFE 900-6a indeed was able to prevent re-growth of *Listeria* but did not reduce the number of survivors to nisin. *P. acidilactici* PAC 1.0-M2 showed only a weak inhibitory effect on the re-growth of listerial cells.

The behaviour of the nisin-resistant strains of *Lact. sake* and *Ent. faecium* was also studied at 10°C as these protective cultures may find application in minimally processed refrigerated foods. At 10°C, similar inhibitory effects of *Lact. sake* and *Ent. faecium* were observed on the re-growth of *L. monocytogenes* exposed to nisin. A sufficiently high inoculum level was found to be a prerequisite for a successful suppression of listerial re-growth. Figure 4 shows the results obtained with *Ent. faecium* BFE 900-6a. The protective culture had to grow to a certain cell density (about 10^8 per ml) to exert an inhibitory effect on the survivors to nisin (Fig.4). When the inoculum was too low the strain needed too much time to reach this high cell density and multiplication of the nisin-resistant listerial cells could not be stopped in time (Fig.4).

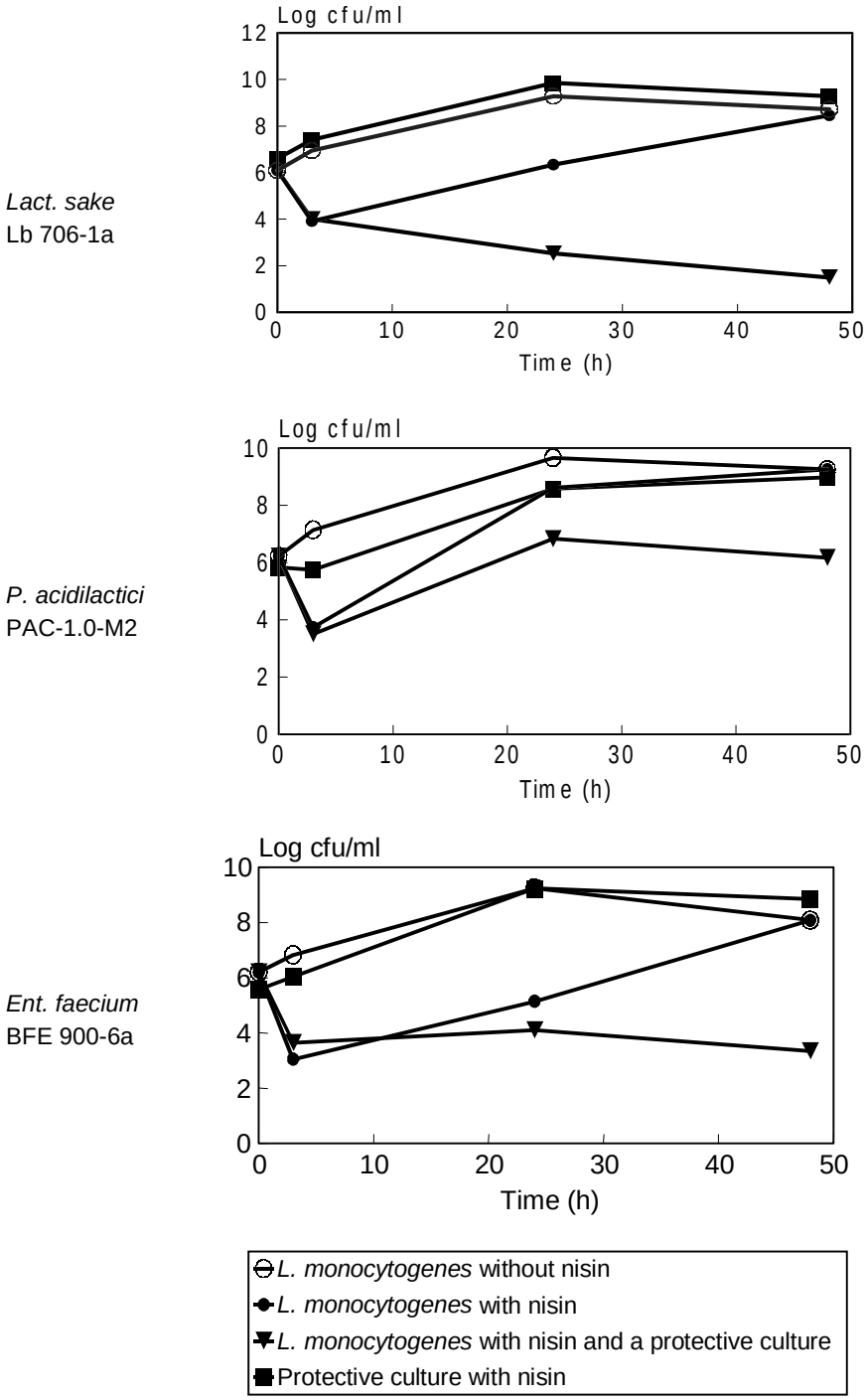
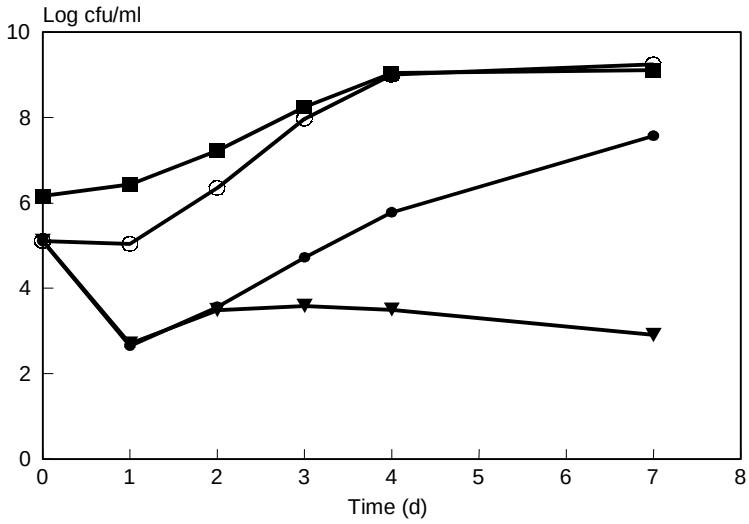
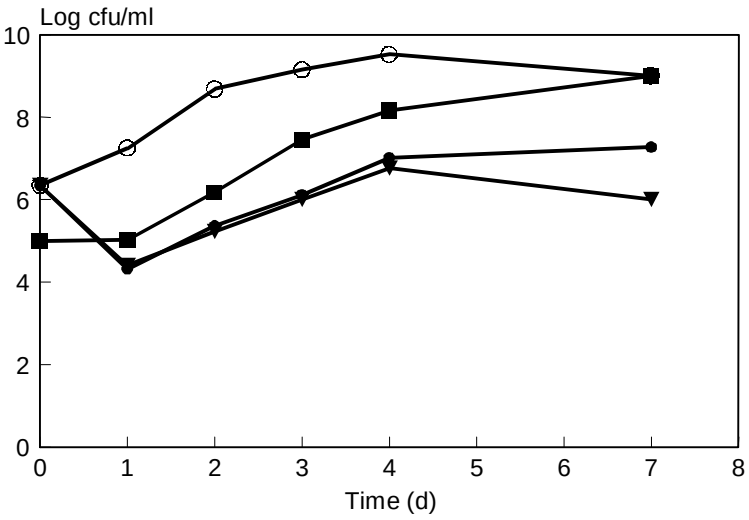


Figure 3: Growth of *L.monocytogenes* Scott A in combination with different protective cultures in the presence of nisin (100 IU/ml) at 30°C

High inoculum of *Ent. faecium*



Low inoculum of *Ent. faecium*



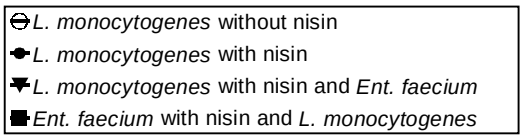


Figure 4: Growth of *L. monocytogenes* Scott A in combination with *Ent. faecium* BFE 900-6a in the presence of nisin (100 IU/ml) at 10°C.

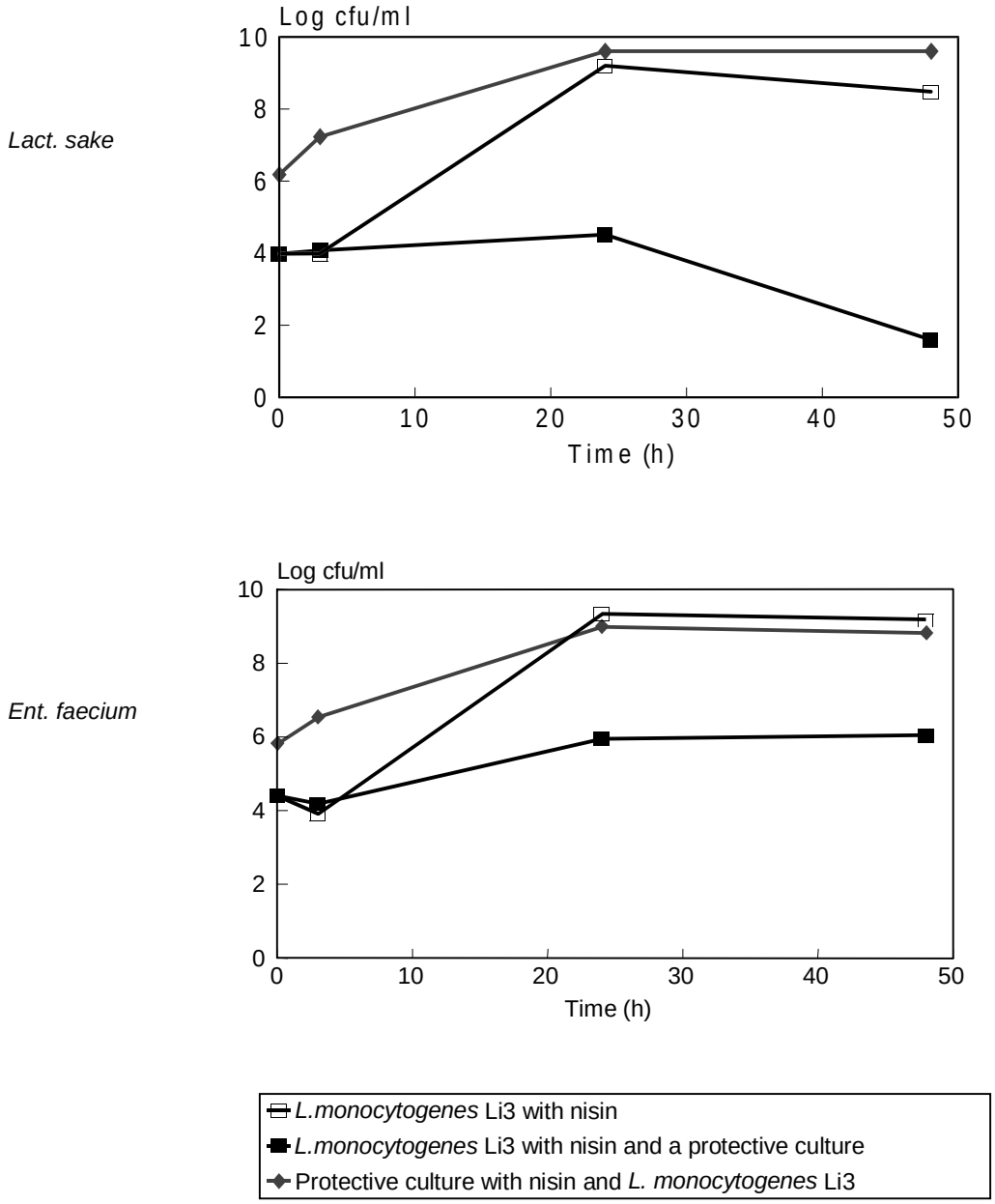


Figure 5: Growth of the nisin-resistant *L. monocytogenes* Li3 in the presence of nisin (100 IU/ml) and *Lact. sake* Lb 706-1a and *Ent. faecium* BFE 900-6a at 30°C.

These results clearly demonstrated that cells of *L. monocytogenes* surviving certain nisin concentrations are inhibited from growing by appropriate protective cultures provided that they are used at a relatively high inoculum and that they are able to grow rapidly at the relevant temperature.

The effectiveness of the protective cultures against nisin-resistant *L. monocytogenes* was confirmed by experiments using a nisin-resistant mutant of *L. monocytogenes* Scott A.

This mutant (Li3) was obtained by exposure of *L. monocytogenes* Scott A to nisin at 100 IU per ml and by isolation of survivors from agar plates. It was resistant to nisin concentrations of 500 to 1000 IU per ml. It is remarkable that strain Li3 did not show cross-resistance to other bacteriocins. The mutant was still sensitive to sakacin A and enterocin B.

Nisin at 100 IU per ml did not reduce viable numbers of Li3; by contrast, the nisin-resistant strain grew to a high cell density within 24 h at 30°C in the absence of a protective culture (Fig.5). This growth was inhibited by the protective lactic acid bacteria added at a high inoculum. Again *Lact. sake* Lb 706-1a was more effective in suppressing listerial growth than *Ent. faecium* BFE 900-6a (Fig.5). With *Lact. sake* less than 100 cells per ml survived after 48 h whereas *Ent. faecium* did not prevent an increase of *Listeria* viable numbers by about 1.5 log units.

A non-bacteriocinogenic variant of *Lact. sake* Lb 706 was used in some experiments to demonstrate that the inhibitory effect of the protective culture was mainly due to the bacteriocin. *L. monocytogenes* Li3 was inhibited by both the bacteriocinogenic and the non-bacteriocinogenic culture (Fig.6). The bacteriocin producing strain, however, showed a much stronger inhibitory effect on the resistant mutant of *L. monocytogenes* indicating an essential role of the bacteriocin in controlling listerial growth.

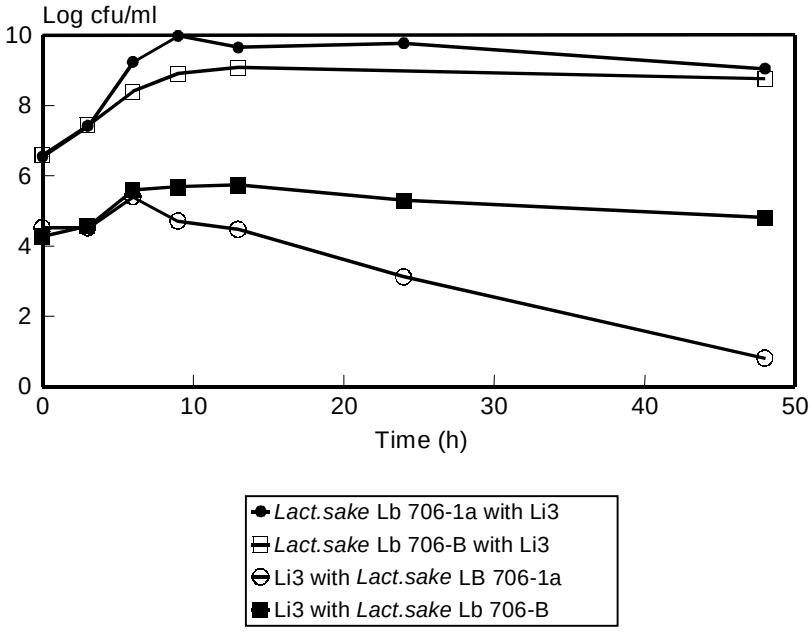


Figure 6: Inhibition of the nisin-resistant *L. monocytogenes* Li3 by the bacteriocinogenic *Lact. sake* Lb 706-1a and the non-bacteriocinogenic *Lact. sake* Lb 706-B

Conclusions

The results obtained in these studies indicate that the efficacy of nisin against certain food-borne pathogens like *L. monocytogenes* may be improved by a combination with a starter or protective culture showing antagonistic activity against the same target strains. Cells surviving action of nisin can be inhibited by the bacteriocin or other metabolic products of the protective culture. Moreover, bacteriocins such as sakacin A, enterocin B and pediocin PA-1 were shown to enhance the bactericidal effect of nisin under certain conditions. All observations were made “in vitro“.

The effectiveness of the protective culture in food systems still has to be demonstrated. It will depend on its adaptation to the food environment and its competitiveness against the natural indigenous microflora of the product.

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Session 5:

Consumer Perception and Transfer Strategies

Is innovation in the food industry market or R&D led?

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Abstract

A sub-group of the research project on Structural Change in the European Food Industries analysed product and process innovation in the food industries through a series of company case studies involving food manufacturers in six EU countries. The companies were all quite successful, but placed no great emphasis either on R&D or market research. The more successful ones appeared to be better focused in the sense that they displayed a dominant orientation to the product, process efficiency or the market and acquired or developed internally a set of competencies appropriate to the orientation, supported by relevant skills in the other areas, either internally or bought in. The study also finds different patterns of innovation among private label as opposed to branded manufacturers, international as opposed to domestic firms, and according to ownership of the firm, but that company size had little impact.

Introduction

Evidence suggests (Traill, 1987, Traill and Grunert, 1997) that the food and agribusiness industry, traditionally low-tech, is becoming more technology intensive, as measured by its R&D to sales ratio (the traditional measure of research intensity). Most industry analysts argue that the combined forces of the biotechnology revolution, pressures arising from globalisation (that require firms to maintain better process control and exploit economies of scale), the need to ensure food safety and nutritional quality and produce a new generation of functional foods, and consumers' demands for convenience and quality, will all increase the demands for research in the industry.

At the same time, the changes in consumer demand and in food-related lifestyles have resulted in a finer market segmentation, a more rapid rate of product turnover and the development of trans-national segments of food consumers. Firms must respond not only by introducing more new products but by better targeting them, to foreign as well as domestic consumers. This suggests the need to upgrade marketing competencies as well as scientific R&D.

A review of the academic literature on innovation (see e.g. Grunert *et. al.*, 1997) suggests two main lines of pursuit. One, most closely associated with the industrial economics profession, has stressed the links between R&D and technological innovation, often indicated by patents, and has investigated relations between R&D, patents and market structure and firm size. The other line of investigation, most closely associated with the marketing profession, has taken the wider view of innovation as the detection and fulfilment of unfilled wants of potential customers. This suggests that successful companies are likely to have strong market skills--in the language of the marketing profession, they will have a high degree of market orientation (a concept usually measured by the

extent that a company bases its activities on information about consumers, through collecting relevant data, digesting it, disseminating it within the company and reacting to it).

More recent literature (e.g. Burgelman and Sayles, 1986; Grunert *et. al.*, 1997) suggests that successful companies achieve a balance and integration between their R&D and marketing activities to ensure the introduction of technologically innovative products that the market demands. However, the literature provides little information on what is meant by 'balance' and 'integration' and how is it achieved. The literature (little of which is specifically food industry oriented) also says little about the wider role of networks and clusters in innovation, of differences between large and small companies, of the effect of alternative distribution channels or the impact of internationalisation.

In a first attempt to test the model and address these other questions, the research project *Structural Change in the European Food Industries* carried out a series of 12 case-studies of food manufacturers in 6 EU countries. These have been written up in full in Traill and Grunert (1997). On the basis of the cases, a new model of the innovation process was proposed (Grunert et.al. 1997). Using 3 of the case studies for illustration purposes, the model was slightly refined in Grunert and Harmsen (1998). The aim of the present paper is to summarise the case studies and tease out some additional insights into the innovation process. On the basis of the case studies, a number of hypotheses are formulated.

The case studies

Twelve case studies were undertaken during 1986, two each in Denmark, France, Germany, the Netherlands, Sweden and the UK. The case companies were selected to provide a mixture of large and small, private label and branded and to give a coverage of various product sectors. Each firm was visited by at least 2 researchers from different countries and the interviews, which were recorded, were based on a semi-structured questionnaire. The number of people interviewed and their positions varied by company, but generally included the owner, chief executive or manager of the strategic business unit, and some combination of directors/managers responsible for R&D, production, marketing, corporate strategy and new product development. Once written up, the cases were cleared by company representatives to eliminate (rare) factual errors and/or trade secrets inadvertently divulged in the interviews. A major difficulty was in assessing companies' degree of success. In fact, all of the companies were considered to be successful, at least, the parts examined in the case studies. In all instances, it was claimed that sales of the unit studied had grown rapidly, but it was usually not possible to confirm this or link it to profitability as many of the firms were privately owned or were business units of larger firms and did not publish separate accounts.

For the sake of brevity, only three representative case studies are presented in this paper, but a summary of all the cases is given in the appendix.

Tholstrup Cheese is medium-large, with 650 employees and a turnover of 110 million ECUs (MECU), mainly selling branded cheese in Germany and Denmark, but also exporting throughout the world. It is the largest privately owned dairy company in Denmark, a leading dairy producing country, but is much smaller than Denmark's two giant co-operatives, MD Foods and Kløver Moelk. It employs a niche strategy, focusing on specialist cheeses and places great emphasis on introducing new products that incorporate a high degree of innovation. The new product development process is fairly formal and high level, involving, among others, the managing director and the director of sales and marketing in a Product Development Group (PDG). NPD relies heavily on the intuition of the PDG that considers itself to be the authority on cheese. The company does

not consider itself to be market oriented (consumers, and indeed retailers, are not thought able to suggest truly innovative products), though it does test new products on consumers and monitors their progress following introduction. Other than product development, the company undertakes no R&D. Two thirds of new products require some process modification (e.g. new equipment or new packaging) and some process innovation is carried out to reduce costs of existing processes, provided it can be guaranteed that this does not impinge on product quality.

Groko is a medium sized Dutch company (employment around 220, turnover 52 MECU) which manufactures frozen vegetable-based consumer products. It is a subsidiary of the Danish multinational Danisco, and was previously owned by American multinational Campbells, but throughout it has operated relatively independently. Around half of its products carry its own brand, the other (faster growing) part is sold under retailers' private label. Germany, the Netherlands and Scandinavia are its largest markets. Its strategy is to imitate new products of the major branded companies and sell them at a lower price (in an interesting example, Unilever introduced a new product that proved very successful in the German market. The German-based multinational retailer, Aldi, asked Groko to imitate it and the imitation was introduced by Aldi to the Dutch market before the original Unilever product). New product ideas thus arise from monitoring other companies' products and from requests by retailers. A new product development committee assesses new product plans and an internal panel tests them against the imitated originals. Groko considers its primary expertise to be its flexibility and efficiency in scaling up new recipes into new production processes, which often requires process innovation. Production and packaging are fully automated. Also important are its ability to operate in different countries, with different languages and customs. The company is not very market oriented in the traditional sense--it does no market research and little market testing, though it does monitor competitors. The company does not undertake any scientific research.

Bulmer, based in the Southwest of England, is the world's largest cider manufacturer, though with 1200 employees and 320 MECU turnover, it is far from being an industrial giant (cider consumption is a peculiarly British tradition, though the company does sell in more than 30 countries). Through NPD and aggressive marketing, the company is largely responsible for transforming cider from a regionally consumed undifferentiated product in the early 1980s to a highly differentiated market that was one of the most dynamic segments of the UK alcoholic drinks market by the early 1990s. The company sells private label as well as branded products in order to take advantage of economies of scale, and to avoid losing that market to competitors. This company conforms well to the traditional model of innovation. It is highly market oriented, with a marketing department that collects market information and monitors markets, carries out formal statistical modelling for forecasting and the estimation of price and income elasticities, and disseminates key information within the company. It also has a large R&D facility (1% of turnover) with links to many universities, which monitors scientific advances, regulatory developments and their implications, and carries out fundamental long-term research. The R&D department is also responsible for technology transfer and the creation of new products from a commercial brief. The main source of new product ideas is extensive consumer research (market intelligence, focus groups etc), though the technical director is of the view that only R&D produces really innovative product ideas. Almost by definition these cannot come from observing the market (note the similarity with the view of Tholstrup Cheese). The new product development process is highly formalised and new products are subject to extensive testing prior to launch.

Assessment of the innovation model

Based on these cases, a number of observations can be made. First and foremost, few of the companies are involved in research to any great degree and few of them are truly market-oriented, though they all monitor sales and some carry out extensive consumer testing of new products. Thus the originally hypothesised model of successful innovation being the outcome of balance and integration between R&D and market orientation is not supported by the cases, even though most of the case companies have been highly successful (in terms of sales growth). Grunert *et.al.* (1997) propose an alternative model, slightly refined in Grunert and Harmsen (1998). This suggests that every successful firm has a dominant 'orientation' which permeates throughout the company, forming the company culture and guiding its behaviour. They result in the gradual build up of what Grunert *et. al.* define as a core competence, a concept which corresponds closely to what, in the resource-based theory of the firm would be called a set of intangible, inimitable (in the short run), unsubstitutable resources that form the basis of a firm's competitive advantage (Barney, 1991). Grunert *et.al.* (1997) suggest three orientations that dominate in different firms in the food industry: product orientation (the company culture is dominated by product quality and a 'love of product', as seen for example in Tholstrup Cheese[1]); process orientation (in which the company culture is dominated by issues such as flexibility and efficiency, as for example in Groko); and market orientation, where the dominant culture is to produce what the market wants (e.g. Bulmer). The firm's dominant orientation demands a set of core competencies (product, process or market), but to be successful, the firm needs to acquire competencies in the other two areas as needed. Such supplementary competencies may be outsourced, unlike the core competence. Thus, for example, Tholstrup Cheese's activities are guided by its product orientation, but for success it needs certain competencies to incorporate efficient processes and ensure that new products succeed (e.g. the market competence skills for product testing); Groko's core competencies relate to its process orientation (particularly, competence in process innovation), but are supported by competencies in market monitoring and product development; and Bulmer backs up its market competencies with product and process competencies particularly derived from its R&D capabilities. Indeed, in general R&D in the food industry may be seen as supporting the development of product and process competencies rather than a specific orientation guiding company behaviour. Whether this is true in high-technology industrial sectors is open to question.

Hypothesis 1: Successful firms have a single dominant orientation, product, process or market.

The impact on innovation of branded Vs private label supply

In the cases, a further major discriminating factor in the type and nature of firms' innovations is the distribution channels they use. Almost exclusively, private label suppliers are process-oriented--their success depends upon production and distribution efficiency, speed and flexibility. Interestingly, these firms introduced the largest number of new products, though in general the products were not highly innovative. One might be tempted to argue that ensuring a continuous flow of new but similar products is for these companies a process competence. However, there are a number of different means by which companies achieve this result. Some work very closely with retailers, some

[1] This view of the product oriented firm is different from the usually negative connotations attached to so-called product-led marketing strategies. The new definition, in successful firms at least, stresses creativity linked to deep product involvement. The old view is almost the opposite-a conservative company, interested only in 'pushing' its product to an uninterested public.

specialise in imitating branded products (Groko), while still others develop products independently and then seek to interest retailers (Royal Greenland).

Hypothesis 2: Private label suppliers are process oriented but place a high emphasis on both NPD as well as process innovation.

Product introductions for branded manufacturers are far more expensive than for private label suppliers, so they introduce less new products and need to pay more attention to making sure they don't fail.

Hypothesis 3A: Successful branded manufacturers are predominantly product or market oriented.

Hypothesis 3B: Those branded manufacturers that rate product quality most highly are less innovative than those that rate quality less highly (because they are unwilling to make changes that jeopardise product quality).

Sector of activity and innovation

Many of the companies studied do not fit clearly into the product categories used in official statistics, so it is hard to deduce relationships between sector of activity and innovativeness. The more innovative case companies appear to be in the fastest growing industry segments (ready meals, specialist cheeses, chilled prepared foods, convenience foods), but this is partly because the companies themselves have often been responsible for driving growth in their market (e.g. Bulmer). It is difficult to determine cause and effect, but it is tempting to propose the following:

Hypothesis 4A: Product innovation in process oriented firms occurs in fast growing markets whereas;

Hypothesis 4B: In product and market-oriented firms, NPD drives market growth.

Product and process innovation and scientific research

Scientific research has been seen to be important in only four, widely different, case companies. Others work closely with machinery suppliers to design new equipment, still others simply buy new process innovation embedded in new machinery investment. Automation is seen as the fundamental need of the industry, though some companies will limit automation if it detracts from product quality or flexibility. The food industry has been characterised as an industry which does not do its own research but brings through to the market-place the benefits of research conducted further upstream, be it in information technology, biotechnology or process engineering (Pavitt, 1984; Christenson *et.al.,* 1996). So far as process innovation is concerned, this seems to be born out by the case companies. So far as product innovation is concerned, this is far less clear (though outside of the case studies, anecdotal evidence suggests that ingredient suppliers are important in product innovation).

Hypothesis 5: R&D expenditures are more closely correlated with NPD than with process innovation.

It is also worth pointing out that the two companies who rate long-term research most highly, Bulmer being one of these, make significant efforts to leverage their internal capabilities through a network of research linkages with universities and other research organisations.

Ownership and innovation

A range of ownership patterns was evident in the case companies, including privately owned, co-operative, subsidiary of MNE, subsidiary of domestic company, and publicly quoted company.

Almost by definition, co-operatives objectives limit them to activities that utilise the product concerned as raw material. This may limit the extent of their product innovation and cause an emphasis on process innovation. However, the cases show that co-operatives can respond to the restraints in different ways, and we would suggest that, in order to be successful, they must develop a strong product orientation in the sense discussed by Grunert et al (1997).

Hypothesis 6A: Co-operatives are less product innovative than other forms of ownership. They may weakly be expected to compensate by being more process innovative.

Among the privately owned case companies, there appeared to be a strong emphasis on the product, though there were counter examples, even in the cases (e.g. Brioche Pasquier). Given the earlier discussion, such companies may be expected to resist undue product proliferation, so placing a lesser emphasis on product innovation. Subsidiaries and public companies are more likely to be innovative than co-operatives and private companies, partly because of shareholder pressure, partly because successful private companies are often bought by larger public companies.

Hypothesis 6B: Privately owned companies are more likely to be product oriented than public companies which are more likely to be process or market oriented. However, private companies have a lower rate of new product development.

Company size and innovation

The case studies do not suggest any clear relationship between company size and innovativeness--in fact, the two least innovative companies are Neumarkter and Royal Greenland, respectively the smallest and largest of the 12, though this is insufficient evidence to suggest an optimal size for innovation.

Hypothesis 7: There is no relationship between company size and innovation.

International orientation and innovation

Economists and others have long argued that competing in foreign markets is more demanding than competing at home (Krugman, 1995; Baily and Gersbach, 1995), but among the case companies the links are weak. The cases do suggest that companies involved mainly in small national/regional or local markets are less innovative. As far as testable hypotheses are concerned, matters are complicated because a company supplying all of Scandinavia say may appear international but be serving a smaller, more homogeneous market than a firm supplying, say, the whole of France. Therefore the following hypothesis is more precise, but less readily testable than one based on internationalisation *per se.*

Hypothesis 8: The larger and more heterogeneous the market served by a firm, the more innovative it must be.

Degree of innovation

Tholstrup and Bulmer both suggested that really innovative products could not be developed simply by watching the market, a process which leads only to minor improvements and revisions to existing products and to repositionings. This suggests that products with a high degree of innovation come either from high competence/expenditure in R&D and/or, that most intangible of all assets, imagination/creativity.

Discussion

The title of this paper asks the question: is innovation in the food industry is R&D or market led? The evidence from the case studies undertaken in the course of the research project suggests the simple answer 'no'. While most of the first analysed undertook development work, few of them undertook any research. And while most of the firms would test market new products, few of them systematically analysed existing and potential consumers and built their product development activities around the findings. There are good reasons for firms behaving in this way. Most process innovation is embedded in new machinery investment and much product development involves trial and error recipe changes rather than sophisticated research. If you are producing to retailers' specifications or imitating competitors' products there is little need for a profound understanding of the final consumer.

The study suggests that the most successful firms have a dominant orientation towards the product, process efficiency or the market and this orientation permeates the entire culture of the company and determines the internal skills and competencies that firms acquire[2]. R&D itself cannot be considered a dominant orientation[3], but is a competence that supports both product and process orientations. In the same way, market research is a competence that supports a market orientation. The study also suggests that firms that do not develop a dominant orientation risk having a poor (unfocused) innovation performance.

The research began with a hypothesised model and finished with a new hypothesised model and a number of other hypotheses. To test these hypotheses requires a quantitative study based on analysis of data from a reasonable statistical sample of food manufacturers.

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[2] In fact it is something of a two way process--the skills and competencies possessed by the firm may in part determine the firm's orientation.

[3] One can imagine R&D being a dominant orientation in, say, pharmaceutical or IT companies.

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Appendix: Summary of case studies

Company	Size	Sector	Market	Ownership	Innovation Strategy	Market orientation
Tholstrup	Medium/large: 600 employees	Dairy	Branded products in Germany, Denmark	Private	Fundamentally new product every 3 years	Relatively informal. Not used in idea generation, but extensive consumer testing of new products
Brioche Pasquier	Large: 1300 employees	Brioches, croissants, pies	France, retail chains, mainly branded	Private	Industrialize traditional products	Close relationships to retailers, some consumer testing of products
Groko	Medium: 220 employees	Frozen vegetable based consumer products	Mainly private label, and mainly to EU countries	Subsidiary of MNE	Imitate leading brands more cheaply	Close relationships to retailers. Monitor competitors' products.
Neumarkter Lammsbrau	Small: 83 employees	Ecological beer	South Germany	Private	?	Monitoring of societal trends
Skåne Erik	Small-medium: 120 employees	Processed meats	Sweden and some N. Europe exports Branded products	Subsidiary of MNE and large national firm	Aim for 5 new products per year	Use of market research agency. Some product testing
Bulmer	Large: 1200 employees	Cider	Mainly UK. Brandedand private label	Public company, but 53% of shares family owned.	Ensure high product quality at low cost. Spread risks.	High on all counts.
Reiter	Medium/large: 550 employees	Processed meats/ ready meals	South Germany	Private	Extend range of ready meals	Sales monitoring.
Royal Greenland	Large: 3000 employees	Seafood/ready meals	Branded in Denmark and UK plus private-label exports to other countries	Private	Broaden product range in ready meals	Limited contact with final consumers
Skånemejerier	Medium/large: 700 employees	Dairy products	Branded and private label in Sweden plus limited exports	Co-operative	Develop technologically sophisticated products	Fairly high in local markets
Johma	Medium	Salads	Branded, mainly in Netherlands	Subsidiary of MNE	Boost sales through new products	High
Magdis	Medium: 180 employees	Fresh, chilled pizzas, pies, sandwiches	French retailers	Subsidiary of MNE	Ensure low cost status retained	Limited - mainly competitor monitoring
Pennine	Medium/large: 600 employees	Chilled ready meals	Private label to UK retailers	Subsidiary of large domestic company	Continuous turnover of new products	Limited - reliance on retailer

Company	R&D	External linkages	Networks and clusters	Formalization of innovation process	Innova-tiveness	Comments/other	Basis of success
Tholstrup	No	Minimal	No	Yes, through product development group	High	'If consumers want it, it is not new'	Creativity, flexibility
Brioche Pasquier	Significant. Mainly process linked. Linked to universities for monitoring and research	Strong links to retailers, but not for NPD	?	?	High	Convenience and price are main innovation driver	Logistics of flexible delivery of fresh produce.
Groko	Limited	Ingredients,equipment and machinery suppliers. Contracts with growers	No	NPD meetings regularly	High	Imitation and price are main drivers	Flexibility, low cost production
Neumarkter Lammsbrau	No	Communication with bar and restaurant owners	No	No	Low	Environment main driver	Product quality
Skåne Erik	No	Informal with machinery suppliers	No	Little formality	Medium	Convenience main driver	Efficient production; competitive prices
Bulmer	Yes, for product and process innovation	With outside R&D teams, plus government, EU, trade, associations, etc.	No	Yes	Medium	Legislation is an important innovation driver. The market does not come up with really new ideas - R&D is needed for that	Market orientation plus promotion
Reiter	No	Informal with machinery suppliers	No	Recently formalized through 'quality circle'	Low/ medium	Convenience main driver	Product quality
Royal Greenland	None internal	With retailers and caterers	No	Increasing formalization	Low/ medium	Convenience main driver	Broad product range, access to rare raw material
Skånemejerier	Yes, mainly external. Monitoring is an internal function	Strategic alliances with Swedish and Danish companies plus links with machinery suppliers	Links to local machinery suppliers and universities	Quite formal	High	Substantially new products originate from R&D	Niche in supplying high-tech products
Johma	Yes, internal plus links to other organizations	Ingredients and machinery suppliers	No	Formal	High	Health, convenience are important drivers of innovation	Production quality and marketing
Magdis	Low internally. Some external linkages	Limited	No	Relatively informal	Medium	Convenience and quality main drivers	Industrialization with 'home-made' appearance
Pennine	No	Informal	No	Formal	Medium	Convenience and product turnover are main drivers	Speed and flexibility in satisfying customer requirements

A consumer-led approach to marketing of foods in the EU: The case of yoghurt

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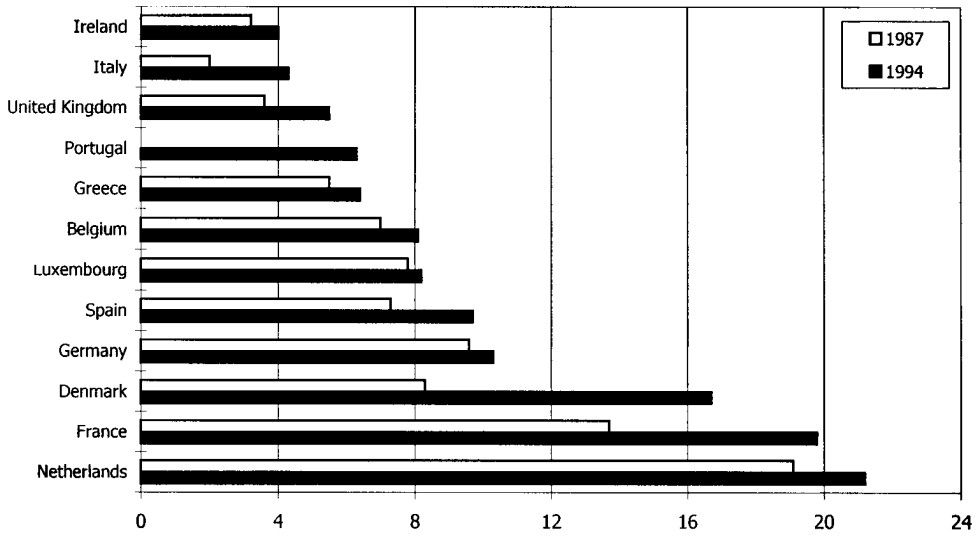
Developments in the EU yoghurt market

The overall demand for dairy products is expected to increase moderately in the EU, but the market for yoghurt is likely to grow faster thanks to the possibility for adding value through differentiation and new product development.

The dairy market in the EU has experienced a trend towards fresh, low-fat and (perceived) healthy products. As a consequence, yoghurt consumption has been characterised by consistent growth throughout the 1990s in all EU countries. and represents one of the most dynamic components of the dairy sector. The recent growth of yoghurt consumption in mature markets (e.g. France) indicates that there are still market opportunities to be identified and exploited. Another factor suggesting untapped demand opportunities is the difference in the quantities consumed across the EU countries. Yoghurt consumption averages around 10kg per capita per year, ranging from about 5kg in Italy and Ireland to over 20kg in the Netherlands.

Figure 1
Change in yoghurt consumption, 1987-1994 (kg per head).



Consumer preferences

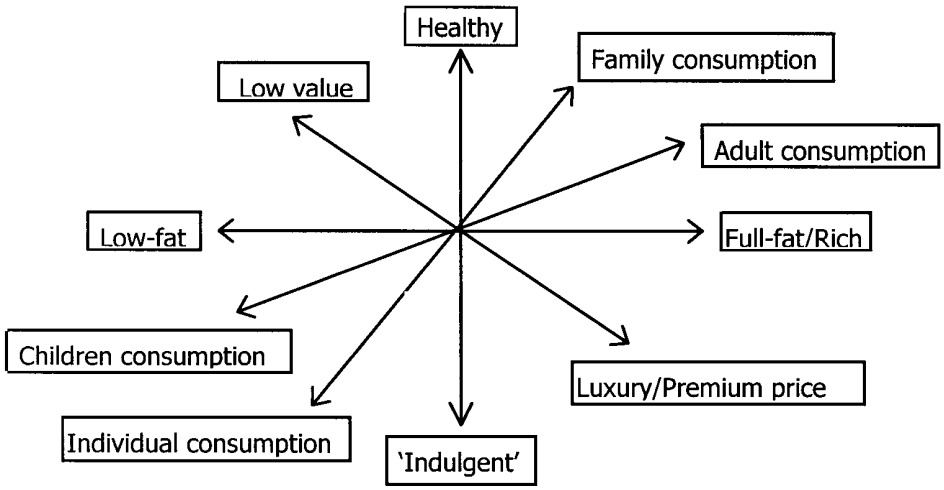
Demographic factors are a major influence on yoghurt consumption. In particular, consumption is higher among the younger age groups, women, households with children, and the higher strata of income and education. In general, consumer preference for yoghurt is mainly dictated by the

perceived healthiness of the product on the one hand and taste on the other. The differences in consumer behaviour across countries are attributed to culture and tradition. These are considered responsible for different preferences with respect to various yoghurt characteristics (e.g. mild/sour taste, thick/thin texture, pack-size, consumption occasions), as well as consumption levels.

Product differentiation

The yoghurt market shows a high degree of product differentiation, either by yoghurt attributes or by the information given to the consumer through branding and market communication. The spectrum of product differentiation includes the characteristics shown in Figure 2.

Figure 2
Dimensions of yoghurt differentiation



Product development

The market shows a high level of product development activity, although core product innovation appears rather modest. New products seem to result mainly from reformulation, new flavours and new packaging. Growth through new product development is mainly expected in the indulgence/luxury end of the market, but the 'health' factor remains important. Furthermore, the market for low-fat, probiotic and organic products is expected to grow in the future.

The supply side and market competition

The EU yoghurt market as a whole is dominated by a few multinational companies that account for about 60% of the total market and either produce yoghurt in, or export it to, a number of European countries. Medium to large companies serve either national or regional markets and their activities increasingly relate to the supply of retailers' private labels. Other small to medium companies have to take advantage of the remaining market opportunities. For these companies, the identification of regional, national or cross-national segments, which may be too small to be of interest to the large companies, could be of considerable importance. They may include niche markets, which the SMEs are well equipped, and flexible enough, to work in. The price of yoghurt differs significantly from country to country due to different pack sizes, retail structure, private labels' share of the market,

manufacturing structure, the level of market competition, and advertising expenditure. The main competitive factors are:

- price;
- quality;
- access to distribution and good relationships with retailers;
- efficient logistics;
- wide product range;
- adequate investments in communication.

Means-end chains

An innovative new approach, viz. the means-end chain approach is used to study and quantify what a food product means to consumers. The means-end chain model consists of the three levels: attributes, benefits, and values. Attributes stand for the relatively concrete and tangible characteristics of the food product. Examples include the amount of fat in milk, colour of tomatoes, amount of sugar in soft drinks, caloric value of desserts, etc. Benefits refer to what the product is perceived as doing or providing to the consumer. Benefits can be rather direct, functional benefits of product use (good taste, preservable, wholesome, etc.) but they can also be more abstract socio-psychological benefits of consumption of the food product (makes you feel a good parent or a sophisticated consumer). As discussed above, values are intangible, higher-order outcomes or ends (fun and enjoyment in life, security, self-respect, etc.).

The key idea underlying means-end chains is that product attributes are means for consumers to obtain desired ends, i.e., values, through the benefits yielded by those attributes. The three levels attributes, benefits, values, are hierarchically linked in that in the mind of the consumer certain attributes (A) lead to particular benefits (B), which contribute to satisfaction of specific values (V): A ⇨ B ⇨ V. Food product attributes are means for consumers to obtain desired ends, i.e., values, through the benefits yielded by those attributes. For example, yoghurt containing less fat (attribute) is perceived to be good for one's health (benefit), which contributes to fun and enjoyment in life (value). In this sequence, we move farther away from the food product per se and closer to the consumer, thus linking the food product with the consumer.

Thus, the means-end chain approach explicitly establishes the relation between product and consumer: attributes lead to benefits which contribute to value attainment. In this way, we are able to understand how food products gain personal relevance and meaning. An attribute is important if it leads to a desired benefit while the perceived benefit derives its importance through the extent to which it is linked to one or more personal values.

One of the key advantages of this approach is that it links explicitly physical attributes of food products to needs of food consumers. This increases the actionability of the results for successful product development as well as for effective and targeted communication strategies.

Means-end chain segments

Identification of means-end segments

Using national surveys of over 3,000 consumers in eleven EU-countries, consumers' means-end chains were identified and used as basis for pan-European market segmentation. Building upon modern segmentation techniques, a statistical model is developed that effectively identifies means-end segments in a correct way. The model uncovers groups of consumers from multiple countries and, at the same time reveals the means-end chains for each of these segments. The segments were also related to other consumer characteristics, to obtain additional profiles of the segments.

Four segments, among which one truly pan-European segment (Y4), characterise the consumer yoghurt market in the EU (see Table 1). A single country (Germany) dominated another segment (Y1) and the remaining two segments were represented by consumers from multiple EU-countries (Y2 and Y3). Based on their means-end structure and other characteristics, the segments are labelled as: 'healthy and vital' (Y1), 'sensible and secure' (Y2), 'healthy and innovative' (Y3), and 'top quality seeker' (Y4).

Table 1: Segment sizes of the yoghurt segments

	Segment Y1 *´Healthy and Vital'*	Segment Y2 *'Sensible and Secure'*	Segment Y3 *'Healthy and Innovative´*	Segment Y4 *Top Quality Seeker'*
Belgium	18%	12%	9%	62%
Denmark	3%	48%	27%	23%
Germany	45%	3%	26%	25%
Great Britain	7%	41%	26%	26%
France	22%	2%	3%	72%
Greece	13%	28%	7%	52%
Ireland	12%	41%	18%	30%
Italy	10%	6%	5%	79%
Netherlands	14%	32%	18%	36%
Portugal	28%	35%	5%	32%
Spain	9%	25%	4%	63%
Total	21%	17%	16%	46%

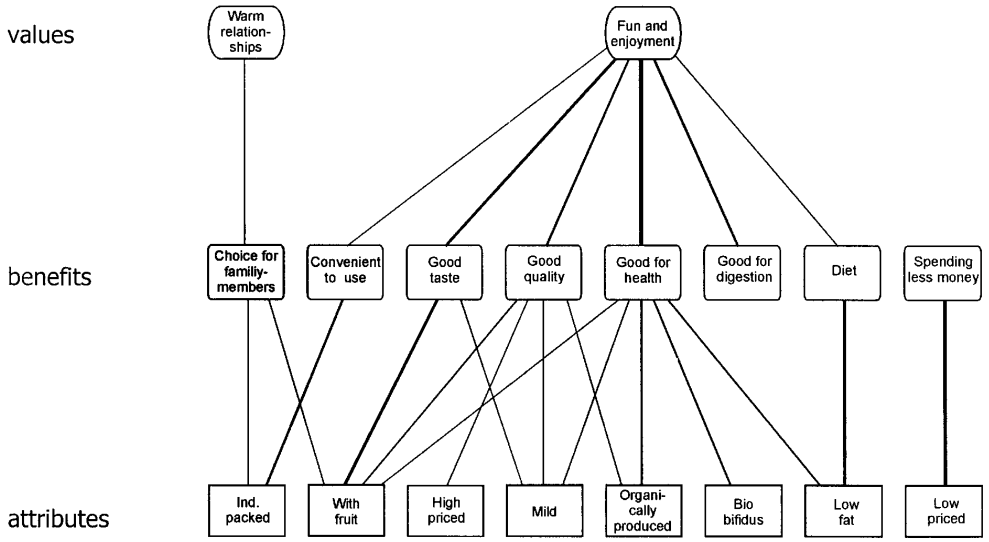
Segment Y1 -Healthy and Vital'

The 'healthy and vital'-segment has a high proportion of German consumers. The resulting means-end map in Figure 3 displays a rich structure of linkages (the thickness of lines indicates the strength of the relations). Multiple linkages connect to 'good for your health' and 'good quality', however, these linkages are moderately strong. In this segment the attributes 'with fruit, 'high

priced'. 'mild' and 'organically produced' serve as quality cues for yoghurt. 'Fun and enjoyment in life' is an important value in the MEC-map, connecting to many benefits.

The healthy and vital consumers prefer yoghurt as a snack rather than as a dessert or for cooking and consider quality and healthiness as the most important benefits. They are relatively older with lower disposable income and less formal education. They appear to be sensitive to price, more responsive to promotions and quite traditional in food consumption.

Figure 3
Means-end map for segment Y1 -'Healthy and Vital'[1]



[1] The links between attributes and benefits, and between benefits and values, are indicated by lines. The thickness of the lines reflects the perceived strength of the links.

Segment Y2 - 'Sensible and Secure'

The 'sensible and secure'-segment is quite well represented among the Danish, British and Irish, whereas the Italians, French and Germans are markedly absent. These consumers do not have such a rich structure as compared to the other segments. 'Good for one's health' links to many values. In this segment health may serve as a claim for low-fat yoghurt. The four values may be used to substantiate this claim in advertising campaigns.

Consumers in this segment use yoghurt more often for cooking than as a snack or dessert. Even though yoghurt does not matter much to them, they value its convenience, and suitability as a diet food and as an alternative to unhealthy snacks. The 'sensible and secure' consumers are quite conservative in their behaviour and not likely to buy foreign foods. They are less sensitive to promotions, but more likely to stick to one brand. They tend to live in small communities, buy yoghurt in small shops or mini-markets, and spend little on it. They are relatively older, have lower disposable income and less formal education, and watch a lot of TV.

Figure 4
Means-end map for segment Y2 - 'Sensible and Secure'

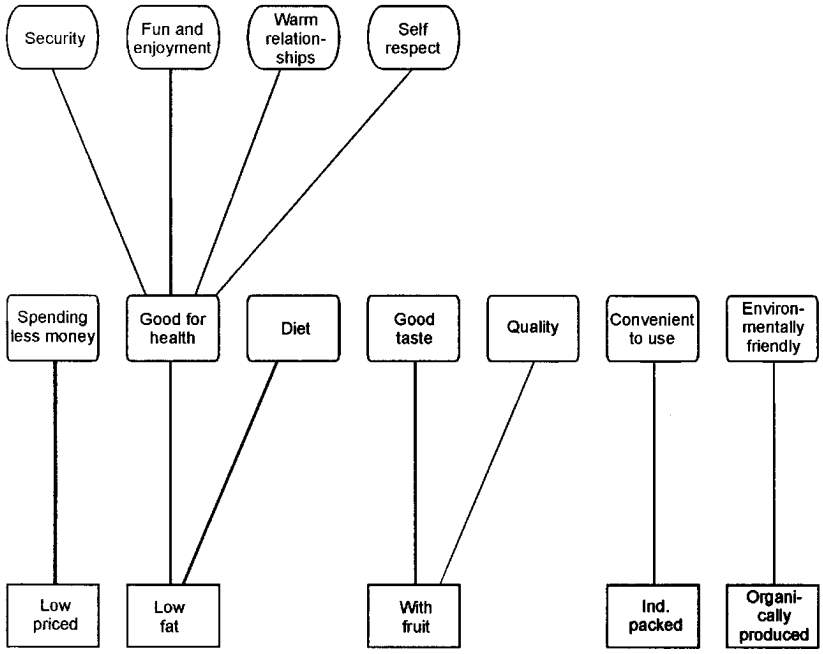
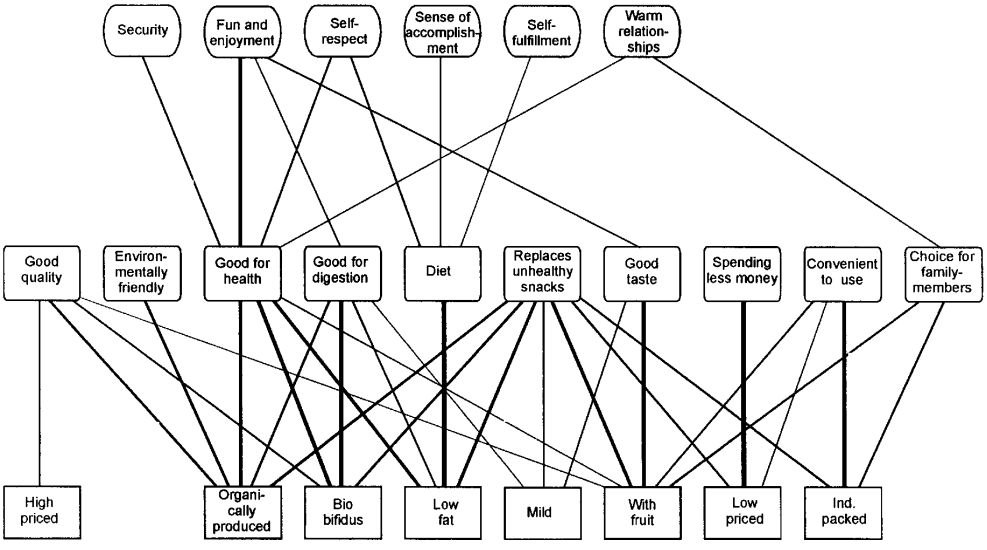


Figure 5
Means-end map for segment Y3 - 'Healthy and Innovative'



Segment Y3 - `Healthy and Innovative`

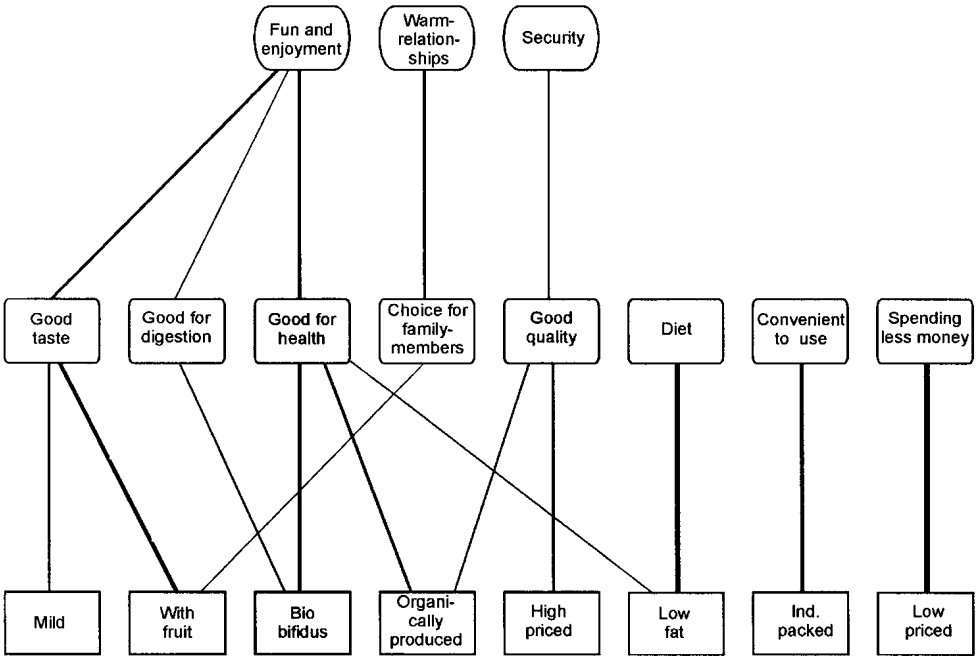
The 'healthy and innovative' segment comprises a minority in each country. In Great Britain, Germany and Denmark its size is about 25% of the population. Many strong linkages arise in the means-end maps, especially at the AB-level. The benefit 'replaces unhealthy snacks' has many connected attributes, but is unconnected with values. It may serve as an important benefit in this segment, but the benefit cannot be communicated through higher level values. Another important benefit is 'good for the digestion'. which has a very strong link with 'bio-bifidus' and some less strong linkages with 'organically produced', 'low-fat' and 'mild'.

Consumers in the 'healthy and innovative'-segment prefer yoghurt as a snack rather than as a dessert or for cooking. The most important yoghurt benefit is, in fact, its suitability as an alternative to unhealthy snacks. These consumers prefer low-fat yoghurt with bifidus in multi-packs with assorted flavours. They are innovative in food consumption, like to experiment with new products and are more receptive to promotions. They are relatively younger with higher disposable income and more formal education, shop frequently, and tend to buy yoghurt in the supermarket.

Segment Y4 - 'Top Quality Seeker'

The pan-European 'top quality seeker-segment exhibits relatively strong linkages between 'individual packed' and 'convenient to use'. and between 'bio-bifidus' and 'good for one's health'. At the BV-1evel the link between 'good quality' and 'security' is unique for this segment.

Figure 6
Means-end map for Segment Y4 - 'Top Quality Seeker'



Consumers in this segment are involved with yoghurt and know a lot about it. The most important yoghurt benefits are healthiness and quality. They are willing to pay a price premium for good quality, and would rather not buy low priced yoghurt. They are environmentally conscious and like to change. They buy yoghurt in hypermarkets or large stores and spend more than the other segments on it. They tend to live in cities, are relatively younger with a higher level of disposable income and more formal education.

Targeting European yoghurt consumers

The segment profiles are related to the marketing mix elements to produce comprehensive guidelines for the development of pan-European strategies. The structure of the means-end chains describing a segment provides the basis on which product concepts and market communication can be developed to target that segment. Consumers' behaviours and personality characteristics help to define where a new yoghurt product should be sold, at which price and which promotional tools can be used.

New product concepts and comprehensive marketing strategies were developed for each of the four pan-European segments identified with respect to yoghurt. Below are two examples. Validation of the results with companies and consumers supported the viability and usefulness of the segments and the marketing strategies developed to target them.

*Targeting Segment 2, the **'Sensible and secure´** yoghurt consumers...*

- with a low-fat, low-calorie, plain or fruity yoghurt, individually packed
- sell through mini-markets, corner shops
- at a price just below the market average
- target with advertising on TV, during entertainment programmes, with famous international spokespeople, with images of healthiness linked to security, selfrespect, warm relationships and fun
- stress convenience, versatility in cooking

Yoghurt B is so versatile!

`B´ is a very low-fat, low-calorie yoghurt. It is available both plain and with fruit in 200g individual pots. On each pot of plain yoghurt B a different recipe suggestion is available to use it to prepare straters, salad dressings and desserts.

~

Yoghurt B is available in local shops and mini-markets.

~

Yoghurt B is advertised on TV during your favourite programmes. You will find out more about B's healthy properties. One of your favourite actors will show you how versatile B is and how many delicious low-fat dishes you can prepare with it. B is convenient to use especially if you want to watch the calories in your diet or simply eat healthily. Taking care of your health can make you feel more confident about yourself, so that you can enjoy life more.

*Targeting Segment 4, the **'Top quality seekers'**...*

- with a fruity, bio-bifidus, organic yoghurt in a luxury recyclable pot, in multi-packs either single or assorted flavours
- charge a premium price and sell in hypermarkets and superstores
- advertise on TV during films and in cinemas, stressing its organic production as an indicator of good quality associated with security
- stress indulgence - a real treat

Yoghurt D - Naughty but nice!

D is an organic fruit yoghurt and contains additional healthy bifido bacteria. D is creamy and mild in taste. It is available in 2x125g glass pots you can recycle. D is a full-fat yoghurt, completely natural, without artificial additives.

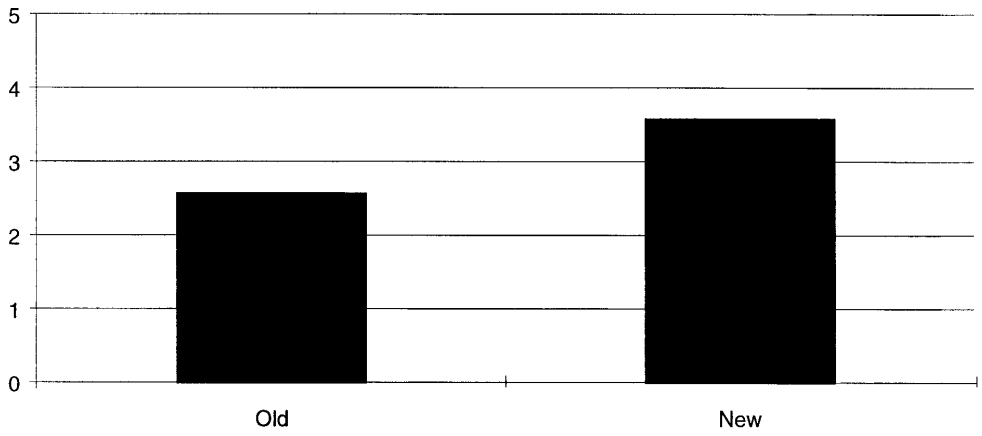
~

D is advertised on TV and in cinemas for you to find out more about its good quality and naturalness. If you want a guaranteed high quality, D is certainly worth a little extra money. D adds to healthy properties of yoghurt a luxurious range of exotic flavours. With D you can indulge yourself at any time of the day without giving up on health. Try it as a dessert to offer to your family and guests, they will enjoy it.

The results show that the means-end approach is a powerful and actionable basis for segmentation in the EU and for identifying the relations between yoghurt and consumers in each segment. It helps dairy companies to develop differentiated products that better meet the needs of the consumers. This will enable consumers to make purchase decisions which result in a greater degree of consumer satisfaction, while providing a stronger competitive position to the food suppliers.

Consumer reactions to the new yoghurts

Consumers were asked to evaluate the four new product concepts as opposed to two existing well-known brands. Overall, the new products seem to positively differentiate themselves from the 'old' ones. The difference in the preference between new yoghurt concepts and the existing ones is shown by how many consumers are prepared to pay.



* 'Willingness to pay' ranges between 0='Would not buy at any price' and 5='Would buy at the highest price'.

Figure 7: Consumer reaction to new yoghurt concepts*

Potential for pan-European marketing strategies for yoghurt

The Means-end Chain approach to market segmentation proves to have important implications for the development of new yoghurt concepts and for market communication, as the associations between product attributes, the benefits from consuming the product and values can be used to communicate product features and benefits. Communication and the way a product is positioned in the market is particularly important in the case of yoghurt, given its nature of branded and highly differentiated product.

The results of the project indicate that standardised communication may be used to target European consumers in various countries. Through the project, European consumers have indicated further ways in which yoghurt brands can be positioned, leading to a better differentiation of products based on the benefits important to consumers. For example, a yoghurt could be more strongly positioned as a 'healthy substitute to less healthy snack foods' or as 'being a versatile ingredient for cooking'.

The development of new yoghurt concepts should start with the understanding of consumer needs and preferences, but in this particular market the interaction between R&D, marketing and production is very important, given the role played by technology and biotechnology in the new product development process.

Apart from the fact that a new product could hardly be exactly the same in many countries, due to different pricing conditions for example, one potential problem to the development of pan-European strategies is identifiable in the heterogeneous nature of product definition. Labelling legislation differs from country to country: what can be called yoghurt in one country should be defined a fermented milk product in another, according to national regulations. A harmonised specification for yoghurt ingredients and production methods would probably make economies of scale in production more feasible.

Acknowledgement:

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The FLAIR-FLOW information system

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Abstract

A rapid transfer of research results from researcher to end-user is of paramount importance for the utilisation of the results and for generating innovation. This is especially important for the very small companies (< 25 persons) who may lack technical expertise and have difficulty in implementing new techniques and technologies. FLAIR-FLOW EUROPE is an ongoing trans-European dissemination project charged with the diffusion of results from European food R & D programmes to the food industry, health professionals and other end-users in 18 countries. The operating procedure and output of the project is described with emphasis on its benefits to, and interaction with, food SMEs. The scope and extent of EU-supported R & D programmes is also outlined.

Introduction

Reaching the end-users with results of research and development (R & D) is always a major challenge. There is often an imbalance between the amount of money spent (a lot) on carrying out the R & D, and that spent (relatively little) on ensuring effective and adequate dissemination of the results to end-users. In carrying out research there are five main steps: (i) planning the project, (ii) seeking funding, (iii) doing the research, (iv) disseminating the results, (v) seeking exploitation and end use. Of the five, the last two are by far the most difficult and are often neglected by scientists as they finish one project and move on eagerly to a new one.

This paper describes the FLAIR-FLOW EUROPE (F-FE) information system (Anon.,1994; Gormley, 1992, 1994, 1996a) which is a series of sequential projects (F-FE I, 1991-1993; F-FE II, 1994-1996; F-FE III, 1997-2000) charged with the dissemination of food research results from European Union (EU) programmes, and notably from the FAIR, AAIR and FLAIR programmes of Directorates General (DsG) XII and XIV. The information is disseminated to the food industry, health professionals and consumer groups in the 15 EU countries and in Norway, Iceland and Switzerland via a series of 18 national networks, the internet, and other procedures.

EU-supported food R & D programmes

The EU Commission supports an extensive food R & D activity under its FAIR (ongoing), AAIR (recently completed), and FLAIR (completed) programmes and also through the activities of COST (Gormley, 1992) and FAR. It also promotes a series of measures whereby food SMEs can participate, in partnership, in R & D activities relating to their specific requirements, i.e. the so-called CRAFT SME initiative which embraces exploratory awards and/or cooperative research.

The FAIR programme has circa 138 transnational food research projects involving 850 scientists and technologists. Corresponding numbers for AAIR and FLAIR were 72 projects (700 researchers) and 33 projects (400 researchers) respectively. The 243 research projects embrace the areas of food science and technology, food quality, food safety and human nutrition/wholesomeness, and represent a formidable dissemination challenge to both the participating scientists and to FLAIR-

FLOW. The details of the food R & D component of the 5th Framework Programme are currently being framed by the EU Commission with the help of groups of experts, and a call for offer will be made in the spring of 1999. The estimate for the key action Health, Food and the Environment in the Commission Proposal is in the order of 500 million ECU.

Objectives and operation of FLAIR-FLOW Europe

FLAIR-FLOW EUROPE III (1997-2000), II (1994-1996) and I (1991-1993) are sequential projects with FLAIR-FLOW III being built on the strengths of II and I. The aim of FLAIR-FLOW III is to disseminate the food research results emanating from the ongoing EU FAIR programme, and also some carry-over results from the now-completed AAIR and FLAIR programmes. FLAIR-FLOW is funded by the EU FAIR (DG XII) and INNOVATION (DG XIII) programmes. Experience from the FLAIR-FLOW II project indicated that the structure embracing national dissemination networks (and network leaders) in each of the 18 participating countries was highly effective. The dissemination procedures used, i.e. user-friendly 1-page technical documents, their reproduction in trade journals, focused workshops, [including RE-TU-ER (ready-to-use-European research) workshops], the internet, and other routes, were very fruitful (Figure 1) and are repeated, with upgrading, in FLAIR-FLOW III. In effect, FLAIR-FLOW is a 'matchmaker' and when end-users require more information they fax or e-mail the researcher named on the end of the 1-page technical document thus establishing a person to person link. The objectives of FLAIR-FLOW are:

- to reach food SMEs (small to medium sized enterprises), health professionals and other end-users in 18 European countries (15 EU countries with Norway, Iceland and Switzerland) with the results from EU sponsored food research programmes in user-friendly form; this will include the identification of new dissemination procedures. The names, institutions and fax numbers of the FLAIR-FLOW network leaders in the 18 countries are given in Table 1.
- to extract the more vital/strategic results from the FAIR/AAIR programmes for end-users. This will embrace the preparation of manuals by consultants (separate mini contracts awarded by the EU Commission).
- to obtain feedback from food SMEs as to the usefulness and uptake of the RTD information coming from European food research. This activity includes the formation of a platform of food SMEs.
- to promote innovation in the European food industry through the diffusion of RTD results to food SMEs and by bringing the actors together, i.e. the technology providers (researchers) on the one hand, and the technology users (food SMEs and other end-users) on the other.
- to quantify output and feedback in the FLAIR-FLOW project.
- to form dynamic interactive links with the network of European INNOVATION Relay Centres, with the network of FAIR Focal Points, with the Bureau Européen des Unions Consommateurs (BEUC), with the European Federation of Associations of Dieticians (EFAD), and with other relevant groups.

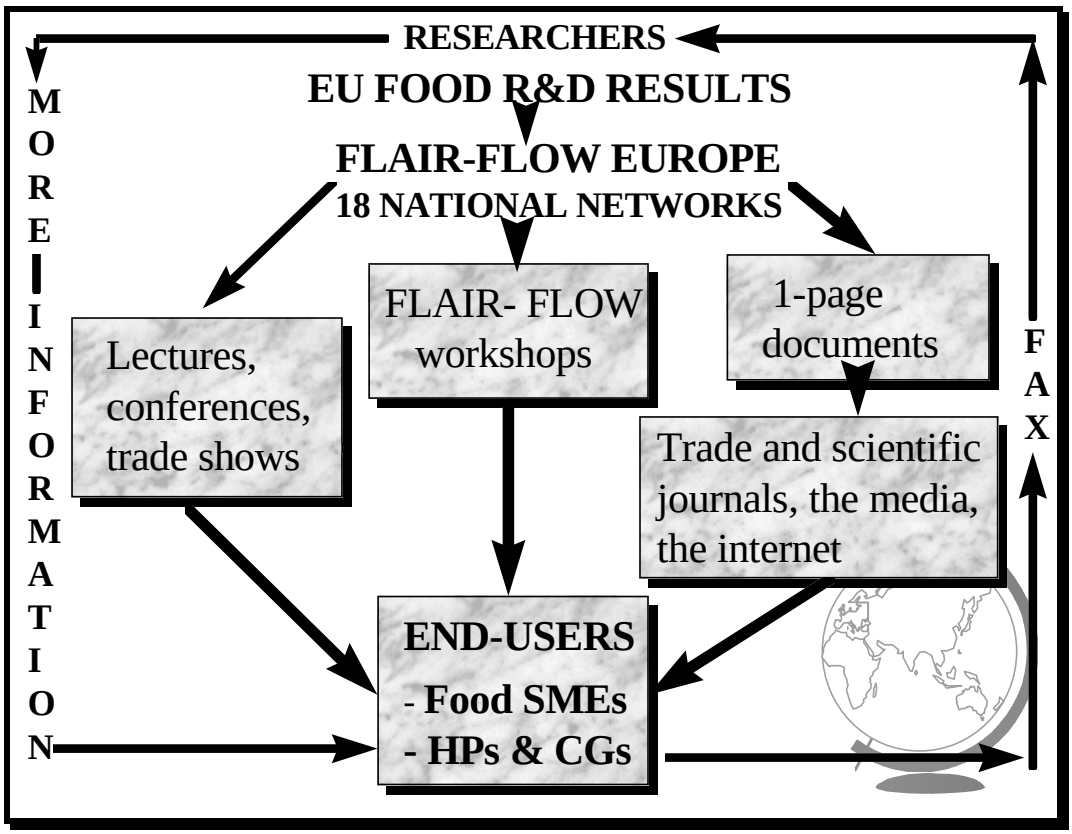

FIGURE 1: *FLAIR FLOW*: Routes for information transfer
SMEs = Small to medium enterprises
HPs = Health professionals
CGs = Consumer groups

From these objectives, it is clear that FLAIR-FLOW is both a dissemination and a research project. The latter refers to identifying new dissemination routes, the pinpointing of food SME R & D requirements, the assessment (by SMEs) of the quality and usefulness of the FLAIR-FLOW project output, and the identification of ways to encourage food SMEs to use the results from EU-sponsored food research, i.e. the project also promotes and encourages innovation in this important industry sector.

TABLE 1: List of FLAIR-FLOW national network leaders

Country	Leader	Organisation	Fax No.
Austria	W. PFANNHAUSER	University of Technology, Graz	43.316.873 69 70
Belgium	E. COOLS	Fédération des Industries Alimentaires	32.2.733 94 26
Denmark	F. HOLM	Food Group Denmark	45.86.201 222
Finland	A-M SJOBERG	VTT Biotechnology & Food Research	358.9.455.2028
France	F. MOLEGNANA	Pole Europeen Agro-Alimentaires	33.472 383 041
France	J.F. QUILLIEN	Inst. Nat. de la Recherche Agron.	33.02.9895 6042
Germany	E. KOENIG	Centre for Agricultural Documentation	49.228.954 81 49
Germany	W. SPIESS	Federal Research Centre for Nutrition	49.724.722 820
Greece	Y. TOTSIOU	SPEED Ltd.	30.1.822 57 55
Iceland	H. EINARSSON	Icelandic Fisheries Laboratories	354.562.07 40
Ireland	G. DOWNEY	The National Food Centre	353.1.8059 550
Italy	C. LERICI	Universita di Udine	39.432.501 637
Luxembourg	W. LORIG	LUXINNOVATION	49.651.8103.413
Netherlands	H. van OOSTEN	Agricultural University, Wageningen	31.(0)317.483 342
Norway	A. TANDBERG	Norwegian Food Research Institute	47.64.970 333
Portugal	T. ALMEIDA	Escola Superior de Biotecnologia	351.2.590 351
Spain	J. ESPINOSA	Instituto del Frio	34.1.549 36 27
Sweden	B. HEDLUND	Swedish Inst. for Fd & Biotechnology	46.31.832933
Switzerland	I. STUDER-ROHR	Swiss Federal Institute of Technology	41.1.632 1123
United Kingdom	S. EMMETT	Ministry of Agric., Fisheries & Food	44.171.921 11 67
Project Leader	R. GORMLEY	The National Food Centre, Dublin	353.1.8059 550
Project Administrator	P. MORIARTY	The National Food Centre, Dublin	353.1.8059 550

FLAIR-FLOW output

Two hundred and eighty seven 1-page technical documents on research results from EU sponsored food research programmes have been issued in the period January 1991 to October 1998 and are collated in booklets F-FE 114/93, 236/96, 274/97 and also on the FLAIR-FLOW web site http://www.exp.ie/flair.html (see below). Articles based on these are published Europe-wide in trade and scientific journals and amount to 3200. FLAIR-FLOW workshops (160 in number) have been held in the 18 countries and over 8500 persons attended, a high proportion of them from food SMEs; a further 70 workshops are planned. Twenty six are classified as RE-TU-ER workshops and are described in greater detail below. Three seafood workshops were also held with the cooperation of DG XIV (Fisheries) and a booklet (F-FE 199/96) on EU seafood projects was distributed. These outputs and activities of FLAIR-FLOW are highlighted in three technical circulars (Gormley, 1993, 1996b, 1997).

The RTD (research and technical development) needs and opinions of 809 European food SMEs were canvassed in 1994 via interviews and questionnaires (Gormley, 1995). One of the questions related to the percentage of companies making 'large' investments in five areas with the following results: new equipment (82%); processes (64%); hygiene (51%); safety (39%); and quality (65%). Hygiene and safety were split as two separate questions and the percentage of companies making large investments in safety was surprisingly small. This begs the question would the percentage have been much larger in 1997 (i.e. three years after the survey) when, presumably, companies were more aware of the importance of food safety due to the BSE and *E. coli* outbreaks. In a second survey (Gormley, 1995), 105 companies were interviewed as to their research, development and technology needs. The percentage responses were as follows: food quality (20%); product development / improvement /modification (18%); technology (13%); food safety (8%) and other areas (41%).

RE-TU-ER workshops

The concept of RE-TU-ER (ready-to-use European research) workshops was activated to help transfer information to very small food companies who are lacking in technical staff. Emphasis is on near-market rather than pre-competitive results and focused data are presented as leaflets and simple manuals. Five sets of RE-TU-ER workshops (22 workshops in all) are proposed (Table 2) and some will take place in up to five countries . Five have been held to date and manuals on 'Managing the cold chain for quality and safety' and on 'Microbial control in meat' are available on request from the author. One RE-TU-ER workshop (Lyon, June 1998) was a pilot activity on 'training the trainers' where data on microbial control in meat were presented to 30 veterinarians (involved in teaching) who are currently 'redelivering' the data (in French) to meat SMEs in different regions of France. Feedback is being obtained on a 3-monthly basis from the veterinarians. Attendees at all FLAIR-FLOW workshops complete a questionnaire on the usefulness of the presentations and handouts, and also their opinions on SME needs in food R & D.

TABLE 2: List of topics (and countries) for RE-TU-ER workshops

Workshop topic	Country
Ready-to-use vegetables	NL, AT, FI, IT, NO
Fresh fish quality	DK, ES, SE, IS
Batch retorts	BE
Low fat meat products	AT, DE
Cold chain	IE, ES, GR, PT, FR_2
Cheese from sheep milk	GR
Microbial control in meat	FR_2, SE, UK, IT

The internet

FLAIR-FLOW EUROPE has a web site at http://www.exp.ie/flair.html. The design of this site has been overhauled recently with the twin aims of simplicity and rapid access to disseminated information. Visitors to the site are presented with a single screen introductory page. This outlines the goals and structure of FLAIR-FLOW. At the bottom of this screen, they are presented with two options -

(a) access to more detailed information about the project organisation (national networks, dissemination mechanisms, quantification of output and national contact points),

or

(b) access to the complete collection of disseminated 1-page documents.

The latter option has been improved by the addition of a free text search facility. This allows the site visitor to specify any single term or combination of terms which are used to search the complete text of every 1-page document mounted on the site. The result of this search is a list (by title) of those documents in which the term or terms were detected; each of the documents thus identified may be viewed in its entirety by a single mouse click on its title. Documents may then be printed or downloaded for off-line perusal or archiving. To maintain its currency, the site is updated at least once every month with new 1-page documents.

The site also offers links to each of the 18 national FLAIR-FLOW network leaders through their listed fax and/or e-mail addresses. Where these exist, links to their host organisation's web sites are also included. The site was visited 6330 times (hits) in the period February - May 1998 and 6034 files were accessed. An example of the breakdown of 'hits' is given in Table 3 (for February 1998).

Ireland was the most frequent visitor but the figure was inflated by the fact that the web site is operated from Ireland. The unresolved category (Table 3) is high and is due to the fact that some visiting sites use the '.com extension'. Traditionally this was a USA commercial extension (no longer the case) and so the 42% unresolved category represents a number of countries, including other sites in the countries mentioned in Table 3.

TABLE 3: Visits (% by country) to the FLAIR-FLOW web site for

February 1998	
Country	Visits (hits) (%)
Ireland	20
Belgium	8
Italy	6
Great Britain	3
Finland	3
Denmark	2
Australia	2
Switzerland	2
Other countries	8
FLAIR-FLOW network	4
Unresolved	42

The system also quantifies the documents being searched and the four most requested 1-pagers in February 1998 were: F-FE 57/92 (HACCP user guide; 28 hits); F-FE 206/96 (Food poisoning microorganisms - some of the answers; 23 hits); F-FE 191/95 (Virgin olive oil - the facts; 21 hits); and F-FE 94/93 (Lactic acid bacteria in the food industry; 19 hits).

Platform of food SMEs

A platform of 10-20 food SMEs has been formed in each of the 18 countries with a current collective strength of circa 213 companies. It is envisaged that the number will increase to 250. The function of the platform is:

- to advise on an ongoing basis on the content and usefulness of the 1-page technical documents.
- to assist with the identification of workshop topics.
- to pinpoint the factors/requirements which enable food SMEs, and especially very small enterprises, to take on board and utilise R & D results, and to devise procedures to facilitate greater exploitation of results.
- to advise the network leaders on current and future food SME needs in the areas of food research, technical development, and innovation.

The SME food platform is viewed as a major innovative aspect in that F-FE III will have a large food SME component ‘on-board’ as part of the project who will give a critical appraisal of the project output in addition to injecting innovative ideas and suggestions.

The breakdown of the platform by product area (Table 4) indicates that companies producing bakery products, meat and meat products, and dairy products predominate. The food platform has five assignments before the end of the current FLAIR-FLOW project in September 2000. The first of these is a prioritisation [from 1 (most required) to 9 (least required)] of nine R & D areas and also the identification of potential topics for workshops. The R & D areas are: packaging technology, food safety/risk perception, environment/energy, process technology, nutrition (health), quality management, cleaning/ disinfection, consumer preference, and raw material/ingredient optimisation.

TABLE 4: Product areas of companies in SME food platform

Product area	Number of SMEs
Bakery products	36
Meat & meat products	34
Dairy products	29
Prepared consumer foods	25
Fruit & vegetable products	20
Fish	17
Ingredients	11
Alcohol-containing products	7
Poultry	4
Soft drinks	4
Other	26
Total	213

The benefits for food SMEs

The benefits for food SMEs and other end-users from the FLAIR-FLOW information system include:

- comprehensive dissemination of results from EU food research programmes via 1-page technical documents (and their reproduction on the internet and in trade/scientific journals), workshops, and by focused lectures and posters.
- the RE-TU-ER workshops have particular application to the very small food companies and bring them 'on-stream' for the uptake and use of research results.
- a 'matchmaking' role whereby researchers and end-users are brought together on a person-to-person basis thus ensuring diffusion of results and increasing the potential for innovation.
- food SMEs have the opportunity to voice their opinions on their R & D requirements to the EU Commission thereby inputting into programme development.
- the contacts made through FLAIR-FLOW increase the awareness of food SMEs of the special research and technological development initiative offered by the EU Commission, i.e. the so-called CRAFT initiative involving exploratory awards and/or cooperative research.

Other activities

FLAIR-FLOW has formed links with Bureau Européen des Unions de Consommateurs (BEUC), the European Federation of Associations of Dieticians (EFAD), the FAIR Focal Points, and the INNOVATION Relay Centres. These links provide additional routes for the dissemination of research results to national consumer unions and consumer groups, to health professionals, and to a wide range of SMEs who are 'clients' of the Focal Points and Relay Centres. FLAIR-FLOW interacts closely with its 'sister' dissemination projects The NF2000 Network (non-food research; web site http.//www.nf-2000.org) and AQUAFLOW (aquaculture research, web site http.//www.aquaflow.org).

FLAIR-FLOW has a number of satellite activities, i.e. items under the umbrella of FLAIR-FLOW but funded by separate contracts by the EU or by national funds. These include the ongoing fax-on-demand system for the 1-page and other technical documents which is operational in Italy and France, and a small-enterprise-exchange activity involving bakers from Sweden, Denmark and Germany which took place in 1996. Potential satellite activities for the future include small-enterprise-exchanges in the fish and cheese areas, and the formation of a 'mini FLAIR-FLOW' for eastern and north-eastern European countries interested in receiving the FLAIR-FLOW outputs.

Conclusions

FLAIR-FLOW is a dynamic project based on national networks in 18 countries and charged with the dissemination of EU-supported food research results to food SMEs and other end-users. One page technical documents, their reproduction in trade/scientific journals and on the internet, and 80 focused workshops are the main dissemination routes.

The success of FLAIR-FLOW to date is excellent as indicated by follow-up requests for more in-depth information by end-users. However, there is no room for complacency as dissemination is a huge task both in terms of the volume of information requiring diffusion, and the huge number of food SME and other potential end-users in Europe.

For more information contact your national network leader (Table 1) or visit the FLAIR-FLOW web site at http://www.exp.ie/flair.html

Acknoledgements

The following are acknowledged:

- the FLAIR-FLOW core team (26 persons)
- the 300 national network members
- the coordinators/partners in EU-sponsored food research projects who supply their results for dissemination
- the EU Commission

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Food science and nutrition in poland and its relevance to european union sponsored research

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Abstract

Education and scientific research systems and funding policy in the years preceding the democratic change in Poland are presented. The reforms of education and research systems initiated in 1990 are discussed and the present situation of Food Science and Nutrition (FSN) in Poland is shown. Teaching of food science, food technology, food engineering, biotechnology and nutrition is conducted in faculties and departments affiliated with agricultural universities and polytechnics as well. Research on FSN is done in universities and polytechnics, and in institutes of Polish Academy of Sciences and R&D units. It is estimated that more than 300 full and associated professors are involved in teaching and research programs on FSN. Main directions of research are presented, and it is shown that the FSN research conducted in Poland is very relevant to that done in the countries of the European Union. Limitations and constrains for the development of the FSN research and education are discussed. Finally, some suggestions for the cooperation between Polish FSN and the European Union are proposed.

Introduction

The state of Food Science and Nutrition in Poland is very much dependent on education and scientific research systems and funding policy. Higher education system is changing continuously since 1990. For more than 20 years number of students in Poland was strictly limited and embraced 10-20% of young people at the university age. The percentage of people with higher education became stabilized at the level of 7%. However, small number of students strongly selected created conditions for high quality education. Relatively small number of very good students made it possible for Polish universities to participate intensely in scientific research. Moreover, Polish higher education was relatively open to broad international contacts. It resulted in well educated graduates of uniform, 5-year studies ending with master's degree.

After the democratic change in 1989 the demand for higher education increased in Poland. In modern, market economy higher education turned out to be the best insurance against unemployment. In the years 1990 - 1994 the number of students entering universities increased twice, and the total number of students by the factor of 1.5. The number of academic teachers practically did not change. Very low salaries in science and education are not encouraging young talented people to stay in universities as teachers. In consequence the load for academic teachers increased to the level of 14.1 students per teacher in 1994.

The reforms of education in Poland are directed toward:

- limiting the number of directions of studies. Detailed knowledge, in particular in professional terms, in very rapidly changing economy and technology may become an obstacle in getting job or in adjusting skills to the needs of a new job,

- a diversification of levels of education. At present two-level studies are introduced. The first level ends with the so called license or professional title of an engineer. The second level graduates are granted with the master's degree,

- an internationalization of studies by curricula and language of lectures. Graduates of Polish universities should be able to take jobs in international firms world-wide. Hence, their communication abilities will be as important as skills and knowledge. Programs to support cooperation with the European Union - such as TEMPUS - have already paid a positive role in this respect.

The reforms of education are aimed to maintain the quality, to meet market demands and to create conditions for individual aspirations fulfillment.

A high demand for continuous education exists in Poland. Nowadays it concentrates on the most popular areas such as finance, management, law and computer science. It is expected that within next few years the demand for these type specialists will be fulfilled and interest in continuous education in other areas will increase.

The research policy in the years preceding the democratic change resulted in a specific system of scientific research in Poland. The state consequently supported science as one of fundamental elements of economic and social progress, but the links between science and economy were weak. The results of research were mostly not implemented into practice because the lack of competition, and the economy of shortages did not stimulate technical progress.

There was no strict division between branches of science in Poland as it was experienced in other former socialist countries. Polish Academy of Sciences was suppose to have exclusive position in the field of basic research and the state financed R&D institutes had to have the monopoly in applied research and its implementation in the industry. As it has been already stated Polish universities were intensely involved into basic and applied research.

The lack of the fixed, strict division of research resulted in relatively high level of education and research in Poland. In the years 1981 - 1992 Poland ranked on average 17 in the world as far as the number of scientific publications was concerned. It was ranked 21 as far as the quotations in the most important scientific journals and reviews were concerned.

All the above mentioned elements resulted in a specific, but high intellectual potential and human capital in Poland. The skills and intellectual potential of educated people, probably, fruited in a relatively short recession and relatively rapid economic growth in Poland after the democratic change.

Transformation process of the system of research in Poland has begun in 1990. Its main objectives were as follows:

- to change the system of research administration,
- to introduce competition for budgetary resources,
- to treat all research participants equally.

In January 1991 the Committee for Scientific Research (CSR) was established. This is a body composed of government representatives and elected representatives of scientists in Poland, and

making decisions in the field of research and scientific policy and financing of science. The Act on the Establishment and Tasks of the CSR introduced a new system of financing of research in Poland. According to this system all research participants, that is institutes of Polish Academy of Sciences, state institutes and R&D units and departments of universities conduct statutory research. Financing of the statutory research is based on the yearly assessment of these units. The level of research in these units is categorized and, in consequence, the higher the category the better financing of research is assured.

The competition for budgetary resources was introduced by the system of tenders for research projects (grants). Tenders are organized every six months. Each project is reviewed by minimum two referees and discussed on opinion-formulating panel. On the average one project out of five is qualified for funding. Besides of the research projects the CSR is also financing, on a competitive basis, targeted projects. These are R&D projects financed in 50% by enterprises interested in implementing the research results. Targeted projects are assumed to be a transmission system of the results of scientific research to practice.

Tenders for research projects are open to all scientists and research institutions on the equality basis.

Another important element of the new system is the funding of investment projects. These are the projects targeted to improve research infrastructure by investment in equipment and new facilities such as new laboratories, computer centers, etc.

Funding of science in Poland is at very low level. In 1992 it was 0.64%, in 1994 - 0.55% and in 1998 - 0.48% GNP. At this level of financing a clear definition of disciplinary priorities of scientific policy was needed. The CSR proposed in 1993 very general disciplinary priorities connected with:

- protection of health and natural environment,
- agriculture and food processing,
- modern technologies, especially those important for defense,
- supporting infrastructure for education, science and transfer of technology to economy.

Two documents have been approved in 1994 by the Council of Ministers which formulated research priorities in Poland. „Guidelines for Innovation Policy in Poland" and „Strategy for Poland" presented preferred directions for R&D projects many of them directly or indirectly connected with food science and nutrition. Directions targeted to food science and nutrition are as follows:

- energy and material saving technologies and machinery for the agricultural production and agricultural food processing,
- agricultural food processing, and particularly the development and utilization of new technologies and biotechnology including healthy and dietetic models of nourishing the society, and pro-ecological production methods,
- research on economic and organizational conditions for the development of agricultural production and food processing.

Food science and nutrition

Education in Food Science and Nutrition (FSN) in Poland before the World War II was a part of the agricultural programs at universities. In this period university research was mainly concerned with technical microbiology for the dairy and fermentation industries. Small industry and handicraft plants did not carry on their own research studies. After the war reconstruction of laboratories and staff as well as new investments were done mainly during the years 1950-1956. Thereafter a continuous development of FSN took place as an effect of the general research and science policy in centrally planned economy.

At present several institutes and universities conduct teaching and research in the field of FSN. Research on FSN is done in Faculties of Food Technology and Faculty of Human Nutrition and Home Economics being a part of agricultural universities. Some hygienic problems of meat processing are dealt with in Faculties of Veterinary Medicine which are also affiliated with agricultural universities. In technical schools, which in Poland are called polytechnics, there are faculties and departments conducting research on food technology, food chemistry, food biotechnology and food engineering. Research concerning post-harvest technologies and storage of agricultural products is also done in some Faculties of Agricultural Engineering. In some Academies of Economy research on food processing and quality of raw materials and food products is conducted. Institutes of Polish Academy of Sciences conduct research on food processing , properties and quality of food products. Most of the state institutes and R&D units are commodity oriented (Table 1).

Table 1: Research and teaching institutions in Poland conducting programs on food science, food technology, food microbiology and hygiene, food engineering, biotechnology and nutrition

	Institution	Number
1.	Agricultural universities, faculties	7
2.	Agricultural universities, departments	12
3.	Agricultural universities, laboratories	3
4.	Polytechnics, faculties	1
5.	Polytechnics, departments	8
6.	Schools of economics, departments	8
7.	Schools of medicine, departments	7
8.	Schools of medicine, laboratories	2
9.	Academies of physical education, departments	1
10.	Academies of physical education, laboratories	1
11.	Institutes of Polish Academy of Sciences	3
12.	Research and development units	21

Food Science and Nutrition is well developed in Poland as regards the staff and the institutions. In 1994 it was estimated that some 690 people were directly involved in research in the field of food technology. In 1998 more than 300 full and associate professors were employed in research in the field of FSN. Food Science and Nutrition in Poland has vivid international contacts, including most of the countries of the European Union.

On the basis of the research priorities formulated by the Council of Ministers Polish Academy of Sciences in cooperation with the CSR estimated the research done in Poland in the years 1991-1993. FSN as the part of agricultural sciences was also evaluated and the main research problems dealt with during the years 1991-1993 were as follows:

- chemical and physical basis for quality evaluation of raw materials and final products. Proteins and biopolymers and their interactions with biologically active food constituents were investigated. Physical properties of raw materials, semi-products and foods, especially rheological and thermal were measured. Water relations in foods and the effect of food processing on the state of water in foodstuffs were investigated. Raw materials of plant and animal origin were evaluated for their suitability for processing. Quality of food from the hygienic, toxicological and nutritional point of view was the subject of numerous research. New analytical methods, including microbiology, were developed,
- engineering and technico-technological aspects of food processing. Research done in this field included: optimal utilization of raw materials, water and energy conservation and management, reduction of wastes and optimization of final product quality. Moreover, unit operations such as heat and mass transfer, mixing of non-newtonian liquids, computer simulation and process optimization were studied. Dynamics of food processing processes was also investigated to formulate basis for automation and on-line control,
- biotechnology and food microbiology. Collections of microorganisms and tissue cultures were surveyed in order to select the best strains for biotechnology use. Genetic engineering was applied to construct new strains for potential industrial applications. New technologies in fermentation and dairy industries were adopted. Research on production of enzymes and probiotics was carried on,
- human nutrition. Research was done on food consumption and economic and social conditions affecting food intake. Food habits and nutritional status of different groups of population in Poland as well as ethiology, pathogenesis and cure of diseases originating from inappropriate nutrition were studied. The effect of antinutritional components of raw materials, toxic elements and pollutants present in food on the health of selected categories of population was investigated. Some work was done to verify and update nutritional recommendations and standards in Poland.

The above mentioned main research directions were evaluated as being in line with general world research trends in the field of FSN. Moreover it was stated that the directions fit very well to the research programs developed by the European Union for the years 1994-1998.

Limitations and constrains

Development of FSN in Poland and its progress is under the threat due to many limitations and constrains. These impediments can be divided into economic and psychological in nature.

Economic limitations are the most important and common for research in Poland. Funding of science in Poland is at present at such a low level that various fields and scientific institutions fight for survival and just maintain their most elementary functions. Low level of funding of science brings about the following effects:

- the equipment becomes outdated,
- a brain drain which has been growing in strength since the beginning of the 1980's. In the last 10 years emigration and change of profession included 25% of the sum of Polish scientists,
- lack of interest of young talented people in scientific career leads to generation gap expansion and the scientific staff senescence.

Research in the field of FSN could be funded, in part, by the industry. However, economic situation of most of food processing companies is rather weak and they are not interested in long-term, perspective research. The demand is for simple solutions needing no- or little investment such as new recipes, modifications of technology with slight equipment modernization, reduction of wastes, conservation of energy and reduction of water use. This type of demand is not promoting ambitious, basic research which fruits with application results in sufficiently long time. Short term, simple research financed by food industry does not change much the present situation of FSN in Poland. The lack of feedback with practice, on one hand, leads to the loss of much of interesting results which cannot be implemented into practice, and, on the other hand, many of solutions offered by science cannot be verified in industrial applications.

Food sector is very interesting to foreign and multinational investors. At present many food processing companies in Poland are either owned or in joint-venture with international consortia. It is sad to say but foreign and multinational consortia dominating on the Polish food market do not use Polish R&D possibilities. As a result FSN depend almost entirely on financing from the state budget, that is in the form of statutory funding and grants. Although there is no official division of the Polish science into branches still statutory research in Polish Academy of Sciences and R&D units is much better than in universities. In consequence, in the situation of general poverty of science in Poland, some laboratories are much better equipped than others.

The psychological limitations arise from some habits, customs and tradition. Teaching of Food Technology in Poland is traditional, commodity oriented. Since Food Science is a multidisciplinary field of studies such an approach to teaching and research results in a weak cooperation and co-ordination both between institutions and between particular disciplines. Commodity oriented research is interesting to small industry and handicraft but, as it was already stated, it does not change the present situation of FSN in Poland. Moreover, the cooperation between better equipped units and those having less sophisticated equipment is obstructed by the lack of interest in the same commodity or technology.

Scientific information in Poland is in a fatal situation which brings about large fund losses and is a threat to the development of FSN. Data base systems on research conducted in Poland are in embryonic state. Hence, Polish researchers have a very good information on research done in the World thanks to data bases such as FSTA, CAB or Internet, but they do not know what kind of research is done by their neighbors. The lack of information makes cooperation even more difficult.

Cooperation with the European Union science and research

Polish FSN is well prepared professionally and scholarly to cooperate with research units of the European Union. Besides the very low funding of research the results published by Polish scientists are of high quality and interest to the world science. It is sufficient to say that some 25-30% of the total number of papers published yearly in the field of food science is published in the world renowned journals and periodicals. More than 25% of the total number of oral and poster presentations are presented on international symposia and conferences. Polish FSN scientists organize international conferences and are members of many advisory committees of international symposia. Many of them are members of editorial boards of international world renowned journals. Many Polish scientists are invited as visiting professors to European and American universities and research units as well as to the developing countries.

Participation of Polish FSN in the European Union programs such as TEMPUS, COST and INCO-COPERNICUS during the last few years showed that this type of cooperation fruits in very good results and mutual benefits. Hence, the future share of Polish FSN in the European Union funded research should increase as well as in the aid programs offered to the countries of Central and Eastern Europe.

Expanded cooperation and broader participation should consider education and research as well. Education programs should be directed toward promotion and development of university teaching in the field of FSN compatible to those programs taught in the European Union. The quality and the education level in Poland, although high, must be recognized by the European Union countries in order to enable graduates to take job anywhere on the position they are qualified to. In another words, the scientific and professional degrees granted by Polish universities must be recognized by the members of the European Union.

Research programs based on the priorities approved by the Council of Ministers are very much in line with the research priorities of the European Union. Food quality approached from different points of view, nutrition, health and standard of living are as much important in Poland as much in the European Union countries. More detailed description of research directions in the field of FSN formulated by Polish Academy of Sciences is as follows:

- effect of raw material quality and processing on chemical composition and nutritional quality of foods,
- factors affecting technological properties of raw materials,
- functional and technological properties of food additives,
- biotechnology and its use in food production and processing,
- antinutritional components and their interactions with other components of food,
- contamination and contaminants in food,
- physiologic, psychologic and sociologic factors determining the choice and acceptance of food,
- development of analytical methods useful in quality assay and assurance,
- lactic acid fermentation bacteria and their use in prophylactics and therapeutics,

- water relations in foods,
- biodegradable packaging,
- management of food processing wastes with the use of biotechnology,
- wasteless technologies,
- management of energy and water in food processing,
- ethiology and pathogenesis of diseases caused by faulty nutrition,
- epidemiology and the state of nutrition of different categories of the population and their effect on health.

Besides the above mentioned strategic directions it seems appropriate to enumerate few more which might be of interest to both parties, that is the European Union and Polish FSN. These are:

- transgenic plants, their properties and suitability for processing,
- specially designed foods such as for diabetics, sportsman, elderly, etc.,
- natural food additives obtained either from non-food raw materials or with the use of biotechnology,
- food engineering and food plant design in the aspect of total quality management, hygiene and quality assurance.

Another aspect which in the research programs should be considered is the technology transfer. The experience of the Member States of the European Union should be more broadly used.

In conclusion it should be stated that Polish FSN is well prepared to scientific cooperation with universities and R&D units of the European Union. Experience of the last few years showed that the cooperation on a partnership basis is very fruitful and beneficial to all. There is a large number of research topics, in the field of FSN, of mutual interest and the conditions for cooperation are very good. Research done in Poland in recent years is very relevant to those directions which are preferred by the European Union countries and this creates even better atmosphere for scientific co-operation.

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What are the strengths of EU RTD programmes in the food sector compared with national RTD programmes?

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Introduction

Food related European RTD began to take shape in 1986 following implementation of the EUs Second Framework Programme (1987-1991) with the development of the FLAIR programme. Prior to this date the only food related research activity was under the auspices of COST which had supported two concerted action programmes on "The Physical Properties of Foods" (COST 90 and 90bis) and "Food Biotechnology" (COST 91 and 91bis). In addition a small programme on "Agri-Food Quality" had been supported under the agriculture research budget of DG VI.

The innovation of the FLAIR programme was that it embraced for the first time the breadth of scientific topics which fall within the scope of a food research programme, and provided the resources to make research collaboration at the European level an effective reality.

Other food RTD programmes followed on in subsequent framework programmes with increasing resources being made available.

EU Food related RTD Programmes

1987-1991	FLAIR	25 million ecu
1990-1994	AIR	59 million ecu
1994-1998	FAIR	97 million ecu

This decade of investment is now beginning to have the effect of creating European added value and making an impact on advances in the field which individual national programmes would have been unable to achieve. Any international programme will take time to build up a momentum and to involve the very best scientific teams across Europe, particularly if those teams are well supported under national programmes. Any European research project requires extra effort in seeking out appropriate partners. Careful planning and attention to detail is also required to ensure that the partners bring added value to the project and this is essential in order to be able to justify the extra costs associated with collaborative research. In spite of the extra effort required, the situation now exists whereby the vast majority of laboratories with an interest in food research are actively and enthusiastically involved with the EU research activities.

The nature of food research

Food research presents a series of challenges which are not met with in every scientific discipline. Both the physical and chemical properties of food are complex and present challenges to the development of predictive modelling and the testing of hypotheses that do not exist with simpler systems. In industries such as the petrochemical, pharmaceutical and electronic sectors, the raw materials are of constant composition and the required product outputs are much more stable and

precisely definable. Food is a much more complex system. As biological materials they are subject to degradation and contamination. Physical, chemical and biological factors interact to affect the quality and organoleptic acceptability of food. Apart from this there are particular psychological attitudes to the acceptance of food, which requires the involvement of physiologists and psychologists to understand in order to ensure that any innovation is acceptable to consumers.

Since a person will consume in excess of a tonne of food in their lifetime, food is the most important contributor by far in determining the environmental contribution to the health of an individual_ But any health effects that might result from a particular diet will be influenced by other factors, such as genetics or infection, to determine health outcomes. Unravelling the importance of these interactions in terms of identifying health benefits or risks is a challenge which will only be effectively met through the encouragement of multi-disciplinary research involving food chemists, biochemists, molecular geneticists, microbiologists, toxicologists, epidemiologists and clinicians.

The nature of these challenges is such that few universities or institutes in EU Member States have either the correct infrastructure or resources necessary to develop scientific understanding to the point where it can be used effectively by industry or regulators. It is noteworthy that in the food industrial sector it is mostly the large, multinational companies that undertake research. In the past few years these companies have taken the decision to become more interactive with the public sector research base rather than attempting to undertake all of their R&D in house. They are all very active in participating in the EU food programmes. There is no doubt that they recognise the importance of collaborative, multi-disciplinary research in order to make progress in many areas. Such companies are not influenced by national boundaries in seeking partners. Rather they will wish to select the most appropriate scientific partners to achieve their goals.

Research goals - European added value

The broad goals of many food research programmes can be defined in terms of providing information with which to (1) ensure public protection and (2) stimulate industrial competitiveness.

Within the first goal the main priorities are to:

- Ensure that food is safe
- To guarantee that food is of the quality demanded by the consumer
- To provide information which will assist in promoting healthy eating.

The second goal additionally requires:

- Technologies to produce safe, and desirable foods competitively
- Information to ensure that non-tariff barriers to trade do not apply in, a common market.

None, of these goals are mutually exclusive and research pursued with public protection in mind can also provide the opportunity for innovation, However more and more of the issues which research is required to address are trans-national in scope. Apart from the increasing globalisation of trade in foods, and the raw materials used in its manufacture, the regulatory environment is influenced through international negotiations.

In the case of food safety issues the regulatory activity within EU Member States is based on Community rules. This is essential if serious barriers to trade, either imaginary or real are to be avoided or resolved. Perceived health risk poses the greatest challenge since it is frequently the subject of differences in scientific interpretation. Uncertainty in the data leads to differences in reactions amongst different Member States, who are subject to widely varying political pressures, priorities from activists, and genuine public concern. At the global level World Trade Organisation rules allow the restriction in the free movement of goods where issues of public health arise. It is precisely in the areas of scientific uncertainty where the greatest challenges to the effective application of these rules will apply.

Competitiveness - the research challenges

The development of the single market has had the effect that more companies have expanded their investment throughout the EU. In terms of the market there is a growing demand for convenience foods where the price and quality are important consumer determinants of choice. The pleasure, enjoyment and social interaction in eating, which is a European characteristic, is increasingly being influenced by concerns over health and well-being, and where the foods are in conformity with an individual's moral and ethical views. All of these pressures on the market are causing a rapid change in demand within a market where there is no extra demand due to a more sedentary life and zero population growth.

The most innovative food companies will only be able to respond to these trends by technological means This will require a much greater focus on the benefits that R&D can bring to the sector than has been the case in the past. The issue is whether such companies can benefit more from the existing national investment in R&D, or whether a dynamic and imaginative European research programme is essential to offer opportunities for investment that would not be possible otherwise..

The ability of the market to exploit advances in R&D can best occur through recognition of where there are technological strengths, and to capitalise on them. Technological weaknesses also need to be defined in order to strengthen them.

Technical strengths in Europe include:

- Excellence in food science and technology
- A unique approach to food & nutritional studies Good engineering skills

Technological weaknesses in Europe are generally:

- a poor exploitation record
- a lack of multi-disciplinary interaction
- an under investment in some key skills

In theory it could be possible for national authorities to recognise these issues and adjust their R&D programmes accordingly. However few EU Member States have sufficiently large R&D resources to achieve this aim. Even if new facilities are created the skill base has to be created and this can be more easily achieved through trans-national co operation and utilisation of training grants which are an important feature of Community R&D programmes.

At present there are very important areas of research skills that, if applied to the food sector, could make important advances. These include:

- The application of sensor technologies developed for non-food uses to the sector
- Process control programming
- Consumer and behavioural science
- Material sciences in respect of food packaging needs
- Material sciences in respect of the plant cell wall

These areas cover skills not traditionally reflected in the food research institutes or university departments of food sciences in Europe. An effective transfer of technology to the food sector can only be achieved through multi-disciplinary, collaborative research. The EU programme can play an important role in catalysing this interaction and overcoming the lack of specific skills in any one country that are limiting progress,

Research for consumer protection

Nowhere is the need for European collaborative research more justified than in those areas where European legislation is operating. This also embraces areas where the nature of the scientific challenge is such that no individual Member State has the technical or financial resources to bring the research to a successful conclusion in a reasonable time frame for use or exploitation.

Nearly all of the issues in relation to the safety of food, such as the development of novel foods, the utilisation of chemicals in the production of food, and the microbiological quality of food, are the subject of EU controls. All of this proposed legislation depends on the use of scientific risk assessment as a basis for controls. Any other approach, other than a scientific one, would result in a lack of consistency in regulation.

Similarly an increasing amount of legislation on food authenticity is required for labelling purposes, and is Community based. The development of methods to determine the origin of a product, or a definition of the genuineness of a claim, such as the authenticity of oils or wine, requires the application of methods that are at the cutting-edge of analytical methodology and uses facilities which are unavailable in all countries. Collaborative research enables these facilities to be shared.

Risk assessment

Risk assessment provides an excellent example of the essentiality of the need for European collaborative research. There is a great deal of mistrust by consumers on the safety of technological processes when applied to food production. The issues of greatest concern vary throughout the Community and require a common understanding if consumers are to have faith in the process of regulation. To achieve this common understanding it is essential for scientific experts to collaborate. There are frequently differences of view expressed between experts within the Community. This is occurring because the scientific basis on which risk assessment is undertaken involves a process of extrapolation and assumption.

The processes of risk assessment have principally been developed for assessing the need for control over exposure to toxic chemicals in the environment. The precautionary principle is applied which frequently leads to an overemphasis of risks and is not applied to determine the presence of any benefit. The process does more to raise concerns, especially if there are divergent the opinions amongst experts, than to enable true risks from exposure to chemicals in foods to be assessed.

The uncertainties of determining true risks are, in part, the result of an inadequate data base on the metabolism and mechanism of action of particular food components. The biological activity will vary depending on whether or not there are genetic differences in response in the extrapolation of data from animals to humans, or even within humans.

Research to improve the methodologies that are currently being applied is essential if more resources are ultimately to be devoted to improving the public health. An effective public health policy in Europe requires as much resource being applied to determining benefit as it does to risk, especially to develop methodology to allow true risk to be assessed. The priorities for legislation or consumer education need to be decided on the basis of identifying the most important risks or benefits.

Examples of the research that needs to be pursued in order to achieve these goals will illustrate the nature of the challenge faced, the multi-disciplinarity of the work required, and the need for collaborative effort throughout Europe.

Examples of research needs for risk-benefit assessment in relation to food

- Detailed analysis of the validity of extrapolating the results of animal tests to humans for specific toxicological effects
- Developing de-minimus concepts based on biological no effect levels Studies on effects at low dose exposure
- Development of a single approach for risk assessment for genotoxic and nongenotoxic effects
- Improved approaches for comparative risk assessment
- Novel approaches to risk benefit analysis (including m~ for assessing true benefits)
- Safety assessment methodology for whole foods

European focus on nutritional research

Nutritional information has long been applied as a basis for the marketing of foods and in the development of new food products. However the focus of nutritional interest has shifted from a definition of nutrient needs for healthy growth and development, to that of the nutrient needs for optimal health status and healthy ageing. In this process the definition of what is a nutrient has also altered since there are compounds in foods, which protect against the initiation and development of disease, which are not essential nutrients.

Whereas some information is available on which to assess the required intakes of essential. nutrients for healthy growth, there is almost no information available with which to recommend optimal intakes of nutrients. The lack of this information is a serious limitation on the development of healthier food products. At present it is incumbent on manufacturers to demonstrate benefit if they

wish to make any claim. The nature of the research challenge is such that it is unreasonable to require them to do so since it is almost impossible to protect much of this intellectual property as the basis for recouping costs. The potential benefits to the public health require a significant input of resource from the public sector.

Ultimately any claim on the benefits associated with consuming a particular food will require the demonstration of its efficacy in human intervention studies. Such studies are very difficult to undertake and extremely expensive. Most of the adverse health effects against which new healthier foods might be directed are diseases associated with ageing and intervention studies would have to be very long term if they had to demonstrate protection against the development of such diseases. There is a crucial need to develop biomarkers which can be effectively used as early indicators of disease in order to make human studies more practicable.

The EU food programmes have given strong support for nutritional studies with a gradually increasing expenditure over successive programmes. But the nature of the problem is such that even greater resources will have to be made available if the issues are to be effectively tackled.

The Commission have clearly recognised that the importance of research on diet and health. The area has been given strong emphasis, backed by substantial resources, in their proposals for the Fifth Framework Programme under the key action Improving the quality of life and the management of living resources".

The benefits of studying diet and health issues at the EU level are:

- there are very great strengths in research capabilities in the biological sciences in Europe which can be capitalised on
- facilities for certain types of investigation are only available in specialist centres
- that there is a great diversity of dietary habits amongst the Community
- human nutritional studies require resources which are not available in any one country
- collaborative, multi-disciplinary research is required as centres of expertise are widely scattered throughout the EU

Conclusions

Expenditure of food research in Europe so far has been low compared with some advanced technological sectors e.g. the pharmaceutical sector. However the scientific and technological hurdles are even more of a challenge if it is accepted that foods are likely to play a crucial role in determining the overall health status of the population. This is because unlike the pharmaceutical sector the products must be manufactured at low cost and high volume. Like the pharmaceutical sector, the food sector is of enormous economic importance in Europe.

The crucial importance of a European food research programme has been illustrated with a few examples of where the need for multinational, interdisciplinary and collaborative research is essential for effective exploitation. There is a need for a close partnership to be fostered between the industry and academia. Industry needs to increase its technological awareness and academics need to be aware of the constraints on innovation which operate in industry.

An innovative European R&D programme is an essential requirement in building up public confidence, improving public health and maintaining a strong and competitive industry. The challenge will be to concentrate the resources that are available in key areas and on innovative ideas, rather than to disperse the resources widely. This is a challenge that the emerging work programme for the EU's Fifth Framework Programme will need to reflect.

Measurement of consumer attitudes and their influence on food choice and acceptability (AIR-CAT)

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Abstract

A changing European food market demands insight into consumer attitudes and their influence on food choice and acceptability. This multidisciplinary area needs to bring together scientists from all regions of Europe and with very different scientific backgrounds. The primary objectives of this concerted action has been:

- To establish a base with state of the art methods for measurements of consumer attitudes
- To review and test existing methods in practical applications in collaboration with European food industries
- To perform comparative studies between laboratories on food products where attitudes play different roles for consumer behaviour in the community countries, such as transgenic foods, irradiated foods, foods with different additives, declarations and process technologies, foods with different origin declarations, ecological foods and foods with strong health connotations (such as high-fat foods).

The members of the action have published more than 130 publications related to aspects of how consumer attitudes can be measured and how food choice behaviour is related to acceptability, during the last 4 years. Studies have been conducted related to methodological aspects as well as particular studies related to specific food items and regions for food production. The paper will give a brief selection of relevant results from experiments reported through the action.

A base with state of the art methods for measurements of consumer attitudes

Transgenic and genetically modified (GM) foods represent a great concern for the European food industries. The public debate concerning these foods has made it clear that acceptance of these foods is going to be a problem of some proportion. Measuring the attitudes, reflected in the public concern, and how attitudes change over time is equally a challenge that has to be met in order to understand the consequences of the introduction of new foods and production practices. In a meeting in June 1998, in Vienna, methods for measurements of attitudes towards genetically modified foods and transgenic foods were focused, while novel and functional foods will be the focus at the final plenary in October 1998 in Dijon.

Dr. Lynn Frewer from the Institute of Food Research in Reading, UK, has studied attitudes towards GM, and related this to models for risk assessment and perceived control (Frewer et al 1995; Frewer et al 1995; Frewer et al 1997). It seems that consumers are more concerned about food related hazards when the cause for concern is not possible to see or control by the consumer, as is typical

for GM foods. Information alone cannot remove this concern, as the trustworthiness of the information source is closely linked (Frewer et al 1996). People with a vested interest in the area are less likely to be trusted as is the case for government officials in the UK. Similar, representatives for the food industry have less convincing power than independent media journalists. These are examples of how risk assessment models can be utilised in evaluation of consumer communication. Unlike the models for risk assessment used by Lynn Frewer, qualitative research often is needed when models are not available for quantification of findings.

Qualitative techniques

At a workshop on qualitative methods the programme gave full attention to the concept of qualitative research, illustration of applications of the methods and to the discussion of qualitative vs. quantitative methods, and relevance to consumer behaviour and food choice measurements. Advantages and disadvantages of the methods were outlined and discussed.

Although there has been considerable interest in the application of qualitative methods to food choice research in recent years, many researchers remain sceptical about the validity of such methods. To a large extent this reflects confusion regarding the objectives of qualitative research techniques and approaches to the analysis and interpretation of qualitative data. The workshop illustrated the use of a wide range of qualitative methods to food quality research. In doing so it helped to improve the understanding of those participants who had not previously utilised any or some of the methods which were discussed.

The following distinctions between the two methods were presented:

> "Qualitative research techniques are open-ended, dynamic, flexible, provide a depth of understanding, tap consumer creativity, go beyond the rational or superficial approach, and provide a rich source of ideas.
> In contrast, quantitative methods offer numerical data, an opportunity for statistical analysis, allows for comparisons between subgroups, replicability, taps individual responses and are less dependant on interviewer skills and orientation."

The most common qualitative methods used in marketing research consist of observation, mystery shopping, group discussions and individual in-depth interviews. Qualitative research covers a broad range of possible research methods. Whereas quantitative methods aim to derive numerical data which can be tested statistically, can be used to test hypotheses and, in some cases, to derive estimates of numerical values for populations, qualitative research methods have a different underlying rationale, and also seek to answer different types of questions. Qualitative techniques tend to be more open ended and flexible. The aim is to provide an in-depth understanding of how people think about things. One issue is that qualitative methods are often much less structured than quantitative methods. This may mean that the results appear less reliable in that they cannot be consistently reproduced. However, proponents of qualitative methods would argue that this does not present a problem, since the results are not meant to be representative of population responses. Another issue is that the quality of the data obtained is dependent upon the skills of the researcher. Qualitative methods can include group discussions or focus groups, individual in-depth interviews, or observation of behaviour. In market research qualitative techniques are often used as a preliminary to more quantitative techniques in order to define the issues which are important and to generate

ideas for further research. They can, however, be used in their own right to explore various issues and are extensively used in social sciences, for example, sociology and anthropology and, indeed, can be used in combination with quantitative techniques in order to explain and interpret the results found from quantitative studies.

Qualitative techniques used in combination with quantitative techniques

Many of the researchers within the AIR-CAT project use various forms of qualitative techniques in combination with quantitative measures of attitudes. An example is research carried out in Germany on marketing of milk (Albensleben and Zielberg 1997) which involved a combination of in-depth interviews and quantitative conjoint analysis studies. In this case the in-depth interviews were used to explore the issues which were important to consumers, since it is necessary in the conjoint analysis to include factors which are both relevant and important to consumers. The initial in-depth interviews offer an excellent way of determining which factors should be included. This study also allowed the comparison of the types of conclusions which would be drawn from in-depth interviews as against the quantitative study. In many cases the conclusions from the qualitative in-depth interviews were the same as those from the quantitative studies. In some cases the quantitative analyses yielded information over and above that from the qualitative interviews and, where the quantitative analyses supported that from the qualitative interviews, it would add confidence to the interpretation of the qualitative responses.

In work on the Theory of Planned Behaviour (Sparks 1994; Raats et al 1995; Daillant- Spinnler and Issanchou 1995), beliefs are usually elicited from a representative group of people. However, it has been suggested that, if beliefs were elicited from the respondents themselves, rather than using a representative sample of beliefs, this might give better prediction of attitudes, intention and behaviour. This type of individual belief elicitation was used in one study investigating purchase intentions of familiar and novel cheeses. Individual beliefs were found to be better predictors of purchase intention for familiar cheeses. However, surprisingly for novel cheeses, in some but not all cases the general modal beliefs were found to give better results. This might be because in the case of novel cheeses people's individual beliefs are less reliable than in the case where they are familiar with the food.

One of the methods investigated in the AIR-CAT project is that of Means End Chains and, in particular, the technique of laddering which is used to derive attributes, consequences and values from a group of consumers concerning foods or food issues. Although qualitative in nature, the protocol for laddering can be specified fairly exactly and it can be used in a more or less structured way. One example of the use of the laddering technique is in a study where the aim is to develop health and hedonic attitude scales. A study on consumer attitudes and behaviour with respect to organic pork, (Arvola 1998) was run in two phases, qualitatively (laddering study, British and Danish researchers, and quantitatively (theory of planned behaviour and means-end chain, British, Danish and Finnish researchers). Laddering revealed four realms of considerations common for both countries: animal welfare, budgetary restraints, health, and enjoyment. The quantitative part suggested, again, that the predictive models are mainly similar in all participating countries. Some differences were also found, e.g., Danish subjects showed somewhat more negative beliefs and intentions to eat organic pork than the others. This is possibly due to larger availability of organic pork in this country that may have led to more pragmatic and less idealistic attitude to this type of product.

A further method used by a number of participants in the AIR-CAT project is Repertory Grids. The repertory grid is essentially a qualitative method, although it lends itself to quantitative analysis through, for example, the use of Generalized Procrusted Analysis (GPA). This has been used in a number of applications. One application was the study of the credibility of sources of risk information, where repertory grids were used alongside more conventional elicitation techniques in order to determine the types of attributes which differentiated between trusted and non-trusted sources (Frewer et al 1996). Analyses of the repertory grid responses were compared with the results from a large scale survey, using more conventional attitude measures. The interpretations of the results were very similar between the more qualitative and quantitative approaches.

Qualitative approaches can also be incorporated into more experimental approaches and in the same area of research on trust in sources of risk information, thought listing procedures were incorporated into a study of the effects of information when various attributes, for example sources of information, persuasive content, were systematically varied. Whilst thought listing is a qualitative technique and requires interpretation of the responses and, in this case content analysis and coding, it allows for a much more subtle exploration of the effects of experimental manipulations than would be the case for many more conventional quantitative measures.

Qualitative methods can be used very effectively to achieve practical objectives, for example the promotion of vegetable consumption (Kilkast et al 1996). This was done by one of the partners within the project, using in-depth interviews and group discussions with high and low vegetable consumers, in order to determine the types of issues which would need to be addressed in order to increase vegetable consumption. This research showed that people consuming few vegetables felt guilty about their eating habits but did not have the self-confidence to change their habits. Factors shown to be crucial were the influence of friends, demonstration of recipes and overcoming perceived barriers of family support. Taste was overwhelmingly important when compared to convenience or whether the vegetables or vegetable dishes were considered to be healthy.

Quantitative techniques and integration of techniques

One of the most important things to emerge from the research within the AIR-CAT project is the integration of the different types of techniques and methods. Integrating the different methods gives the potential benefits from the different types of approaches and allows greater confidence in results obtained.

Preference mapping: An effective tool for translating consumer preferences to sensory product characteristics

Preference mapping includes a set of statistical procedures based on principal component analysis, cluster analysis and multiple polynomial regression, which can help food industries to improve a product or to develop successful new products.

To conduct a study using preference mapping techniques does not only refer to a choice of a statistical method for analysis of data. During the 3rd plenary meeting in Barcelona, Hal MacFie presented the critical points to take into account when conducting a preference mapping study. The first of which is the selection of products required in the analysis. The second point is the selection of consumers. A specific attention must be paid to the recruitment strategy. According to Hal MacFie between 120 and 200 consumers should participate. The third critical point is the experimental

design and especially the order in which the products are presented. As well as Pascal Schlich (Schlich 1995; Schlich et al 1996; McEwan 1996), Hal MacFie recommends to use Mutually Orthogonal Latin Squares designs. Moreover, Hal MacFie suggested a presentation of a dummy sample, as neutral as possible from a hedonic point of view, to avoid bias from the first sample (i.e. the fact that the liking score is higher when the product is in the first position). Another critical experimental point is how many questions can be asked . There has been much debate on this point but experiments have been carried out to test what will occur when consumers are asked a number of ancillary questions. From these results Hal MacFie recommends that if the only measure of interest is “overall liking” then ancillary questions should not be asked. If however, there is to be an advertising campaign emphasising sensory attributes then it might be appropriate to ask questions about these sensory properties to “put them in the minds of” the consumers. Concerning data analysis, Hal MacFie suggests to use internal preference mapping, i.e. a PCA of the matrix containing the consumer scores, and to regress the objective sensory data generated by a trained panel on to the product coordinates on each preference axis. Moreover, this partner has developed, in a joint project with a food company, a method using bootstrapping technique to calculate confidence intervals around each product on the preference plot.

Preference mapping is a useful tool to examine the acceptability response at an individual level and, thus, to see how individuals cluster into groups, while classical techniques require complete design. To evaluate a great number of products represents a difficult task for the consumer and this difficulty is likely to affect the quality and validity of the data. Philippe Callier and Pascal Schlich (Schlich et al 1996) have proposed to use incomplete block designs, where each consumer would taste only a subset of the studied products. This work has been done in collaboration with a French food company. A technique of classification was set up to establish homogeneous clusters on incomplete data. This technique, called CLIP for Clustering of Incomplete Preferences, was applied on simulated and real data in order to study its validity. This analysis led to recommendation tables for incomplete block design preference mapping studies.

Methods for children

Products especially designed for children (Wolfe 1990) are more and more important in the market. Moreover, the choice of products consumed by the whole family is very often influenced by the children. However, product development and marketing of children’s food products differ from product development and marketing of adults’ food products.

Two contributions on this topic were presented in 1997, one by Jean MacEwan during the 3rd plenary meeting and one during the workshop on qualitative methods in food quality research by Joan Ferguson (1997) who has 22 years of practical experience as a qualitative consumer researcher for the Unilever Research food laboratory. Information given by both speakers was very useful for all participants who had questions from food companies about product development and marketing of children’s food products.

Both speakers emphasised that products produced and marketed for children must be acceptable to both children and parents who are focused on a different set of requirements. Concerning qualitative research, both gave common recommendations and permits to be aware of the most important issues: age group differences, attention span limitations, peer group pressure and about the moderator: attention should be paid on the image and body language, on the need to use simple

words and to listen carefully to responses. There were interesting exchanges during discussions. It was stressed that sensory attributes are important and that among the sensory characteristics the importance of flavour for children often has been underestimated: children under 7 are not only visual oriented. For quantitative measurement, use of a scale developed in Africa for illiterates was suggested.

Sensory methodology

Several studies have been carried out on sensory methodology. Some examples are presented here.

Papers written by Jean MacEwan (McEwan 1997; McEwan and Colwill 1996) addresses some of the issues associated with undertaking consumer product acceptability trials by different methods: in-house, hall and home tests. This particular study illustrated that potentially different conclusions may be reached on analysis of the three data sets, particularly with respect to the most acceptable samples. This key point on consumer acceptance trials has been included in the first draft of the AFNOR standard on hedonic tests. A table with advantages and limits of each type of data collection method was included.

Most of the preference measurements are based on first impression only. However, brief measures of preferences obtained in a laboratory may differ considerably from preferences obtained after a long experience with the product. This is especially true for food products. There are two methods for measuring consumption behaviour under domestic conditions measurements. The first is the observation of consumption behaviour in a natural context (a dining hall for example). The second is a diary method in which subjects have to complete a diary at home each time they consume a product. However, these two procedures have some disadvantages, such as the price, the quantity of products required, the duration of the study, and, for the diary method, the involvement of the subjects who have to complete a form for every consumption. The aim of the study conducted by Christelle Porcherot and Sylvie Issanchou (1995) was to test the validity of this laboratory method by comparing two methods measuring the dynamics of liking: a home use test and the laboratory boredom test developed by Köster (1990). The results of this experiment did not permit to conclude definitely about the predictive value of this simple and rapid laboratory boredom test. However, some results observed in the laboratory conditions could be clues for results obtained in the home condition. Moreover, this method can be a very useful tool to help food companies to determine the ideal size of a one portion food packaging.

Applications to food products

Besides all this methodological work, several studies have been performed on different food products in co-operation with food industries. Moreover, most of the results are published and could be used by industries even if they have not participated in the studies. Some examples are given below.

Milk marketing in Germany

Reimar von Alvensleben (1997) presented at the workshop on qualitative methods in food quality research the usefulness of using a combination of qualitative and quantitative methods in two applications to milk marketing problems in Germany. The first case study concerned a dairy farmer

with a production of 1.8 million kg per year, who wanted to differentiate his offers from the competing ones in the following: conversion to certified milk, filling in earthenware bottles, free delivery and price level. The explorative interviews and conjoint analysis revealed that the offer would be far from the ideal product and the dairy farmer was advised not to pursue his concept further. A second case study concerned the marketing of organic milk. The problem was that as direct selling from farms comes to its limits, it seems to be increasingly necessary to market organic milk via dairy companies. The acceptance of organic milk in supermarkets therefore has to be assessed. The most important result of the qualitative analysis was that the image of organic milk is not much better compared to conventional milk. In quantitative terms, the importance of the label 'organic' in the process of decision is very low, most attention is put on packaging. Advice concerning further development of sale of organic milk was given. It was indicated that effort should be put on packaging, communication, developing of the distribution channels and product differentiation.

Organic foods

During the 4th plenary meeting several talks were devoted to consumer perception of organic foods. The presentations concerned studies conducted in different European countries: one in the Netherlands, one in Germany and France by Alvensleben et al (1998) and one about a collaborative study in Denmark, France, Germany and Great Britain conducted by Brunsø et al (1996). Thanks to these presentations it is possible for producers to get information about similarities and differences within and between European countries and for researchers to obtain useful advice about methodology for further studies. In the Netherlands, organic food buyers considered themselves more responsible for their own health and were more likely to undertake preventive health action than the general population. Wholesomeness, absence of chemicals, environment friendliness, and a better taste were the primary reasons to buy organic foods. The customers of health and natural food stores found appearance, ease of preparation, and fitness for slimming less important than the nation-wide sample. The differences between buyers and non-buyers suggest that organic food consumption is part of a life style. It results from an ideology, connected to a particular value system. Reimar von Alvensleben performed a longitudinal analysis and thus, was able to study age and cohort effects. The analysis showed, for example, that young people are more critical than old people towards conventional foods and that young people of today are less critical than young people 10 years ago. A recent study conducted on young people in Kiel and Toulouse revealed that organic food is spontaneously associated with health in Germany and with nature in France. From a methodological point of view, this technique of spontaneous association appeared particularly useful because it permitted to obtain not only positive associations, but also neutral and negative associations, which are generally forgotten when questionnaires are designed by the experimenter.

Fat reduced meals

Dave Mela (1996) started his presentation by summarising main points about physiological effects of fat and fat reduction. Fat is energy dense but has weak effect on satiety, increase fat consumption does not increase oxidation and there is an efficient conversion to body fat. Fat reduction decreases nutrient bioavailability, fat substitutes may also have gastrointestinal or other specific side effects. Dave Mela stressed the necessity to carry out experiments in real conditions and presented one study where consumers' behaviour about their choices of the products they bought were recorded during 10 weeks and one study conducted in a restaurant. In the first study, it was observed that consumers increased their choice of reduced fat versions, but neither weight nor calorie intake

decreased. In the second study, it was observed that when consumers knew that one dish was a fat reduced version, they compensated their calorie intake within the meal. The conclusions of this work were that benefits would depend on actual composition of reduced fat-version products and on wilful effort from the consumers.

Hans Jørn Juhl presented a study conducted by MAPP in collaboration with the National Food agency of Denmark about factors influencing the fat content of sauces (Juhl et al 1998). Since 1991 the National Food Agency of Denmark has been responsible for conducting public nutrition education in Denmark. It was decided to focus on two major sources of fat in the Danish diet, and thus, to recommend a cut-down of the amount of fat used as spread on sandwiches and a stop in sauce making with high-fat ingredients. To be more efficient in the communication regarding sauce making, it was decided to carry out a study to determine factors of importance for the fat content of the sauces prepared at home. A total of 885 sauces collected from 200 consumers over the course of a week were collected and analysed. Participants filled in a self-administered questionnaire. Regressions were performed in order to relate fat content to variables related to the sauce, to the context, to the person responsible for the preparation, to the person consuming the sauce. Two different models were used: an error component model which can make allowance for repeated observations for the same respondent and a latent class model allowing to obtain groups of consumers. With this last method 2 segments of consumers were found. However, it was stressed that one should be very careful about the size of the two segments considering the method of consumer selection used in this study. Hence it is necessary to make a broad campaign. The analysis suggests however that the sauce gets specially fatty when fish is served. Another essential result is that sauces based on finished or semi-manufactured ingredients are more fatty than the traditional home made Danish sauce, and this should be considered seriously by the industry.

Low fat foods

Paivi Kähkönen (Kähkönen et al 1995; Kähkönen et al 1997*;* Kähkönen et al 1996) presented results of several studies conducted to compare normal and low-fat versions of different food products (e.g. yogurt, chocolate, sausage, fat spread). She particularly studied the impact of information on sensory and hedonic expectations and the effects of these expectations on the ratings of sensory and hedonic characteristics after tasting in presence of information (actual ratings). The conclusion from the different experiments is that reduced fat foods are expected to taste differently from regular versions - that actual sensory ratings tend to assimilate to sensory expectations - that information has an effect on expected pleasantness, but taste is more important than expectation on actual hedonic rating and, that effect of information about the fat content depends on product type. She also observed different behaviours according to consumers and it appeared that the Personal Involvement Inventory Scale could be a useful tool to obtain segments of consumers according to their reaction towards information about fat content.

Food related lifestyles

By the end of the year 1996, several cross-cultural studies (Brunsø and Grunert, 1995; Grunert, 1993; Grunert and Kristensen; 1994) on food perceptions and preferences were underway among the AIR-CAT partners. These included research on the effect of health information on food perceptions. The study suggests that British consumers are more concerned about health issues than French consumers and it was observed motivational effects on weekday food practices among

British, Danish and Finnish female consumers. The study shows a range of common reasons to choose beef and, additionally, varying emphasis according to the culture, from family wishes and preferences (Finns), to social aspects (Danes) and ease of preparation (British respondents) and appreciation of various sensory properties of a specific food product, dry-cured ham. A study demonstrated a strong positive association between the desired sensory properties and the subjects' previous experience of the type of the product observed among French and Belgian student populations studied by questionnaires developed by the back-translation technique. Back-translation was observed to identify errors but not to solve them and even after careful translation, various cultural factors can affect the way the expressions are understood (food-related lifestyles among German, French, British and Danish consumers). Overlapping characteristics were observed, but each country also showed specific tendencies, such as being hedonistic (France), careless (UK, Denmark) or conservative (Germany, UK).

Inclination to eat foods low in animal fat was guided by several predictors common for all three countries. These predictors were attitudes, health-conscious identity, perceived moral obligations and past behaviour. Some predictors were different, e.g., British and Finnish respondents were guided by their subjective norms, whilst Danish respondents were not. Danes and Finns were significantly (and negatively) affected by perceived difficulty of getting foods low in animal fat, but British consumers were unaffected by this factor. Overall, however, the results do not demonstrate major differences in food choice prediction in the three countries.

Conclusion remarks

Among the methods for measurement of consumer preferences and attitudes which have been reviewed and developed by the participants of the programme, some have particularly held food companies' attention. Thanks to the communication through presentation of works and through exchanges, the level of knowledge and expertise of the participants has increased. This knowledge is being transferred to European food industries through collaborations, through courses and through our participation in scientific conferences.

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Mealiness in fruits - Consumer perception and means for detection

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Abstract

Mealiness is an important internal quality parameter of fruits, and is characterized by a texture deterioration of the fruits during inappropriate storage, resulting in soft, dry and mealy fruit. This phenomenon is of particular significance for fruits such as apples, peaches, nectarines and tomatoes which are characteristically juicy when fresh. It is estimated that 30% of the apples in the Northern European countries are affected by mealiness to some degree. Because of the considerable economic loss and consumer aversion associated with mealy fruit, there is need for better understanding and control of the mealiness phenomenon.

In this contribution an overview is given of the results obtained so far in the framework of project (FAIR CT95-0302) which is funded by the European Union. The overall objective of this project is to enhance the consumer's quality of fresh fruits by prevention or elimination of mealy products through respectively improved treatment, and the use of instrumental, objective measurement and monitoring techniques. The project team consists of 7 partners covering different areas such as storage technology of fruits, sensory profiling, consumer studies, instrumental measurement techniques, and NIR spectroscopy.

Introduction

Because of the health benefits, much effort nowadays is directed towards increasing the consumption of fruits. The attractiveness of fruits with respect to the purchasing behaviour of consumers is affected by visual appearance, but the expected internal quality is of equal importance. *Mealiness* is such an important internal quality parameter, which is characterized by a texture deterioration of the fruits during inappropriate storage, resulting in soft, dry and mealy fruit. This phenomenon is of particular significance for fruits such as apples, peaches, nectarines and tomatoes that are characteristically juicy when fresh.

In this contribution the results will be reviewed which were obtained so far in the course of EU FAIR project “Mealiness in fruits. Consumer perception and means for detection” (FAIR-CT95-0302) which is co-ordinated by the author. The overall objective of this project is to enhance the consumer's quality of fresh fruits by prevention or elimination of mealy products through, respectively, improved treatment, and the use of instrumental, objective measurement and monitoring techniques. The partners are: the Katholieke Universiteit Leuven (K.U.Leuven, Belgium, co-ordinator), the Flanders Centre for Postharvest Technology (VCBT, Belgium), the Agrotechnological Research Institute (ATO, The Netherlands), Bran+Luebbe (Norderstedt, Germany), the Institute of Food Research (IFR, U.K.), the Instituto de Agroquimica y Tecnologia de Alimentos (IATA, Spain), and the Universidad Politecnica de Madrid (UPM, Spain).

The results described in this paper were extracted from the publications which were generated in the framework of this project and which are included in the reference list at the end of this paper. For further details the reader is referred to the original publications. Although three fruits are considered

in the project (apple, peach and tomato), in this article only the results obtained for apple will be discussed. Ortiz et al. (1998) and Costell et al. (1998) summarized the research results related to mealiness (or 'wooliness') in peaches.

Definition of mealiness based on sensory assessment

Mealiness is term which is commonly used by consumers but which is not defined very well in the literature. As a common, useful definition was required for the purpose of the project, we decided to start with sensory results in the first place, and to relate these in a further stage to histological observations and instrumental measurements. As we knew from experience that mealiness is typically associated with apples (notably Cox's and Starking) which are stored at high temperature and high humidity conditions, we defined a protocol to produce mealy apples. The protocol consisted of storing the apples at ambient temperature and 80% RH, as a higher RH would lead to physiological breakdown. Apples with different degrees of mealiness could then be produced by increasing the storage time (up to four weeks). Usually three different storage times were considered. In addition, some partners (UPM, IATA) produced mealy apples by storing them at 5°C for different times. Several varieties were considered by different partners, but the following common varieties were chosen to cover a wide range of mealiness susceptibility: Schone van Boskoop, Jonagold and Cox's Orange Pippin, and in a later stage also Top Red.

For the sensory profiling by the IFR, IATA and ATO, attribute terms were already available from previous research, derived from literature or generated in panel discussions. In further discussions and by applying suitable multivariate techniques the total number of attributes was typically reduced. Both peeled and unpeeled apples were considered, but their sensory profile did not differ fundamentally. The lists included attributes such as granularity, juiciness and toughness, which (or the lack of) are thought to be related to mealiness. One important result was that the term mealiness does not seem to be used as such in English, but probably corresponds to a combination of lack of hardness and a granular, floury texture. Also, lack of hardness was not equivalent to mealy, as soft but juicy apples were not perceived as mealy.

In an experiment with Jonagold, Boskoop and Cox apples, which were exposed to various degrees to the mealiness enhancing conditions, no clear distinction could be made anymore between the attributes granular and floury on one hand, and pulpy on the other hand. It was assumed that this was due to the fact that the bitter, unripe, acidic and green Boskoop apples distorted the response of the panel, and Top Red – a variety which is very susceptible to mealiness – was used instead in further sessions. It was found that the protocol which was outlined above indeed resulted in mealy apples.

Histological basis of mealiness

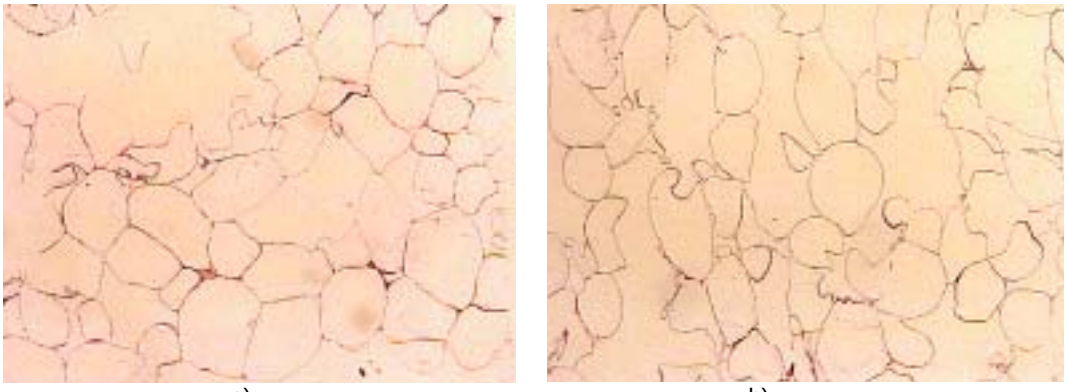
In a next stage of the project we investigated how apples which were perceived as mealy were different from non-mealy apples at the histological level.

It is commonly accepted now that mealiness is related to the relative strength of the cell wall compared to that of the middle lamella. If the cell wall is stronger than the middle lamella, the tissue will yield between the cells and the cell contents will not be released during mastication. If the cell wall is weaker than the middle lamella, the yielding will occur through the cells and as a result the liquid content will be released. In the former case the sensory perception is that of a dry, chalky

granular texture, and in the second case, that of a juicy product. This was confirmed by the results of Harker and Hallett (1992), who followed physiological changes associated with the development of mealiness of apple fruit during cool storage. Changes in the tensile strength of apple tissue were related to the way in which cells separated from each other. Application of the tensile test following cool storage of low maturity apples resulted in the rupture of individual cells at the fracture surface and the subsequent release of cell contents and collapse of the cell wall. However, when tensile tests were applied to cool-stored, more mature fruit, neighbouring cells were pulled apart, leaving undamaged cells at the surface. Hatfield and Knee (1988) concluded from their studies of mealy fruit that cells tend to become more rounded, and as a consequence the amount of intercellular spaces in mealy apples is higher than in fresh fruit. Reeve (1953) studied the cell size and percentage of intercellular spaces for five cultivars of apples using light microscopy. However, he concluded that there was no consistent correlation between structural features of intercellular spaces or cell size and the ease of cell separation.

As in the sensory analysis a clear difference in the susceptibility to mealiness was observed for different cultivars in this project, we decided to investigate whether these differences are due to differences at the histological level, since published data are often inconsistent. Three Belgian cultivars were used, all commercially important: ‘Boskoop’, ‘Cox’s Orange Pippin’ and ‘Jonagold’. To this end, microscopic images were made to visualise the difference between fresh and mealy apples, and to establish the relationships between mechanical tests and microscopic observations for the three cultivars.

In Figure 1 light microscopic images of the tissue of a fresh and mealy Jonagold apple are shown. It is clear that the mealy tissue contains more air voids and the cells are only loosely interconnected. For all three cultivars it was found (De Smedt et al., 1998a) that in fresh apples, the cells tend to break when a force is applied, whereas in mealy apples the cells tend to separate instead of break. Using four cell shape parameters (area, perimeter and two roundness parameters), it was possible using discriminant and principal component analysis techniques to discriminate between fresh and mealy ‘Cox’s Orange Pippin’ and ‘Boskoop’ apples but not between fresh and mealy ‘Jonagold’ apples. This confirms sensory results obtained by ATO, which indicate that Jonagold apples become less easily mealy than Cox’s apples. The quantitative results confirmed earlier observations by Hatfield and Knee (1988) that cells of mealy tissue are more round than those of fresh tissue.



a) b)

Figure 1: Light microscopic images of the tissue of a fresh (a) and mealy (b) Jonagold apple. The mealy tissue contains more air voids and the cells are only loosely interconnected.

Instrumental measurement methods

From the sensory and microscopy results it was clear that no single mechanical parameter would be sufficient to quantify the degree of mealiness. As it was shown that mealiness was associated with a combination of loss of crispness, hardness and juiciness, a confined compression procedure was suggested by UPM to measure mealiness (Barreirro et al., 1997b). In this test a sample is compressed in a cylindrical probe, and the breaking force, force/deformation ratio and juice area of the spot accumulated in the filter paper underneath the probe is measured. Based on this method a mealiness scale was established for Golden and Top Red apples. A good correlation was obtained between sensory and instrumental scores of mealiness for these apple varieties.

The use of ultrasonic wave propagation to measure mealiness was also investigated. Both transmission velocity and attenuation were considered. Although ultrasonic wave propagation is basically a nondestructive technique, the attenuation – even at 50 kHz – was so large that it was necessary to carry out the measurments on fruit samples. Also, plexiglas adapters were used in order to concentrate the wave and obtain a higher input signal. The existing laboratory setup was changed in order to keep the force with which the probes are forced to the sample at a constant level of 5N by connecting them to a universal testing machine. In this way more consistent attenuation readings could be obtained. A first series of experiments indicated that the direction of the sample has an influence on the wave propagation velocity in the sample. It was found that both parameters correlated relatively well with the storage time of apples in mealiness inducing conditions.

NMR imaging was also evaluated by K.U.Leuven and UPM as a technique to measure mealiness. It was found that the variability of the T2 values inside an apple is larger than in between apples. However, a difference between the average T2 value of fresh apples and that of apples stored in mealiness enhancing conditions was noticed. Also, the images of mealy apples showed also a regional variation of contrast which was not the case for non-mealy apples. This variation of contrast was similar to the NMR images of apples with internal breakdown although the contrast was smaller (Barreiro et al., 1997a).

Near infrared reflectance measurements at ATO and Bran and Luebbe indicate that mealiness could be measured in a nondestructive way. However, the calibration models contained many principal components and need to be improved further.

As cells of mealy tissue do not break during mastication, the flavour compounds are also not liberated. This would explain the fact that the aroma of mealy apples was perceived by the sensory panels as smaller than that of fresh apples. It was therefore decided to investigate the aroma compounds of Cox and Jonagold apples by means of gas chromatography. It was observed that the concentration of aroma compounds in the static headspace even increased in mealy apples while the aroma determined by sensory panel clearly decreased. Further, sugars and acids were measured by means of HPLC. A linear relationship between malic acid content and sensory mealiness was perceived.

A collaborative measurement session was organised by the IFR, Reading, UK (November 1996) to correlate physical properties measured by instrumental means with sensory attributes with the participation of K.U.Leuven, B+L, and UPM. At this meeting Boskoop, Jonagold and Cox apples with three different degrees of mealiness were analysed using trained sensory panels, confined compression and the acoustic impulse response technique. The data were analysed using principal

component analysis. It was shown (Barreiro, 1998b) that the first principal component involves both sensory and instrumental texture parameters, whereas the second component was associated with sensory attributes sweetness, and unripe. Boskoop apples were clearly segregated according to this axis. Further, the sensory descriptors dealing with juiciness were highly correlated with the juice area measured under confined compression. The sensory descriptor stale was much more correlated with the sensory attributes dealing with juiciness than with any other sensory parameter. Also, the variables stiffness and frequency measured through the acoustic impulse response technique showed a significant correlation with the sensory attributes of juiciness. The sensory descriptor floury, though mainly aligned with the 1st principal component, shows a significant correlation with the sensory attributes dealing with juiciness. This fact confirms that the floury sensation in the mouth is due to the combination of a loss of texture and juiciness.

A statistical model was established between the sensory attributes crispiness, floury, and juiciness assessment at first bite and during chewing, in relation to the readings obtained from the confined compression test and from the acoustic impulse response technique. Reasonable correlation coefficients of 0.85 for juiciness and 0.71 for crispiness could be achieved. Therefore the statistical models do not allow for continuous prediction of the sensory attributes that define mealiness. However, the instrumental parameters involved can be used to identify different commercial mealiness stages.

Sensory analysis and consumer's acceptability

An important objective of the project was to investigate the consumer's acceptability with respect to mealiness, and how this affects his purchasing behaviour.

As a first step, it was investigated whether there are cultural differences with respect to the perception of mealiness in Europe. Hereto the repertory grid method was used (Adani and MacFie, 1998). This method was originally developed to identify the constructs that people use to structure their perceptions of the social world. The basis for this method is the elicitation of 'constructs' which can be described as a way in which two things are alike and, in the same way, different from a third. Repertory grid studies were conducted in five languages (English, Flemish, French, Spanish or Danish respectively). Twenty-five consumers from each area took part in the studies. The criteria for recruitment required consumers to eat apples at least once a week, have the requested language as their first language and be between the ages of 18 and 60. An attempt was made by the recruiters to achieve an equal balance of males and females and to achieve a balance across 3 age groups. Boskoop, Cox and Jonagold apples with varying degrees of mealiness were used. The consumers were asked to both generate constructs and to rate them. In the end the consumers were asked to complete two psychological questionnaires. The interviews were conducted in the consumer's native tongue by almost always one native interviewer. The data were analysed using generalized procrustus analysis (GPA) to explore the relationship between samples and attributes on a multivariate space.

The consensus plot from the repertory grid study is seen in Figure 2. The sample means for the consumers within each country where plotted, and their distribution around the global mean is a measure of how different they are. From the map it can be seen that no one consumer group differed significantly from the others in terms of how they perceived the individual samples. The position of the groups around each sample is fairly tight. Also, consumers perceived the differences between samples quite similarly. Overall there is no structure in the way that the consumer panels are positioned around each sample. Therefore it can be concluded from this plot that the consumers perceived the

differences between the samples similarly so that there is a cross-cultural consensus with respect to the perception of mealiness. However, although consumers from different language groups perceive mealiness similarly and associate it with the texture of Cox apples, they describe their perceptions differently. While the descriptor mealy is well understood and reliably used by Danish, Flemish and French speaking Belgium consumers the Spanish use the term floury in place of the word mealy and the British consumers used the descriptors coarse, spongy and dry/crumbly instead. Overall the texture of Cox was described as soft, dry, coarse, floury, mealy and spongy by the consumers and granular and floury (texture) and fluffy (internal appearance) by the Sensory Panel.

Preference mapping is a form of multidimensional scaling in which all consumer preferences are represented. 150 British consumers were asked to rate the apples for how much they liked them on a 9-point hedonic labelled box scale, followed by two psychological questionnaires which assessed the tendency for an individual to engage in and enjoy thinking, and how aware he or she is of his/her internal sensations. The main conclusion was that consumers in general disliked Boskoop apples. The variety differences masked the differences in mealiness.

From a Madrid consumer survey involving 768 consumers, it was found that in general mealiness is considered a negative factor of the fruit although there are a 50% of the people who don not know the name of the phenomenon. This indicates the need of information and education to the consumer. Mealiness is perceived as unpleasant by most consumers, but as pleasant by some elderly people (30%). The most vaulted quality aspects of fruit are (in descending order): taste, aspect and firmness. The favourite tastes in apple are juiciness, acidity and sweetness, all of them equally appreciated. In peach they are juiciness and sweetness. Preference in apple firmness are crunchiness and hardness, while for peaches hardness and firmness are preferred. Women prefer acid apples while men prefer sweet apples. Young people (under 30 years old) also prefer acid apples, but middle-aged people prefer sweet apples.

Influence of pre- and postharvest factors on the development of mealiness

At IATA, the effect of variety, harvest period, storage temperature, storage time and their interactions on the sensory perception of Top Red and Golden Delicious apples was investigated. It was observed that the effects of harvest period and storage time are better studied in connection with variety as there were interactions. The differences due to harvest period were more important in Top Red than in Golden (crispy and floury), whereas storage time affected more to Golden (astringency and sweetness). Floury, the attribute probably most related with mealiness, showed and interesting interaction among variety, storage time, and storage temperature. In Golden, the attribute floury was almost not detected after 3 months of storage but was clearly detected after 6 months. It was also more pronounced when stored at 2ºC than at 0.5ºC. On the contrary, in Top Red the attribute floury was detected at 3 months with a similar level than in Golden at 6 months, and was also more pronounced at the higher temperature of storage. However, after 6 months the attribute floury increased considerably and the assessors were not able to distinguish between storage temperatures. Since Starking (included in the training phase and showing the greatest mealiness) was not included among delivered samples, the assessors did not score floury at the maximum of the scale. Nevertheless, they were able to distinguish more precisely between mean differences of mealiness. This probably would have been more difficult in the case of including extremely mealy apples, due to their masking effect on lower differences. MANOVA results clearly separated varieties, storage time in each variety and the first harvest period of Top Red apples.

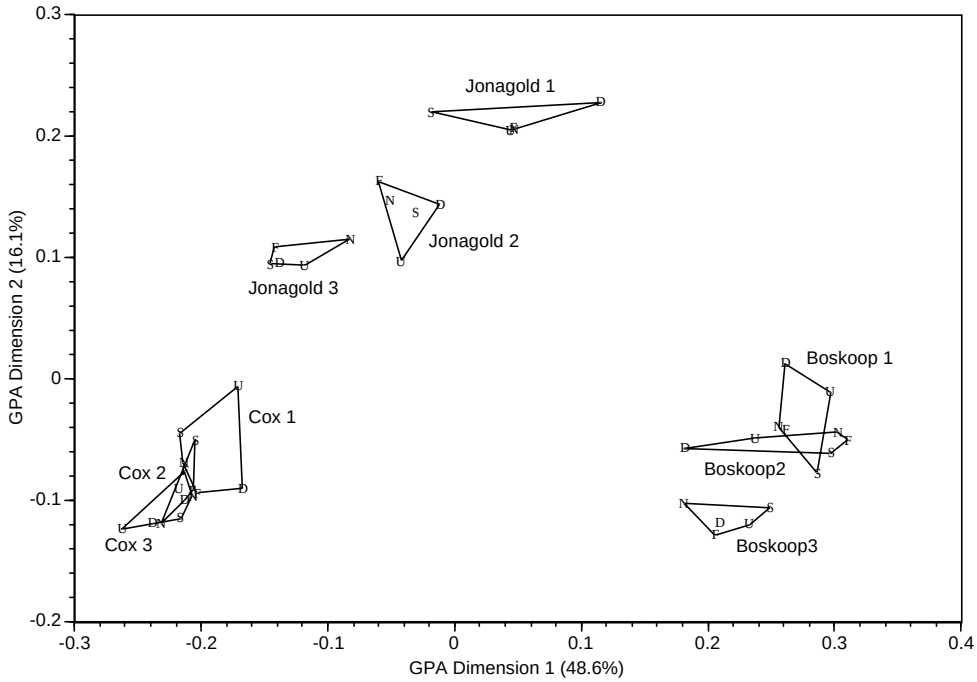


Figure 2: GPA consensus plot from repertory grid study of apple mealiness. Showing variations among the different consumer panels. 1 = 'fresh', 2 = 'mid-point', 3 = 'mealy'.

UPM carried out shear resistance and confined compression tests on duplo samples, and found that in Golden apples the storage time combined with fruit size is the most significant factor in relation to the development of mealiness. In the case of Top red apples there is an important effect of storage time combined with harvest date (only early harvested apples remained acceptable after 6 months of storage while more than 50% of apples were extremely mealy for common and late harvest dates.

Conclusions

In this project several aspects of the mealiness phenomenon are investigated. A protocol to induce mealiness in apples was established, and based on this procedure fruits with different levels of mealiness were produced to train a sensory panel to detect and score mealiness. The histological basis beyond the development of mealiness was investigated, and it was confirmed that the phenomenon is related to a change in the shape of the fruit tissue cells and in the relative strength of the middle lamella with respect to the cell wall. Several instrumental techniques have been developed to measure mealiness, and the best results were obtained using a combination of crispness, hardness and juiciness measurements by means of confined compression. The consumer studies indicated further that in general consumers of different European countries perceive mealiness similarly.

It is expected that using the results of this project fruit growers and retailers can monitor the degree of mealiness by means of the instrumental techniques and improve the quality of their products by minimizing mealiness through appropriate storage conditions. The consumer studies can be extended to other horticultural products as well. The project is still ongoing, and further results are expected to be published in the literature.

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Consumer attitudes of modern biotechnology in the Agro-Food sector

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1 Introduction

In the Agro-Food sector of the European Union the first products based on modern biotechnology or genetic engineering have entered the market (e. g. recombinant chymosin for cheese production, tomato puree, herbicide resistant plants). In the coming years an increasing influence of biotechnology on agriculture, food production and food processing is expected. However, the application of modern biotechnology in agriculture and the food industry is still intensively discussed in the EU countries. In this context the question arises how consumers in the different countries of the EU assess the application of modern biotechnology in the Agro-Food sector.

Several population polls in the EU as well as in different member states have been carried out in order to analyze consumer attitudes towards modern biotechnology (e. g. European Commission 1997, Marlier 1993, Hampel et al. 1997). In this context the Eurobarometer surveys initiated by the European Commission achieve particular interest because they allow a cross country comparison of the attitude of the population concerning the application of modern biotechnology in different application fields. The Eurobarometer surveys and representative opinion polls in general which are based on a questionnaire methodology have been criticized for the lack of texture and meaning which can be attached to the statistics, especially in the absence of product knowledge which requires a high degree of abstraction by the interviewees (Tait 1994).

Another possibility to analyze consumer attitudes towards modern biotechnology represent focused group discussions which have been applied in this field several times (e. g. Martin and Tait 1992, Hamstra 1991, Hamstra 1993, Zimmerman et al. 1994, Grove-White et al. 1997). This approach provides enhanced insight into the motivations and attitudes behind the acceptability of genetically modified food but does not allow to include a high number of interviewees due to practical reasons.

In addition to these methodologies, participatory approaches aiming at providing discourse on the use of modern biotechnology like consensus conferences or citizens' panels, can be regarded as another possibility to get information about the attitude of consumers towards modern biotechnology. A consensus conference is a forum, in which a group of lay people asks experts to a controversial scientific or technical issue, assesses the experts' opinions and tries to reach a consensus which is published in a final report. This instrument was developed in Denmark in the mid-80ies and used in the field of modern biotechnology several times (Danish Board of Technology 1987, 1993, 1995). In the meantime consensus conferences on modern biotechnology have been carried out in additional countries (like United Kingdom, Netherlands, Switzerland) as well (e. g. Joss and Durant 1994, 1995, Joss 1995). In Germany a modified approach was used from the Academy of Technology Assessment in Baden-Württemberg which organized so-called "Bürgerforen", in which lay people and experts discussed about the consequences of the use of modern biotechnology in food production and food processing (Akademie für Technikfolgenabschätzung in Baden-Württemberg 1995).

Summing up the applied methodologies to get information concerning the attitude of consumers towards the application of modern biotechnology in the Agro-Food sector it can be concluded that questionnaire-based approaches which include a representative number of people do not allow to analyze the attitudes of different application areas of modern biotechnology in detail. In contrast, focused group discussions as well as participatory approaches like consensus conferences or citizens' panels provide information on motivations behind the acceptability of genetically modified food but does not allow to include a high number of people due to practical reasons.

Besides the currently used methodologies, an additional approach is suggested which allows to collect information on consumer acceptance and attitudes as well. This represents the so-called Delphi methodology, in which (a relatively high number of) selected experts are asked about their estimation of future developments with the help of a written questionnaire.

2 Design of the Delphi survey

In a project which was financially supported by the Commission of the EU the impacts of modern biotechnology on the Agro-Food sector in five different countries of the EU (Germany, The Netherlands, Italy, Spain, Greece) have been analyzed using the Delphi methodology. The specific feature of this method is that selected experts are asked to answer a questionnaire two (or more) times, whereby the results of the first round are presented in the questionnaire of the second round. By proceeding in this way the experts are able to examine their views in the light of the other experts' opinions and if necessary, correct any deviations. This procedure supports a consensus mechanism and majority opinions are favored.

Because the pure considering of technical aspects is not adequate for the analyzed issue, economic aspects, ecological impacts, the acceptance of modern biotechnology and the legal framework conditions have been taken into account in this Delphi survey for the first time. Besides, the selection of different expert groups has been a new feature in this project because not only scientific experts of research institutions or industry were engaged in the survey but also representatives of other relevant groups (e. g. farmers, retailers) and members of different social groups (e. g. consumers, biotechnology critics, journalists, politicians, educational sector) were regarded as 'experts' in this context.

In total, more than 7,000 experts were asked in the first round of the Delphi survey in the five countries. After the second round more than 1,200 experts have answered the questionnaire. An overview of the number of answering experts after the second round as well as the composition of the expert panels in the five countries involved is given in table 1. In those countries, in which the number of experts of the consumer group was below 30 after the second round, this group was matched with the group of the "critics". This relates to Spain, Italy and the Netherlands so that in the following text this combined group is compared with the "consumer" group in Germany and Greece.

Table 1: Composition of expert panel in five countries

Expert group	Germany		Spain		The Netherlands		Italy		Greece	
	No.	in %	No.	in %	No.	in %	No.	in %	No.	in %
Industry	99	19	43	28	34	17	27	18	17	9
Research institutions	142	27	58	38	51	25	39	26	38	20
Farmers	66	13	8	5	31	15	19	13	22	11
Consumers and users	37	7	16	11	13	6	22	15	66	34
Critics	75	14	21	14	44	22	21	14	49	26
Others	89	17	5	3	31	15	12	8	0	0
Without code	14	3	0	0	0	0	9	6	0	0
Total	**522**	**100**	**151**	**100**	**204**	**100**	**149**	**100**	**192**	**100**

3 Consumer attitudes in different application areas

The experts participating in the Delphi survey were asked to express their personal attitude to 30 statements dealing with future scientific and technical developments in the field of Agro-Food biotechnology. The aggregated results expressed by the representatives of consumer organizations (and biotechnology critics in Italy, Spain and the Netherlands) towards the application of modern biotechnology in food processing, plant production and animal production are presented in figure 1. At a first glance, it is obvious that there are significant differences between the five countries as well as in the assessment of the different application areas. On the one hand, we find the rather critical consumers in Germany and the Netherlands, on the other hand, the Spanish and - to a lower degree - Italian representatives of consumer organizations appreciate the use of modern biotechnology. The Greek interviewees show a rather unique answering behaviour which does not seem to be very differentiated compared to the other countries.

The general results of the Delphi survey are basically consistent with the results of the Eurobarometer survey of 1996 (see figure 2) if the ranking of countries according to their degree of acceptance of modern biotechnology is compared between the two surveys. Some minor differences occur during this comparison: In particular, the positions of Italy and Spain are exchanged with respect to Eurobarometer and the rank of Greece in the Delphi survey appears to be higher. In this context it is interesting that in both surveys a rather high proportion of the Greek interviewees have not been able to answer the questions (like in the Eurobarometer 1996) or express an "indifferent" attitude towards modern biotechnology application (like in the Delphi survey).

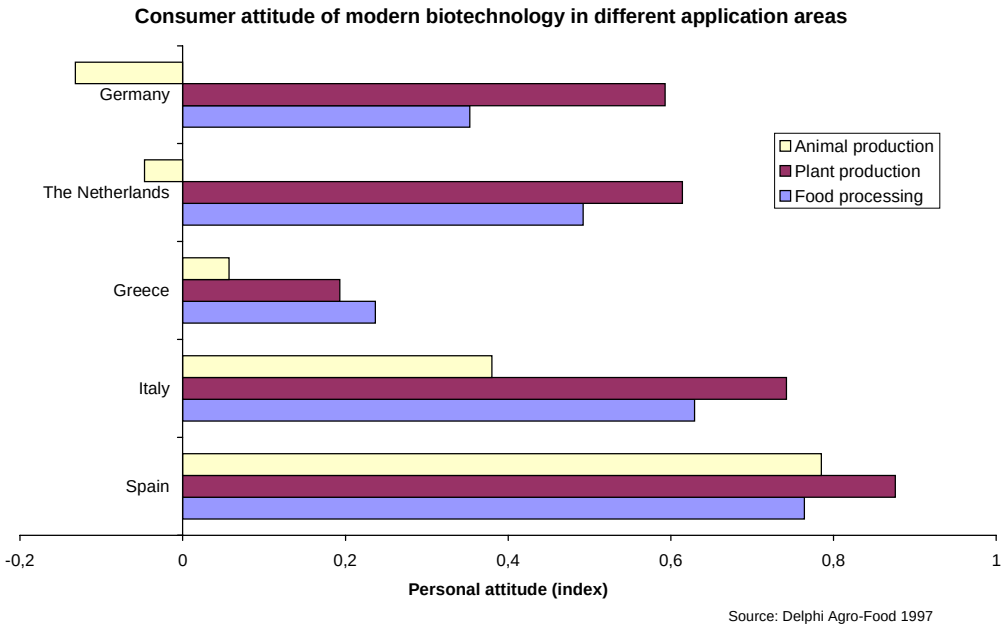

Figure 1: Consumer attitude towards the application of modern biotechnology in different application areas

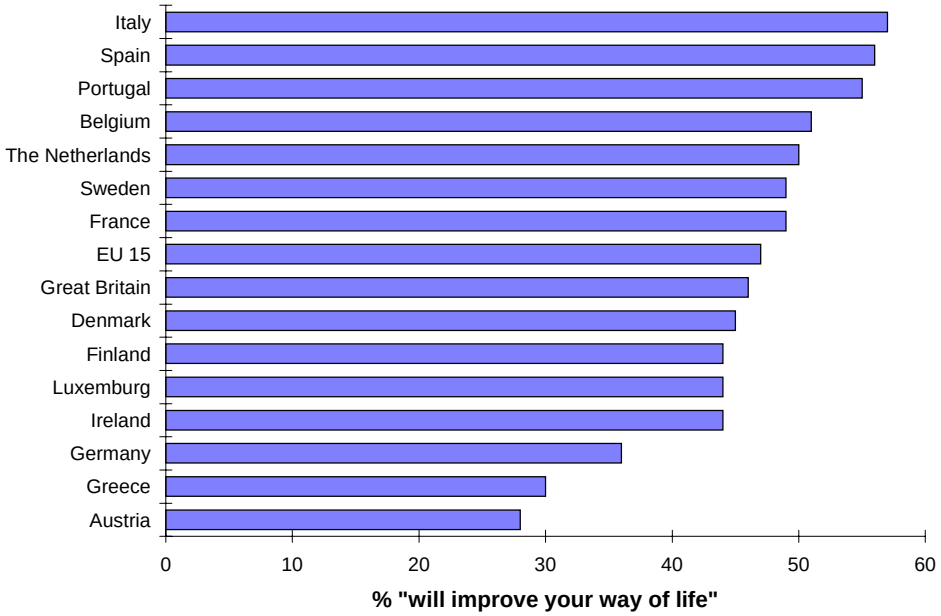

Figure 2: Anticipated effects of modern biotechnology according to Eurobarometer 1996, Source: European Commission 1997

While the use of modern biotechnology in plant production and food processing is assessed rather positively from the majority of the consumer group in all countries, the application of this technology in animal production meets much more hesitations in all countries with the exception of Spain. In the latter country the experts from consumer organizations as well as those for societal aspects highly appreciate the use of modern biotechnology in all three areas. In contrast, the German consumer group can be regarded as being the most critical among the five included countries. This relates in particular to genetic engineering of animals, of which the German experts of consumer organizations express more negative personal attitudes than positive attitudes resulting in a negative acceptance index for animal production (see figure 1). Compared to their German colleagues the representatives of Dutch consumer organizations have a slightly more positive view in particular towards the use of modern biotechnology in food processing. The assessment of the different application fields of modern biotechnology are in line with the results of the last two Eurobarometer surveys (European Commission 1997, Marlier 1993) as well as population polls on national level (e. g. Hampel et al. 1997).

Since 30 statements have been included in the Delphi questionnaire which deal with the future scientific and technological development of Agro-Food biotechnology, it is possible to analyze the answering behaviour of the experts of consumer organizations in the five countries in further detail. The profiles of the personal attitudes of these groups in the five included countries are shown in figure 3. The German and Dutch consumer groups show a rather similar profile which is mainly characterized by the high level of differentiation between the different application areas. The use of modern biotechnical methods and techniques for monitoring and analytical purposes is highly appreciated by the asked experts, while genetic engineering and cloning of animals is rejected by a high majority of representatives of German and Dutch consumer organizations (see figure 3). Interestingly, the use of modern biotechnology to develop feed additives or animal vaccines is seen as positive as genetic engineering of plants - at least by the Dutch interviewees.

Compared to the Central European countries all Mediterranean countries show a rather moderate level of differentiation between the different applications of modern biotechnology. This relates in particular to the attitudes of the Greek representatives of consumer organizations who follow the general trend found in all countries but with very low variations between the included application areas. One reason for this rather unique answering behaviour might be the aspect that a relatively high proportion of the Greek experts mentioned some problems while filling in the questionnaire - a phenomenon which is found in the Eurobarometer study in this country as well (European Commission 1997). In Spain the differentiation is rather moderate as well: Only the cloning of farm animals shows a small deviation from the highly positive acceptance index in all other application areas (see figure 3). The Italian respondents show the same principle - a phenomenon which might be influenced by the cloning of the sheep "Dolly" which was published between the first and second round of the survey and caused an intensive debate on the ethics of animal biotechnology in both countries.

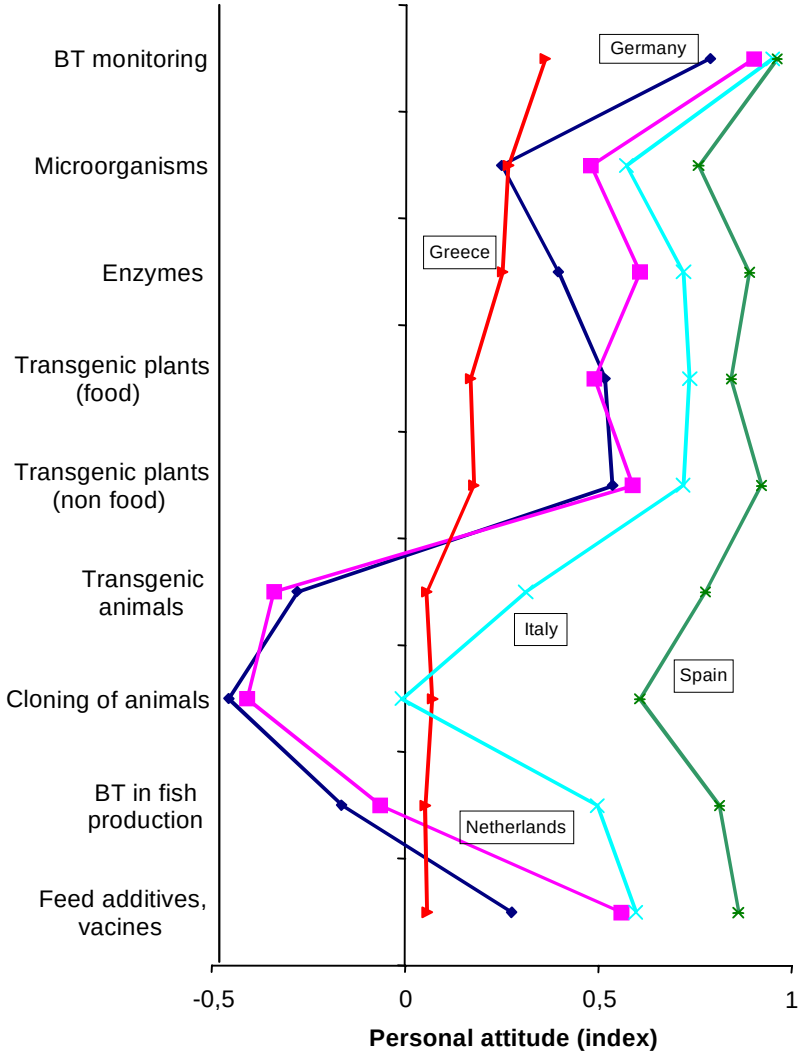

Figure 3: Profile of consumer attitudes in different countries

The role of food traditions

A major factor among those contributing to shape the social environment for the development of Agro-Food biotechnology is the attachment of consumers to their traditional tastes and foods. In particular, the share of respondents, who see this development as not realistic (in other terms, who see food traditions as a non-influencing factor) is much higher in Germany and the Netherlands compared to Italy, Greece and Spain (see table 2). Two indications can be drawn from this finding:

- People's attachment to food traditions is considered much stronger in Mediterranean countries.
- Such attachment is seen as a possible constraint to the development of a market for the products of food biotechnology.

Table 2: Modern biotechnology has failed in producing food and beverages satisfying traditional taste preferences of large consumer groups in your country (statement 47)

Time of realization	Germany	Greece	Italy	The Netherlands	Spain
0-5	12 %	39 %	6 %	7 %	35 %
6-10	27 %	41 %	44 %	7 %	25 %
11-15	12 %	7 %	19 %	0 %	0 %
> 15	6 %	4 %	0 %	0 %	0 %
No realization	15 %	2 %	6 %	63 %	0 %

Another statement relevant in order to evaluate the role of food traditions is statement 45 ("In your country most of the beer is produced with genetically engineered yeast."). Here the situation observed for statement 47 is reversed, as the consumer representatives in beer-drinking countries (Germany and the Netherlands) are clearly more sensitive on this issue. In fact, they tend to deny the realization of this development in a higher share with respect to respondents in wine-drinking countries (see table 3).

It seems clear that in countries where beer is already seen as an industrial product without a national tradition, the acceptance of genetic modification in brewing is facilitated. On the contrary, in countries where beer is a traditional, still perceived as craft-made product, consumers' acceptance is more controversial. Conversely, in countries where food production is more industrialized, the acceptance of food biotechnology in general is less influenced by attachment to food traditions. This confirms that the role of attachment to food traditions is effectively important.

On the other hand, no remarkable differences are observed between the countries as concerns the time period, in which food traditions are expected to have an influence on the market development of Agro-Food biotechnology. Only a negligible minority of the experts in each country expects that such influence would last for more than ten years. On the whole, the final success of food biotechnology in getting accepted by the consumers seems taken for granted by a majority of the consumer experts in all countries.

Table 3: In your country most of the beer is produced with genetically engineered yeast (statement 45)

Time of realization	Germany	Greece	Italy	The Netherlands	Spain
0-5	12 %	51 %	29 %	21 %	33 %
6-10	39 %	36 %	57 %	19 %	48 %
11-15	15 %	5 %	0 %	6 %	10 %
> 20	21 %	0 %	0 %	16 %	0 %
No realization	3 %	3 %	0 %	25 %	0 %

4 Impacts of modern biotechnology applications

In addition to the acceptance of consumers to specific scientific and technical developments, it is important to analyze the assessments of the impacts of modern biotechnology in the view of consumers. This analysis is mainly based on a ranking of the statements according to the personal attitude of the consumer and critics representatives. Summing up their estimations, the following areas can be identified which got the highest support in the Delphi survey:

- Monitoring and control activities

This area includes two different segments: The first refers to monitoring and control techniques based on modern biotechnology in food production and processing. This relates in particular to the use of rapid test systems based on modern biotechnology to identify pathogens in plant production and animal husbandry as well as improvements in hygiene monitoring and the on-line control of quality parameters in food processing with the help of modern biotechnology.

The second area refers to monitoring and control of the impacts of Agro-Food biotechnology. In this context, especially the investigation of possible long-term health impacts of Agro-Food products is highly appreciated. The same applies - in most countries to a smaller extent - to the improvement of the reliability of risk assessment of field trials with genetically engineered plants.

- Positive contribution of biotechnological approaches to the environment

A very positive attitude is generally observed in all the countries toward those applications which produce some direct benefit for the environment. These include enzyme systems which are specifically developed to improve the environmental performance of conventional food processing procedures, the reduction of emissions and waste from animal production as well as the conversion of organic agricultural waste into marketable products with the help of modern biotechnology.

In a second step those developments and implications of Agro-Food biotechnology are identified which are mostly rejected by consumers and critics. This can be done by comparing the statements with the highest proportion of "negative attitude" in the different countries. By proceeding in this way, the following conclusions can be drawn:

- The respondents' concerns are primarily related to the possible negative impacts of modern biotechnology on human health. Environmental concerns appear slightly less important, though coming immediately after possible health impacts. These results go in line with recent research on consumer acceptance of Agro-Food biotechnology in the Netherlands (Hamstra 1994, 1993).
- Worries about possible economic impacts are third in this "hit parade of public concerns" about Agro-Food biotechnology. These include concerns about possible job reductions and concerns about possible marginalization of SMEs, which is probably associated by a concentration process in the Agro-Food industry. A price increase for food products improved by the application of modern biotechnology is clearly rejected in every country.
- While in Germany and the Netherlands the most critical application area concerns the genetic engineering of farm animals - what is underlined in other studies as well (e. g. Hampel et al. 1997, Hamstra 1993) -, in the three Mediterranean countries social acceptance is highly referred to specific framework conditions of Agro-Food biotechnology.

In addition, the attitudes expressed toward those developments directly related to consumption of genetically engineered food in general are less positive. In this context it is interesting to observe that among the less welcomed developments are the following statements as well:

- Most consumers in your country have quickly got used to all kinds of food and beverages produced with the help of genetic engineering.
- A restaurant chain or catering service specialized in offering trendy meals with genetically engineered ingredients open branches in almost all large cities in your country.
- Food made with the help of genetic engineering achieves a turnover share of 30 % or more of all the food consumed in your country.
- In your country most of the beer is produced with genetically engineered yeast.

All such statements directly refer to the market development of food biotechnology and deal directly with the habituation of consumers to gene food products - an area which is identified as being decisive for the future market development. This clearly indicates that in general, the introduction of genetic engineering in food production and processing is not arising enthusiasm in each of the included countries but is seen as a development which cannot be avoided.

5 Conclusions and summary

The results of the Delphi survey show that the degree of acceptance of Agro-Food biotechnology varies considerably from one country to another. This is an indication that cultural factors play a major role in shaping the personal attitude towards modern biotechnology. In line with the results of the three Eurobarometers on biotechnology, in the Delphi panel the most "optimistic" countries are Spain and Italy, while Germany confirms its very low degree of acceptance and the Netherlands appears to have an intermediate position.

In line with previous opinion surveys, the genetic modification of microorganisms appears slightly more accepted than genetic modification of plants. Both application areas of modern biotechnology are much better accepted than genetic modification of animals which is generally considered very negatively (except when it is used for medical purposes). Negative attitudes toward genetic engineering of farm animals are much more frequently observed in Central European countries and in Greece compared to Italy and Spain. The results of the Delphi survey seem to confirm what has been found by previous population surveys in the EU. This finding is surprising to some extent as it shows that the level of knowledge and the familiarity with the concerned issues are not so decisive in shaping the main attitudes. Instead, cultural factors seem to be prevailing.

The majority of the experts asked in the Delphi survey appears concerned about the "freedom of choice" of the consumer, i. e. the consumer should be made aware of the available options through proper information and be allowed to make a rational choice on the basis of cost-benefit considerations. All developments involving a broad diffusion of gene food products receive a moderate consensus. Positive attitudes seem to be linked to the indication of clear and specific benefits for human health and the environment. This confirms that the existence of ethical purposes is a key factor in order to obtain the social acceptance of genetic modification. This expectation, however, is accompanied by a feeling of inevitability of the final adoption of genetic engineering techniques in food production and processing.

The most supported applications of Agro-Food biotechnology are those concerning monitoring and control activities and those involving some benefit for the environment. The perception of possible risks is clearly indicated by the very high support given to epidemiological research on possible long-term impacts on human health due to the application of modern biotechnology. This suggests that the social acceptance of Agro-Food biotechnology is also linked to the satisfaction of the public demand for control.

The attachment of consumers to their national food traditions is seen as an important factor in the process of acceptance of food biotechnology. A rejection of the products of modern biotechnology resulting from such attachment is seen as less likely in the Mediterranean countries compared to Central Europe. This relates in particular to specific food products laden with symbolic meanings (like beer) than to food in general. However, on the whole, the final broad introduction/adoption of the new food (bio)technologies is seen as inevitable.

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