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# 7. Genetically modified microorganisms and their potential effects on human health and nutrition

Atte von Wright[a,*] and Åke Bruce[b]

[a]Institute of Applied Biotechnology, University of Kuopio, P.O. Box 1627, FIN-70211 Kuopio, Finland (tel.: +358-17-162087; fax: +358-17-163322; e-mail: atte.vonwright@uku.fi)

[b]National Food Administration, Uppsala, Sweden

## 7.1. Introduction

Microorganisms (bacteria, yeasts and filamentous fungi) can be used in food production either as integral parts in the preparation of various fermented[1] foodstuffs or to produce food additives and processing aids (organic acids, flavouring agents, food enzymes etc.). In fermented foods the microorganisms can either be dead, inactivated or viable. When genetically modified (GM) micoorganisms (GMMs) are used, these alternatives, naturally, have rather different safety implications. Owing to the plasticity of microbial genomes and existing gene exchange mechanisms, genetic containment, especially, is fundamentally different for GMMs than for other genetically modified organisms (GMOs). The following is an attempt to analyse the impacts of GMMs on human health and nutrition taking into account their various actual or potential uses.

## 7.2. Contribution of transgenic technologies to food microorganisms

### 7.2.1. Background to the use of microorganisms in traditional fermented foods

Food fermentations represent an age-old technology to improve the keeping quality, safety and nutritional value of perishable foodstuffs; the types of fermented foods differ greatly in different parts of the world and among different food traditions (Cooke, Twiddy & Reilly, 1987; Nout, 2001). While many processes in the developed world are nowadays carried out using defined starter strains with known and predictable properties, spontaneous fermentation, back slopping (inoculation of the fresh batch with material from previous ones) or poorly characterised starter cultures with unknown composition are still used for many products, especially in home or artisanal production.

#### *Lactic acid bacteria*

The organisms responsible for lactic acid fermentation, the lactic acid bacteria (LAB), represent a variable group of bacteria; however, they share certain common characteristics (Axelsson, 1998). They are Gram-positive rods or cocci with a microaerophilic metabolism and produce lactic acid as the main fermentation end product, either alone (homofermentative LAB) or together with carbon dioxide and ethanol or acetic acid (heterofermentative LAB). The common food-associated LAB genera are *Lactobacillus*, *Lactococcus*, *Leu-*

* Corresponding author.

[1] 'Fermentation' in this context means modification of food and food components by various microbial processes, rather than anaerobic energy production from an organic compound, in the physiological sense.

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*conostoc* and *Pediococcus*. Physiologically, such genera as *Streptococcus*, *Enterococcus*, *Carnobacterium*, *Vagococcus, Oenococcus*, *Tetragenococcus* and *Weissella* are also typical LAB, although their applications to foods are more limited and usually restricted to certain types of products (for example *Streptococcus thermophilus* in thermophilic cheese and yoghurt starters, enterococci in many endogenous food products and oenococci in red wine ripening). Ecologically, LAB are commonly found in energy rich habitats, such as decaying plant material, milk and the gastrointestinal tract and other mucous membranes. They are traditionally used in the manufacture of a wide range of foods, including the production of fermented milks, ripened cheeses, sauerkraut, pickled vegetables, brined olives, semi-dry or dry sausages, cured meats and sourdoughs, and so on. (Nout, 2001). In addition to traditional food uses LAB are being increasingly used as health promoting or probiotic bacteria in functional food products (von Wright & Salminen, 1999).

#### *Other bacteria*

Members of the genus *Bifidobacterium*, anaerobic Gram-positive bacteria that belong to the normal intestinal microflora, comprise another group of probiotic bacteria that is increasingly being used in various functional formulations, although the bacteria do not have traditional food uses (Ballongue, 1998).

In comparison to LAB, other bacteria have a less general but nevertheless important role in food manufacturing processes. Propionic acid bacteria are used in Swiss type cheeses to produce gas and aroma compounds (Lemee, Lortal, Cesselin, & van Heijneoort, 1994; Østlie *et al*., 1995; Shaw & Sherman, 1923). In meat fermentations certain staphylococci (*Staphylococcus carnosus, S. xylosus*) are also utilised, as well as other Gram-positive cocci, such as *Kocuria* (formerly *Micrococcus*; Montel, 2000). Bacteria of the genus *Brevibacterium* are essential for the proper ripening and surface texture and pigmentation of certain types of cheeses (Weimer, 2000).

#### *Yeats and filamentous fungi*

In contrast to bacteria, yeasts and filamentous fungi or moulds are eukaryotic microorganisms with a cellular organisation that resembles that of plant or animal cells. While moulds are aerobic organisms, yeasts have both aerobic and anaerobic metabolism, with ethanol and carbon dioxide as the main fermentation end products. The well known, main applications of yeasts in wine making, brewing and baking are based on the fermentative capability of these organisms.

While wild yeasts associated with grapes, fruit and berries are responsible for the fermentation of many traditional wines and drinks, it is the brewers' yeast, *Saccharomyces cerevisiae* with various variants and subspecies, that is widely used in production of wine, beer, cider, bread and other bakery products. Other yeasts associated with food include *Candida* (Hommel & Ahnert, 2000) and *Kluyveromyces* (Batt, 2000). The latter are able to degrade lactose, which makes them useful in certain dairy products (e.g. kefir; Özer, & Özer, 2000). The making of kefir and sourdough (Salovaara, 1998) provides good examples of the complex microbiological interactions between the yeasts and LAB that are needed to produce the desired technological properties and flavour of the final products. In addition to food fermentations, one yeast, *Saccharomyces boulardi*, is used as a probiotic (Elmer, Surawicz, & McFarland, 1996).

As their metabolism is aerobic, the application of moulds in food processing is based not on actual fermentation but on the action of the various enzymes and metabolic end products that they produce. In western societies *Penicillium* coated fermented meat, sausages and hams and, especially, blue cheeses and surface ripened cheeses, made with the typical starter organisms, *P. roqueforti* and *P. camemberti*, are probably the best known examples of the use of moulds in food production (Blank, 2000). In the far east there is a rich tradition of using different types of moulds (mainly of genus *Rhizopus*) to produce soybean, peanut or bean based products, such as various types of tempeh and soy-sauce (Gandjar, 2000).

### 7.2.2. Details of transgenic technology in microorganisms used in food fermentations

#### *Bacterial starter cultures*

Traditionally, bacterial starters for specific processes and products have been selected from among natural variants and spontaneous mutants. At present, genetic and molecular biological studies and the level of sophistication of the available genetic techniques vary greatly among different bacterial groups used in food fermentations. Much research has been focused on LAB, especially on the lactococci (von Wright & Sibakov, 1998). The detection of lactococcal metabolic plasmids, many of which code for important functional properties, such as lactose fermentation, proteinase activity, aroma production, phage resistance, production of exopolysaccharides, restriction-modification systems and so on (McKay, 1983), triggered early genetic studies in the 1970s. Studies since then have culminated in the sequencing of the total genome of *Lactococcus lactis* IL1403 (Bolotin *et al*., 2001). The possibilities for actual genetic modification of lactococci began with the

development of transformation techniques, first protoplast transformation (Kondo & McKay, 1982; von Wright, Taimisto, & Sivelä, 1985) and subsequently electroporation (Harlander, 1987; Holo & Nes, 1989). Electroporation also proved to be the method of choice to transform other species of LAB such as *Streptococcus thermophilus* (Mercenier, 1990), *Lactobacillus plantarum* (Bates *et al*., 1989; Scheirlinck, Mahillon, Joos, Dhaese, & Michielis, 1989) and even the industrially important but poorly transformable *Lb. delbrueckii* subspecies *bulgaricus* (Serror, Sasaki, Ehrlich, & Maguin, 2002).

Concomitant with the introduction of transformation methods has been the rapid progress in the development of cloning vectors. The first generation vectors were based on small lactococcal plasmids on which antibiotic resistance markers had been cloned (Gasson & Anderson, 1985; Kok, Van Der Vossen, & Venema, 1984). Subsequently integration vectors have been developed, based on either homologous integration (Bhowmik, Fernandez, & Steele, 1993; Leenhouts, Kok, & Venema, 1991; Scheirlink *et al*., 1989; Scott *et al*., 2000) or phage attachment sites (Bronsted & Hammer, 1999). Since antibiotic resistance markers have been considered unacceptable in any eventual food applications, several food grade cloning systems, often combined with limited host range plasmid replicons, have been designed. The selection can be based on bacteriocin resistance (Froseth, Herman, & McKay, 1988; von Wright, Wessels, Tynkkynen, & Saarela, 1990), on thymidylate synthase (*thyA*) gene (Ross, O'Gara, & Condon, 1990), on components of the proteolytic system (X-prolyl dipeptidyl aminopeptidase; Mayo *et al*., 1991) or on multicomponent systems based on the instability of the selective marker in the absence of selection (Emond, Lavall, Drolet, Moineau, & La Pointe, 2001). Complementation systems based on plasmids carrying *LacF* in the background of *LacF*-deficient host strains have also been introduced (McCormick, Griffin & Gasson, 1995), as have ochre or amber suppressors against a background of nonsense mutants in purine biosynthesis genes (Dickely, Nilsson, Hansen, & Johansen, 1995).

LAB-specific promoters and signal sequences involved in bacterial secretory pathways have been characterised and used to produce and secrete homologous and heterologous proteins in lactococci and lactobacilli. Examples include the production of clostridial endoglucanase in different lactobacilli (Bates *et al*., 1989; Cho, Choi, & Chung, 2000) and of bacillar alpha-amylase in lactococci (van Asseldonk, Simnons, De Vos, & Simons, 1993). The S-layer signal sequences could, apparently, be potentially useful for the optimisation of heterologous gene expression in LAB (Kahala, & Palva, 1999; Savijoki, Kahala, & Palva, 1997).

In comparison with LAB, fewer genetic studies have been undertaken on other food-associated bacteria. Transformation systems and vectors have been described for both propionic acid bacteria (Jore, van Luijk, Luiten, van der Werf, & Pouwels, 2001) and brevibacteria (Bonnassie *et al*., 1990; Haschiguchi, Kojima, Sato, & Sano, 1997). Some studies on the genetic modification of bifidobacteria have also been reported (Kullen & Klaenhammer, 2000; van der Werf & Venema, 2001).

### *Yeasts and filamentous fungi*

*Saccharomyces cerevisiae* is probably biochemically and genetically the best-known eukaryote. Recombinant DNA technologies have long been applied to this organism, and its complete genomic sequence is known (Goffeau *et al*., 1996). The vectors and selection markers used in yeast genetics differ in many respects from those used in bacteria (Sandhir, Garg, & Modi, 2000). While vectors based on the episomal yeast 2 μ plasmid resemble prokaryotic plasmids, vectors carrying the yeast origin of DNA replication and a chromosomal centromeric region (the YCp-plasmids) are recognised by the yeast mitotic spindle, in cell division, ensuring precise partitioning. Yeast artificial chromosomes (YAC) also contain telomeric sequences, and they can be linearised after the cloning event, providing a means to clone lengthy DNA inserts. Yeast selection markers typically either suppress or complement auxotrophic mutations in the host (e.g. *URA3* for defective uracil synthesis) or are based on the *CUP1* gene, which conveys copper resistance (Henderson, Cox, & Tubb, 1985). These kinds of markers are less problematic from the safety point of view than bacterial antibiotic resistance genes. However, many yeast vectors are *Escherichia coli*-yeast shuttle vectors, containing a prokaryotic origin of replication and an antibiotic resistance gene, allowing for selection in a bacterial background.

In filamentous fungi, although vectors are available (Bowyer, Osbourn, & Daniels, 1994; Gems *et al*., 1994), transformation efficiencies are generally low and the resulting genetic constructs represent integrations of the vector into the host genome. Linear, telomere containing plasmids have been suggested as more efficient vectors (Barreau, Iskandar, Turcq, & Javerzat, 1998).

## 7.2.3. The use of viable GMMs in foods

Although recombinant DNA technologies can be applied to a wide range of bacteria used in traditional fermented foods, the actual use of GMMs in food products has been slow to materialise. One of the reasons, at least in Europe, has probably been the uncertainty about the consumer reaction to GMMs associated with food. Furthermore, most of the food starters contain many species and strains, and the final flavour and tex-

ture of the food products are based on processes that are controlled by many different genes, the functions of which are still poorly known. Consequently, modification of the activities of single genes in an individual strain will, as yet, seldom have effects on the fermentation process that would be predictable enough to make rational strain design possible. However, research in this area continues, and specific applications, for example to control food contaminants and pathogens, remain a possibility.

With LAB, probably the best genetically characterised group of food bacteria, the emphasis has been on the elucidation of fundamental aspects of physiology and metabolism, such as the proteolytic systems (Kunji, Mierau, Hagting, Poolman, & Konings, 1996) or lactose utilisation (De Vos & Vaughan, 1994). Improved sensory properties or accelerated ripening of cheese using autolytic strains and the use of metabolic engineering to stabilise the production of diacetyl ('butter aroma'; Maxel-Henrixen, Nilsson, Hansen, & Johansen, 1999) or to improve yoghurt taste by shifting pyruvate to the synthesis of alanine (Mollet, 1999) have all been mentioned as possible applications of LAB gene technology in food. So far, these applications have not been used in actual food production.

Probiotic bacteria could be genetically modified to enhance their health-promoting properties, provided, of course, that the genes controlling these properties were known. The successful treatment of murine colitis using a GM *Lactococcus lactis* strain, which produces Il-10 (Steidler *et al.*, 2000) gives an indication of potential human applications. The development of oral vaccines based on GM food microbes (Steidler *et al.*, 1998; Wells, Wilson, Norton, Gasson, & Le Page, 1993) is another application on the borderline between foods and medicines.

In conclusion, the full-scale application of GM bacteria in food processes has not yet been realised. Proposed food related applications have been mainly directed to improve the technological and sensory aspects of foods or to introduce some new, specific, health promoting properties, rather than to optimise nutritional qualities.

Although GM yeasts intended for food applications have been approved in UK for more than 10 years they have not yet been introduced in the market (Robinson, 2001). However, genetic modifications of yeast for a wider substrate range (such as starch, lactose or xylose), flavour improvement and elimination of by-products have been proposed as potential future applications with great promise (Ostergaard, Olsson, & Nielsen, 2000; Sandhir *et al.*, 2000). Genetic modification of brewers' yeast has been advocated to introduce new enzymatic activities (glucoamylases and beta-glucanases), to modify flocculation properties and to optimise flavour development in beer (Hammond, 1995).

### 7.2.4. Microbial food enzymes produced using GMMs

The production of enzymes probably represents the most common application of GMMs in food production, so far. Currently there are at least 30 different enzymes, many of them in food use, that are produced by GMMs.[2] Genetic modification offers the possibility of increasing the yield of the desired enzyme either by introducing multiple copies of the corresponding gene into the production organism or by influencing the regulatory sequences. A major strategy is to introduce the gene encoding the enzyme in safe and efficient microorganisms.

Enzymes produced in this way include various polysaccharide degrading or carbohydrate modifying enzymes (amylases, glucanase, pectin lyase and esterase, hemicellulase, glucose isomerase and oxidase, etc.), proteases, peptidases and lipases (Robinson, 2001). Examples include an amylase for production of maltose syrups, a lipase for interesterification of fat and acetolactate decarboxylase for maturation of beer. Members of the genus *Bacillus* are a prolific source of robust enzymes, including alfa-amylase and glucose isomerase, for use in various industrial applications, including the food industry. Other production organisms include other bacteria (*Streptomyces*), yeasts, and filamentous fungi (*Aspergillus, Trichoderma*). In most cases the genes themselves are of microbial origin; calf stomach chymosin, produced in GM *Aspergillus niger* or *Kluyveromyces lactis*, is a notable exception.

With the exception of chymosin in cheese, invertase in confectionery and glucose oxidase used as an antioxidant in soft drinks, most enzymes are used as processing aids rather than as additives; that is, they are inactive, degraded or removed from the final product (Engel, Takeoka, & Teranishi, 1995).

An example of an enzyme with possible applications in both the flavour and food additive area is cyclomaltodextrin glycosyltransferase. Cyclodextrins are used for stabilisation of volatile substances (e.g. flavours and spices), modification of physical properties (e.g. reducing bitterness and masking unpleasant odours), and selective absorption (removal of cholesterol from egg or butter; Pedersen, Jensen, & Jørgensen, 1995).

### 7.2.5. Other food components produced by GMMs

#### *Flavours*

Food flavours are complex mixtures of individual flavour ingredients; they are often natural constituents of food and can be produced through physical means,

[2] *US policy on Biotechnology*, available [October 2002] at http://usis.usemb.se/biotech/enzymes.htm.

chemical synthesis or, more recently, through modern biotechnology, including fermentation, enzymolysis, and cell and tissue cultures, with and without the use of genetic modification.

Fermentation systems employing unmodified organisms have long been recognised as sources of flavour ingredients such as fatty acids, methyl ketones, carbonyl compounds, lactones and esters. A variety of bacteria, yeast, and fungi have been identified as useful organisms for the production of flavour ingredients, and genetic modification may facilitate the development of new systems useful in industrial production. An extensive range of flavour ingredients has been produced by various microorganisms (Berger, 1995).

Microorganisms that are used in traditional food biotechnology and possess GRAS (generally regarded as safe) status in the USA are preferred candidates for genetic modification aimed at supporting the formulation of volatile flavours. The underlying metabolic pathways associated with the cultivation of these organisms on natural substrates are well investigated. Dairy starter strains form carbonyls, fatty acids, and amino acids via glycolytic, lipolytic and proteolytic reactions. Genetic modification has led to strains with increased production of nonvolatile flavours, such as amino acids, 5′-nucleotides and sweet-tasting proteins.

The flavour industry has explored two areas of biotechnology for the production of flavour ingredients: plant tissue culture (with or without genetic modification); and GMMs. Modern biotechnology has also been applied indirectly, through the use of enzymes produced through genetic modification in conventional food processing, for example cheese production and the subsequent use of various cheese related products to produce flavour ingredients.

At the present time, the use of GMMs in combination with fermentation and enzymolysis holds the most promise for the production of flavour ingredients, through either direct expression or the bioconversion of appropriate substrates. Plant tissue culture may, in future, become a significant method of production of flavour ingredients, although at present this application is hampered by limited capabilities, difficulties in purification and low yields.

### Food additives

Organic acids produced by microbial fermentations are important compounds in food technology (Bigelis & Tsai, 19951). They serve as food ingredients or as precursors for food ingredients. The major organic acids produced and used in the largest volumes function primarily as food acidultants.

Citric acid is an important commodity chemical, which is manufactured by industrial fermentation. As a food ingredient it has unrestricted status and is GRAS. *A. niger* is currently used for large-scale production; *Candida* species are also used but in much smaller volumes. Most industrial citric acid is produced in large fermentors, through submerged fermentation under rigorously controlled environmental conditions. Most citric acid fermentations rely on strains of *A. niger* that have been chosen for their high productivity and adaptability to industrial fermentors. However, transformants of *A. niger* have been obtained that show 20- to 30-fold over expression of enzymes that are key in the formation of citric acid.

Lactic acid is used in many food and non-food applications. The lactic acid strains used in industrial fermentations are usually *Lactobacillus delbrueckii* incl. subspecies, but other *Lactobacillus* species are also good for commercial fermentation. Mutants of lactobacilli can be generated by spontaneous or induced mutagenesis. For a long time, *Lactobacillus* strains suitable for industrial lactic acid formation were considered nontransformable and thus not suitable for genetic modification.

The enzymatically synthesised class of cyclic oligosacharides known as cyclodextrins (see above) are manufactured and used for flavour and aroma encapsulation in an increasing number of countries worldwide.

### Amino acids

Amino acids have traditionally been used as animal feed and human food additives. The use of pharmaceutical grade amino acids for parenteral and intravenous feeding solutions has increased. Applications of amino acids include the food industry (about 2/3), feed additives (about 1/3), medicine and cosmetics, and starting materials in the chemical industry. Plant proteins have an important market but are often deficient in essential amino acids such as L-lysine, L-methionine, L-threonine, or L-tryptophan. These amino acids are synthesized by microorganisms from carbohydrate-derived precursors (Malumbres, Mateos, & Martin, 1995).

With the exception of some microorganisms that secrete glutamic acid, wild strains normally do not produce high amounts of amino acids. It is therefore necessary to modify the cell metabolism or metabolic regulation of microorganisms in order to achieve overproduction of these acids. Today, a number of microorganisms are used to produce amino acids by fermentation, the major group being coryneform bacteria. These strains are used in several industrial processes, including production of amino acids, production of nucleotides (flavour enhancers), bioconversions (including steroid and terpenoid conversions), formation of pigments and flavour in cheese, production of antimicrobial and antitumour agents and degradation of hydrocarbons and haloalkenes.

Strategies to isolate strains for the overproduction of amino acids were initially based on the screening of natural isolates that already produced appreciable amounts of such metabolites. Later, mutant microorganisms, usually produced using chemical mutagens, were developed and selected to improve yields.

Until now, most of the genetic studies have focused on isolating and characterising biosynthetic mutants, with the aim of developing strains that produce high levels of selected amino acids. Recently, cell fusion and genetic modification have been introduced into the field of amino acid fermentation. Several strategies have been used to obtain strains overproducing amino acids.

- Genetic removal of feedback control mechanisms
- Altered channelling of intermediates toward different pathways
- Increased concentrations of the substrate of a regulatory enzyme that competes with the feedback effectors
- Decreased concentration of one effector or of end products in branched pathways
- Stimulation of the cellular uptake of the precursors
- Inhibition or inactivation of the enzymes concerned with the degradation of the amino acids produced.

The three aromatic amino acids (tryptophan, phenylalanine and tyrosine) are produced by microbial fermentation but often with low yield. L-Tryptophan production by direct fermentation from glucose is normally carried out by *Escherichia coli*, *Bacillus subtilis* or corynebacteria strains, as much is known about amino acids biosynthesis and regulation mechanisms in these organisms.

Lysine occurs in most plant proteins although only in low concentrations. It is used mainly as a feed additive. As such there is a worldwide market for lysine, which is, economically, one of the most important amino acids. At present, the industrial production of lysine is carried out by microbial processes; high levels of amino acid overproduction have been achieved using GM strains. Species of the genera *Corynebacterium* and *Brevibacterium* are the best lysine producers.

## 7.3. Outcomes and impacts of use of GMMs in food products and food-related processes

### 7.3.1. Potential effects of food-related GMMs on humans

#### *Interactions with human microflora*

Many of the eventual applications of GMMs in food would lead to a situation in which viable GMMs would be consumed in considerable quantities by humans. This necessitates consideration of the possible interactions between the GMMs and resident human microflora. Each human being harbours a numerous and multifaceted microbial community, associated with the skin and various mucous membranes. The main interactions between microorganisms associated with food and endogenous microflora occur in the gastrointestinal tract, where microbial counts also reach the highest numbers. In order to evaluate these interactions the physiological role of microflora and their significance to the host is first reviewed, below.

Human gastrointestinal microflora are estimated to consist of up to 400 species, many of which are difficult or impossible to cultivate routinely (Mikkelsaar, Mändar, & Sepp 1998; Willis & Gibson, 2000). The numbers and species vary greatly in different intestinal locations. Owing to its low pH, the stomach is normally almost devoid of actual resident organisms. The presence of bile acids and relatively fast transit times keep bacterial numbers low in the small intestine. In the colon, bacterial densities reach up to $10^{12}$ bacterial cells per gram of luminal content; yeasts or other fungi normally represent only a non-significant fraction of the total microbial count. The major bacterial genera, reaching densities of $10^{10}$ or more, are *Bacteroides*, *Bifidobacterium*, and *Eubacterium*, while numbers of groups such as *Lactobacillus*, *Enterococcus* and *Enterobacteriaceae* are several orders of magnitude lower.

Metabolic activities associated with microflora are apparently significant but very difficult to study or estimate. The saccharolytic activity of the colonic microflora results in the formation of short chain fatty acids (SCFAs), such as butyrate, acetate and propionate, making a marked contribution to the energy metabolism of the host (Willis & Gibson, 2000). Intestinal bacteria can modify both endogenous compounds and ingested xenobiotics. Bile acids and their derivatives can be deconjugated or dehydroxylated to secondary bile acids (Eyssen & Caenepeel, 1988). Microbial beta-glucuronidases, beta-glucosidases, nitroreductases and azoreductases deconjugate or activate xenobiotics to more toxic derivatives (Mallet & Rowland, 1990). Small molecular weight microbial metabolic end products, some of which have or may have harmful consequences, include ammonia, hydrogen sulphide, indoles and phenolic compounds (Willis & Gibson, 2000).

The gastrointestinal tract is associated with an extensive immune response system (Mayer, 1998), consisting of gut-associated lymphoid tissue (GALT) and diffuse lymphoid cells distributed along the intestine. This system normally ensures tolerance to indigenous microbes and common foods and protects against pathogens.

The extent to which microorganisms, whether GMMs or conventional, associated with fermented foods can influence the intestinal microflora depends on their ability to survive in the gastrointestinal tract or even to colonise it. While different stress factors, such as acidic conditions in the stomach, the presence of bile acids, anaerobic conditions and human body temperature,

effectively select against many types of microorganisms, food-associated bacteria with fermentative metabolism could survive and, theoretically, even thrive in the intestinal tract. Apparently there have been no human trials on the colonising potential of GMMs. Studies have focused on different conventional probiotic strains, as verification of their intestinal survival and physiological effects is required for the documentation process of such functional foods. The colonisation of the human intestine by certain probiotic strains such as *Lactobacillus rhamnosus* GG (Alander *et al*., 1999; Saxelin, Elo, Salminen, & Vapaatalo, 1991), *Lactobacillus salivarius* UCC118 (Dunne *et al*., 2001), *Lactobacillus casei* F19 (Mattila-Sandholm *et al*., 1999) *Bifidobacterium lactis* Bb12 (Satokari, Vaughan, Akkermans, Saarela, & De Vos, 2001) and *Bifidobacterium infantis* UCC35624 (von Wright *et al*., 2002) has been unequivocally detected. Although in some cases the administered strain has been detected in the faeces several weeks after the cessation of administration (Dunne *et al*., 2001), colonisation in most cases is, apparently, transient; the strain gradually disappears after the end of feeding (Alander *et al*., 1999). The term 'persistence', rather than colonisation, has been suggested to describe this type of behaviour (ILSI, 1999).

Studies on probiotic microorganisms also provide the best documented evidence that an external microorganism can modify the functions of intestinal microflora and their interactions with the host. The observed effects include alleviation of the symptoms of lactose malabsorption, prevention and alleviation of different types of diarrhoea, modification of faecal enzymatic activities, and decreases in faecal mutagenicity (Salminen, Deighton, Benno, & Gorbach, 1998). Probiotic intervention also affects immunological functions, enhancing the production of IgM and IgA antibodies in individuals challenged with vaccines, or viral or bacterial infections (Fukushima, Kawata, Hara, Terada, & Mitsuoka, 1998; He, Tuomola, Arvilommi, & Salminen, 2000; Isolauri, Kaila, Mykkänen, Ling, & Salminen, 1994; Isolauri, Joensuu, Suomalainen, Luomala, & Vesikari, 1995). It is noteworthy that these effects have been reported with types of bacteria that actually represent a minority of the intestinal microflora. This indicates that the number of bacteria is not necessarily the decisive factor in determining their physiological activity. Even the viability of the strains does not seem to be an absolute requirement for probiotic associated effects (cell adhesion, immunomodulating activities) to be apparent (Ouwehand & Salminen, 1998).

### Gene transfer among and between food-associated and intestinal microbes

Yeasts of the genus *Saccharomyces* are perfect fungi, with a complete sexual life cycle; they are therefore capable of genetic exchange through mating. However, many other yeasts (*Candida*) and filamentous fungi present in fermented foodstuffs are imperfect, lacking the sexual phase of the life cycle; their ability to be involved in genetic exchange is, therefore, limited (Sutton, 2000). With bacteria, the known mechanisms of natural gene transfer are transduction or phage-mediated DNA-transfer from an infected cell to another host, transformation or direct uptake of DNA from solution through the cell wall, and conjugation or the transfer of DNA from the donor to the recipient by direct cell to cell contact. All these mechanisms might be involved in the genetic exchange among food-associated microbes or between them and intestinal microorganisms.

In LAB, transduction and conjugation occur frequently (von Wright & Sibakov, 1998), while—with the exception of streptococci—natural transformation is apparently less frequent. No studies on the gene transfer mechanisms of other food-related bacterial groups appear to be published. Conjugation, including interspecific and intergeneric conjugation, could, especially, be a potentially important mechanism of gene transfer between food-associated and intestinal strains. Well documented cases of conjugative transfer of broad host-range plasmids and transposable elements between enterococci, lactobacilli, lactococci and *Listeria* have been reported, some of them in *in vivo* animal experiments (Fitzgerald & Gasson, 1988; Morelli, Sarra, & Bottazzi, 1988; Gasson, 1990; Schwatrz, Perreten, & Teuber, 2001).

Among the intestinal bacteria, conjugative transposable elements appear to be very common in *Bacteroides* and *Clostridium*. There is evidence that extensive transfer of antibiotic resistance determinants has occurred both within the genus *Bacteroides* and between *Bacteroides* and other intestinal genera (Shoemaker, Vlamakis, Hayes, & Salyers, 2001).

Streptococcal strains are among the bacteria in which transformation occurs naturally. It has been possible to transform an oral streptococcus, *Streptococcus gordoni,* with plasmid DNA exposed to human saliva (Mercer, Scott, Bruce-Johnson, Glover, & Flint, 1999; Mercer, Karen, Melville, Glover, & Flint, 2001). While the role of transformation in intestinal conditions is currently difficult to estimate, it is of interest to note that experimental transformation of *Escherichia coli* and *Bacillus subtilis* in different food matrices has been detected (Bräutigam, Hertel, & Hammes, 1997; Bauer, Hertel, & Hammes, 1999), indicating that there might be genetic flow between food-associated microorganisms and various unrelated contaminants.

In conclusion, it appears that the intestinal microbial community shares a considerable gene pool, probably mainly by conjugative DNA-transfer mechanisms. Moreover, the food-associated microorganisms may, in this respect, form a continuum with the intestinal microflora, and this should be taken into account when evaluating the safety of eventual food GMMs.

#### 7.3.2. The case of eosinophilia-myalgia syndrome

While the hazards identified in food applications of GMMs have mainly been theoretical and have not, so far, materialised as actual risks, unintended and unforeseen effects remain a safety concern. There has been speculation as to whether the eosinophilia-myalgia syndrome (EMS) might be an example of an unintended effect (Belongia, Mayeno, & Osterholm, 1992). During the summer and autumn of 1989, an epidemic of a new, multisystem illness occurred in the USA. The disease was characterised by severe muscle pain and profound eosinophilia. An association with tryptophan consumption was suspected early on. The major clinical features formed the basis for the name of the new disease: eosinophilia-myalgia syndrome. It mainly affected fascia with variable involvement of other tissues. After one year about 1500 EMS cases had been reported, including 27 deaths. Response to treatment was poor and over 50% of patients remained symptomatic after one year of follow-up.

It was soon suspected that EMS was triggered by a contaminant that was present in some lots of manufactured tryptophan. Epidemiological investigations demonstrated that EMS was most probably due to exposure to a contaminant in tryptophan manufactured by one company. The company used a strain of *Bacillus amyloliquefaciens* to synthesize tryptophan from precursors. Following fermentation, the amino acid was extracted from the broth and purified using a series of filtration, crystallisation and separation processes. In December 1988 the company introduced a new strain of bacillus species, which had been genetically modified to increase the synthesis of intermediates in the tryptophan biosynthesis pathway. In 1989 the company also processed some fermentation batches with a reduced amount of powdered activated carbon in one of the purification steps and eliminated the reverse osmosis step. These changes did not seem to alter the quantitative purity of the tryptophan produced, which was maintained at 99.6% or greater; however, 60 impurities were identified, including 1,1′-ethylidenebis(tryptophan) (EBT; Mayeno *et al.*, 1990). Epidemiological investigations have indicated that the presence of EBT was associated with the incidence of EMS.

Chemical analyses supported the epidemiological findings. High performance liquid chromatography demonstrated separate unique patterns for tryptophan manufactured by different companies and even between different batches. One peak was significantly associated with the case lot and with the manufacturing changes described above. The chemical structure of this peak was determined to be EBT, a molecule that is structurally similar to tryptophan. Results from animal studies suggest that EBT may cause pathologic changes in fascia that resemble EMS.

Although it is not known whether changes in the production organism or in the process were the ultimate cause of the EBT contaminant, the case demonstrates the need for a new safety assessment of products whenever there has been a major change in the manufacturing conditions, including introduction of transgenic technology. Such an evaluation should be done on a case-by-case basis, applying such studies as are appropriate, taking into account the nature and intended use of the product.

### 7.4. Regulatory aspects

According to the present European Union (EU) regulatory system, foods containing, consisting of or derived from GMMs, in addition to being subject to directive 2001/18/EC (previously 90/220/EC), fall automatically within the scope of the Novel Food Regulation 258/97 EU (Hugget & Conzelmann, 1997). Thus their safety should be evaluated before eventual introduction to the market. However, it should be noted that food additives and flavourings fall outside the regulation. The final regulations on labelling and traceability of GMOs and products derived from GMOs are still under consideration within the EU. Propositions put forward by the Commission and the Parliament are being discussed in two committees of the Parliament and are not likely to enter into legislation until 2004.

### 7.5. Methods for safety evaluation of food-associated GMMs

#### 7.5.1. General principles to determine risks and benefits

Hitherto there has been very little experience of the toxicological evaluation either of complex foods or even of individual microbial strains. Therefore the concept of substantial equivalence (see Chapter 8) has been introduced by the World Health Organization/Food and Agriculture Organization (WHO/FAO; WHO, 1995) in order to facilitate safety assessment. If a new food or food component is found to be substantially equivalent to an existing food or food component, it can be treated in the same manner with respect to safety. The toxicological evaluation can then be focused on those aspects in which the novel food or food component clearly differs from the traditional one. Although this concept has occasionally received criticism, the recent WHO/FAO consultation on the safety assessment of foods derived from GMMs[3] also recommended a case-

[3] FAO/WHO (2001). *Safety Assessment of Foods Derived from Genetically Modified Microorganisms* (Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology), Geneva: World Health Organization. Available [January 2003] at http://www.who.dk/foodsafety/Publications/mtgrpt.

by-case comparative approach, using the concept of substantial equivalence. It should be emphasised that determination of substantial equivalence is not a substitute but a starting point for safety assessment.

In its guidelines for the safety assessment of novel foods, the International Life Science Institute (ILSI) has introduced the SAFEST concept (Safety Assessment of Food by Equivalence and Similarity Targeting), in order to clarify further the requirements and studies needed to establish the safety of a product (ILSI, 1995). If a novel food or food ingredient is substantially equivalent to a traditional foodstuff it belongs to SAFEST Class 1, and no further safety studies are needed. Foods that are 'sufficiently similar' to a traditional reference food and do not differ from a traditional counterpart except in certain identifiable aspects belong to SAFEST Class 2, requiring further studies and eventual testing. SAFEST Class 3 is reserved for foods for which no substantially equivalent or sufficiently similar traditional reference foods can be found; consequently, the most thorough safety evaluation should be focused on foods in this class.

### 7.5.2. Viable GMMs in foods

ILSI (1999) has applied the SAFEST concept to specific cases of GMMs in foods and designed decision tree approaches to determine SAFEST classifications for particular GMMs.

Several levels of genetic modification could be involved in eventual food applications of GMMs, each being different from the point of view of substantial equivalence and thus requiring a different approach in safety assessment. At least the following cases should be considered.

- The GMM strain contains only DNA from the same or a closely related species. For example, a dairy starter strain could be transformed by a natural plasmid of a related strain coding for some functional property (e.g. phage resistance) that would make the strain more useful in some specific application. Here the substantial equivalence of the resulting strain to the traditional one is obvious, and the risks can be compared to those of any other starter strain with similar functions.
- The GMM has been modified to change, either qualitatively or quantitatively, the metabolic end products present in the food. Here the safety assessment is more complex, as both the safety of the intended metabolites and the possible unintended effects that the altered metabolic shifts may have caused have to be taken into account. Even when the metabolic pathways that have been experimentally modified are very well known and characterised, the controversial case of tryptophan production by a GMM (see above) shows that unintended effects should not be overlooked.
- New enzymes or other novel proteins have been introduced. In these cases the properties and quantities of the new proteins are, naturally, of crucial importance. Proteins from known safe food sources and in quantities not different from the conventional should not present a particular problem, while proteins with no previous history in food use or derived from sources that are known to cause problems in some consumer subgroups need special attention. Allergenicity is one of the obvious risks that should be evaluated, according to the principles outlined by FAO/WHO.[4] In most cases the toxicological evaluation of the purified proteins would probably suffice, although if the novel enzymatic activities modify the food matrix, these changes might deserve attention in some cases.
- The physiological properties of the organism have been changed in a way that affects its colonisation potential. In theory, for example, changing the pH or thermotolerance of a strain, with consequent improved survival in the gastrointestinal tract, might lead to disturbances in the composition and functions of the intestinal microflora. Such situations are difficult to predict, and gastrointestinal models, animal experiments and eventual human trials would probably be required for the safety assessment.
- The GMM represents a species with no history in food use or a species with known pathogenic strains. In this case the non-pathogenicity and non-toxicogenicity of the organism should be separately verified, in addition to evaluating the safety of the actual genetic modification.

#### *Aspects of genetic containment*

As noted in Section 7.3.1, containment of the genetic modification, in the case of a viable food-associated GMM, is a special and complex concern, since the organisms, in most applications, are consumed alive, and some could survive in the intestinal tract. Conjugation in gut conditions is known to occur, and among the food-associated microorganisms, especially among the LAB, there are several species and strains in which conjugative plasmids, sex factors and transposons are

[4] FAO/WHO (2001) Evaluation of Allergenicity of Genetically Modified Foods: Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology, (22–25 January 2001), Rome, Italy, Food and Agriculture Organization, United Nations, available [December 2002] at http://www.fao.org/es/esn/food/allergygm.pdf.

known (von Wright & Sibakov, 1998). While it is reasonable to assume that the probability of conjugation depends both on the relatedness of the GMM to the intestinal microflora and on its residence time in the gastrointestinal tract, the possibility of intergeneric conjugation in the gut cannot be ruled out, as the observations with *Bacteroides* cited above (Shoemaker *et al.*, 2001) indicate.

In animal experiments, DNA mixed into the food matrix (Schubbert, Rentz, Schmidt, & Dorfler, 1997) or from ingested food itself (Einspanier *et al.*, 2001; Hohlweg & Doerfler, 2001) has been shown to enter the blood stream and tissues and has been found to be associated with somatic DNA. While the significance of this phenomenon to the biosafety of the host is difficult to evaluate, and food will inevitably contain DNA, this finding indicates that the aspect of bacterial transformation by foreign DNA should also be taken into account.

In conclusion, the possibility of a transgene being introduced into an intestinal bacterium probably cannot ever be completely ruled out. The safety implications of such genetic exchanges depend on the nature of the gene. The use of antibiotic resistance markers, which might be transferred into gut bacteria and potential pathogens, has received much attention in the discussion on GMOs. Such a possibility is, naturally, much higher for GMMs than for GM plants, for example. As noted above, several food grade vectors that do not contain antibiotic resistance genes have been designed and would undoubtedly be used in eventual food applications. With transgenes other than antibiotic resistance markers, the safety evaluation should, again, be done on a case-by-case basis.

#### 7.5.3. Food enzymes

The starting point for the safety evaluation of food enzymes has been the establishment of the non-pathogenicity and non-toxigenicity of the production organism (Pariza & Foster, 1983). The actual safety testing is mainly based on animal oral toxicity testing and on checking for the presence of known mycotoxins, if the production organism is a mould. A decision tree approach, designed to take into account also the genetic modification of the producer organism has been proposed (Pariza & Johnson, 2001).

#### 7.5.4. Flavours, additives and other food ingredients

In most cases flavours, additives and other food ingredients do not contain either the production organism or modified DNA. Consequently, the general principles of the safety evaluation of chemical compounds could be applied to these substances. However, the possibility of unintended contaminants resulting from the production organism or process should be considered.

### 7.6. Knowledge gaps

#### 7.6.1. Food fermentation

As pointed out in Section 7.2.3, the successful application of GMMs in food fermentations is hampered by the lack of knowledge about the impact of specific genes on the fermentation process. This is particularly true with complex properties like aroma, ripening and texture, which are often also greatly influenced by environmental factors. It might even be questionable, whether transgenic technology is the optimal methodology to improve many traditional processes or whether, in many cases, conventional strain selection and adjustment of process conditions might also be future methods of choice. Instead, introduction of new, nutritionally valuable or health-promoting properties into fermented foods by designed GMMs would be a rather attractive line of future research and product development. However, in these areas there are many uncertainties, not the least in the safety and regulatory aspects.

#### 7.6.2. Interactions with host microflora

The interactions of GMMs with host microflora and associated host functions are crucial for many of the eventual benefits and potential hazards. The role and functions of human microflora in health and disease are still poorly understood. This concern was also raised by the WHO/FAO expert consultation on the safety assessment of foods derived from GMMs.[5]

### 7.7. Conclusions

Food fermentation is an ancient practice and still valuable today. Introduction of GMMs could improve existing processes and lead to novel products with improved nutritional status and safety margins. The safety assessment of any future GMM applications should be rigorous, as with any other novel foods, recognising that no application can be expected to be completely risk free. The identified hazards should be compared with those that are accepted for conventional food products, and they should be weighed against the expected benefits.

The safety evaluation of processing aids or food additives produced by GMMs should follow the same guidelines and practices that are applied to conventional products. However, special emphasis should be directed

[5] FAO/WHO (2001). *Safety Assessment of Foods Derived from Genetically Modified Microorganisms* (Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology), Geneva: World Health Organization. Available [January 2003] at http://www.who.dk/foodsafety/Publications/mtgrpt.

to the elimination of unintended effects every time a production organism or process is modified, whether by recombinant DNA-techniques or conventional methods.

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