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Safe use of genetically modified lactic acid bacteria in food. Bridging the gap between consumers, green groups, and industry

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Abbreviations: DRI: daily recommended intake
EFFCA: European Food and Feed Culture Association
GRAS: Generally Recognized As Safe
LAB: lactic acid bacteria
NICE: nisin induced controlled expression system
PMS: post-market surveillance
QPS: Qualified Presumption of Safety

Within the European Union (EU), the use of genetically modified organisms (GMOs) in food production is not widely applied and accepted. In contrast to the United States of America, the current EU legislation limits the introduction of functional foods derived from GMOs that may bring a clear benefit to the consumer. Genetically modified lactic acid bacteria (GM-LAB) can be considered as a different class of GMOs, and the European Union is preparing regulations for the risk assessment of genetically modified microorganisms. Since these procedures are not yet implemented, the current risk assessment procedure is shared for GMOs derived from micro organisms, plants, or animals. At present, the use of organisms in food production that have uncontrolled genetic alterations made through random mutagenesis, is permitted, while similar applications with organisms that have controlled genetic alterations are not allowed. The current paper reviews

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the opportunities that genetically modified lactic acid bacteria may offer the food industry and the consumer. An objective risk profile is described for the use of GM-LAB in food production. To enhance the introduction of functional foods with proven health claims it is proposed to adapt the current safety assessment procedures for (GM)-LAB and suggestions are made for the related cost accountability. A qualified presumption of safety as proposed by SANCO (EU SANCO 2003), based on taxonomy and on the history of safe use of LAB applied in food, could in the near future be applied to any kind of LAB or GM-LAB provided that a series of modern profiling methods are used to verify the absence of unintended effects of altered LAB that may cause harm to the health of the consumer.

Ever since human domestication took place approximately 10,000 years ago, parts of our food supply have been cultured in order to support survival of the human population. In our current times, the main objectives of agriculture are still the same, the assurance of efficient food supply in the first place, and the increase of quality of the supplied foods for the consumer in the second place. Modern biotechnology, using recombinant DNA technology, offers ample opportunities to further improve food production in the expanding world of today.

Within the United States of America there is not much public resistance towards the use of genetically modified organisms, GMOs (Box 1), in food. However, in other areas of the world, for instance Oceania and Europe, the production of novel foods using GMOs is subject to strong debate. For example, within the European Union the scientific community states that there is sufficient legislation to guarantee that such novel foods are safe for the consumer and the environment, but green groups in many cases state an opposite opinion. The support for the use of GM crops and foods among the European population has recently increased in some countries. Yet at the same time a majority of Europeans do not support agri-food biotechnology (Gaskell et al. 2003). In the current debate about the application and safety of GMOs in the food industry no clear distinction is made between GMOs derived from animal, plant, or microbial origin. Moreover, ethical aspects could play a role in the debate about GMOs from animal or plant origins, but these are considered less relevant in the discussion about genetically modified micro-organisms. Apart from this, the current European legislation on novel foods and the use of GMOs in food production processes, as laid down in the regulation EC No 258/97, amended in regulation 1829/2003, directive 2001/18/EC (revised directive 90/220/EC), directive 90/219/EC, and Commission Recommendation 97/618/EC, generates a framework for what should and what should not be permitted by law. The legislation, however, is not yet completely clear on a number of important scientific matters. First of all, the legislation predominantly focuses on the methodology rather than on the end product and hence, holds too strongly to the definition of GMOs. Consequently, organisms in which the genetic material has been altered by recombinant DNA techniques in a way that does occur naturally, for instance by point mutations or small deletions, are considered to be GMOs. Secondly, foods with a new structure which date from before 1997 are not considered as novel foods. Thirdly, self cloning (Box 2) of non-pathogenic micro-organisms is not considered to lead to a GMO as long as containment of the organism is guaranteed (directive 90/219/EC). However, the same self-cloned micro-organism when used in products (deliberate release) is considered to be a GMO (directive 2001/18/EC). Moreover, the market introduction of foods with organisms that have been improved via classical breeding or random mutagenesis is currently acceptable without profound safety analysis[1], but it could be questioned whether these foods are more safe than GMO-derived novel foods. The new regulation 1829/2003 (amending 258/97) regulates the approval and the labelling, and distinguishes between products from GMO, and products which are produced with help of GMO. Foods, ingredients, additives, flavours as well as feedstuffs, which contained or are GMO, or which were produced from GMO are in the scope of the regulations. The labelling of these products is also process-oriented. Products produced with help of GMO are not in the scope of the regulations. It is still unclear if the regulations can be applied on the use of GMO (micro-organisms) within fermentation (Jany and Schuh, 2005).

The rationale for food safety regulations is the assurance to prevent any adverse effects on human health or on the environment. The starting point for a food safety approach is the comparison between the new foods and the conventional counterparts that have a history of safe use, both for the food produced and for the micro-organism that is modified. This approach leads to identification of new or altered hazards relative to the conventional counterpart (substantial equivalence, Box 3) is essential (OECD, 1993).

This document will analyse the current situation within Europe with regard to the safe use of novel foods derived from genetically modified lactic acid bacteria (GM-LAB). The application and safety assessment of genetically modified yeast and fungi are not discussed; neither is the use of GMOs derived from plants (Kuiper et al. 2001) or animals. Genetically modified microbial inoculants for use in agriculture, such as biological control agents, biofertilisers, or phytostimulators is reviewed elsewhere (Amarge, 2002; Morrissey et al. 2002). An overview will be given of the specific genetic techniques that generate GM-LAB and the advantages that the novel foods fermented with GM-LAB may bring to the consumer or the producer. Moreover, the current safety assessment procedures are considered and further suggestions are made for an adequate risk analysis when GM-LAB are applied in food production. Careful analysis of the properties of GM-LAB combined with a profound safety assessment procedure for foods that are produced through the actions of GM-LAB, are essential for comparing the safety of such products with traditional foods. Finally, based upon the

Table 1. Types of DNA modification methods and the acceptability to be used in food production.

Modification of DNA	Directed genetic alteration	Un-controlled genetic alteration	Acceptance of contained use, 90/219/EC	Acceptance of deliberate release, 2001/18/EC
Spontaneous mutations	-	+	+ non-GMO	+ non-GMO
Induced mutations	-	+	+ non-GMO	+ non-GMO
Mutations via insertion elements	-	+	+ non-GMO	+ non-GMO
Conjugation	+	-	+ non-GMO	+ non-GMO
Transduction	+	-	+ non-GMO	+ non-GMO
Self-cloning	+	-	+ non-GMO	- GMO
Non-self-cloning	+	-	- GMO	- GMO

principle of assuring food safety for the consumer, it could be argued to make no distinction between foods produced by LAB that have been altered in an uncontrolled way, for instance by random mutagenesis, or a more directed way, such as GM-LAB. This could imply that all foods fermented by LAB should be subjected to a safety assessment procedure, before commercialisation can occur. Recently, such issues have been considered for non-GM-LAB in a draft document introducing the concept of Qualified Presumption of Safety (QPS), which will be discussed below (EU, 2003). Furthermore, we will examine the value of the principle of a long and safe history of use of traditionally used LAB and discuss whether this could be a starting point for the acceptance of GM-LAB.

LACTIC ACID BACTERIA AND GENETIC ENGINEERING

LAB have a long history of use by man for food production and food preservation. LAB are Gram-positive, non-spore forming bacteria and naturally present in raw food material and in the human gastro-intestinal tract. The heterogeneous group of LAB includes the rod-shaped bacteria like lactobacilli, and cocci such as streptococci, lactococci, pediococci and leuconostocs. LAB are widely used as starter cultures for fermentation in the dairy, meat and other food industries. Their properties have been used to manufacture products like cheese, yoghurts, fermented milk products, beverages, sausages, and olives. These food-grade bacteria can also improve the safety, shelf life, nutritional value, flavour and quality of the product. Moreover, LAB can be used as cell factories for the production of food additives and aroma compounds. It is further assumed that LAB may function as probiotics and contribute to the general health of the consumer upon consumption. The use of probiotics falls currently within a grey area between food and medicine and many health claims assigned to probiotics are not yet scientifically proven. Another application - the use of LAB in the production of proteins for application in health care or for development of new vaccines (Mercenier et al. 2000) – is more related to pharma than to food. In the future it is predicted that knowledge about the interaction between LAB and the human host will open new avenues for developing LAB which support human health.

The uncontrolled genetic alterations of LAB that occur during random mutagenesis may lead to strains with improved traits. These may be either attractive for the manufacturer of fermented foods, or have benefits for the consumer. Both directed and uncontrolled genetic alterations result in a change of the genetic code of the micro-organism that may affect the transcription and translation processes and, consequently, may influence metabolic processes in the cell. However, it seems that in the current legislation regarding the use of GMOs in food industry, the nature of the DNA modification could dictate the necessity for the novel food product to be subjected to a safety assessment procedure (Table 1).

Uncontrolled genetically altered LAB

Spontaneous mutations may occur in LAB by natural events such as insertion sequence elements (Visser et al. 2004), radiation, erroneous DNA replication or transcription, and other factors. The level of such mutations depends upon the growth conditions. By screening of natural isolates of LAB, strains with improved fermentation characteristics can be selected. The frequency of mutations can be further increased by exposing LAB to mutagenic conditions such as UV light or chemicals like N-methyl-N'-nitro-N-nitrosoguanidine (NNG), or ethyl methyl sulphonate. Specific screening for desired traits may result

Table 2. Overview of lactic acid bacteria with controlled or uncontrolled genetic alterations.

Host strain	Donor strain	Gene involved	Intended effect	Modification technique	Reference
Lb. bulgaricus	Not applicable	*lacZ*	limited lactose fermentation	IS mediated deletion	Mollet and Delley, 1990
L. lactis	Not applicable	*ldh* and others	increased carbon dioxide production	spontaneous and induced random mutagenesis	El Attar et al. 2000
L. lactis	Not applicable	*ldh* and others	increased acetoin and diacetyl production	NNG induced random mutagenesis	Boumerdassiet al. 1997
L. lactis	Not applicable	*aldB*	increased diacetyl production	NNG induced random mutagenesis,	Monnet et al. 2000
L. lactis	Not applicable	*aldB*	increased diacetyl production	Spontaneous random mutagenesis	Goupil et al. 1996
L. lactis	Not applicable	*ribC*	increased riboflavin production	induced random mutagenesis	Burgess et al. 2003
S. thermophilus	Not applicable	gal operon	fermentation of galactose	spontaneous random mutagenesis	Vaughan et al. 2001
L. lactis	Not applicable	*glk, ei*$^{man/glc}$	no glucose fermenting capacity	spontaneous random mutagenesis	Thompson et al. 1985.
L. lactis	*L. lactis*	*aldB*	increased diacetyl production	double crossover homologous recombination	Swindell et al. 1996
L. lactis	*Lb. helveticus*	*pepN, pepX, pepC, pepI*	modulation of proteolytic system for enhancement cheese ripening	food grade vector cloning	Joutsjoki et al. 2002
L. lactis	*L. delbrueckii*	*pepI, pepL, pepW, pepG*	modulation of proteolytic system for enhancement cheese ripening	NICE System	Wegmann et al. 1999
L. lactis	*Peptostreptococcus asaccharolyticus*	*Gdh*	increased production of alpha-ketoglutarate	Vector cloning	Rijnen et al. 2000
L. lactis	lytic phage *phi31*	phage inducible promoter	expression of lethal three-gene restriction cassette LlaIR+,	Vector cloning	Djordjevic et al.1997
L. lactis	lytic phage *phi31*	anti sense phage RNA	silencing of phage genes	Vector cloning	Walker and Klaenhammer, 1998
L. lactis	lytic phage	anti sense phage RNA	silencing of phage genes encoding structural genes	Vector cloning	Kim et al. 1992
L. lactis	Not applicable	*pip*	inactivation of phage infection protein	double crossover homologous recombination	Monteville et al. 1994
L. lactis strains	*L. lactis strains*	lacticin encoding genes	lacticin production	Conjugation	O'Sullivan et al. 2003
L. lactis strains	*L. lactis strains*	lacticin encoding gene	lacticin production	conjugation (plasmid stacking)	Mills et al. 2002
L. lactis	*S. thermophilus*	abiA, abiG	abortion of cells upon phage induction	Vector cloning	Tangney and Fitzgerald, 2002
L. lactis and others	*Pediococcus acidilactici* and others	*lcnC, lcnD*	lantibiotic production	Vector cloning	Horn et al. 1999
L. lactis	phage	*lytA, lytH*	production of lysin and holin	NICE	de Ruyter et al. 1997
L. lactis	*S. thermophilus Sfi6*	EPS gene cluster	altered EPS production	Vector cloning	Stingele et al. 1999
L. lactis	*S. thermophilus Sfi39*	EPS gene cluster	altered EPS production	Vector cloning	Germond et al. 2001
Lb. gasseri	*L. lactis*	folate gene cluster	introduction folate biosynthesis pathway	Vector cloning	Wegkamp et al. 2004
S. thermophilus	Not applicable	*pgmA, gal U*	inactivation of phosphoglucomutase	double crossover homologous recombination and vector cloning	Levander et al. 2002
L. lactis	*B. sphaericus*	*ldh, alaD, alr*	rerouting of pyruvate to L-alanine	double crossover homologous recombination, vector cloning	Hols et al. 1999
L. lactis	*L. lactis*	riboflavin gene cluster	overexpression riboflavin biosynthesis pathway	Vector cloning	Burgess et al. 2003
L. lactis	*L. lactis*	folate gene cluster	overexpression folate biosynthesis pathway	Vector cloning	Sybesma et al. 2003b
L. lactis	*L. lactis*	*glk, pfnABCD, pfcBA, genes* lactose-PTS and tagatose-6P	inactivation of glucose fermenting system and introduction of lactose fermentation	double crossover homologous recombination, vector cloning	Pool et al. 2003
L. lactis	*L. lactis*	*galA, aga*	introduction a-galactosidase activity	food grade vector cloning	Boucher et al. 2002
Lb. plantarum	*B. subtilis*	*phyC*	introduction phytase activity	Vector cloning	Kerovuo and Tynkkynen, 2000
Lb. plantarum	*L. amylovorus*	*amyA*	introduction a-amylase activity	chromosomal integration	Fitzsimons et al. 1994
Lb. plantarum	*B. stearothermophilus, C. thermocellum*	α-amylase gene, *celA*	introduction a-amylase and cellulase activity	single homologous recombination	Scheirlinck et al. 1989
S. thermophilus	*S. thermophilus*	*glyA*	increased acetaldehyde production	Vector cloning	Chaves et al. 2002
Lb. fermentum	Not applicable	*ldhD, ldhL*	increased mannitol production	double crossover homologous recombination	Aarnikunnaset al. 2003
Lb. helveticus	Not applicable	*ldhD*	production of pure L-(+) lactic acid	double crossover homologous recombination	Kyla-Nikkila et al. 2000
Lb. delbrueckii	Not applicable	EPS genes	altered EPS production	Chemically induced random mutagenesis	Welman et al. 2003
S. thermophilus	*S. thermophilus* bacteriophage	anti sense phage RNA, helicase gene	silencing of phage genes	Vector cloning	Sturino and Klaenhammer, 2002
Streptococcus mutans	*Zymomonas mobilis*	*ldh, adh*	prevention of dental caries	double crossover homologous recombination, gene replacement	Hillman, 2002
Lb. delbrueckii	Not applicable	β-galactosidase gene	Increased b-galactosidase activity	EMS or NNG induced random mutagenesis	Ibrahim and O'Sullivan, 2000

L. lactis	*Eimeria tenella, L. plantarum*	M1Pase gene, *MtlD*	increased mannitol production	NICE	Wisselink et al. 2005
L. casei ATCC 334	*L. casei LC202*	*dhic*	increased alpha-keto acid dehydrogenase activity	Vector cloning	Broadbentet al. 2004
L. plantarum	*Lactobacillus sake*	promotors and regulatory genes	increased gene expression	Vector cloning	Mathiesen et al. 2004
L. lactis	*L. lactis*	Prt+ and Lac+derivatives of *L. lactis* MG1363	increased proteolytic and acidifying activity	conjugation	Picon et al.2005
L. lactis	*S. simulans*	Lss	production of lysostaphin	NICE	Mierau et al.2005
L. lactis	*P. stipitis*	XYL1	production of xylitol	Vector cloning	Nyyssölä et al.2005
L. lactis	Not applicable	unknown	increased oxidative-stress tolerance	spontaneous mutations (natural selection)	Rochat et al.2005
L. lactis	*L. lactis*	*nisRK, nisFEG*	increased nisn Z production	Vector cloning	Cheighet al. 2005
L. lactis	*Z. mobilis, L. lactis*	pdc, nox	increased acetaldehyde production	NICE	Bongers et al. 2005
L. lactis	*G. stearothermophilus, L. Brevis, L. lactis*	*sgsE, slpA, usp*	production of excreted S-layer protein	NICE	Novotny et al.2005
L. lactis	*E. coli*	gshA, gshB	increased glutathion production	NICE	Liet al.2005
L. lactis, L. paracasei	*L. paracasei*	groEL	increased stress tolerance	NICE	Desmond et al. 2004
L. lactis	Not applicable	unknown	increased L(+)-actate production	random mutagenesis	Bai et al. 2004
S. thermophilus	Not applicable	deoB, gst, rggC, and unknown	increased oxidative-stress tolerance	random insertional mutagenesis	Fernandez et al. 2004

EMS: ethyl methanesulfonate; NNG: N-methyl-N'-nitro-N-nitrosoguanidine

in the identification of new strains with improved utility for the food industry.

Examples of LAB selected after uncontrolled genetic alterations

Selection of strains that have been subjected to uncontrolled genetic alterations is used in the dairy industry to improve certain intrinsic characteristics of the fermented end product, like flavour, structure, nutritional value, or phage resistance. Spontaneous mutations in single genes can lead to altered lactose metabolism, citrate uptake, and increased proteolytic activity. In *Lactobacillus bulgaricus* a spontaneous insertion sequence (IS) element-mediated deletion of the *lacZ* gene altered lactose metabolism resulting in limited fermentation capacity. As a consequence, yoghurt, made with these altered *L. bulgaricus*, is not suffering from post-fermentation acidification during the shelf life period (Mollet and Delley, 1990). A gene inactivation method for several lactic acid bacteria has been described that is mediated via IS elements that may integrate into a gene, resulting in inhibition of function (Maguin et al. 1996). According to current legislation, however, strains with inactivated genes obtained by this latter IS-based strategy are considered to be GMOs.

L. lactis strains that are involved in the fermentation of Roquefort cheeses were randomly mutated, and selection was based on carbon dioxide production that improves the quality of the cheese (El Attar et al. 2000). In a similar way LAB defective in lactate production, but with increased production levels of the butter flavour compound diacetyl were selected after random mutagenesis with the mutagen NNG (Boumerdassi et al. 1997). The selection of a spontaneously mutated *L. lactis* strain that overproduced diacetyl, responsible for the butter flavour in many fresh dairy products, is described by Monnet et al. 2000. A stronger and more direct selection procedure was applied by the temporarily introduction of a plasmid that generates valine prototrophy in the bacterium (Curic et al. 1999). After selection of the desired mutation in the *aldB* gene, encoding α-acetolactate decarboxylase, the plasmid was removed, leading to strain that did not contain any foreign DNA. Nevertheless, under the actual EU legislation this strain is still considered as a GMO after release in the environment (Henriksen et al. 1999). This last case is a perfect example of possible inconsistencies in the current legislation.

The nutritional value of fermented foods can also be improved by (over) production of healthy components, like vitamins, in the fermenting bacteria. To increase vitamin B2 (riboflavin) production levels in the dairy starter bacterium *L. lactis*, bacterial cells were exposed to increasing concentrations of roseoflavin. This resulted in the selection of a roseoflavin-resistant strain with deregulated riboflavin biosynthesis and increased production levels of riboflavin, up to 1 mg/L. Food products obtained from fermentation with this modified *L. lactis* will contribute strongly to the daily recommended intake (DRI) of 1.3 mg for riboflavin. Partial analysis of the genome of this mutant revealed a mutation in a supposed regulatory sequence just upstream of the first gene of the riboflavin gene cluster (Burgess et al. 2003).

LAB can also be selected for the removal of undesirable compounds from raw food materials. In traditional yoghurt fermentation, the undesirable sugar lactose is degraded only partially. One of the degradation products is galactose which may be harmful for people suffering from galactosemia and which also may cause cataract problems

(Liu et al. 1998). Vaughan describes the selection of spontaneous galactose-fermenting mutants of *Streptococcus thermophilus*, that contain up-mutations in the *gal* operon and that may assist in removal of undesired galactose from the food matrix (Vaughan et al. 2001). The selection of a double mutant strain of *L. lactis* with an inactivated glucose uptake system deficient in glucokinase could also reduce the concentrations of lactose and galactose during fermentation. Moreover, this strain could also be used as natural sweetener due to the excretion of the undegradable lactose moiety glucose (Thompson et al. 1985). An overview of LAB selected after random modifications of DNA is given in Table 2.

Controlled genetic alteration of LAB

An alternative for random mutagenesis is targeted or directed modification of the DNA. Renault (2002) has evaluated the potential benefit to society and the possible risks associated with the use of genetically modified lactic acid bacteria in food and health applications. It was concluded that risk assessment and expected benefits will determine the future use of modified bacteria in the domains of food technology and health. Controlled genetic modifications vary from single base pair substitutions, mutations, insertions of genes into the chromosomes, or removal of DNA from the chromosome (deletions) resulting in inactivation of specific enzymes. A summary of the practices of directed genetic alteration of LAB is given in Table 3. According to the current legislation, all such directed genetic alterations lead to strains that are considered as GMOs, except for strains obtained by self-cloning, transduction and conjugation (Table 1).

A variety of techniques has been developed to generate GM-LAB, such as cloning systems, chromosome modification systems and expression systems (de Vos, 1999). The most popular transformation system for generating directed genetic alterations in LAB is electroporation with self-replicating vectors. Alternative systems are conjugation and transduction (Gasson, 1990). Besides efficient cloning systems, adequate expression systems have been developed allowing the controlled expression of homologous and heterologous genes. Controlled constitutive expression is possible by using a system of synthetic promoters (Solem and Jensen, 2002), whereas a nisin induced controlled expression system (NICE) allows the gradual over expression of genes (Kleerebezem et al. 1997). Other systems are controlled by promoters based on sugar utilisation, e.g. the lactose operon promoter (Payne et al. 1996), by presence of salt, *e.g. gadC* promoter (Sanders et al. 1997), pH decrease (Madsen et al. 1999), or temperature up-shifts (Nauta et al. 1997) (reviewed by de Vos, 1999). Targeted gene replacement or removal and inactivation of genes can also be applied via (non-replicating) vectors using the natural event of crossing-over during cell division and DNA replication (Leenhouts et al. 1996). Compared to the use of replicating vectors in GM-LAB that result in new or enhanced cellular behaviour, the deletion of genes after double cross-over events (by using a non replicating plasmid) does not result in the addition of any DNA to the genetic content of the cell.

A specific aspect related to the application of vectors in industrial strain improvement is the use of selection markers. The use of antibiotic resistance markers might result in transfer of antibiotic resistance from one organism to another. As a consequence, the practical value of antibiotics that are used for human health or in veterinary practice will be severely reduced. Hence, food-grade resistance markers are preferred. Currently there are many food-grade selection markers for vector cloning in LAB. For instance, transfer of the α-galactosidase gene (*aga*) and a gene coding for a putative transcriptional regulator from the LacI/GalR family (*galR*) of *Lactococcus raffinolactis* ATCC 43920 into *L. lactis* and *Pediococcus acidilactici* strains modifies the sugar fermentation profile from melibiose negative (Mel(-)) to melibiose positive (Mel(+)) (Boucher et al. 2002). A similar food-grade vector is based on complementation of the lactose operon in *L. lactis* NZ3600 or *L. casei* by introduction of *lacF*, or *lacG,* respectively, enabling growth on lactose (MacCormick et al. 1995; Platteeuw et al. 1996, Takala et al. 2003). An alternative system is based on a suppressor tRNA allowing growth in milk of a purine auxotrophic strain (Dickely et al. 1995). A newly developed food-grade marker is characterised by the requirement of D-alanine in the medium to enable growth of the micro-organisms (Bron et al. 2004). An overview of general strategies for constructing food-grade markers has previously been reported (de Vos, 1999). As mentioned before, the current use of these food-grade markers if constructed via self-cloning techniques is restricted to applications with contained use.

Other targeted modifications of the genetic content of DNA may occur via conjugation and transduction (Gasson, 1990). These processes are considered natural events. According to the current legislation, bacteria that are changed by using these transfer systems are not considered as GMOs (Table 1).

Examples of GM-LAB engineered via directed genetic alterations

Directed mutagenesis is widely applied in research to improve fermented food products. The following paragraphs will provide an overview of successful examples of metabolic engineering aimed at the improvement of certain characteristics of food products, like flavour, structure, shelf life, nutritional value, or product performance in general. A schematic overview of GM-LAB is given in Table 2.

The flavour and flavour stability of buttermilk was improved by inactivation of the *aldB* gene encoding α-acetolactate decarboxylase (Swindell et al. 1996). The over

Table 3. Practices of directed genetic alterations of LAB.

• Improved LAB made by introduction of plasmids via the natural event of conjugation or transduction.
• Improved LAB made by passage (introduction and subsequent elimination) of a recombinant plasmid.
• Improved LAB constructed by deletions of DNA as a consequence of double cross-over recombination.
• Improved LAB constructed by self cloning that does not change the total genetic make-up of a LAB cell.
• Insertion of DNA from micro-organisms with a long tradition of safe use in the food industry.
• Insertion of DNA from micro-organisms without a long tradition of safe use in the food industry
• Insertion of DNA from other organisms

expression of peptidase genes in LAB via self-replicating plasmids could also enhance flavour formation and cheese ripening. Peptidolytic activity can be increased by overproduction of peptide degrading enzymes originating from the same species or from other species. An example of the self-cloning strategy is the overproduction of an aminopeptidase (PepN) by *L. lactis* (Gasson and de Vos, 1994). GM-LAB with increased proteolytic propertied are generated by the over expression of the genes encoding PepN, PepX, PepC and PepI peptidases from a highly proteolytic *L. helveticus* strain (Joutsjoki et al. 2002) or PepI, PepL, PepW, and PepG from *L. delbrueckii* (Wegmann et al. 1999) into *L. lactis*. Moreover, the expression of *gdh* from *Peptoniphilus asaccharolyticus*, encoding glutamate dehydrogenase, into *L. lactis* increases the production of α-ketoglutarate (Rijnen et al. 2000). This enhances the degradation of amino acids, which also benefits the cheese ripening process.

Food fermentation processes that, because of size, cannot be operated under strict axenic conditions, may suffer from bacteriophage infections resulting in lysis of starter bacteria. The transformation of industrially important strains with phage resistance genes from other LAB could generate new phage resistant strains. Engineering of starter strains aimed at inhibiting phage development is possible by creating a system of lethal gene induction upon phage infection (Djordjevic et al. 1997) or by production of antisense RNA inhibiting translation of phage RNA (Kim et al. 1992; Walker and Klaenhammer, 1998). The inactivation of a phage infection protein, *pip*, involved in phage adsorption and DNA injection resulted also in a phage resistant *L. lactis* strain (Monteville et al. 1994). The lactococcal abortive infection mechanisms AbiA and AbiG were introduced into *Streptococcus thermophilus*, and a range of phages capable of infecting this host were sensitive to these mechanisms (Tangney and Fitzgerald, 2002). The quality of food fermentation processes could be further improved when they are protected from spoilage by other bacteria. The expression of the food-grade lantibiotics in dairy starter strains could prevent spoilage from Gram positive bacteria during the process of cheese ripening (Horn et al. 1999). The production of lantibioticsto prevent growth of *Listeria monocytogenes* and *Lactobacillus fermentum,* during the production of cheese was also achieved via the non-GMO approach of conjugation (Hugenholtz and de Veer, 1991; O'Sullivan et al 2003). Bacteriophage-resistant dairy starter cultures were also selected after the natural event of transduction (Hill et al. 1989; Gasson, 1990) or after conjugation (stacking) of plasmids with phage resistant genes (Mills et al. 2002).

The event of phage induced cell lysis is undesirable during early stage of cheese making, but cell lysis in a later stage of the fermentation can actually decrease ripening time and enhance flavour due to the release of many enzymes. The controlled production of lysin and holin by bacteria has created the possibility to induce cell lysis at desired states of food fermentation (de Ruyter et al. 1997).

Another important feature of food products is texture. Complete gene clusters, encoding exopolysaccharide producing enzymes have been transformed from one LAB strain to another one. The newly generated strains could influence viscosity and texture of the fermented product (Germond et al. 2001). In *S. thermophilus* the phosphoglucomutase gene was inactivated resulting in improved exopolysaccharide production enhancing the viscosity of the fermented food product (Levander et al. 2002). Engineering of exopolysaccharide production in *L. lactis* was also achieved by using a self-cloning strategy.

Genetic engineering using genes from non related micro-organisms could also be used to produce high added value products, such as l-alanine. By introduction of a *Bacillus subtilis* (formerly *B. natto*) alanine dehydrogenase gene into a *L. lactis* strain deficient in lactate production, pyruvate conversion was pushed in the direction of alanine. The subsequent inactivation of the host gene encoding alanine

racemase leads to the production of the stereo specific and thus highly valuable l-alanine (Hols et al. 1999).

The nutritional value of fermented foods can be improved via use of GM-LAB. It was shown that by overexpressing the complete riboflavin gene cluster (four genes) via a multi copy plasmid and strong promoter, riboflavin production reached levels as high as 8 mg/L (Burgess et al. 2003). With this production level in fermented dairy products, average servings would already contain the DRI of riboflavin. Another important vitamin in the human diet is folate. This B-vitamin decreases the risks of neural tube defects in newborns and lowers the concentration of homocysteine in the blood, which is a risk factor for the occurrence of cardiovascular diseases. Nowadays, a large part of the population in both developed and under-developed countries does not meet the DRI of folate (200-400 µg). By the overexpression of all the genes encoding for the folate biosynthesis pathway in *L. lactis*, folate levels ranging from 5 - 8 mg/L can be achieved (Sybesma et al. 2003a). This could result in fermented food products that require an intake of less than 100 ml to meet the DRI for folate. It should be stated here that both riboflavin and folate-overproducing strains were developed as proof of principles, and in the current state contain plasmids with chloramphenicol resistant markers. These strains could only be applied in food fermentations after the substitution of the antibiotic resistance marker with a food-grade marker. Another line of research focuses on control of the bioavailability of folate produced in fermented foods. In nature, folate predominantly exists as polyglutamyl folate, however folate is absorbed as monoglutamyl folate. Several studies show that the bioavailability of monoglutamyl folate, which does not need enzymatic deconjugation, is higher than that of polyglutamyl folate. The overexpression of the first enzyme in the folate biosynthesis pathway increases the production of monoglutamyl folate (Sybesma et al. 2003a; Sybesma et al. 2003b).

Recently, the engineering of a *L. lactis* strain was described that contributes to the formation of lactose-free and naturally sweetened foods. By the deletion of three genes involved in glucose uptake and degradation, and introduction of the genes for lactose-PTS and tagatose-6P pathway, the strain can grow on lactose. The galactose-moiety of lactose is used as a C-source, whereas the glucose moiety is completely excreted (Pool et al. 2003). This GM-LAB obtained by self-cloning has identical properties to the earlier selected non-GM-LAB of Thompson (Thompson et al. 1985). However, the genetic background of the latter strain was spontaneously altered and, except for the desired mutations, it is likely that several non-anticipated mutations have occurred (de Vos and Hugenholtz, 2004).

An example of the use of recombinant DNA technology in the development of probiotic strains is in removal of undesirable sugars from the gastro-intestinal tract. α-Galactosides such as raffinose and stachyose, that are present in raw agromaterials like soy, cause digestion problems in a lot of people. Because humans do not produce intestinal α-galactosidases, these sugars pass into the lower GI-tract where they are fermented by gas-producing bacteria, rendering intestinal pain and flatulence. The expression of genes encoding enzymes that are able to degrade raffinose, stachyose, etc, such as α-galactosidases (Boucher et al. 2002; Silvestroni et al. 2002), in certain LAB, results in the (extracellular) production of sugar-degrading enzymes. Consequently, upon passage of these sugars through the GI tract, they will be degraded by the probiotic LAB and not by the resident gas-forming bacteria (Piard, 2003).

In the feed industry, strain improvement can also be successfully applied. *Lactobacillus plantarum* is used as an inoculum in grass silage and vegetable products due to its pH lowering effect, which allows preservation of the products (Seale, 1986; Daeschel et al. 1987). The cloning of the phytase gene, *phyC*, from *Bacillus subtilis* in *Lb. plantarum* has recently been described (Kerovuo and Tynkkynen, 2000). Phytases are enzymes which hydrolyse phytate. These enzymes have a significant value as feed additives. The application of a phytase-producing silage strain in feed preparation may improve the nutritional value of feed and, at the same time, reduce the environmental phosphate pollution following consumption by monogastric production animals. Another GM-LAB designed to improve the nutritional value of feed is an amylolytic *L. plantarum* silage strain with improved starch degrading capacity (Fitzsimons et al. 1994).

RISK FACTORS FOR USE OF GENETICALLY MODIFIED LACTIC ACID BACTERIA

The process of introducing novel traits into LAB by the addition, substitution, removal, or rearrangement of defined DNA sequences, including the DNA sequences used for the maintenance or transfer of the new DNA into the recipient strain could lead to the generation of LAB with novel genetic properties and a modified cellular behaviour. It is anticipated that this will result in the intended effects. However, unintended effects, that could be a consequence of the modification, could also occur in the newly generated LAB. A distinction can be made between the predictable and unexpected effects that differ in their origin. Predictable effects are a foreseeable and direct consequence of the intended genetic alterations and unexpected effects are caused by unintended genetic alterations, or they may occur as a consequence of predictable effects.

The predictable unintended effects

The predictable unintended effects could occur as a consequence of directed genetic alterations. For example, by insertion or modification of genes that are located in an operon, the expression of adjacent genes that are present in the same operon may be affected (polar effects). Also the expression of the inserted gene and subsequent production

of a protein may result in potential toxicity or allergenicity of this protein, especially when posttranslational modifications in the recipient micro-organism may be different from the original host of the new protein. Another kind of predictable unintended effect may arise as a result of the synthesis of a chimeric protein (fusion protein), due to the insertion of foreign DNA. These new proteins may provoke a different type of cellular behaviour, but they might also lead to toxicity or allergic responses by the consumer because of their different chemical structure. Careful analysis of the genomic organization prior to and following the directed genetic alterations could be used to avoid the occurrence of such predictable unintended effects. For instance, the use of well-characterised loci for the insertion of the new DNA may prevent the effects on expression levels of adjacent genes.

It should be noted that despite the fact that strains obtained via random mutagenesis are not considered as GMOs, the occurrence of such predictable unintended effects is very likely to occur within such mutant strains as well. In addition, it is possible that DNA elsewhere in the genome will also be affected by random mutagenesis. This could result in synthesis of modified enzymes and altered metabolite production (unexpected unintended effects), which might affect the health of the consumer. Therefore, it is not unlikely that certain GM-LAB may have a lower risk profile than LAB isolated after random mutagenesis.

The unexpected unintended effects

The occurrence of unexpected and unintended effects could be a consequence of unintended genetic alterations. However, these effects could also be caused by the direct intended effect or by the predictable unintended effects of the alterations. The overproduction of certain desired metabolites or blocking pathways preventing the production of specific metabolites in the cell may result in unexpected and undesired cellular responses. For instance, a LAB defective in lactate dehydrogenase capacity may produce other acids and alcohol under certain environmental conditions. Increased concentrations of desired metabolites may also result in feedback inhibition of parallel biosynthetic pathways in the cell. Hence, a careful analysis of alterations in metabolism is desirable. Recently it was shown that inactivation of *ldhD* (responsible for the formation of D-lactate) in *Lactobacillus johnsonii*, primary and secondary end products were not easily redirected in high amounts to other pathways (Lapierre et al. 1999). Another unexpected and unintended effect may occur when the overproduction of one product inhibits the production of another compound. For instance, the biosynthesis of the vitamins folate and riboflavin starts with GTP as a substrate. An increased flux through the riboflavin biosynthesis pathway, as found with *L. lactis* producing increased riboflavin levels (Burgess et al. 2003), might reduce the concentration of GTP available for folate biosynthesis. As a consequence, folate production levels may be decreased.

Another class of unexpected unintended effects is the change of the microbial population when GM-LAB are applied in mixed cultures. Several starter strains may grow simultaneously or in succession. For instance, during yoghurt fermentation, *S. thermophilus* grows prior to *L. bulgaricus* which only starts growing after acidification of the milk by the *S. thermophilus*. During the fermentation of sauerkraut, heterofermentative bacteria such as *Leuconostoc mesenteroides* initiate the fermentation, and after 3 to 7 days it is usually succeeded by the more acid-tolerant homofermentative *Lactobacillus* species, while *Lactobacillus plantarum* completes the fermentation (Lu et al. 2003). Hence, the synthesis of new metabolites, specifically those with antimicrobial activity, may disturb the balance in a mixed culture resulting in unintended changes in the final product.

The phenomenon of gene transfer can probably be seen as one of the most important unexpected unintended effects. In the previous section many examples are given about self-replicating vectors to introduce additional DNA in the LAB cell. The production and consumption of GM-LAB will result in their contact with micro-organisms in the environment, including the intestinal microbiota of the consumer. Consequently, transfer of DNA between these micro-organisms may occur (horizontal gene transfer) (Jain et al. 2002; Doolittle et al. 2003). Transfer between the GM-LAB and the consumer has not been demonstrated (Genereux and Logsdon, 2003). Horizontal gene transfer is not restricted to plasmid-based DNA, as other modes of gene transfer such as those mediated by transposons, phages, or naked DNA, can also take place (Droge et al. 1998). In case a selective advantage is conferred by transferred DNA, the likelihood of stable gene transfer may be higher. For this reason the use oftransferable antibiotic resistance markers in fermenting micro-organisms should not be allowed, because these could provide a selective advantage following transfer to intestinal micro-organisms as soon as consumers are treated with antibiotics. This is a realistic scenario since there is evidence indicating that gene transfer of antibiotic resistance genes has occurred to bacteria that are present in traditionally fermented foods (Teuber et al. 1999). In contrast, the inactivation of genes by deletion does not result in the addition, but in the omission of DNA. Consequently, any discussion about potential gene transfer in GM-LAB with inactivated genes is void. It should be noted that the potential for the occurrence of unintended effects is not restricted to the use of recombinant DNA techniques. Rather, it is an inherent and general phenomenon that can also occur in the development of strains using traditional genetic techniques and procedures, or from exposure of micro-organisms to the intentional or unintended selected pressure. An overview of these concerns and other potential effects of food-related genetically modified micro-organisms is provided by von Wright and Bruce (2003).

CONSIDERATIONS FOR RESTRICTIONS IN DEVELOPMENT GM-LAB

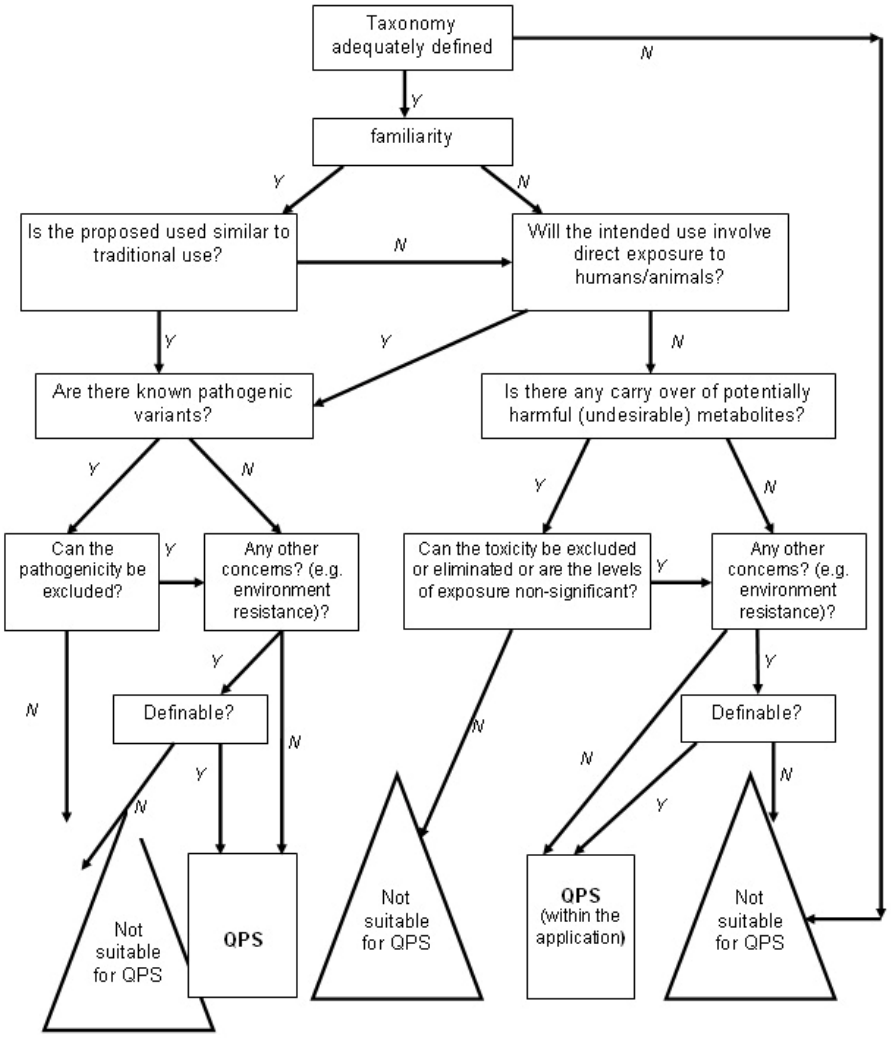

Figure 1. A general scheme for the assessment of suitability for QPS status of micro-organisms (as proposed by EU, 2003).

Partially following earlier recommendation (Wymer, 1998), and to ensure the safety of humans and the environment, the following restrictions regarding the uncontained use of GM-LAB are recommended: 1. Only LAB with a qualified presumption of safety (see below, EU, 2003) should be used as a recipient; 2. The use of transferable antibiotic resistance markers should not be permitted; 3. The expression of distantly related genes in LAB is acceptable under contained use only, unless the inserted gene will not induce growth or survival advantages to other micro-organisms after potential horizontal gene transfer; 4.The strain engineering should never result in production of toxic or allergic compounds or generation of a pathogenic strain that may affect human health.

Food-grade micro-organisms

The use of micro-organisms in food production is accepted when they have a long history of safe use. However it is not scientifically defined what is a long history and what is a safe use. Moreover, a recent draft document (EU, 2003) suggests that only micro-organisms that have a long and safe history of use in food and that also have a qualified presumption of safety status (see below) should be applied in the food industry. These strains could be used as recipients for genetic modification. The use of pathogenic micro-organisms should not be allowed. However, the history of safe use in food should not be an everlasting guarantee that the strain could always be applied in food fermentation processes. If new research shows that strains with a long history of safe use are producing toxic components in levels that may harm human health, these strains should not be accepted anymore for use in food fermentations. For instance, certain LAB have an unblemished history of safety in food fermentation, but, as was discovered later, may produce unfavourable amines under some conditions (Ten Brink et al. 1990; Cantoni et al. 1994; Faeth and Radler, 1994). Evidently these strains should not be used in preparation of foods without a

profound safety assessment, that, especially in this case, also investigates the actual concentrations of the harmful compound to which the consumer will be exposed.

Transferable antibiotic resistance markers

The use of transferable antibiotic resistance markers in LAB used in food production may increase the risk of their transfer to human intestinal flora, and cannot be allowed. The use of safe and sustainable food-grade selection markers is recommended (de Vos, 1999). However, contrarily to current legislation, the use of antibiotic resistance markers in intermediate strains that are used for the generation of new bacterial strains with improved characteristics, and that are completely removed before the final production strain is developed, should not be rejected on the basis of reasons related to antibiotic resistance. For instance, the selection of the earlier described GM-LAB with inactivated α-acetolactate decarboxylase (Curic et al. 1999) should be allowed, because the vector containing the antibiotic resistance gene was removed from the selected strain. It is noteworthy that antibiotic resistance could also occur spontaneously. Well known examples include resistance to streptomycin and rifampicin that are a consequence of point mutations in rRNA or RNA polymerase genes, respectively.

A specific case is resistance to vancomycin. Strains carrying transferable vancomycin resistance genes should not be used for food fermentations. Related Gram-positive bacteria like enterococci can spontaneously acquire vancomycin resistance. In such vancomycin resistant strains, spontaneous mutations in genes encoding cell wall synthesizing enzymes may result in altered peptidoglycan intermediates bearing D-alanine–D-lactate termini that confer vancomycin resistance (Arthur et al. 1996; Baptista et al. 1997). Similarly, LAB that produce D-lactic acid show endogenous resistance to vancomycin. These naturally occurring phenomena however, are not a reason to exclude such strains from consideration as recipients in constructing GM-LAB, since this endogenous resistance is not transmissible.

Specific consideration needs to be given to tolerance to antibiotics that is caused by certain classes of transporters. Many of these are naturally present in LAB and may be essential for their survival in certain environments, where they could be involved in export of toxic compounds. It is conceivable that these transporters also export structurally related compounds, such as some antibiotics. This is likely to be the case for the endogenous tetracycline resistance found in many lactobacilli. In most cases the level of antibiotic resistance is far from that found in clinically relevant antibiotic-resistant pathogens. Hence, there appears to be no need for specific precautions when such antibiotic tolerant LAB strains are used as recipients. It should be further noted that the eventual selective pressure on these transporters caused by antibiotics used during medical treatment is not as dominant as the selective pressure caused by all the (natural) compounds that exist in nature. Therefore, it is likely that the risk of antibiotic resistance transfer via genes coding for transporter proteins from harmless bacteria to clinically important bacteria is much higher in nature than in the situation where fermenting bacteria are consumed.

Expression of homologous or heterologous genes

The potential event of horizontal gene transfer following expression of foreign genes in LAB used in food fermentations is one of the major concerns that affect the safety assessment for consumer and environment. When the applied genetic elements originate from LAB that have a long and safe history of use in food, it is often said that no new risks are introduced. Although this argumentation is very logical, it does not mean that fermented foods are by definition safe. Therefore, adequate safety assessment remains necessary. However, it could eliminate the risk of negative consequences for the environment. For microbiota in complex natural habitats it is generally assumed that all genetic information is already omni-present, but that natural constraints drive selection. Thus, fear of negative consequences after the release of engineered LAB (containing genetic elements of other LAB species in the environment is unfounded. Evidently this is also valid for strains that have been subjected to spontaneous DNA modifications, or deletions of DNA.

The introduction of more distantly related genes in micro-organisms is likely to occur in nature at much lower frequencies compared to the transfer of related genes. The cloning of such genes on a plasmid can be regarded as a potential risk factor since horizontal gene transfer could lead to selective advantage in specific niches. Therefore, some precautions are suggested for the expression of heterologous genes in LAB from non-microbial origin. As long as heterologous genes are only inserted in the chromosome via double cross over, and the inserted heterologous gene does not lead to evolutionary advantages and survival of such strains, there are no reasons to fear the consequences of potential gene transfer. Containment of such engineered strains may prevent interactions with the environment. In addition, survival in the environment can be limited by using host strains with a strict dependency on growth factors or special temperature or pH -requirements (Steidler et al. 2003).

Genes that may cause pathogenicity (*e.g.* generation of virulence factors) or that encode for enzymes that are known to be involved in synthesis of toxic or allergic compounds, or precursors of toxic or allergic compounds, should *a priori* be excluded as targets for genetic engineering. Finally, one should consider avoiding the use of sequences that mediate integration into other genomes in the development of genetic constructs. Although in nature such sequences widely exist, and their use in strain development could be regarded as safe, it is better not to

accelerate and support the process of gene transfer by the use of these sequences.

CONSIDERATIONS OF THE SAFETY ASSESSMENT PROCEDURES FOR FOODS DERIVED FROM TRADITIONAL LAB AND GM-LAB

Introduction

With the exception of those LAB not previously used in the preparation of a human food within the EU, LAB for food use are not subject to EU regulation. Implicit in this absence of any formal requirement for a safety assessment is the recognition that there has been a long history of presumed safe use. The long and safe history of LAB usage is in itself an arbitrary criterion and may need further consideration. If the long history of safe use is based on the absence of reports of the occurrence of adverse effects on consumers of fermented foods, it can be questioned whether this could also apply to certain GM-LAB. This would include LAB obtained by self cloning, or LAB that were subjected during their production process to recombinant DNA techniques, but that did not maintain the foreign DNA. This would imply the immediate acceptance of all such non-pathogenic GM-LAB for food production. However, is this whole debate not made for the assurance of safety for the society? Before regulation about food safety was an issue, society decided autonomously that it wanted to consume certain fermented foods, probably based on positive experience. These facts could be a convincing social, but less scientific, argument that LAB with a long history of safe use may be accepted in food fermentations until adverse effects are reported. The European Food and Feed Culture Association (EFFCA) has produced a list of starter species for which a documented history for use in food manufacture exists. Documented history of use is defined by the EFFCA as cultures sold for human consumption in quantities exceeding 100 kg of freeze dried culture. Although for many species belonging to LAB the safety documentation is sufficient, it is remarkable that the list also contains species that are potentially pathogenic.

Qualified Presumption of Safety

Within the EU it is proposed to introduce a system similar in concept and purpose to the GRAS (Generally Recognized As Safe) definition used in the USA, which could be applied to micro-organisms and their products. Such a system should lead to a listing of qualified micro-organisms that will not have properties that may adversely affect human health or the environment (EU, 2003). For this purpose, Qualified Presumption of Safety (QPS) is being introduced as an assumption based on reasonable evidence. It aims to provide a qualified generic approval for non-genetically modified micro-organisms, without requiring all organisms used in food production with a long history of safe use to be subjected to a full and unnecessary safety review. A case-by-case safety assessment then could be limited to only those aspects that are relevant to the organism in question. A pre-requisite for QPS would be that identity is unambiguously established at the taxonomic level claimed. Appropriate and state of the art biochemical and molecular biological methods must be applied to realise this. For organisms not commonly used in food production or without a long history of safe use, this implies the need for experimental data on the genetics of the taxonomic unit and the growth and biochemical characteristics of the component strain under a variety of relevant environmental conditions. For some groups of organisms, such as those used as biocontrol agents for plant protection, a consideration of the impact on the wider environment may be appropriate. However, this could exclude organisms considered either to be of uncompromised origin and regularly introduced into the wider environment or originating from soil or water. In both cases the organisms are naturally occurring and therefore free of any need for an environmental impact assessment. The QPS could only be valid for bacteria that enter the food chain and that are free of any acquired resistance to antibiotics of importance in clinical and veterinary medicine. The presence of antibiotic resistance determinants would not exclude their safe use for production purposes provided that only the fermentation products are retained in the final product. A decision scheme relating to the concept of QPS has been proposed and is presently subject to discussion (Figure 1) (EU, 2003). It is worthwhile mentioning that this QPS decision protocol might be subjected to modifications before its implementation. The decision step questioning other concerns related to safe use of micro-organisms, which are not involved with pathogenicity or toxicity, is currently not well defined.

Many industrial strains will be the product of mutagenesis and selection programs designed to improve their phenotype for a particular purpose. These cryptic mutations will not affect taxonomic status. As a consequence, following the QPS decision tree, strains that are not produced via genetic modification, but contain altered DNA sequences that may cause predictable or unexpected unintended effects, will be accepted for use in food preparation without a further safety assessment. The next paragraph considers whether such strains of LAB should be subject to similar scrutiny as GM-LAB.

Should traditionally fermented foods be subjected to similar safety assessment procedures as novel foods?

Due to the long history of use of many LAB, the resultant fermentation products are obviously not systematically assessed for safety. Moreover, food products containing improved strains obtained after random mutagenesis are not assessed either. It could be reasoned that the omission of adequate safety assessment in the past does not render the fermented foods safe for the consumer and the environment in the future. However, in the debate about safety of food derived from genetically modified microorganisms, the point is not to demonstrate that novel foods are completely

risk-free, but that the risks are comparable to those associated with traditional foods. The safety assessments that have been conducted thus far are primarily aimed at discrete chemical entities such as food additives, or specific chemical or microbial contaminants that pose identifiable hazards and risks.

It is likely that unintended effects could always occur in the development of strains using traditional (non-recombinant DNA) techniques, or from exposure of micro-organisms to selective pressure or certain environmental conditions, or just by natural events. This would justify that each new natural LAB strain should undergo the same safety assessment procedure as engineered strains. Whether this is a viable approach is currently being investigated by analysing differences at the global transcriptional level in strains isolated by random mutagenesis and by directed mutagenesis (Renault, 2001). The development of GM-LAB for use in food should be focused on minimizing the occurrence of unintended effects with adverse consequences on human health. Risk assessment studies using new technologies should focus on global response in cellular metabolism as a tool to identify unintended effects.

It could be considered that in the future the decision to grant a QPS status to strains that are isolated after spontaneous or induced random mutagenesis, or to assess those strains using a profound safety assessment procedure as obliged for novel foods, would be taken after the performance of representative comparative profiling studies. These may include transcriptomics, proteomics, and metabolomics, between the mentioned strains and their natural conventional counterparts. In case of no relevant differences between the strains, the QPS protocol could be applied. In analogy with the outcome of these studies, the profiling of engineered strains obtained by directed genetic alterations may result in a more scientific food safety assessment procedure for novel foods derived from these GM-LAB, in which not the technical aspects, but the final products are assessed[2].

Consideration of safety assessment procedures of GM-LAB and novel foods

The safety assessment of food produced by GM-LAB should comprise analysis of the intended effect, the nature of the modification, and detectable unintended effects that may occur in the micro-organism or in the food that is producedby the fermenting micro-organism. Furthermore, issues that are specific to the use of recombinant GM-LAB include the genetic stability[3], the potential for gene transfer (see above), the colonisation of the gastrointestinal tract and the persistence therein, interactions that the recombinant-DNA micro-organism may have with the gastrointestinal flora or the mammalian host, and any impact of the recombinant-DNA micro-organism on the immune system. The development of modern genetic techniques could enhance the safety assessment, and the follow up by post market surveillance may also contribute to the safety assessment and increase the consumers' trust. Furthermore, the safety assessment procedure should encompass the obligations stated in directive 2001/18/EC and Commission Recommendation 97/618/EC (summarized in Table 4).

Toxicity and allergenicity

Because the nucleic acid techniques enable the introduction of DNA that can result in the synthesis of new substances in the LAB, specific attention should be paid to the toxicity and allergenicity of the newly synthesised compounds (proteins and metabolic products). When a substance is new to foods or food processing, the use of conventional toxicology studies or other applicable studies on the new substance will be necessary. In the case of proteins, the assessment of potential toxicity and allergenicity should focus on amino acid sequence similarity between the new protein and the known protein and anti-nutrient activities (*e.g.* protease inhibitors, lectins) as well as stability to heat or processing and to degradation in appropriate representative gastric and intestinal model systems. Appropriate oral toxicity studies may need to be carried out in cases where the protein present in the food is not similar to proteins that have been previously consumed safely in food, taking into account the biological function of the protein in the donor organism where known. At present there is no definitive test that can be relied upon to predict allergic response in humans to a newly expressed protein. However, it could first be assessed whether a newly expressed protein is one to which certain individuals may already be sensitive as well as whether a protein new to the food supply is likely to induce allergic reactions in some individuals as could be predicted by amino acid sequence homology and sera screenings.

The Codex Alimentarius (2003) proposes that IgE-cross-reactivity between the newly expressed protein and a known allergen should be considered a possibility when there is more than 35% identity in a segment of 80 or more amino acids. The development of international serum banks would enhance efficient testing for allergic potential of the newly expressed proteins. Strategies such as stepwise contiguous identical amino acid segment searches may also be performed for identifying sequences that may represent linear epitopes. The size of the contiguous amino acid search should be based on a scientifically justified rationale in order to minimise the potential for false negative or false positive results. Moreover, resistance to pepsin degradation has been observed in several food allergens and was found to correlate with the allergic potential of the protein under scrutiny. Therefore, the resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergic.

Potential toxicity of substances that have not been safely consumed in food before should be assessed case-by-case

on the basis of the identity, concentration, and biological function of the substance and dietary exposure. The type of studies to be performed may include evaluations of metabolism, toxicokinetics, chronic toxicity/carcinogenicity, impact on reproductive function, and teratogenicity. It is important that in such studies the stability of recombinant DNA in lactic acid bacteria is also considered. It is also important to ascertain the impact of changes on the overall nutrient profile and to verify to what extent the modified nutrients are bioavailable and remain stable during processing and storage.

Recombinant LAB that remain viable in foods may interact with the immune system in the gastrointestinal tract. Closer examination of these interactions will depend on the types of differences between the recombinant-DNA micro-organism and its conventional counterpart.

Modern DNA techniques, genomics, transcriptomics, proteomics, and metabolomics

The rapid development of genetic and biochemical techniques can be applied to increase reliable, fast and profound safety assessment of the fermenting micro-organisms and the derived foods, in accordance with the concept of substantial equivalence. It is preferable that the unintended effects could be foreseen at an early stage so that potential harmful effects could be analysed beforehand. The presence of new proteins or the change in concentrations of certain proteins could point to the altered activity of biochemical pathways that may result in changed metabolite content. The analysis of the complete set of metabolites in the cell could also provide information about potential toxicity and about the general nutritional value.

Genome wide analysis of modified strains and of their natural counterparts, for instance by using fingerprint techniques, or by complete sequencing of the genome, can reveal all loci in the genome where mutations have occurred that could result in unintended effects. Transcriptome analysis, by using DNA array or DNA chip technology, could provide information about altered expression patterns of the new micro-organisms compared to the wild-type strain. The observation of altered expression levels of genes not involved in the targeted effect of the genetic engineering could be a starting point for the analysis of the reaction products of the enzymes coded for by the genes of interest. This might be done by studying the literature and by application of bioinformatics, and, if a harmful compound may be produced, subsequent biochemical analysis could be performed to measure the presence of these metabolites. More relevant for food safety assessment is an analysis of the proteome and metabolome of the modified micro-organisms. Proteome analysis, by using 2D-gel electrophoresis, 2D-HPLC or advanced mass spectrometry, provides information on altered protein concentration and content produced by the modified micro-organisms. Likewise the outcome of the transcriptome analysis, the reaction products of the new proteins or increased protein concentrations themselves could be predicted and analysed.

The value of proteomics has already been proven in genetically modified rice that was engineered to produce more storage proteins. SDS-PAGE analysis revealed an unexpected increased level of prolamins (Kubo, 2000). Metabolome analysis, by using a wide range of chromatographic and spectroscopic techniques, could provide information about the presence of new metabolites or altered concentrations of existing metabolites. Increased concentrations of known metabolites could be compared with current acceptable daily intake levels to assure the consumer's safety. Likewise, decreased concentrations of vital components could reduce the nutritional value of a new product. The identification of new proteins, or altered protein levels, new metabolites, or altered metabolite levels, could be a starting point for further toxicological studies. To strengthen the safety assessment, transcriptome, proteome and metabolome analyses under food processing and GI-tract conditions should be performed in case the new engineered strains have potential for survival in the human gut. The outcome of these data analyses will also provide information on the occurrence of the intended and, if present, of the unintended effects. The conclusion of the analysis should be the assurance that neither the intended effects, nor the unintended effects, have adverse effects on human health, because no harmful components or proteins with allergic characteristics are being produced.

In the near future it is possible to develop a universal proteome and metabolome reference frame consisting of all proteins and compounds produced by fermenting micro-organisms. The proteome and metabolome reference frame should contain information on potential allergenicity of proteins, based on amino acid sequence comparison with known allergens, and information about acceptable maximum and minimum levels of all compounds present in food. The changed protein and metabolome content of any new engineered strain can then be easily compared with the reference frame. Comparison of the proteome and the metabolome of the new engineered strains with the reference frame could be a starting point for further food safety assessment procedures and could ensure that further safety assessment remains proportional to the perceived risks. For instance, if it could be verified that proteins or metabolites are not produced in harmful concentrations and that the nutritional value is not adversely affected, no further safety assessment would be required for the fermenting micro-organisms and the fermenting food.

Post-market surveillance

A post-market surveillance (PMS) on novel foods, involves the observation of (health) effects on the consumer over a longer period of time after release of the novel food on the market. It could be questioned whether, after approved pre-market safety assessments of genetically modified foods, the use of post-market surveillance would still be

Table 4. Essential information for safety assessment of novel foods. (As laid down in Commission Recommendation 97/618/EC of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients and the preparation of initial assessment reports under Regulation (EC) No 258/97 of the European Parliament and of the Council).

I. Specification of the novel foods.
II. Effect of the production process applied to the novel foods.
III. History of the organism used as the source of the novel foods.
IV. Effect of the genetic modification on the properties of the host organism.
V. Genetic stability of the GMO used as novel foods source.
VI. Specificity of expression of novel genetic material.
VII. Transfer of genetic material from GMO.
VIII. Ability of the GMM to survive in and colonize the human gut.
IX. Anticipated intake/extent of use of the novel foods.
X. Information from previous human exposure to the novel foods or its source.
XI. Nutritional information on the novel foods.
XII. Microbiological information on the novel foods.
XIII. Toxicological information on the novel foods.

necessary. However, theoretically, novel foods may contain undetected anti-nutrients or toxicants which may lead to (delayed) adverse effects on the health of humans and animals. Moreover, various other factors may also be involved in determining the safety of food in the long run, such as the consumption conditions and the genetic profile of the consumer. Therefore, to further protect the general public, including specific sub-populations such as immuno-compromised individuals, infants and the elderly, and to provide additional information on long term effects, PMS could be applied in the form of a recording and investigation system by national health services, perhaps similar to the adverse drug effects reporting systems used by physicians. Up to now only a few food additives have been subjected to PMS, including the artificial sweetener aspartame (Butchko et al. 1994) and the fat replacer olestra (Slough et al. 2001). Recently within the EU, PMS has started on a novel food related to the use of phytosterol-esters in yellow fat spreads that is assumed to have cholesterol lowering effects in the consumer (Lea, 2002). However, it may be difficult to introduce PMS over a longer time period if the food consumption pattern of individuals is not consistent. It should be taken into account that some consumers might be exposed to newly introduced components from different sources during their daily consumption pattern and consequently might be at risk because of overexposure to certain compounds. PMS could also be used to verify the conclusions about the absence of potential consumer health effects, and it could eventually monitor changes in nutrient intake levels that could occur by the introduction of foods that are likely to significantly change the nutritional status.

Although the application of PMS to food products raises significant technical issues, particularly for active surveillance, passive surveillance based on spontaneous reports could be utilised. Apart from this, it could be considered that for novel foods derived from GM-LAB that are substantially equivalent to LAB with a QPS status, PMSwould not be necessary.

An effective PMS contains the following elements (Health Council of the Netherlands, 2002): i) Setting up a government supported line for all consumer complaints associated with health and foods. This would enable any side effects produced by a product to be traced, provided that they arise soon after consumption and that they attract attention. One such example is food allergies. This monitoring system will not reveal any causal relationships; however, it can be used to generate a hypothesis. In this way it would alert people to health problems, leading on to a focused follow-up study; ii) Continuous monitoring of consumption of foodstuffs, carried out jointly by government and industry. One precondition is that this must

facilitate a detailed breakdown of data on individual products into information on their ingredients (down to the molecular biology level in the case of genetically modified foods); iii) Long term epidemiological, prospective, cohort studies into the relationship between chronic diseases and diet; and iv) An active market monitoring program carried out by companies on novel foods that contain bio-active ingredients. The aim here is to check the accuracy of the presumed (safe) intake of the target group (Health Council of the Netherlands, 2002).

It may be concluded that PMS could only prove its value by signalling hazardous events resulting in immediate withdrawal of the concerned food. PMS could not be seen as a safety assessment, but as an early warning system that operates better as the adverse effects are stronger. Moreover, it could be employed if increased confidence by the consumer to use novel foods evolves, and if the principle and reasoning for setting up PMS is well communicated to the consumer.

Suggestions for cost accountability of safety assessments for novel foods

The development, approval and subsequent market release of novel fermented foods involves many economical issues for industry, consumers and society in general. Investments in research and development by the food industry will provide opportunities for employment and the development of new scientific technologies. The consumption of novel foods with added value will benefit the consumer. The introduction of products with proven health claims, may especially contribute to the generation of a healthier community, and this is expected to decrease public health costs.

The costs associated in the development of novel foods can only be worthwhile in a society that accepts and buys these novel foods. The introduction of traits that decrease production costs for the manufacturer could be the impetus to reduce the price as a way to generate an additional advantage for the consumer.Conversely, the introduction of traits that benefit the consumer might lead to added value of the product that may justify a price increase to the consumer. Moreover, novel traits could also be developed that have a lower environmental load and that bring advantages to the society as a whole. It is expected that any obligation to perform a profound safety assessment, before the launching of any novel food, could significantly add to the initial costs to be made by industry. The question is, who should cover these costs: the industry, the consumer, national authorities, or supra national authorities?

Finally, although each food producer is responsible for the safety of the products placed in the market, in many countries food safety is principally an issue for the regulating authorities. Therefore, it could be proposed that public money could be used to set up the complete infrastructure required for food safety assessment as stated in the paragraphs above, including development of profiling techniques based on transcriptomics, proteomics, and metabolomics. Consequently, it would also be the task of regulatory authorities to control and conclude the whole safety assessment protocol. On the other hand, the industry might be expected to participate as well in the process of assessing novel foods derived from GM-LAB. After approval of the novel food, PMS would remain acombined matter for the society and the industry.

The high costs associated with the development and assessment of novel foods may be a limitingfactor for the industry to develop novel foods, especially those with interesting traits based on health benefits for the consumers. Therefore the industry should be allowed to promote novel foods with scientifically proven advantages over traditional foods by use of labelling and other forms of publicity. Currently the possibilities for promoting scientifically proven functional foods are limited and not harmonised within various EU countries. It is evident that the consumer should be protected by prohibiting the labelling of untrue claims. However, not permitting the labelling of true health claims is equally wrong (Katan and de Roos, 2003). The future EU regulation, as proposed in its working paper of 2002 about nutritional and functional claims (EU/SANCO, 2002), seems to provide a good solution for both consumer and industry by allowing and regulating the labelling of health claims.

THE CONSUMER'S PERCEPTION

Consumer's perception is an extremely important factor for successful introduction of new technologies in the market. Leisner (2005) described the obstacles for the introduction of pasteurization and starter cultures in the late 19th century to indicate that the gap between industry, consumers and pressure groups is not an entirely new issue.

Genetic alteration of plants and animals is sometimes seen as interference with nature that could have unknown and potentially disastrous interactions with human genetics and natural ecosystems. Moreover, many consumers are extremely aware of health threats caused by unknown risks in the food supply. Green groups have been raising several questions about the environmental risks of genetically altered crops and animals. Environmental risks include the evolution of GM-plants into "super weeds," cross-pollination introducing herbicide resistance into existing weeds or introducing undesirable genetic traits into neighbouring crops, and harm to non-target populations caused by toxins introduced to create insect resistance.

In the present work we have shown that the consequences of development and use of GM-LAB are different compared to GMO from plant or animal origins. The potential intended and unintended effects and related risks can be predicted more accurately and can be verified. In combination with profound safety assessments, safety for consumers and safety for the environment can be assured.

The occurrence of unintended effects is not unique for GM-LAB, but also occurs in LAB used in conventional food fermentations. Logically, this is no reason to ban GM-LAB, but it could be a reason to analyse the unintended effects of traditionally used strains in food fermentation by genomics-based profiling methods. A further response to the public concern is to strengthen the pre-marketing data requirements and to introduce post-launch surveillance program to confirm safety, as described above.

Besides an approval of the safety assessment, the GM-LAB designed for use in food production is probably more acceptable for the consumer if a clear correlation with consumer benefits can be made. Therefore, labelling on food products should emphasise the benefits (eventually health claims) of the novel foods. Evidently, product labels should also inform the consumer about the nature of the (genetically modified) bacteria, the risk assessment procedure, and the nature of the conventional counterpart. However, it is questionable why, according to the new EU legislation, food and feed products and additives that are derived from GM-LAB should be labelled with such a characteristic when there is no DNA or protein derived from a GM-LAB detectable in the final product[4].

A parallel strategy that could be adapted to increase trust among consumers relates to the liability of the manufacturers in case of damage caused by novel foods. Despite profound pre-market safety assessment that is expected to be conducted before the introduction of novel foods onto the market, unpredicted adverse effects directly related to the consumption of the novel food can theoretically not be excluded. The industry and its products would probably gain support if they accept the liability for such events. However, it would not be reasonable to establish an unlimited liability for the industry. Therefore the liability for predictable adverse effects associated with the consumption of novel foods could be placed onto the consumer as long as the labelling includes warnings for the specified adverse effects that could occur in sub-populations. Moreover, the free choice of the consumer should be respected. Thus, alternative similar products, although they will not possess the introduced advantageous traits, should be widely available on the market.

A complex and harmonised legislation and regulation system seems at present times the only correct response to consumer demand for protection from the perceived harms that could be caused by GMOs. However, it remains to be seen if the consumers would accept that as a guarantee for the safe consumption of novel foods. For the future it could be recommended to set up governmental sponsored research programs with the ultimate goal of further reducing the uncertainty and mistrust within society. Educating the public on these matters may help to overcome the negative emotions related to the use of certain GMOs.

CONCLUDING REMARKS

The low degree of trust by the general public in the (food) industry, in the regulatory authorities and in the (food) scientific community is a major element contributing to the general negative perception of GMOs in the EU. The set up of an infrastructure by the regulatory authorities to subject all fermenting micro-organisms and fermented foods to a new and profound risk assessment procedure might be the extra step that is currently needed to convince all parties involved that novel foods are as safe as traditional foods. A distinction within the discussion and legislation between food safety assessment procedures for fermented foods (dealing especially with LAB), and GMOs derived from plant and animal origin, is necessary in the current controversy. As a starting point, the QPS protocol (EU, 2003), based on taxonomy and on the history of safe use of LAB applied in food, could in the near future be applied to any kind of LAB or GM-LAB provided that a series of modern profiling methods are used to verify the absence of unintended effects by altered LAB that may cause harm to the health of the consumer.

Objective safety assessments of GM-LAB could be a way to improve the current legislation. It is expected that knowledge of genome evolution and profiling studies will reveal that in many organisms the genetic material, that is naturally or spontaneously changed, has been modified more than can be established by directed alterations, including genetic modification. Direct genetic engineering, including self-cloning, generates targeted modifications that can be much better controlled and evaluated than strains that are exposed to random mutagenesis approaches. If such strains are accepted in food fermentations via QPS status, the acceptance by society of GM-LAB obtained via directed genetic alterations could be improved.

In line with current legislation, safety issues should dominate the acceptability of GM-LAB in food industry. A targeted approach should be based upon analysis of key compounds in the new food, such as proteins, carbohydrates, fats, vitamins, minerals, as well as upon analysis of toxicity and allergenicity. A non-targeted approach should use to profile methods to detect global responses to the new food. Global expression profiles will reveal whether changes additional to those expected have occurred. The expression of any new or altered gene in LAB, that is either randomly mutated or self cloned, may affect the transcriptome, proteome, and metabolome profiling and could tell if the expression of the new gene results in any (undesirable) side effects. The assessments of foods derived from modern biotechnology should be reviewed to ensure that the emerging scientific information is incorporated into the risk analysis.

In conclusion, broad evaluation of the nature of novel foods derived from GM-LAB, which should be considered as a specific group of GMOs, could be a starting point to bridge the gap between industry, consumers and green groups.

This may lead to acceptance of GM-LAB derived novel foods to provide for a better quality of life in today's society.

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Box 1. Definition of GMO according to Council Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms (repealing Council Directive 90/220/EC) and Council Directive 90/219/EC of 23 April 1990 on the contained use of genetically modified micro-organisms.

A GMO is described as an organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. This means that all organisms which are produced by means of recombinant DNA techniques using vector systems, or techniques involving the direct introduction of genetic material into an organism, or cell fusion techniques, are in principle GMOs. Organisms that have been subjected to processes involving modification of DNA by means of transduction, conjugation, polyploidy induction, cell fusion, or mutagenesis involved by exposure to certain environmental conditions, are not considered to be GMOs. (Self-cloning is not considered as a GMO under Directive 90/219/EC).

Box 2. Definition of self-cloning according to Council Directive 98/81/EC amending Directive 90/219/EC.

"self-cloning" means the removal of nucleic acid from a cell or organism, followed by the re-insertion of all or part of that nucleic acid — with or without further enzymatic, chemical or mechanical steps — into the same cell type (or cell-line) or into a phylogenetically closely related species which can naturally exchange genetic material with the donor species. Accordingly, the temporary introduction of plasmids, the deletion of specific DNA sequences, or introduction of DNA from another micro-organism belonging to the same species fall within the definition of self-cloning.

Box 3. The concept of substantial equivalence according to OECD 1993 (Safety evaluation of foods produced by modern biotechnology – concepts and principles, OECD Paris).

The concept of substantial equivalence is part of a safety evaluation framework based on the idea that existing foods or traditionally used micro-organisms in food production can serve as a basis for comparing the properties of a genetically modified food or genetically modified micro-organisms (GMM) with the appropriate counterpart. Careful risk analysis should investigate the nature and effects of new metabolites and proteins that could be produced by the GMM. The concept of substantial equivalence is not a safety assessment in itself; rather it represents the starting point that is used to structure the safety assessment of both a GMM relative to its conventional counterpart. This concept aims to identify similarities and differences between the old and new micro-organism. The safety assessment carried out in this way does not imply absolute safety of the new product, but it focuses on assessing the safety of any identified differences, so that the safety of the GMM and the GMM derived food can be considered relative to their conventional counterparts.

[1] As long as they are not substantially modified, which would result in the classification of a novel foodsrequiring a profound safetyassessment.

[2] Currently no validated and calibrated protocols for application of modern RNA, protein, or metabolite profiling methods are available.

[3] Microbial genomes are more fluid than those of higher eukaryotes; that is, the organisms grow faster, adapt of changing environments, and are more prone to change. Chromosomal rearrangements are common. The general genetic plasticity of microorganisms may affect recombinant DNA in microorganisms and must be considered in evaluating the stability of recombinant DNA lactic acid bacteria.

[4] For an overview of the recent legislation in the EU about traceability and labeling of GMOs and GMO derived food and feed products see MEMO/02/160 of the European Commission (2003).