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GENETICALLY MODIFIED MICROORGANISMS - CHALLENGES AND LIMITATIONS

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LO 3: GMMs IN FOOD PRODUCTION AND SAFETY

1. Introduction

Nowadays use of technology of genetic modification in global agriculture and supply of food and feed is quite complicated due to the active anti-GM technology policy of Green Peace, Friend of the Earth and other groups of interest. As a consequence of this activity large quantity of GMO-based ingredients manufactured for human consumption has been eliminated by EU. This anti-GMO assumption recently is directed to the use of genetically modified ingredients in livestock production. Through the introduction of genetically modified oilseeds and cereals in animal feed, European Commission in 2001 proposed legislative changes to restart the process of GMO based technologies. If case of success of this policy, it will bring important economic consequences to the EC economy. Thus, the clarification of the questions related to scientific and safety issues associated with GMMs are of crucial importance.

2. Definitions on genetically modified foods' safety

Safety and risk assessment of foods, including those obtained using GMOs, are in general considered within the range of risk assessment.

In order to find a solution WHO (World Health Organization) and FAO (Food and Agriculture Organization) defined for a number of WHO member states some definitions concerning the nature and safety of genetically modified foods.

The following definitions have been specified:

- "**Genetically modified microorganisms (GMMs)**" - microorganisms (bacteria, yeasts or filamentous fungi) in which genetic material (DNA) has been altered through non-natural approach by multiplication and /or natural recombination. This technology is often called "modern biotechnology", "gene technology", "recombinant DNA technology" or "genetic engineering".
- "**Modern biotechnology**" means the application of direct transfer into cells or organelles of nucleic acid, including recombinant one, through *in vitro* nucleic acid techniques and cell fusion (protoplast fusion and hybridization).

Regarding the food and food ingredients produced by means of GMMs, the following classifications were proposed:

- Products consisting of or containing viable GMMs.
- Products consisting of or containing non-viable GMMs.

- Products obtained by means of fermentation processes with GMMs. After post fermentation processing the GMMs have been eliminated.

3. Food safety assessment process

3.1. The principle

Microbial fermentation technology represents a rational means for production of food of high nutritional and hygienic quality, which is also historically proved. Microbial fermentation processes give about ¼ from the overall food production. It comprises foods as bread, sour dough, sour milk and cream, yogurt, cheese, pickles, fermented meet, vinegar, wine and beer. Recent introduction of modern biotechnological approaches in food production requires elaboration of new issues for food safety.

The general principles of safety assessment of GMMs in food with reference to the established principles applied for this purpose are specific to the nature and use of microorganisms in food. These principles are postulated by FAO / WHO; OECD; WHO / ILSI; EC through agreements associated with the safety assessment of novel foods including GMMs and recommendations on use of substantial equivalence. They aim at implementation of integrated stepwise approach and case-by-case study using the concept of substantial equivalence, as well as at development of guidance for the safety assessment process and elaboration of decision trees for determination the extent of testing required in specific cases. According to EC Regulation 1829/2003 the placement of GM food and feed on the market could be authorized after defined scientific assessment of any risks that they could cause on human and animal health as well as for the environment (Directive 2001/18/EC).

3.2. Specific considerations

The elaboration of the assessment procedure for safety evaluation of food produced from GMMs is subjected to the following important principles:

- Consideration of health aspects of human population including disadvantaged ones (immune compromised persons, elderly people and infants).
- Implementation of scientific data as a background of safety assessment and application of good practice in terms of assessment methods. Revision of safety assessment in respect to new data obtained.

- Detailed characterization of genetic modification procedure - i. e. description of deletion/insertion of DNA sequences, the recipient microorganism, the ultimate donor organism, the vectors applied in GMMs construction; the construct; the GMM obtained.

These principles should be applied taking into account several important reasons for safety assessment of food, manufactured trough application of GMMs:

- The way of exposure of humans to the food, or GMM itself.
- Information about possible secondary effect from gene expression, metabolic pathways in host, DNA disruption.
- Detailed characteristics of nutrient media used (macro and micronutrients) and production of side products: endogen toxicants, allergens and physiologically active substances.
- Characterization of inherent differences between microbes and plants and effect of food matrix on GMM.

3.3. Additional elements

The following elements should be taken into account in safety assessment:

- Methodological peculiarities - techniques used for genetic modification; characterization and verification of expected protein expression product of the novel DNA; strain identification and characterization (recipient, donor, GMM itself)
- Host strain - natural habitat; history of use by humans; pathogenic potential; safety and nutritional assessment (potential toxicity and nutritional aspects)
- Environmental events – gene transfer and genetic stability
- GMMs derived food - composition of food containing GMM; effects of processing, cooking and storage
- GMM - interaction between GMM, the gastrointestinal flora and the mammalian host; impact on the immune system

4. Genetically modified foods and human health – the potential risks

4.1. Risks for human health imposed by genetically modified foods' application

The assessment of safety of food obtained with the aid of GMMs is performed generally through the following investigations:

- Direct health effects (toxicity).
- Possibility to provoke allergic reaction (allergenic properties).

- Definition of specific components causing nutritional or toxic effects.
- Evaluation of inserted gene stability.
- Definition of nutritional effects associated with genetic modification.
- Unanticipated effects resulting from gene insertion.

4.2. Approaches for risk assessment

The main consequence of application of genetically modified food on human health could be summarized in three main directions: provocation of allergic reaction (allergenic properties), gene transfer, out crossing and comparative approach.

- **Allergenic properties**: Traditionally developed foods are not generally tested for allergenic properties. The main concern for GMMs food is to prove that the protein product of the transferred gene is not allergenic. Protocols for tests of GMMs foods have been evaluated by FAO and WHO. An allergic effect of GMM foods currently on the market has not been found.
- **Gene transfer**. Possibility for gene transfer from GM foods to cells of human body or microorganisms, occurring in gastrointestinal tract, represents a matter of concern if the transferred genetic material adversely affects human health. This event is relative to the transfer of genes for antibiotic resistance used for construction of GMMs into organisms at macro/micro level, mentioned above. For this reason, the use of technologies without implication of antibiotic resistance genes is encouraging, no matter that probability for this transfer is low. Recent FAO/WHO expert panel recommends this policy to scientific society working in that field.

  An illustration of this event is given by the following example. Antibiotic resistance becomes common and widespread since the corresponding antibiotics were widely used in medicine and agriculture. Regarding this, an agreement exists that any rare transfer events from ingested plant DNA to gut microflora could have no significant effect to human health. But the *bla* – TEM ampiciline resistance gene occurred in some varieties of transgenic maize is already found in ruminal *E. coli* strains and 10 – 50 % human gut strains are already ampiciline resistant. If thus event happened and human pathogens acquired antibiotic resistance through gene transfer it is possible a new route to be opened up by feeding of transgenic material.
- **Out crossing**. This term is defined as gene transfer from GM plants into conventional crops or relevant species. This could happen through mixing of crops derived from

conventional seeds with such received using GM crops, because of indirect effect on food safety and security. There are some events indicating the reality of such risk: for instance, traces of maize type which was used only for feed purposes appeared in maize products for human consumption in the United States of America. For this reason, some countries (Argentina, Canada, South Africa, USA, EU) have applied measures to reduce mixing, including proper separation of the fields where both types (GM and conventional) are grown (maize, soybean, oilseed rape, chicory, squash, potato). At that moment all GM crops available on the international market are designed by using genes from microorganisms. They are characterized by one of three basic features: resistance to insect damage; resistance to viral infections and tolerance towards certain herbicides.

4.3. Safety aspects specific to GMMs

The recombinant DNA techniques used for modification of plants are similar to those used for design of genetically modified microorganisms. As it was already mentioned above distinct genetic characteristics of microorganisms are implicated and should be taken into account for safety reasons. Microorganisms applicable for food production are Gram + and Gram – bacteria, yeasts and filamentous fungi. Their genome and recombinant genetic technologies have differences, although some common techniques exist as well.

Use of homologous recombination in bacteria has a major advantage, because an integration site can be applied by design and undesirable DNA can be easily removed. Thus, a homologous genes system for the selection and maintenance of introduced DNA can be designed together with development of appropriate selection methods, compatible with safe food use. These features facilitate good control over genetic modification procedures.

Recently the safety evaluation of GMMs is enhanced by availability of genome sequence data of some bacteria and yeast. This acquisition of the complete genome sequence for particular microorganism is a realistic scientific base for evaluation and assessment of a particular gene technology. Development of post-genome analytical methodology and technical devices give reliable opportunity for analysis of gene expression at the level of the entire genome. Success of micro array DNA technology allowed investigation of all genes of the genome by means of nucleic acid probes. Thus, the presence of individual genes and gene expression in different strains and environments can be demonstrated. Advance in proteomics allows proteins isolated from the whole cells to be separated by two-dimensional gel electrophoresis and to be analyzed. In this way a comparison could be made between strains from different environments. Using mass spectrometry, an individual protein could also be identified and so facilitate the relating

of separated protein spots to specific genes. Microorganisms used in food processing could be found viable in the end product and could be introduced into the consumer. That is why a potential for interaction (direct or indirect) between organisms and consumer actually exists. For this reason, it is very important to prove with certainty that the microorganisms used in food processing are not pathogenic, toxigenic or allergenic and the genetic modification do not alter their safe status. In this respect the fate of GMMs consumed and their impact on gastrointestinal tract and gut microflora has to be taken into account. Here it is important to note that effect of GMMs should be considered also on the level of the animal health, having in mind the influence on humans through nutrition. One of the most general concerns that have been expressed is the possibility for transfer of modified gene sequences to gut microorganisms or host cells. Rare acquisition of diet-derived DNA fragments cannot be ruled out and the possible impact of genes not normally present in ruminant diets should be considered.

5. GMMs in food production

The specific aspect of food safety issues concerns mainly application of GMMs for food production. Here, a potential gene transfer between the GMM and other microorganisms taking place in food or gastrointestinal tract is discussed. Also the safety of genetic markers used for selection (for example - antimicrobial resistance gene), as well as the potential GMM interaction with the intestinal microflora and the immune response are appraised. In this respect an evaluation of current state of knowledge in this field and prediction of possible health risk measurement based on scientific methods have been made.

5.1. Genetic modification techniques

5.1.1. Classical methods

The classical methods for genome modification of microorganisms are divided into two types:

- Selection of mutations arising spontaneously and induced by different physical and chemical factors of the environment. The spontaneous mutations are consequence of rearrangements in the heritable DNA molecule due to substitution of one nucleotide with another, the addition or deletion of one or more nucleotides or other types of reformations. A lot of spontaneous mutants arise due to the movement of transposable elements to new locations in the double strand DNA. Such kinds of elements are typical for plants, animals and microbes.

- Exchange of DNA between closely related organisms. This type of gene modification in microorganisms concerns introduction of new genetic information by chromosomal or plasmid DNA. This event happens when DNA from the chromosome of the donor microorganism is integrated into DNA of the recipient one. Being self-replicating, plasmids transfer the DNA of the donor into recipient without integration with chromosomal DNA. Thus, plasmid DNA can be transferred to widely divergent organisms compared to the donor ones. The movement of the plasmid could be easily observed because of the marker borne by its molecule (for instance antibiotic resistance). Three different classical types of gene transfer are characteristic of bacteria. It is considered that these three mechanisms occur naturally:
    - DNA-mediated transformation (DNA is transferred as "naked" DNA)
    - Transduction – transfer of DNA is mediated by a virus
    - Conjugation – DNA is transferred during cell-to-cell contact between donor and recipient cells.

5.1.2. Molecular techniques

Recent molecular technological advances in mutagenesis and gene transfer methods expanded considerably the range of microorganisms into which DNA from unrelated organisms can be introduced. The genus barrier and the kingdom barrier are no longer insurmountable obstacles. Current methods used in bacteria allow the inserted gene recombinant constructs to be integrated at specific sites into the chromosome or the plasmids. Nevertheless, the procedures for gene modification should be subjected to safety considerations. The following important features are a matter of discussion: peculiarities of the host microorganism in respect to inserted gene(s), characteristics of the vector and construct; DNA transfer methods.

When bacteria are used as host microorganisms a procedure for safety assessment requires a history for safe consumption of this microorganism either as a food or as a food component. If this evidence lacks, the safety of the host must be established. For the eukaryiotic host the same safety considerations are applied.

The inserted gene(s) could be taken from the same microbial species or from evolutionary more distant organism. The inserted gene products should have a history of safety use in food or its safety should be substantiated. The shorter the inserted DNA fragment, the more reduced/facilitated the procedure for evaluation of food safety would be.

As regards the characteristics of the vector and the construct - if the vector used is a part of the genome of GMM the whole DNA sequence should be characterized including; replicons, promoters, selective markers, linkers as well as any other parts of DNA. The vector must

contain nucleotide sequence from microorganisms with history of safe use in food. The selective markers must be selected very strictly and based on safe use and the antimicrobial resistance marker must be avoided. In case of use, an application of specific methods for removal from GMM genome should be applied (e. g. sequence specific recombination). For the eukaryotes specific cloning vectors like centromeric plasmids, yeast artificial chromosome, plasmids based on killer factor determinants etc. have been designed.

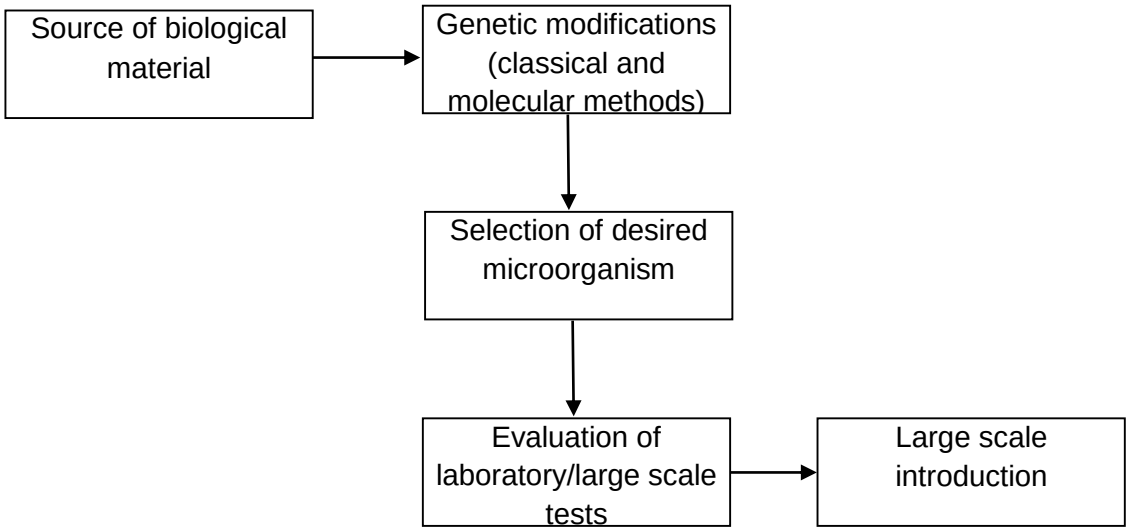
Speaking about DNA transfer methods - recommendation exists for use of methods of DNA transfer of physical, chemical and biological nature, which minimize major genetic rearrangements in host genome. In case of use of integrative vectors, the nucleotide sequence of the flanking regions at the integration site of the chromosome should be characterized. This information is necessary to predict the risk of the methods used. In eukaryotes there are dependable methods for directed integration of *in vitro* modified or composed gene constructs into specific chromosomal sites and for deletion of genes applicable in certain species. On the basis of these methods, transgenic constructs have been made and they are highly stable during vegetative growth of cells. A possibility of recombination by mating with related strains of indigenous microflora exists. When the genetic properties of the used strains are not well known, the insufficient information about possible recombinant events makes it impossible to forecast the mechanism and integration site of the foreign genes. Thus, it is possible the methodology used in genetic manipulation of yeasts and filamentous fungi to enable integration at variable sites, which could cause appearance of different biotechnological properties and genetic stability of GMMs.

5.1.3. Comparison of classical and molecular approaches

The terms “classical” and “molecular”, regarding the methodology of genetic modification of microorganisms, as it was already mentioned, concerns enhancing their genetic variability. This effect is achieved using classical methods by spontaneous or mutagen induced variation, by hybridization or gene transfer. These methods are inaccurate and none directed, less powerful in comparison to the molecular ones for gene modifications. But there is no doubt that there is no conceptual distinction between genetic modification of microorganisms by classical or molecular techniques which cause DNA modifications and gene transfer.

Fig. 1 shows the genetic modification of microorganisms and pathway for introduction into environment. Here both methods are unified in sense that it is no matter classical or molecular breeding methods at the steps of evaluation in laboratory, field or large scale environmental introduction are applied.

Figure 1:



The underlying biological principles characterizing this concept were implicated in the EFSA reports:

- The primary focus for decision making about the introduction in the environment is the product obtained by genetic modification and selection, not the process itself.
- The characterization of the product needs information about the process used. But the nature of the process is not a sufficient criterion to decide whether the product should be more or less overseen.
- The responses of the microorganisms modified by molecular or classical methods are based on the same physical and biological laws. The accumulated knowledge about products of classical modifications could be applied on product obtained by application of molecular techniques in terms of relative safety and risk assessment.

5.2. Molecular methods for control of genetically modified foods

5.2.1. Strains identification methods

The taxonomic status of the host microorganism is considered to be a feature of great importance regarding safety assessment. For this reason, the microorganisms used for genetic manipulations should be well examined taxonomically using proper methodology. It should be adequately characterized from scientific, manufacturing and safety perspective. At present, the most exact tool for proper characterization of the taxonomic status of microorganisms are DNA/DNA hybridization technique and 16S rRNA sequence determination. These methods give crucial information about taxonomical status of the microorganisms under investigation. At present standard physiological/biochemical methods for phenotypic characterization are on

the market and are widely used. The important feature for the strain characterization is also information about its pathogenic properties.

After application of genetic modification procedure the obtained GMM strains should sustain the safe properties of the host microorganisms. The new strain should be characterized by the same methods and accuracy, including phenotypic and genotypic characteristics in order to assess its safety. This precise comparison between the host and GMM could be done using existing molecular techniques: restriction analysis, random amplified polymorphic DNA analysis (RAPD-PCR), amplified fragment length polymorphism (AFLP), protein profiling etc. The analysis can be extended also to genome sequencing.

Other important factors, which should be studied in respect to the safety assessment of GMM are: the effect of the genetic modification on the properties of the host microorganism, the stability of the genetic system, the functional properties of the gene construct.

All these characteristics are important during the process of safety assessment of the products obtained by GMMs and their impact on the environment.

The methods for identification of production strains, contaminating strains or pathogens.comprise techniques applied at both genotype and phenotype level. The genotypic methods include tools such as rDNA sequence analysis, DNA base composition and DNA/DNA hybridization.

DNA sequencing, especially *rDNA sequence analysis* aims at comparative studies of rDNA sequences. This is performed through direct sequencing of parts or nearly the entire 16S or 23S rDNA molecule by PCR using appropriate primers.

DNA base ratio (moles percent G + C) is a classical genotyping method, part of the standard description of bacterial taxa. The range observed is no more than 3 % within a species and no more than 10% within a genus. Among bacteria G + C content varies between 24 and 76 %.

DNA/DNA hybridization is applied for identification virtually to all bacteria, as well as to great variety of yeasts and fungi.

As regards the taxonomic resolution of these methods rDNA sequence analysis and DNA base composition are readily applicable for genus and species identification, while the DNA/DNA hybridization is used only for species characterization. The genotyping methods have useful application for bacteria and yeasts and to some smaller extent to fungi (only rDNA sequence analysis).

The phenotyping molecular methods include cellular fatty acids fingerprinting and total cellular protein electrophoretic patters. These methods are mainly used in bacterial identification and

while the former is applicable to genus and species level, the latter is routinely used for species identification.

5.2.2. Typing methods

Introducing molecular biological techniques a variety of DNA-based typing methods for discrimination of species, as well as isolates of a certain species are designed. The data obtained applying these methods may provide insight in the dissemination and persistence of food spoiling microorganisms or pathogenic ones not only in the foods but also in the environment. Thus, DNA-based typing methods can be used for epidemiological purposes and can help discriminating coincident but independent infections and epidemics caused by a single isolate. This is of special importance since may facilitate the implementation of preventive and hygienic measure.

Genotyping methods are usually categorized depending on technical aspects. According to this the following can be listed:

- DNA sequencing;
- Restriction endonucleases patterns analysis of plasmid and/or genomic DNA (e.g. Restriction Fragments Length Polymorphisms (RFLP), Pulse Filed Gel Electrophoresis (PFGE));
- Probe-based techniques (labeling methods);
- PCR-based techniques (amplification methods such as Random Amplified Polymorphic DNA (RAPD) and Amplified Fragments Length Polymorphisms (AFLP)).

Restriction Fragments Length Polymorphisms (RFLP) explores the natural variability of DNA molecules (chromosomes, plasmids and mitochondrial DNA in eukaryotes) regarding the position and the number of 6- to 8-mer sequence along this molecule. Cutting such DNA with restriction endunucleases results in generation of fragments with different length which can be separated by Agarose Gel Electrophoresis (AGE) and visualized either directly after staining with ethidium bromide (in case of limited number of fragments, e.g. less than 50) or after hybridization with specific labeled probes.

Pulse Filed Gel Electrophoresis (PFGE) is a technique explored in separation of large DNA molecules such as chromosomes. It can be applied also for separation of large DNA fragments obtained after cutting with rare-cutting restriction endunucleases, which generate a limited number of fragments. Being highly polymorphic the chromosomes/large fragments patterns are very useful for strain identification.

Probe-based methods (labeling methods). These methods concern incorporation into or attachment at the end of a nucleic acid fragment a probe. Different variations of the basic method exist depending on variety of factors. As concern the type of the nucleic acid, its size and quantity, 4 methods of labeling can be listed: 3' and 5' end labeling, random labeling by nick translation and random prime labeling. In respect to the labeling molecule, i.e. its nature, radioactive and non-radioactive labeling can be explored. In the radioactive labeling radioisotopes are detected by autoradiography while the non-radioactive labeling utilizes fluorescence, chemo-luminescence or enzymatic reactions.

PCR-based techniques (amplification methods). In these methods the in vitro enzymatic amplification of nucleotide sequence is explored for strain identification purposes. Both basic PCR protocol and its modifications are powerful tools for amplification of a DNA sequence of interest, detection and typing of production, contaminating and pathogenic strains. Among the wide diversity of PCR-based methods targeting direct identification of a given organism in a food product the RAPD and AFLP analyses are the most commonly used.

Random Amplified Polymorphic DNA (RAPD) is a random type PCR technique that is based on amplification of a DNA region without previous information about some target sequence. Designing of 10-mer primers at random and applying of low stringency control amplification conditions a set of amplified sequences can be obtained, which in general is individual specific. This last fact contributes to the use of RAPD-PCR as a reliable method to differentiate among microorganisms populations.

Amplified Fragments Length Polymorphisms (AFLP) is a highly sensitive method for detection of polymorphism among individuals applicable for both inter- and intra-species discrimination. This is PCR mediated RFLP of selected DNA fragments from a pool of such. AFLP screens for polymorphism in the length of the amplified fragments through selective amplification of some of them. The method comprises digestion of genomic DNA with 2 restriction endonucleases followed by PCR amplification of the obtained fragments. The restriction fragments are modified in advance to the amplification with adaptors specific for the endonucleases used in the experiment, thus serving as primer binding sites. The primers themselves are designed in a way that bind to the adaptors and allow very specific amplification due to the fact that only fragments fully matching the primers sequence will be amplified.

5.2.3. Strains detection and tracing methods

Here, only genotyping methods are explored, namely all typing techniques listed above with special emphasis on DNA probes, which discrimination power covers detection at genus, species and strain level in bacteria, yeasts and fungi.

5.3. Molecular methods for detection and quantification of GMMs

5.3.1. GMM detection

The first Genetically Modified Organisms (GMO) to be used for health or industrial purposes were modifies strains *Escherichia coli* and *Saccharomyces cerevisiae* producing insulin. Since this period numerous recombinant microorganisms have been implemented. They are used in industrial bioreactors under confinement conditions and such processes do not generally require carrying out a GMO detection technology.

The outcome of such a specific need appeared with the introduction in the mid-nineties of genetically modified (GM) animals and plants in the natural environment.

GM crops mainly have opened an important debate, particularly in Western Europe, for safety assessment of food and for potential modification of the environment. Food warranted free from GMO has been proposed to reassure the consumers. However, not only raw materials can be used for food preparation but also numerous ingredients from plant origin, which can be present in small amounts in the final product. It is for example the case with lecithin extracted from Soya beans. Lecithin is used as an additive in a lot of industrial recipes in chocolates of chocolate flavored preparations. The target to be analyzed is not the purified lecithin itself (which is identical to the lecithin from the non-GM Soya beans) but some modified DNA sequences or the coded by them recombinant protein(s). The latter can be only presented as traces, thus very sensitive methods for their detection are necessary.

5.3.2. Types of detection methods

Detection of GMO or its derivative can be performed through detecting of the molecule - primary target (the DNA sequence itself and eventually - RNA), specifically connected with the genetic modification or its product (the recombinant protein that can be produced in relation with the genetic change. The greater part of the methods available concern DNA detection, and just few techniques are applied in RNA and protein detection. The reasons for this fact are as follows:

- DNA can be amplified and purified rapidly and efficiently using PCR. Multiplication of RNA and proteins is more complicated and time consuming process.
- DNA is a stabile molecule while RNA is not stabile at all. The protein is easily subjected to temperature denaturation during food processing, thus its stability depends on various external factors.

- If the modifying element is a nuclear DNA there is a linear dependence of its quantity and the amount of the GMO. However such correlation is virtually not observed between the quantity of the GMO and RNA/Protein.
- Since the genetic modification is done at DNA level, it is reasonable to detect this alternation at the same level.

Nowadays all commercialized GMO possesses foreign nuclear DNA.

5.3.3. Protein based methods

The basis of these methods is immunological and lays in the specific binding of the type "antigen – antibody" and classical ELISA assay. The antigen – antibody reaction recognizes the foreign molecule, binds to it and the bound complex thus obtained, is detected usually through a chromogenic reaction. Since the antibody, needed to detect the antigen, can not be developed without access to the purified antibody itself, the latter can be either artificially synthesized in the amino acid sequence is known, or purified from the GMO studied.

Generally the product of a transgene is a small polypeptide or a protein, which can be expressed under a strong constitutive promoter in any tissue and virtually during any time of the life cycle of the plant. In such a way the protein is in enough quantity to constitute an analytic target. However, in more recent GM plants the desired protein is only produced under the control of an inducible promoter (e.g. under stress conditions, during defined period of life-cycle, etc.). In these situation the detection and analysis of the presence of the recombinant protein is often not adequate.

5.3.4. RNA based methods

In these methods a specific binding between the RNA molecule and a primer (RNA or DNA synthetic oligonucleotide) is performed. The primer, complement to the start of the RNA molecule, annealed with it resulting in a double strand heteroduplex similar to DNA. Using reverse transcriptase a DNA molecule is synthesized *de novo*, which can be further amplified by PCR and detected. A disadvantage of this method is the fact that the specific primers can not be designed without knowledge about the RNA composition to be detected.

5.3.5. DNA based methods – PCR application

DNA-based methods primary rely on multiplication of a specific DNA piece by PCR technique [14]. For visualization of the amplification products gel electrophoresis is routinely used. It may be solely performed or coupled with restriction endonuclease digestion (RFLP-PCR). A more sophisticated variant of the basic PCR protocol involves determination of the Tm profile by means of a dye intercalating double stranded DNA and emitting fluorescent light. With

increasing the temperature the two strands of DNA begin to separate and correspondingly – the light emission, which can be measured, decreases. Tm is a specific characteristic of a DNA sequence rather than DNA length. At last, but not leased an alternative is to use probes and perform hybridization with DNA or RNA. If appropriately designed, a probe can discriminate between the native and any foreign sequence. Labeling the probe with radioactive or non-radioactive compounds facilitates the detection of the present molecule. For GMO analysis gel electrophoresis and hybridization techniques are currently the most commonly exploited techniques.

Screening of food samples for the presence of GMOs by use of basic PCR protocol comprises the following procedure;

- extract of DNA from the sample and standards of unknown GMO content;
- assembly of several PCR with specific primers (usually for well known regulatory sequences, as the viral 35SCaMV or Tnos promoters);
- visualization of the DNA fragments on an agarose gel electrophoresis;
- analysis and semi-quantitative assay using image analysis software.

With multiplex PCR-based methods several DNA sequences can be screened for and detected in a single reaction. However, the development of a multiplex assay requires careful testing and approving. The pool of amplification fragments needs to be further analyzed to distinguish between the various amplicons. This can be done with the aid of specific hybridization probes by gel electrophoresis and comparison of the fragments size or using specifically labeled primers.

A great advantage of this technique is the fact that fewer reactions are needed to test a sample for presence of GMO-derived DNA. Additionally if it is necessary to further perform quantification assays it will be good if you know which GMO to quantify since the procedure is relatively expensive. The identification of a certain GMO is important also in the context of our knowledge about the approved and aunapproved GMOs.

Another approach is to apply **PCR-based quantification methods.** PCR-based quantification can be performed both during the amplification process (the real-time PCR) and at its end (end-point PCR).

The end product analyses are commonly based on comparison of the mount of amplified DNA of two DNA targets: the one to be quantified and a competitor (in known small quantity) added to the amplification mixture before the PCR and co-amplified with the target to be quantified. This process is also called competitive quantitative PCR. It is based on the presumption that if

both target DNA and competitive DNA yield the same amount of amplification product, the starting amount of DNA is also assumed to be the same.

In real-time PCR analysis the amount of a product synthesized during PCR is estimated directly by measurement of the fluorescence in a PCR. There are commercially available hybridization probes emitting fluorescence corresponding to the amount of the synthesized DNA. The amount of the synthesized product can be also estimated by the emitting of intercalated fluorescent dye but here it is not possible to distinguish between specific and not-specific products. The advantage of this method is that not only the quantity of the formed product can be followed in dynamics but also the defined number of cycles, which are needed to produce a certain amount of PCR product, can be determined.

The real-time PCR requires more sophisticated and expensive equipment; it is faster than competitive PCR and m

5.3.6. The detection process

The detection process comprises a procedure consisting of the following individual steps [9]:

1. *Sampling*. The sampling strategy involves complex statistics to produce reliable estimate of the quantity of GMO or their derivatives.
2. *Homogenizing*. This step includes homogenization of the sample.
3. *Isolation/purification*. This step concerns isolation and purification of a DNA, RNA or protein. The most critical factors at this step ate the quantity (concentration), purity and quality of the macromolecule to be tested. Here again statistics are involved.
4. *Present/absent analysis.* At this step analyses for determination of presence or not of GNO or its derivative are performed. As mentioned above a range of alternative methods is available, each offering different ability to discriminate between derivatives of different GMOs and different reliability concerning false (+) and (-) results. Another considerations that should be taken into account when choosing a detection method are: the probability the molecule to be detected to be entirely degraded, i.e. no longer detectable; the probability during the food processing the molecule, which the detection method is designed to detect, have been removed; the probability the analysis to be performed on a mixture of GMOs and thus the identification process appeared to be more complicated and time consuming.
5. *Identification.* For identification of the detected molecule usually any (+) result have to be verified in order to omit false (+) reactions and confirm the identity of the found molecule.
6. *Quantification.* The quantitative estimation of the modified material is a sample is performed at this step. Here again statistics are important since quantification always requires standards.

7. *Interpretation of the analysis results.*

5.3.7. Advantages and disadvantages of the detection methods

The limits of the detection and quantification can be categorized in three groups:

1. Absolute limits – the lowest number of copies that must present at the beginning of the first cycle to obtain probability of 95 % correct detection;
2. Relative limits – the lowest relative percentage of genetically modified materials that can be detected;
3. Practical limits –the limits applicable to a defined sample.

The specificity of the currently available DNA based methods can be categorized into four groups:

1. Screening methods detecting wide range of GMO without identifying them;
2. Screening methods for a certain type of genetic modification;
3. Construct specific methods sometimes used for identification of GMOs;
4. Transformation specific methods used for identification of GMOs (still under laboratory development; not commercialized).

5.4. Assessment of gene transfer

5.4.1. Prokaryotic microorganisms

A variety of mechanisms are typical for transfer of DNA within prokaryotes and it can result in transfer of heritable properties.

These mechanisms of DNA transfer give to bacteria an advantage in response to environmental changes by adoption of new genetic information which could provide an efficient tool to sustain unfavorable selective pressure. Such kind of event is the wide spreading of antimicrobial resistance genes throughout microorganisms due to the introduction of antimicrobial agents in agriculture, healthcare, veterinary and medicine. A wide spread mechanism of gene transfer within prokaryotic systems is the conjugation, which is based on the presence of a plasmid in the donor cells or of conjugative transposons in the chromosome. The direct cell-to-cell contact helps these genetic elements to transfer copy(ies) of the plasmid or of the transposon(s) into the recipient cells. In bacteria a lot of plasmids have been identified and some of them lack the possibility for their own transfer. In this case it is facilitated by other plasmids.

The number of plasmids present within the bacterial cells could be different and this feature is common to bacterial populations, inhabiting different niches. These moving genetic elements - plasmids and transposons often can introduce new properties into the cells. A unique

phenomenon in nature (as well as in experimental conditions) is the conjugative gene transfer from bacteria to eukaryotic cells (yeasts, filamentous fungi, animal and plant cells).

Another gene transfer process that is based on active uptake of extracellular DNA by bacteria into their cytoplasm is a natural transformation. This phenomenon was found to be characteristic of a limited number of bacteria occurring in major trophic and taxonomic groups. It was found that this process (transfer) could happen effectively during a specific growth phase of population growth called "competence". The transformation can be accomplished by chromosomal DNA fragments or plasmids. This process can happen in specific physical or chemical conditions characterizing the phase of competence, when foreign DNA may enter bacterial cells. This type of transformation is often used in realization of gene technology.

The third type of gene transfer - transduction is also occurring in the microbial population and communities. Thus far it is mediated by bacterial viruses, which incidentally packed DNA of the last host cell, making it a donor one, and afterwards transmitted it to a recipient cell.

The specificity of the above described three mechanisms depends upon the genetic relatedness of the donor/receptor cells. The gene transfer could occur using these mechanisms within members of one species, but also between members of different species and genera. The so called "horizontal gene transfer" realized by these mechanisms is widely studied and admitted as very important for the genomic structure of bacterial species. The investigations of this phenomenon include also a whole genome sequence analysis.

Investigations in the field of natural gene transfer indicated that various transfer events could occur in natural habitats of bacteria including soil, rhizosphere, phyloplan, sediments, river epitops, foodstuffs, intestinal tract, mammalian oral cavity etc.

After the efficient transfer of foreign DNA into recipient cell it could be introduced into the genome via genomic integration (e.g. homologous recombination), or by formation of plasmid (in case of presence of replication origin). This process could be suspended by different reasons (like lack of nucleotide sequence homology or the presence of restriction endonucleases). It is evident that in case the novel genetic information gives an advantage for the recipient and allows its survival during changes in the natural ecosystem it tends to be preserved on the population level, when the selective pressure is durable. Thus, gene transfer could be considered as a phenomenon typical of the nature of prokaryotic microorganisms. It is a natural response to the changes in selective pressure of the environment, where the circulation of a gene or gene combination as well as the generation of several gene assemblies, give better opportunities in survival of microbial population.

Having in mind the natural character of gene transfer within bacterial community, which may ensure the wide spreading of recombinant constructs, it is preferable to use a chromosomal integration approach during the process of gene engineering manipulations. Inclusion of the genes into constructs bearing the target construct, which could give selective advantages under certain conditions, should also be avoided (e.g. antimicrobial resistance determinants). The procedure of elimination of each gene sequence, which is able to stimulate the random integration into other genomes, should be applied in the process of preparation of a desired construct.

5.4.2. Eukaryotic microorganisms

Eukaryotic cells differ from prokaryotic ones by their more complex structure: they possess well developed nucleus.

The process of gene transfer in these microorganisms (yeasts and filamentous fungi) is also different from those already described for bacteria.

Natural cell hybridization and genetic recombination take place mainly within species possessing sexual or parasexual cell cycles. These events proceed in eukaryotic cells with sexual reproduction through mating, meiosis and sporulation. A gene transfer in microorganisms with parasexual life cycle and anastomosis, nuclear fusion and haploidization by gradual loss of chromosomes takes place. In certain genera interspecific hybridization can also occur between closely related species.

The transfer of synthetic genes from yeast to mammalian cells has been done using yeast artificial chromosomes (YACs). They have an excellent potential as vectors in gene therapy.

5.5. Genetic stability of microorganisms

Microorganisms are genetically much less stable than tightly organized chromosomes in higher eukaryotes. They possess faster growth rate and being unicellular can adapt quickly to the changing environment. Thus, they are able to change genetically easier than higher eukaryotes. As it has been already mentioned above, several mechanisms for horizontal gene transfer have been already identified. Movable DNA particles are responsible for changes of bacterial genetic material, which often leads to appearance of new phenotypic characteristics, inactivation of genes, gene losses and entire destabilization of genome. These movable particles of DNA involve insertion sequence (IS), plasmids, prophages, transposons. A lot of bacterial strains possess a great number of different IS elements and part of them often actively cause transposition.

The genome of eukaryotic microorganisms is also subjected to DNA changes. They could undergo series of rearrangements during the process of growth depending on the physical, chemical conditions and cultivation system. In some cases, continuous cultures are efficient selective tools for stabilization of adopted new DNA sequences. Generally, these changes arise by spontaneous transposition of movable elements (e.g. Ty retrotransposons) and segments of chromosomes. The last event is well manifested in chromosomal length polymorphism.

The phenomenon of genetic variability of microorganisms could affect also the stability of the recombinant DNA in GMMs. This possibility should be taken into account in case of evaluation of genetic stability of GMMs.

The localization of cloned gene(s) (chromosomal or plasmid) strongly influences the genetic stability of the recombinant DNA molecule. It makes the selection of convenient vector system very important for the fate of the recombinant DNA. When high copy number vector or integrated into chromosome recombinant DNA are used, the stability of the new genetic information will depend on basic biological mechanisms. The genetic stability of the transferred genetic material will be like those of the host microorganism. The high stability of the transferred genetic material requires profound knowledge on the location of transposons and IS element insertion, as well as the site for attachment of temperate phages.

5.6. Microbial pathogenicity

In general microorganisms which are applied in biotechnology for production of fermented foods (e.g. lactic, propionic, acetic acid bacteria, yeasts (*Saccharomyces*, *Kluyveromyces*), some filamentous fungi like *Penicillium*, *Aspergillus*), are considered as safe for human use. They have a long story of common life with mankind and are considered generally as safe and nonpathogenic. There are some data about very rare cases of bacteriemia and endocarditis caused by some enteric lactic acid bacteria to patients with severe core disease. These cases by no means denote these microorganisms as food borne pathogens.

The food borne pathogens have invasive and/or toxigenic effect being in food or in the human gastrointestinal tract. Another group of microorganisms - opportunistic pathogens in general are not dangerous for a healthy person but in case of health-compromised people they represent some risk. Recently, using chance given by molecular techniques, the genomes of a lot of food-borne and opportunistic pathogens have been entirely sequenced and the genes responsible for their pathogenicity have been determined. These achievements give an opportunity for identification of similar genetic information in the genomes of different microorganisms used in food industry. Evaluation of data for several sequenced genomes of microorganisms used in

food fermentation indicates two examples (*Saccharomyces cerevisae*, *Lactococcus lactis*) which do not possess known pathogenicity features.

In conclusion, the long term of safe use and the recently obtained genetic evidence show that the genetic background of the majority of microorganisms applied in food industry do not possess pathogenicity islands and other determinants for pathogenicity.

Besides these main considerations for pathogenicity there are some additional effects which should be taken into account.

Some undesired effects in genetically modified microorganisms resulted of genetic manipulation consequences such as metabolic discrepancy, expression of "silent" genes, change of cross-talk between microbe and intestinal immune system can be outlined. These effects concern increase of the amount of common metabolites with non basically toxic effect to non acceptable amount in lactic acid bacteria, yeasts (acetaldehyde, formic acid, biogenic amines); and the fungus *Penicillium camamberti (roqueforti) (*cyclopiazonic acid or roquefortin); expression of some genes coding for toxins; appearance of undesirable immune reactionp; undesirable reactions with other cells (e. g. enterocytes) of the GI tract.

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LO 4: RISK ASSESSMENT OF GMMs AND DERIVED PRODUCTS

1. Risk assessment of GMMs and derived products for human and animal health

1.1. The concept of substantial equivalence to GMMs

GMM and the products derived and applied for human and animal consumption are ranging from a group of single compound to pure cultures of viable GMMs. Purified products like amino acids and vitamins are typical example for the first group, but the probiiotic culture or diary starters represent the second one. An intermediary place is given to both products from genetically modified microorganisms such as dairy products where the viable GMMs persist, as well as products without presence of viable GMMs. The last one could contain traces of the transgenic event, e.g. crude enzyme preparations produced by the lysis of microbial cells. On the basis of these considerations three groups of GMMs or derived food and feed may be distinguished (see Table 1 below).

Table 1. Groups of GMMs

Group	*Description*
Group 1	Defined mixture of compounds or single compounds derived from GMMs (e.g. amino acids, vitamins, pure enzymes).
Group 2	Complex products without viable GMMs and not containing unit length of any cloned (foreign) open reading frames (e.g. lysed cell extracts, some feed enzymes, wine, some beers, etc.)
Group 3	Cultures and products containing viable GMMs or genetically intact cloned (foreign) DNA (e.g. live or heat killed starter cultures and probiotic cultures, some beers, cheese, yogurts, etc.)

Different assessment procedures are applicable when the foods and feeds contain products obtained from mentioned above groups (Fig. 1). The most intense scrutiny is foreseen for products containing viable GMMs. Limited information regarding production system is required to perform a risk assessment on single compound. In case GMMs are not recoverable from a product, but its purification is limited, the required information for risk assessment is more extensive then for the single products. There is necessity to understand the process by

which the GMM has been inactivated in the product and the degree to which traces of the transgenic event could be detected in the product. If alive GMMs persist in a product, the required information will be comprehensive in order to allow a scientific risk assessment.

The scrutiny level of the risk assessment is related to the history of use of the recipient and donor strains (depending on the sequences to be cloned) as well as the modification itself. The procedures for the risk assessment of GMMs will become less scrutiny when the qualified presumption of **s**afety (QPS) of microorganisms in the food and feed chains has been embedded. In such case, the risk assessment should target relevant information, which is not listed in QPS qualification already granted to the parental / recipient / donor strains, or to the taxonomic group with QPS status for the same end-use.

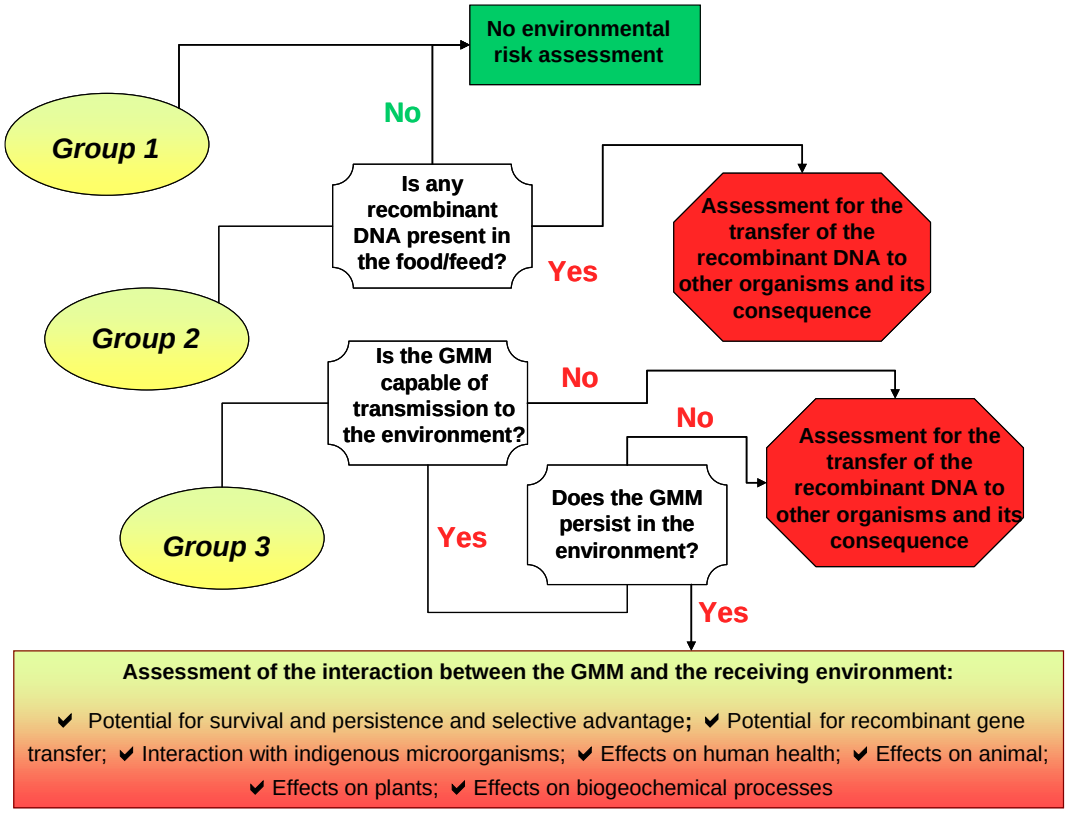

Fig. 1: Approach to the environmental risk assessment of GMMs and their products

1.2 Application of comparative approach

The strategy for GMMs assessment is based not only on evaluation of intended modifications, but also on the unexpected outcomes of the genetic manipulation process. It compares the GMM or GM food or feed with its conventional counterpart. This comparative approach is based on the concept that a conventional counterpart with a history of safe use

can be taken as a referent point for the environmental, food and feed risk assessment of a particular GMM. In order to characterize this, OECD has developed concepts of "familiarity" and "substantial equivalence", which were further worked out by *ILSI* and WHO/FAO. The risk assessment goals to identify new or altered hazards connected to the conventional counterpart. These comparative investigations could be used as a first step of the risk assessment. After, in the second step, both intended and unintended differences should be identified and their environmental and food/feed safety and nutritional impact should be assessed.

The information about concepts of "familiarity", "body of knowledge:", "history of safe use" and "substantial equivalence" is given below:

Table 2. OECD concepts of "familiarity" and "substantial equivalence"

The concept	*Description*
"familiarity" and "body of knowledge"	Majority of GMM strains used for food / feed purposes belong to well characterized microbial species. Such "familiarity" permits the risk assessor to draw on previous knowledge and experience with the introduction of similar microorganisms into food and environment, as well as to the results from risk / safety analysis, performed before scale up of technologies. This term is replaced by the new one – "body of knowledge".
"substantial equivalence"	This concept is based on the underlying principle that an existing microorganism with a "history of safe use" as food or feed can serve as comparator when assessing the safety of GM food and feed. Detailed description is given in ILSI and EFSA Scientific Colloquium on QPS.

Neither the concept "body of knowledge", nor "history of safe use" guarantee creating no harm. In case the parental microorganism has been given a recognized status of QPS, all available information on the history of safe use has already been assessed.

The natural diversity of microbial genome becomes obvious in foods and during their processing as very complex microbial associations it may be a common event. Moreover, chemical and physical factors/characteristics of foods influence gene expression and cause

variations. This peculiarity should be taken into consideration during safety assessment as a variety of data can be obtained in laboratory experiments with foods or in gastro-intestinal tract during ingestion. Thus, a concept for ***substantial equivalence*** should be applied to GMMs themselves as well as to the foods obtained using them. Here must be noted that application of a ***substantial equivalence*** concept should be done very precisely because minor differences can distinct pathogenic and non-pathogenic strains of microorganisms.

The main characteristics of the concept of ***substantial equivalence*** are involvement of a specific analysis of composition and phenotype of GMM and comparison to that of conventional parent strain. In this respect FAO and WHO noted in their report that it was necessary to keep in touch with the progress in new molecular methodologies. Their application provides a powerful tool for obtaining of detailed analytical information and can facilitate a successful comparison between the conventional and genetically modified microorganisms. In this respect to use DNA microarrays and proteomics techniques is especially convenient. Metabolic profiling of microorganisms trough a range of analytic techniques is an advanced approach, possessing special value in assessment of GMMs where metabolic rearrangement is the anticipated result

The application of such kind of technique is restricted by necessity of evaluation of a background in the normal variations and the significance of differences detected. Important steps should be carried out prior to considering these techniques in the routine assessment of safety:

- Validation of methodology for insurance of their reproducibility and robustness
- Achievement of agreement for assessing their performance (i.e. definition of the range of differences in one array/profile which can be considered as “normal variation”)
- Evaluation of each difference in the profile which is not considered as a “normal variation”

1.3. Intended and unintended effects

Intended effects: They are anticipated to be happened as a consequence of the introduction or inactivation of genes or DNA sequence and are tightly connected with the goal of the genetic modification. These intended modifications in the composition of a GMM, which are different in comparison to the parent and could be measured as a single compound (newly obtained protein) or change in the cellular metabolic flux.

Unintended effects: The changes in phonotypical differences between GMM and its isogenic counterpart, which are not expected from the performed gene manipulation. These unintended effects could be due to:

- Integration of the metabolic pathways;
- Genetic rearrangement;
- Metabolic perturbations and pleiotropic effect;
- Synthesis of new fusion protein.

1.4. Exposure to GMMs

In the first place in order to prevent the undesired effect of application of GMM in food production, a pre-market safety evaluation should be performed. Any impact on the food chain should be monitored. During the safety assessment, the following important factors should be taken into account: possibility of consumption of GMM; type of GMM / product / gene; assessing the potential hazard assessment on population level.

The methods for measurement of potential hazard on exposure of the population to GMMs should be determined. In this respect it is important to note the conclusions by previous consultations:

> *"The change in nutrient levels in a particular crop plant may impact on overall dietary intake. In such cases, it is important to determine alterations in nutrient content and bio-availability and their stability with time, processing and storage, as well as to monitor changes in dietary patterns as a result of the introduction of the genetically modified food and evaluate its potential effect on nutritional and health status of consumers. However, an assessment of the impact of nutritional status of consumers is important for all significant dietary changes and not specific to the introduction of genetically modified foods".*

As it has been noted by FAO/WHO it is very difficult to predict a potential long term health effect of any food, including those obtained with the aid of GMMs. It is due to the wide genetic variability in human population and to the complexity of the effects monitoring. It is considered that it is very difficult to identify any effects against background of conventional foods unless specific investigations were planned to answer very specific questions. Thus, the development of specific methods for traceability of GMMs exposure is very demanding.

1.5. Effect on intestinal microflora

A great amount of alive microorganisms (up to 10^{14}) inhabit the human being gastrointestinal tract during its life. The constitution of the microflora numbers about 400 species. It is not well-known as the big part of it can not be analyzed due to lack of adequate techniques. It is considered that the available classical and molecular approaches are not powerful enough to describe all this diversity of microorganisms. The type of microbial population changes subsequently in respect to the composition and amount from oral cavity (where a dominant microbial species are lactic acid bacteria, streptococci and some anaerobic species), to the stomach (transient acid tolerant microbes), to the small intestine (populated by colon - like microflora), to the colon, where microbial entities reach up to 10^{12} grams/ dry weight.

The population colonizing the colon is dominated by anaerobes like *Bifidobacterium, Eubacterium, Bacteroides and Clostridium*. The microaerophilic and facultative anaerobes such as lactobacilli, enterococci, and coliforms are usually less - 3 to 4 orders of magnitude. This endogenous microflora living in gastrointestinal tract represents a main barrier against exogenous microflora tending to be invading GI and assure the so-called colonization resistance.

The composition of the gastrointestinal (GI) microflora in quantitative and qualitative aspects strongly depends on a number of factors including:

- environmental factors - type of diet, the antimicrobial therapy applied, disinfectants, food additives, occupation, climate.
- host associated factors - age, gender, intestinal motility, transit time, pH, bile acids defenses etc.
- interrelation of species in GI microflora - rate of nutrient uptake, oxygen, H^+, H_2S, production of antimicrobial agents, organic acids, NH_3

All these factors have a concerted action on the overall status of the GI microflora.

There are some interactions between GI microfolora and different mammalian host - associated structures and functions. These interactions could be found at different levels: organs, cell and molecular, and could be summarized as: prokaryotic-eukaryotic cross-links at cellular level; production of organic acids, nucleotides etc.; interaction with enterohepatic circulation; development of gut associated immune system (GAIS); influence on intestinal motility and enterocystic mitosis. They depend on the age and the health status of the individual. Thus, the survival of the exogenous microflora (including GMMs) is subordinated to their ability to overwhelm the influence of the indigenous GI microflora and above mentioned host -

associated factors. Also the colonization resistance takes part in evaluation of survival, but these mechanisms are not well understood. Sometimes microbes can leave the gut lumen and appear elsewhere. The term describing this behavior is translocation. To determine the intestinal survival of microbes in vitro is a difficult task. For this reason a selection of suitable animal models, simulating human GI system and experiments with patients are required. These investigations should be supported by reliable strain identification methodology.

In case the introduced GMMs into GI system survive digestion, they could appear as transient one or may establish itself for different time in the gut. This phenomenon is described by term colonization measured with a constant level of microorganisms detected for a relevant period of time.

Long life (permanent) colonization of the GI tract of adults by exogenous microorganisms is very rare. But if it happens, an application of certain probiotic strains indicates that the normal microflora could be recovered in the facies and colonic mucosa for weeks after oral administration. To describe the survival of microorganisms in GI tract for period longer than two intestinal transit times term “persistence” has been involved.

In case of introduction of GMMs in the GI tract, independently of its establishment (detected or not), the possibility exists for interaction with microflora of the mammalian host. The anticipated effect on gut flora could partly depend on the functions expressed by GMM (phenotypic expression) and this way on horizontal gene transfer.

The effect of GMMs on mammalian host could be defined as direct and indirect. The direct one is characterized by total influence on all structures and functions listed above and indirect could be mediated by interaction with endogenous microflora and particularly with their active elements. Both types of interactions (direct/indirect) could be provoked also by non-viable microorganisms as they preserve functional properties (i.e. immuno modulation, chemical binding, cell adhesion). Some secretion of biologically active compounds like toxins, enzymes could also be anticipated.

The possibility for gene transfer has been already discussed. So it is reasonable to consider conjugate transfer between microorganisms in the gut which depends both on the relatedness of GMM to the intestinal microflora and its residence time in the GI tract. This effect could be expected with persistent or colonizing strains. The transient strains are of low influence in this respect. At present there is no doubt that a measurable persistence of DNA in the intestinal tract exists. It has been found that plant and recombinant DNA could enter the blood system, tissue cells and even nuclei of mammalian host.

1.6. Effect on immune system

The conclusion of FAO/WHO Consultation regarding assessment of immuno modulating potential of GMMs is that case-by-case considerations are necessary. For instance, about the allergenic properties these organizations have already made several recommendations.

There are findings indicating the interactions between gut microflora and the status of the immune system. It should be noted that in contrast to GM plants, GMMs are well established in GI tract, thus causing potential immune modulating effects.

2. The future perspectives

The presently available methods for detection of GMOs and their derivatives cannot distinguish between two different ingredients in a foodstuff. The methods can only be used to detect and quantify the content of GMO at species level.

Currently the companies seeking approval of their GMO in Europe are allowed to keep secret the sequence information describing their GMO. In this way scientists lack basic information to design detection methods.

Better methods for isolation of the molecules of interest to be detected and their quantitative and qualitative analysis are needed and recently under development. The majority of the developments are focused on DNA methods that may allow increased specificity through;

- PCR methods targeting the junction between the insert and the integration part;
- GMO specific fingerprinting methods similar to those used for criminals;
- Diagnostic micro-arrays similar to those determining the inheritable disease predisposal.

Finally, proficiency tests have to be organized for industry control authorities and other purchasing GMO analyses by accredited laboratories using international standards.

3. Concluding remarks

The safety evaluation process should be based on:

- case by case basis through implementation of series of well defined questions
- comparative approach to identification of similarities and differences between classically obtained and GMMs foods through application of the concept of substantial equivalence, providing practical means
- introduction of special considerations regarding intrinsing properties of microorganisms through evaluation of the impact of GMMs on the food matrix

- application of the concept of ***substantial equivalence*** both to the GMM and the food produced through examination of additional parameters like pathogenicity and persistence in the mammalian GI tract
- consideration of specific uses and exposures to GMM - GMMs could be an integral part of foods in viable or non-viable form
- evaluation of GMMs should be done in safety and nutritional aspects - microorganisms used in food production possess basic importance for nutritional quality and safety of the product
- evaluation of the effect of GMMs or their parts on the immune system in mammalian host needs additional consideration - microorganisms in GI tract affect the immune system and the implementation of GMM in food production needs specific safety assessment regarding the used system for manipulation
- careful evaluation of possible gene transfer from GMM to gut microflora - genetic material from food has the potential for transfer into gut microflora and mammalian cells in vivo
- strong necessity for history of safe - use in food of host microorganism the used host microorganisms should have a safe status for use in food; a selective marker should be chosen very carefully in respect to safe use, and antimicrobial resistance marker genes should be avoided and excluded in the final GMM

An overview of the suggested future development of the risk assessment process is outlined in Fig. 2.

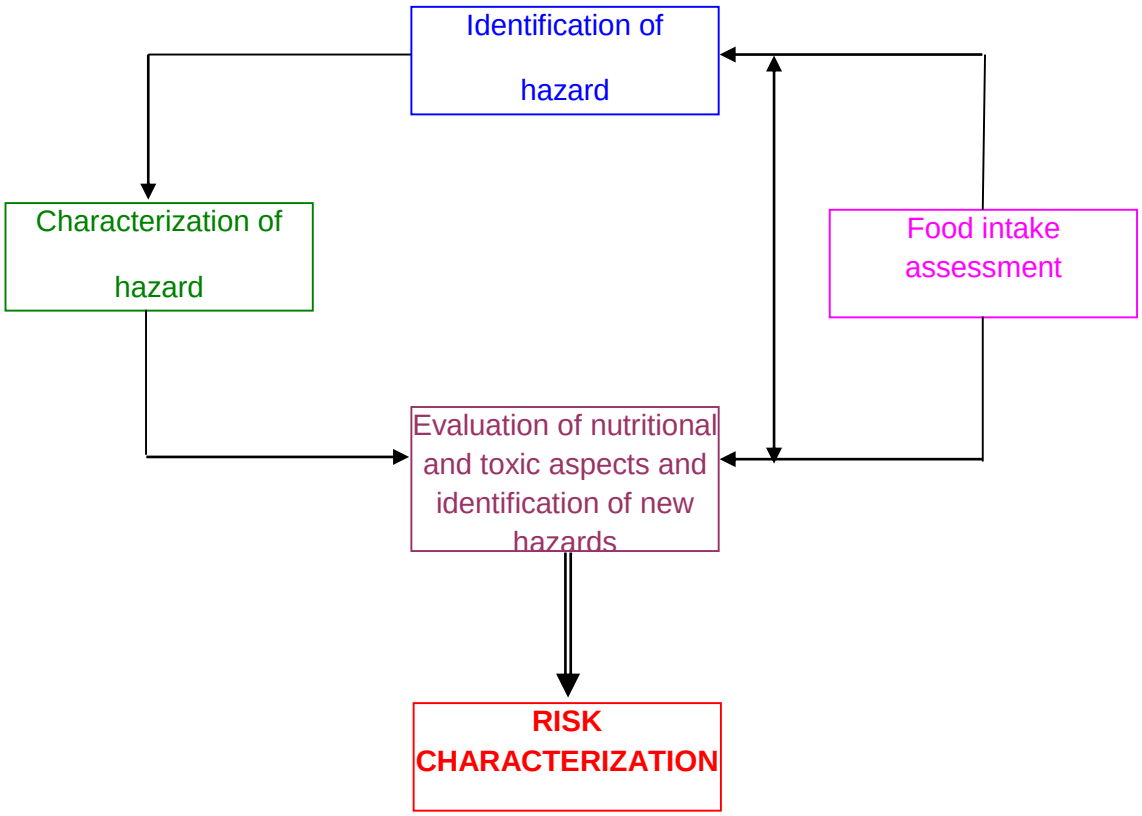

Fig. 2. Overview of suggested future development of risk assessment process.

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