marlon

Work Package 9

Dissemination, Training and Communication

Overarching report

Project Deliverable D09.07

monitoring the safety of animal feed

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Website: www.unitus.it

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1. Background of the MARLON project

Introduction

The primary focus of the recently finished MARLON project was the safety of genetically modified (GM) feeds for livestock animals. The epidemiological tools and other data that the project developed are intended to support the post-market monitoring of GM feed impacts on animals' health. The tool and data are complementary to the pre-risk assessment already to be undertaken for each GM feed intended to be marketed.

Crop biotechnology & regulatory safety assessment

The field of agricultural biotechnology has witnessed some major developments over the last few decades, notably in the commercialization of GM variants of major commodity crops such as soya, maize, cotton, and canola in the mid-1990s. Since then, the adoption of GM crops by farmers worldwide has increased progressively, with 181.5 million hectares of arable land planted with these crops in 2014 [James, 2014]. While most of these crops are grown outside Europe, substantial amounts of the harvested products, either processed or unprocessed, can still find their way into the European food and animal feed supply chains, for example through imports as feed commodities.

Before GM crops can be grown in the field, and products consisting of, produced with, or derived from GM crops can be marketed as food or feed, they need to receive regulatory approval in the European Union and other countries across the globe. Part of the regulatory procedure consists of a pre-market safety assessment according to internationally harmonized guidelines of the Codex Alimentarius, which is a joint programme of the Food and Agriculture Organization (FAO) and World Health Organization (WHO) establishing standards, good practices, and guidelines for food quality and safety. The principles of the safety assessment have also been elaborated in more detail by the guidelines published European Food Safety Authority's (EFSA) Panel on Genetically Modified Organisms (GMO Panel) [EFSA, 2011], which further served as basis for the Implementing Regulation (EU) No. 503/2013 [EU, 2013].

Pre-market safety assessment approach for GM crops

A key step in the pre-market safety assessment is the comparative assessment in which the GM crop is compared to a conventional non-GM counterpart with a history of safe use. It is recognized that since foods and feeds are complex mixtures of substances, both with beneficial and adverse effects (sometimes even from the same substance, depending on the level of intake) it will not be possible to test their safety in exactly the same way as highly purified chemical substances, such as much more limited dose range in laboratory animals.

For the comparative assessment, an extensive range of parameters linked to the composition and physiology/morphology of the plant are tested, including an analysis of key macronutrients, micronutrients, anti-nutrients, and toxins characteristic of the particular crop, usually from parts harvested from the crop during field trials. In addition, plants may be checked for characteristics of plant morphology, reproduction, development, field behaviour, and traits of agronomic interest. In addition, a molecular characterization of inserted DNA and the products derived from newly introduced genes (for example, newly expressed proteins) is commonly carried out. Based upon the differences thus identified between the GM crop and the non-GM counterpart, it is decided if further tests are needed to establish the safety of the GM crop or whether the existing knowledge and data are already enough to conclude on the safety implications of these differences.

Besides the molecular characterization and the comparative analysis of composition and agronomic/phenotypic characteristics, the following items are commonly included in the safety assessment data submitted to EFSA as part of the dossier for regulatory market approval of GM crops:

- Potential toxicity of newly expressed proteins and other substances being different between the GM crop and its counterpart;
- Possibility of newly expressed proteins becoming allergens (i.e. with the capacity to induce allergic reactions) given that all known food allergens are proteins. Moreover, the likelihood of unintended changes caused by the genetic modification to the overall allergenicity of the host crop, if it is known to be allergenic (e.g. soybean), will be assessed
- Any changes in the nutritional properties of the GM crops, such as changes in the levels of key nutrients or in their bioavailability. Some pre-commercial GM crops

have actually been deliberately changed in their nutrient content (e.g. essential amino acids) for feed purposes. Another example from GM crops in the pipeline is the enzyme phytase being engineered into these crops in order to increase the bioavailability of phosphorus and several essential metals.

- Unintended changes caused by the genetic modification. The extensive comparative analysis of compositional, agronomic and phenotypic characteristics will give an indication of whether any other effect than those targeted with the genetic modification might have taken place. Feeding trials with the whole GM food/feed being fed to experimental animals may also be devised for this purpose as additional test, such as in case there is much uncertainty left or there is no good non-GM comparator available. It should be kept in mind that these tests have their limitations, such as a limited dose range (to avoid nutritional imbalance and also based on factors such as bulkiness and palatability) and other considerations such as extrapolation to other species as well as ethical concerns.
- Potential for horizontal gene transfer: The possible consequences of a potential uptake by recipient organisms (particularly disease-causing micro-organisms) of newly introduced plant DNA released from the GM crops (for example, released during digestion of the plant tissues) and incorporation into the recipient's own genetic make-up is considered. The likelihood of a spontaneous transfer of functional plant DNA to a pathogenic bacterium itself is considered negligible, notwithstanding the fact that transfer mechanisms exist among microorganisms where horizontal transfer is a relatively common phenomenon (although these are not relevant for the scenario of GM crops).
- Pesticide residues: In herbicide-resistant GM crops, the application of target herbicide to the GM crop may actually lead to different levels of herbicide residues and other metabolites than in its non-GM counterparts. This is, however, considered during the risk assessment of the specific crop-herbicide combinations under the pesticide regulations, in parallel to the regulatory GM crop approval procedure.

Post-market monitoring

Post-market monitoring of a product which has been allowed to enter the market can focus on possible health effects arising from consumption of the GM food/feed identified

during the pre-market safety assessment, and also on verification of intended uses (for example, for restricted purposes) and intake estimates that have been taken into account in the pre-market assessment. Post-market monitoring is therefore not intended to replace the pre-market safety assessment of the GM crop but may therefore help to verify certain assumptions made during the pre-market phase. Moreover, it is not a mandatory requirement to carry out post-market monitoring and whether to do this or not will have to be decided upon based on the outcomes of the pre-market safety assessment. While the actual performance of such monitoring is a so-called "risk management" activity assigned to the field of decision takers and policy makers, the design of a monitoring plan and its scientific background are still within the domain of the scientific safety assessors advising the risk managers.

For food and feed derived from GM crops, companies requesting market approval for such GM products in the European Union must prepare a dossier with safety data that they submit along with an application for marketing under Regulation (EC) No. 1829/2003 [EU, 2003a]. An accompanying proposal for post-market monitoring should only be provided if appropriate. Interestingly, the Implementing regulation (EU) No. 503/2013 identifies examples of situations for which monitoring may be appropriate, including the verification of actual consumption in case of a changed nutritional composition or value, or if the allergenicity of the GM crop has likely been increased as a result of the genetic modification. Monitoring can also serve the purpose of verifying the intensity and relevance of effects identified in the pre-market stage, including unintended effects besides those targeted by the genetic modification of the crop [EU, 2013].

Two types of post-market activities can be distinguished similar to what is undertaken in the environmental risk assessment of GM crops (under Directive 2001/18/EC [EU, 2001]), namely:

- General surveillance, in which adverse effect reports are recorded (usually passively such as through reporting systems including phone numbers or online systems) and farmers and other stakeholders may be regularly interviewed via questionnaires for any deviating observations they made related to usage of the GM crop. In the European Union, general surveillance commonly must be carried out for each approved GM crop, which may also include surveys of farmers of which the questionnaires include questions about potential adverse effects observed after feeding animals GM crops, as well as solicitation of feedback, i.e.

the reporting back of any effects, if applicable, of which stakeholders in the feed production chain (such as feed processors and handlers) have become aware. Product producers usually have to inform the governmental officials at regular intervals about the reports received, while also needing to immediately report any serious, acute health threats that they become aware of so that appropriate action can then be taken by the government. Interestingly, during general surveillance for the marketing of GM insect-resistant maize for cultivation, import, and processing in the European Union, farmers reported a change in animal performance, probably related to decreased mycotoxin contamination of this maize under specific circumstances, namely decreased insect damage leading to less mould infection of the crop (BioMath, 2013). This kind of surveillance is done within the European Union as part of the monitoring plan that needs to be proposed and evaluated during the pre-market assessment according to the requirements posed by Directive 2001/18/EC on the deliberate release of genetically modified organisms (GMOs) into the environment [EU, 2001]. The European biotechnology industry association EuropaBio has developed a harmonized methodology towards general surveillance for use by its members (particularly companies wishing to market GM crops and therefore preparing market applications) including a sample farmer questionnaire and a webpage with information for production chain operators on which GM products have been approved for marketing with the option to report any possible adverse effects they observe in relation to these products [Windels et al., 2008; EuropaBio, 2009].

- Case-specific monitoring for verification, in the post-market stage, of presumptions about risks, uses and intake of the GM product that have already been made during the pre-market assessment. This need for verification may arise, for example, from limited extrapolation possibilities from field-scale experiments to the market environment. In the European Union, such monitoring has been recommended and required for vegetable oils from GM soybean with modified oil composition based on the changes in intake of polyunsaturated fatty acids, as part of the decision by the European Commission for market approval of these GM oilseed crops with modified oil composition. However, this kind of requirement for case-specific monitoring has hitherto not been imposed for consumption of GM feeds by animals.

Challenges linked to post-market monitoring of GM feed

In brief, the challenges posed to post-market monitoring of GM feed include interdisciplinary research cooperation, the lack of a regulatory history of post-market monitoring of GM feeds, and practical feasibility of relating any observed health impacts with GM feed consumption.

The field of GMO biosafety research has been well-developed over the last two decades, witnessing a flurry of activities and publications, while currently being also the focus of systematic reviews and laboratory investigations in various parallel European Union and nationally funded research projects (*e.g.,* AMIGA, GMO90+, GMSAFOOD, GRACE, G-TwYST, and SAFE FOODS). While various studies previously focused on the performance and health of livestock animals fed feeds derived from GM crops in controlled experiments of limited duration (*e.g.* production cycle), no particular attention had been paid to the possibility of monitoring of such animals for possible health implications of GM feeds being consumed by these animals. As stated previously, there has so far neither been a regulatory requirement for a specific GM crop to be monitored for pre-defined animal health implications in EU regulatory decisions (notwithstanding this option being available to decision makers), while the authors are also unaware of such requirements being posed elsewhere.

In parallel to the GM biosafety research mentioned above, the field of veterinary health surveillance and epidemiology has been receiving a growing interest and its importance for the protection of animal and human health widely recognized. This also relates to the various roles that epidemiology and surveillance can help fulfil such as the early detection emerging, re-merging or exotic animal diseases, provision of reassurance that regions are disease-free (*e.g.* to avoid trade barriers to exported animal products), and research into the behaviour and spread of diseases (with an eye on preventive strategies). There is also an increasing awareness of the linkage between veterinary health for public health given that many of the pathogens causing human diseases that emerged recently have their origins in livestock animals, while the human – animal interface provides for various routes of transmission, such as through food or direct contact. However, the topic of feed-related health impacts have received little interest also in this field while some recent initiatives focus more specifically on feed-related

adverse effect surveillance. A recent publication sought to compare various routinely surveyed production data of various livestock species (pigs, cows, goats, poultry) related to health and performance from before and after the large-scale introduction of GM crops into the feed supply chains over a 30 year period [Van Eenennaam and Young, 2014]. The authors thus observed that the trends in the measured parameters (e.g. milk production, somatic cell count) were comparable and hence not indicative of a potential adverse impact of GM crops.

Another important issue considered within the project is how to be able to relate any observed change in the health of livestock, either in individual or groups of animals, to prior exposure to specific GM feed ingredients consumed by these animals. While information on trade flows and the degree of adoption of GM crops in countries exporting feed commodities to the European Union is available, it still needed to be clarified whether existing data, reporting, and control activities would allow to determine more precisely the presence of specific GM crop-derived ingredients in feeds.

National inspection services, for example, are known to carry out inspections at regular intervals, sampling select feed and food products. This is commonly done for the purpose of verifying the correctness of labelling as the presence of market-approved GM ingredients has to be mentioned on the label of the pertinent feed and food products, and also to test for the presence of unapproved GM ingredients, while the outcomes may require follow-up risk management measures to be taken in case of non-compliance. The question thus arises which kind of data are generated from these inspections and whether they are amenable to exposure estimates.

It was also intended to explore whether there was a possibility to exploit other types of indicator of previous exposure. If foreign, GM-crop-related DNA or protein survived digestion or even were transferred to animal tissues or body fluids, for example, it would be interesting to know if the potential presence of such DNA or protein could then be used as a token of exposure. This might also hold true for any secondary changes in the animals' physiology in response to GM components, if they occurred.

The European Commission's call for research

In 2011, the European Commission's Directorate for Research (DG RES) published a call for research proposals within the "Food, Agriculture & Fisheries, and Biotechnology" theme of the Seventh Framework Programme (FP7) for research and technology development, which also served the aim of building a Knowledge-Based Bio-Economy (KBBE) within the European research area. As one of the topics within the area of food quality and safety, proposals were called for a coordination and support action type of project on "Post-market monitoring of GMOs based on epidemiological studies".

The call text acknowledged that there was a need to develop agreed scientific methodologies for such epidemiological studies and that proposed projects should investigate the feasibility of performing such studies. In particular, they should identify relevant studies, collect relevant data for performing such studies in a database, establish an epidemiological tool based on collection of existing data while meeting the needs of the project, and assess and validate the model for the main circumstances encountered in Europe.

During the subsequent evaluation procedure, the proposal for the MARLON project was selected, and following various administrative preparations, was started in August 2012 as a three-year project.

2. The MARLON project

Introduction

The acronym MARLON stands for "Monitoring of Animals for Feed-related Risks in the Long Term". It is a three-year Coordination and Support Action which started in 2012, with the aim of collating existing relevant background information, knowledge and insights and develop a methodology, including an epidemiological tool and background data, which would enable the monitoring of health impacts of feed consumption in livestock animals. It was not intended to perform original research such as feeding studies or to carry out monitoring within the project itself.

Expected outputs and impact

The expected outputs of the project included in particular:

- An epidemiological tool for monitoring studies that aim to identify potential changes in the health of livestock linked to feed consumption, and verification of the feasibility of applying this tool to real-life scenarios;
- Collection of background data useful for designing monitoring programs, including existing monitoring activities that can provide input data and outcomes of previous research relevant to the topics of health impacts of GM feed and of detection of GM feeds and exposure of animals to such feeds;
- Recommendations, training and guidelines for the monitoring of animal health impacts of feed consumption, including the application of the projects outcomes as well as potential areas for further exploration.

While the project's focus was on GM feeds, these outcomes are expected to have a broader and more general applicability to other types of animal feed as well. Overall, the results of the project are envisaged to further contribute to

- The assurance of the quality and safety of livestock feeds within the European Union;
- A harmonized approach towards the monitoring of GM feeds should this be required as part of a regulatory authorities' decision for their market authorization;

- A greater insight into the relationship between feed consumption by livestock and associated impacts on the health and welfare of these animals.

The project brought together a consortium of 11 partners from 8 European countries, each contributing with its specific expertise in a topic relevant to the work performed, including scientific knowledge and research experience in particular fields of veterinary epidemiology, animal health and nutrition, and GMO biosafety, as well as communication and dissemination, and administrative and legal affairs linked to project activities and reporting. The project also solicited input from three independent scientific advisors, who monitored the progress of the project, and provided feedback and advice, such as during the project consortium meetings which they also attended. The European Commission, which was the main sponsor of the project, had assigned, among their staff, both scientific and financial officers to monitor the project progress. The financial and in-kind contributions received from other institutions (such as but not limited to the Dutch Ministry of Economic Affairs) are also gratefully acknowledged.

Division of tasks and activities

The activities within the project were divided into different Work Packages with specific tasks. These activities can be divided into three major "vertical" strands of research, including "data collection", "epidemiological model development", "feasibility of implementation (of developed methodology)". The horizontal, cross-cutting issues included dissemination & communication, scientific coordination, training, and administrative tasks, such as reporting financial and scientific progress to the European Commission [Figure 1].

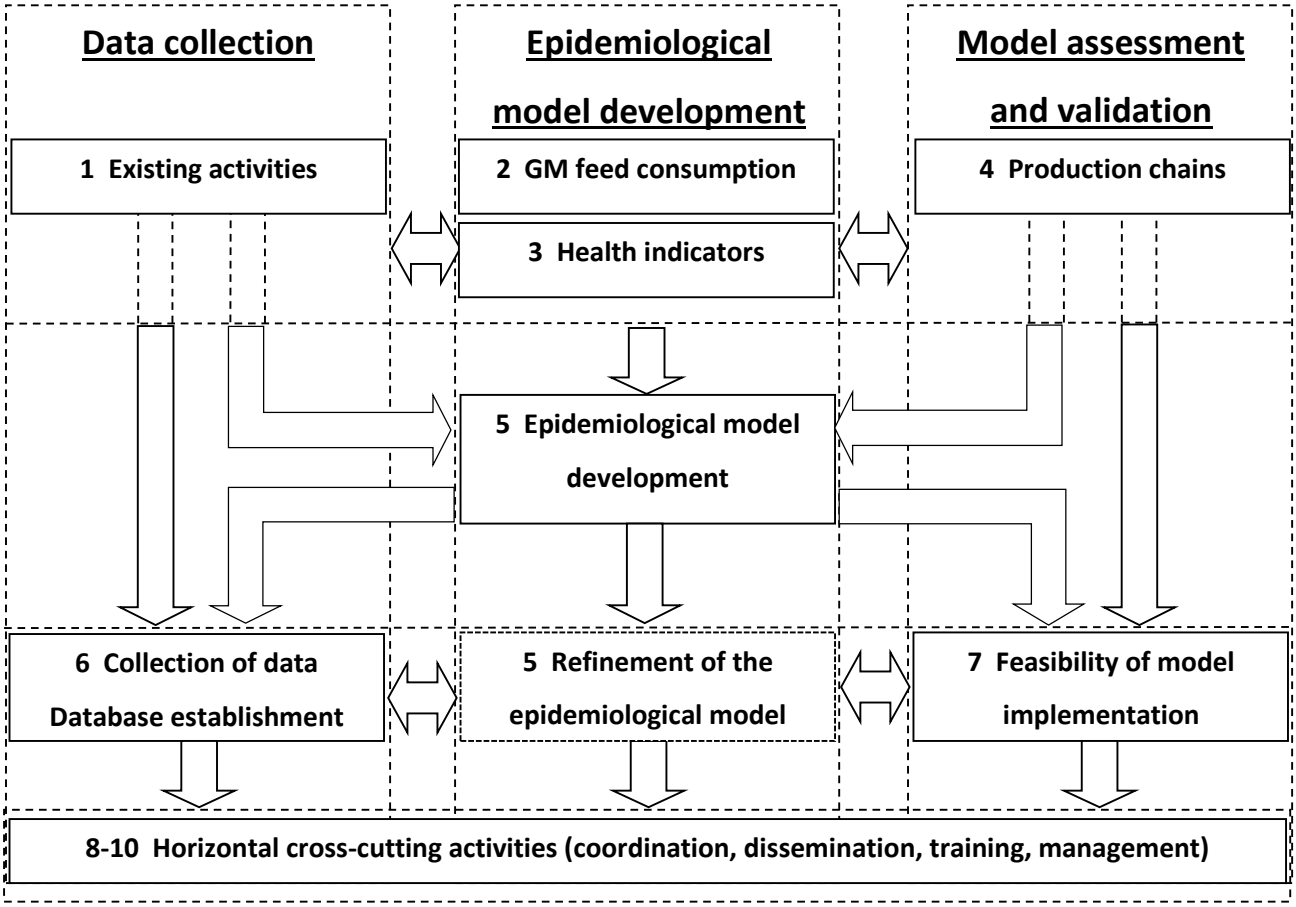

Figure 1: Activities of the MARLON project: three main vertical strands of research and division of tasks across Work Packages 1-10

The outcomes of the various activities within each of the three strands of research are summarized below, while in the next section the recommendations formulated on the basis of these outcomes and the experiences gained with performing the activities are provided.

Data collection

Inventory of existing knowledge and initiatives

Broadly defined, the aim was to collect data useful for the design and implementation of monitoring of animal health for impacts of GM feed or of feed in more general terms. These data included outcomes of scientific studies published in scientific literature or research reports on the impact of GM crop on animal health, frequently in the form of controlled feeding studies in which groups of animals consuming a particular GM crop

ingredient were compared to those consuming a non-GM counterpart. Another type of data of interest is on the fate of novel DNA and protein from GM crops in livestock production (feed, digesta, tissues, fluids and products) and their detection in these products. A further important category of data were the existent monitoring activities for animal health that could provide useful input data for the monitoring of livestock for feed-related impacts. While such potentially useful initiatives were identified, it was not intended, though, to perform the collection of the data generated by these initiatives but rather to list the latter so that they can assist in the design of any future activities that might draw on these initiatives as a source of data.

A review was conducted of articles on the health of livestock animals fed GM feedstuffs published in scientific literature. These articles had been retrieved and selected from a bibliographic search and also from the reference lists within these selected articles. The review thus concluded that few of the selected studies investigated health parameters beyond performance (*i.e.* feed consumption, growth, and body and product composition). The reviewers concluded that for measuring health in livestock, a good indication might be obtained from a combination of parameters, including serum biochemistry, haematology, organ weights, and histopathology (microscopy). Moreover, the authors noted that ideally, the comparator used in the feeding trials should be isogenic and grown under the same conditions as the GM crop. This was actually one of the design items found implicated in various studies, besides the experimental design and statistical analysis. In most cases, the GM crops tested belonged to the so-called first generation with genetically modified traits of agronomic interest, such as insect resistance and herbicide tolerance, which neither are intended to have a modified composition nor target the crop composition such as for improved nutritional value for livestock animals, unlike various GM crops that may enter the market in the near future. The studies thus reviewed did not raise concerns over possible impacts of GM on livestock health.

An inventory was also made of the various parallel EU projects, current and past, that dealt with the issue of GM feed or food in relation to animal health, as well as other initiatives and institutions active in this field at the EU and international level for GMO biosafety and animal health if relevant to the topic of the MARLON project. National research projects, sources of reports of feed controls and inspections in selected EU countries (of the partners), and pertinent research reports on national research initiatives were also identified. An inventory of national (in 3 partners' countries, namely Bulgaria,

Germany and Netherlands) animal health monitoring programs was created. It was observed that while many activities focus on animal health, there were no systems identified that collect data about GM feed and a small number of systems that collect data on feed.

The IPAFEED database

In a following step, the accessible online IPAFEED database (http://www.ipafeed.eu) was established with data on three topics that were posted in three corresponding database sections entitled "GM feed consumption", "DNA and protein detection", and "Health monitoring programs" [Figure 2]. The purpose of the database is to allow for data mining by the biosafety research community as well as other interested stakeholder groups. The extracted and annotated data has been stored within the database in a searchable, user-friendly format, which will facilitate exploration of the background knowledge on the health impacts of GM feeds in livestock animals as well as the fate of transgenic DNA and newly expressed proteins in livestock animals consuming feeds containing these substances.

The first two sections (consumption, detection) contained data from scientific literature studies retrieved through literature searches in a scientific bibliography (Scopus). As the search strings are provided, these searches can be updated so as to include the most recent research outcomes. The studies were scrutinized and data extracted with each of the health parameters entered as a single entry so that the outcomes for a given parameter in different studies can be retrieved separately and across the database. The data on the experiments that investigated the possible presence and transfer of transgenic and newly expressed proteins into livestock animals was treated in a similar way. Data in both sections can be searched using selection criteria such as animal species, GM crop, trait, inserted gene, type of health parameter (for health), and detection methods and type of sample (for transfer studies).

The third component on existing monitoring activities contains the data collected from Bulgaria, Germany and Netherlands, as described above. Entries include data on, for example country, system name, the aim of the monitoring activity, whether or not feed data are collected, livestock species covered by the program, organization in charge of

the program, and website address. The database can be searched on country and animal species.

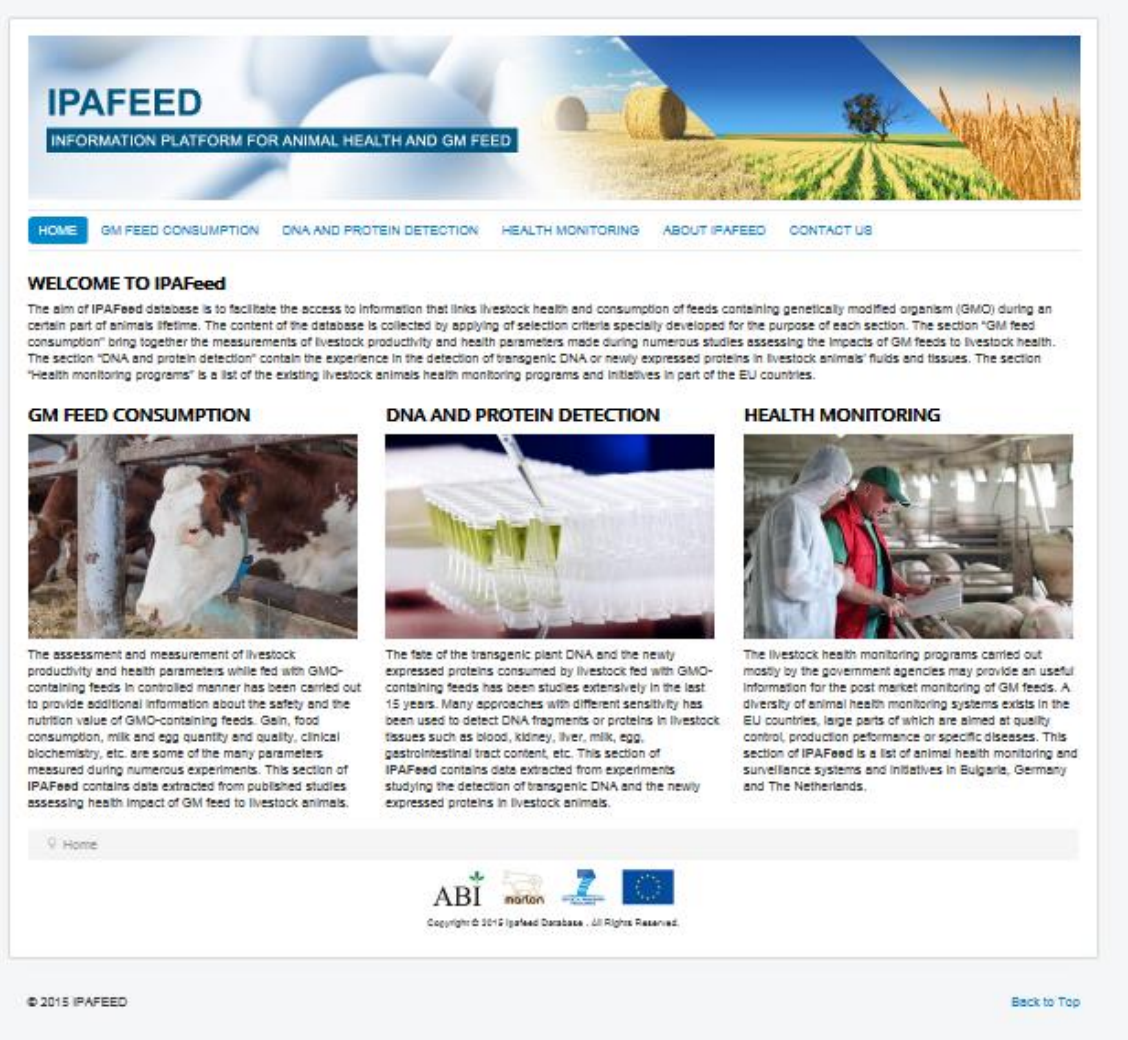

Figure 2: Screenshot of IPAFEED website

The parallel initiatives were also approached in the frame of dissemination activities, such as for the exchange of information on events and outcomes and posting of news items related to this on websites.

Epidemiological model development

Technical options to measure exposure of livestock animals to consumed GM crops

In a review of scientific literature, the feasibility of measuring GMOs in animal samples as a measure or prior exposure to GM feeds was explored. This included a study of the existing knowledge regarding whether the transfer of GM DNA from consumed feeds to animal tissues and fluids occurs, and the possibilities to detect such transferred DNA, including methods that can be used towards this end. Besides DNA, the same was done for the potential transfer of newly expressed proteins from GM crops to animals consuming feeds with GM crops containing such proteins. It was observed that DNA from plants was not completely degraded in the gastro-intestinal tracts of various livestock species and in some cases uptake of DNA fragments into tissues and fluids has been observed. Yet it was also recognized that the design of the studies was highly variable and in a few cases, positive detects were actually due to contamination. The polymerase chain reaction was considered the DNA detection method of choice (amplifying and measuring DNA fragments specific for the analytical target, in this a given GM crop) for this kind of studies. However, for the newly expressed proteins, it was considered unlikely that protein detection methods could measure residues of such proteins in animal tissues, fluids, and excreta based on considerations of the likely absence of antigenic residues in such tissues, and the lack of sensitivity and specificity for detection of the proteins in complex biological materials.

Also the possibility to apply metabolomic approaches to samples from the animals was considered. Such methods would measure possible changes in the array of metabolites (biochemical substances) present in such samples in an indiscriminate, non-targeted way, that is, without defining on beforehand which specific analytical parameters the analysis will zoom into. This was considered to be a possibility for GM crops with nutritional modifications as these are likely to impact on certain compositional parameters that are likely to be changed in the composition of animal products from animals consuming these nutritionally improved crops. However, the review also made a number of caveats before metabolomic techniques can be applied, such as the need to extensively evaluate the use of biomarkers identified through the metabolomic approaches before their use as markers for exposure, excluding the interference by confounding factors.

The review also explored the technical options for DNA-based detection, including novel DNA-based technologies for the "multiplex" detection of multiple approved GMOs in a single analysis. While currently no multiplex methods are routinely used for this purpose, the review identified a number of promising new developments that might be further tested and developed for this purpose. These include miniaturized PCR approaches such as droplet PCR, as well as a combination of PCR to multiply target DNA sequences with next-generation sequencing for identification of the approved an unapproved GMOs within a sample.

Intricately linked with analysis is also the issue of sampling, while the complexity of the feed production chains and factors such as mixing of harvests from different origins that will contribute to the heterogeneity of the consignments and products potentially containing GM crops. While there are directions on how to sample consignments of feed for the purpose of GM analysis, this is at one of the production stages and there are currently no directions at which checkpoints within the production chain samples should be taken. Besides the various stages from seed via field, storage places, transfer between handlers and processors, and commercialization, a main point of sampling is likely to be at the elevators (that is, the facilities receiving, storing, mixing, and dispatching the harvested feed crop grains).

Besides the technical feasibility of measuring the presence of DNA from GM crops in feeds and possibly animal digesta and products, traceability data on the GM crop may provide another option. This is particularly the case for the European Union given its legal traceability requirements for GM food and feed, which entail a duty to keep records (documentation) at every stage of the production chain of the GM crop products received and dispatched. A case study of GM maize was applied to the dairy production chain in Catalonia. It was thus observed that there are extensive documentation requirements in place (besides the GM feed traceability already mentioned) at the various stages of field cultivation, drying after harvest, transport, wholesale trade, feed imports, feed production, and livestock farming. While it will indeed be possible to verify whether or not feeds contain GM crops, most of the more detailed information on which particular GM crop (GM event, variety) was used and at which level it is present in the traded commodity will already be lost at an early stage in the production chain. For example, mixing of different maize lots of different nature can occur after harvest at facilities for drying or transport. This would be even further

complicated if multiple GM crops (instead of the single GM crop, namely a particular insect-resistant maize) were to be grown in the same region at the same time.

Potential health indicators based on four case studies

Until present, there has not been any known instance of specific health effects of GM feeds being identified, which could also lead to a requirement of post-market monitoring of such effects among livestock consuming feeds containing the particular GM crops in question. To explore the possibility to employ health indicators for such monitoring, four cases were conceived based on issues hitherto addressed in safety assessment of GM crops for human food and/or environmental risks (allergenicity, horizontal gene transfer, altered nutritional value), as well as a possible cause for an observation reported during the general surveillance of a GM insect-resistant maize grown in a limited number of European Union countries (lowered mycotoxin content). These cases were thus elaborated within the project, while the possibilities were explored to devise health indicators that are amenable to monitoring for these effects.

The first case was potential changes in the intrinsic allergenicity (for livestock animals) of the host. Whereas allergic reaction to certain crops have been well-described for human patients, there is scarce data on such reactions towards feeds in animals including livestock and details on the prevalence of such reactions among them is also unavailable. There are no reports of allergic reactions in animals fed GM feeds as compared to non-GM feeds. While measuring IgE, the main immunoglobulin class associated with allergy in sera of allergy patients, could be a strategy for post-market monitoring in livestock, these are not always linked to clinical responses. It was thus concluded that for this scenario, case-control studies might be more suitable.

The second case pertained to the potential horizontal gene transfer of inserted DNA from the GM crop to recipient organisms, in particular to bacteria in the gastrointestinal tract of the animal consuming the crop. This has been of a particular concern for antibiotic resistance marker genes introduced into a limited number of GM crops in which they, while there are currently strategies that allow for avoidance of such genes in GM crop development. For the risk assessment it is also important to consider the function the transferred DNA and the background occurrence of this gene in other sources than the GM crop. The likelihood of such a crop-bacteria transfer spontaneously occurring was

considered a rare event, while also taking into account the background occurrence of transfer of genes between bacteria (where this is a more common phenomenon) and the difficulties surrounding the integration (without homologous recombination) and expression of plant DNA in bacteria.

The third case study pertained to lowered mycotoxin levels that had occasionally been reported for insect-resistant maize, which probably relates to the fact that crop damage caused by pest insects provide a point of entry for mould infection and consequently for mycotoxin production by these moulds under specific conditions. An overview was made of the various field studies in which insect-resistant maize had been tested for this, with the notion that also other crops might be prone to mycotoxin contamination due to mould infection after insect infestation. While these studies appear to be consistent in that a lower fumonisin levels can be achieved in GM maize, the outcomes of the field data also showed a mixed outcome for other mycotoxins, including aflatoxins, zearelenone, deoxynivalenol, failing to show a systematic control by the insect resistance trait. Given that the lower mycotoxin contamination is a potentially beneficial side effect of the insect resistance trait (also depending on the fact that no insect control measures are taken in the non-GM counterparts) and therefore not the primary aim of the genetic modification, this could be assessed on a case-by-case basis. Moreover, a more systematic pan-European research study would be needed to verify the effectiveness of GM insect-resistant maize. While aflatoxins and fumonisins are considered to be of interest due to their impact on animal health, only the presence of aflatoxins is regularly checked for in the frame of existing control activities, namely aflatoxin B1 in feed and its metabolite aflatoxin M1 (formed by the livestock animal from B1) in milk

The fourth study was on nutritionally improved GM crops. As stated above, such crops have been recognized as scenario for exposure assessment and post-market monitoring. Guidance that has been developed for the performance of controlled studies on performance of livestock animals, including poultry, pigs, and lactating cows, provides a number of parameters that could also be useful for monitoring purposes, such as feed intake, weight gain, feed conversion, animal product yield and composition, and cell counts in milk.

Among the nutritional modifications considered were elevated levels of essential amino acids and fatty acids, and decreased anti-nutrients (or indirectly, an anti-nutrient-metabolizing enzyme such as phytic-acid-degrading phytase, which increases the

bioavailability of phosphorus from feeds containing phytic acid). For amino acids, it was considered that the GM modification may replace the addition of pure amino acids to feeds as a feed additives. Therefore, if animal diets are balanced by adding amino acids to optimal levels in diets containing the non-GM crop counterpart of the GM crop, the impact of the elevated essential amino acids in the GM crop feed ingredient may be compensated for by this practice and no final impact is expected. Only studies in which the targeted essential amino acid is limiting would be expected to be able to show any potential impact. Performance measures related to amino acid nutrition include production performance in rapidly growing animals needing balanced diets for optimal growth, as well as reduced lean meat content. However, these are syndromes that may also be related to other changed conditions in husbandry practices.

As regards fatty acids, there are no indications of adverse health effects while the variations in feed fatty acid composition caused by the change in the fatty acid profile of the oil is likely to be much lower than that caused by shifts in the various crop ingredients. Moreover, the crop materials used for feeding often include meals left after extraction of the oil so the GM crop trait targeting oil composition is unlikely to have an impact.

With regard to the phytase enzyme, this is also currently being used as a feed additive added separately to feeds in order to enhance the bioavailability of phosphorus and essential metals based on the degradation of the anti-nutrient phytic acid. In the case of phosphorus-deficient diets, effects may become evident from the presence of phytase in the GM crop, as would likely become evident from skeleton malformations and locomotion problems. These effects, however, are not specific for phosphorus deficiency only.

Epidemiological tool development

One of the major objectives of the MARLON project was to develop a tool that supports the design of epidemiological studies with the aim of identifying impacts on the health of livestock that may be attributable to the consumption of GM feeds. Furthermore, it was also intended to verify the feasibility of applying this tool to real-life scenarios.

In more general terms, animal health surveillance may serve the purpose of detecting the impacts of both expected and unexpected impacts of the genetic modification on the health of the livestock animals consuming feeds derived from these GM crops. For unexpected effects, general surveillance as described above (for example, the surveillance that has to be performed within the European Union on GM crops under the legislation for their environmental release, under Directive 2001/18/EC. In addition, plausible hypotheses for health impacts of feeds can be the basis for the design of case-specific monitoring programs in livestock animals. As described above for the collection of existing data, it is recognized that the previously considered data on potential health impacts of GM crops in animals has failed to pinpoint health effects linked to GM technology in general or for specific GM crops being fed to them. Moreover, such a requirement for post-market monitoring in animals related to GM feed has not yet been posed in the European Union and the specific health effects for which such monitoring may be required in future, if it occurs, are also still unknown. The methodology developed within MARLON should therefore be generally applicable to a wide range of impacts as well as the different animal species and husbandry practices in which the GM crop can be used as feed.

As explained above, previous work in the project had already indicated the impossibility to specifically and quantitatively establish exposure estimates for GM crops using existing data available from GMO controls and traceability data. It is therefore not possible to link any observed changes in the health status of animals directly with prior exposure to GM ingredients unless the conditions of different groups of animals that are compared are controlled for this factor. As a generic method, it was therefore proposed to rely on "syndromic surveillance" by following trends in the occurrence of syndromes, including generic health- and performance-related parameters commonly measured in livestock in existing monitoring programs and which are linked to the hypothesized health effects (form example, mortality, abortion in dairy cattle, milk yield and composition, carcass weight). Given the generic nature of the syndromes, once a change in their occurrence has been noted, further investigations will be needed so as to clarify the true causes underlying these changes and whether GM crop-derived feeds are implicated. It was also realized that for some health parameters in a particular scenario (species, husbandry practices, country), existing data may not be complete and therefore a methodology to use expert surveys using questionnaires and interviews was explored in order to elicit

inside knowledge on the background prevalence of certain syndromes as well as the frequency of health checks, reporting loyalty, and other relevant items.

Given that the identification and reporting of health effects involves different stakeholders along the production chain (for example, farmer, veterinary professional, abattoir), the modelling approach employed "scenario trees" that took into account at each stage of the chain the probability of identification and reporting. Using a Bayesian statistical modelling approach, it was thus possible to estimate the overall likelihood that a hypothesized change in the prevalence or severity of a given health syndrome would be picked up as a change in syndrome reports, that is, whether a true change would indeed be observed (or, for example, was too small to be observed above the background variation). Such a modelling tool can prove instrumental when designing monitoring programs that have to provide meaningful outcomes for monitoring of syndromes related to postulated health impacts of GM feeds and feeds in more general terms. The tool is also to become available through the partner's (RVC) website for the wider community and prospective users in particular, which are envisaged to mainly consist of technical experts able to implement and use it for the purpose of health monitoring.

Model assessment and validation

Feed and livestock production chain characteristics

A study of the characteristics of feed and livestock production chains was undertaken within the project, studying a wide range of species (dairy and beef cattle, pigs, poultry, fish) within the countries of the various partners contributing to this research (Bulgaria, Germany, Italy, Netherlands, Spain). In this way, it could be gauged which organization and technical issues need to be considered within these chains before implementing strategies for the monitoring impacts of consumption of feeds (GM feeds in particular) on animal health, the detection of GM feeds, and the measurement of animals' exposure to such feeds. The research therefore focused on issues such as traceability, inspections and controls, and available data on feed usage, as well as organic and non-GMO feed chains. For livestock production, this included, for example, animal identification and tracing, husbandry systems, breeding, and feeding practices. For feed production, this also included a particular study on the maize production chain in Catalonia, Spain, which

is interesting as this is currently one of the major European areas where GM insect-resistant maize is grown on a commercial scale.

As regards the European Union-wide regulatory requirements that are of interest to the topic of this research are the documentation requirements for animals, including their traceability (identification numbers, ear tags), life cycle, health and movements. For example, farmers may have to register their animals, such as poultry holdings above a certain size, and to keep documentation on the numbers of chicken held, found dead, culled, or remaining after sales. Holders of animal holdings may be required to check for the health of their livestock, such as poultry farmers for the occurrence of pathogens like *Salmonella*. They also have to comply with hygiene requirements and holdings may also be subject to inspections by the national animal health inspection services. Such records are also kept in national animal databases with livestock registers containing traceability data for specific animals, such as for cattle and pigs.

Feeding practices on the farm usually involve the use of mixed feeds containing different crop species as ingredients, while this may differ, for example, between animals kept in pastures or as free-ranging animals (for example, with supplementing feeds provided to foraging animals, or to fish fed natural feed such as plankton, algae), as opposed to livestock animals kept indoors (with a more complete mixed feed administered to them). A commonly observed husbandry practice in for example poultry and pig farming is "all in, all out", in which groups of animals are raised and cleared from the facility (towards the slaughterhouse) at the same time. As regards the breeds of animal kept, this can differ from species to species, such as cross-breeds commonly used in pig farming, hybrids in poultry (broiler chicken and laying hen), and Holstein-Friesian breed dairy cows in dairy farming.

Animal transport, including supplier and recipient's details, place of pick-up and delivery, and details on the animals transported also have to be registered and documentation kept for a minimum period of 3 years. The European Union also hosts the electronic TRACES system which tracks the trade and movement of animals.

In addition, slaughterhouse controls are regulated at the European level, requiring inspections to be carried out on the animals to be slaughtered, both before and after their death, including checks by an official veterinarian. Particular attention is paid to notifiable diseases in this regard, for which electronic reporting systems are available.

Examinations will include, among others, general checks for signs of disease, tests for the presence of forbidden substances, and health records to be provided. Besides these controls, there are also a number of health monitoring activities in each country, such as those focusing on specific animal diseases (investigated under the heading of "data collection", see above).

With regard to feed production chains, various types of controls and inspections and record keeping are required by European Union legislation, including requirements for businesses (producers, transporters and traders), national authorities, and European institutions. Businesses must, for example, label the feeds produced with details on the ingredients they contain, production facility, batch number, product name and intended livestock species. Moreover, they must keep records that allow for the traceability of the feeds such as the details of suppliers and clients. Any adverse effects in relation to feed consumption will have to be reported to the authorities, which, in turn, may have to report this through the Europe-wide Rapid Alert System for Food and Feed to notify other European authorities. National authorities do have regulatory frameworks in place for regular control and inspection of feed producing facilities and sampling of feed materials. This is usually done on a select number of businesses or batches of feed or feed ingredient commodities each year, while this selection can be based on indications of an increased likelihood of non-compliance or risks. A similar scenario applies to the inspection of feeds for the presence of GMO-derived ingredients, for which member states (and the "Länder" in Germany) have inspection services carrying out the controls for compliance with legislation on the labelling for the presence of market-approved GM ingredients in foods and feeds according to Regulation (EC) No. 1830/2003 [EU, 2003b] and also to check for unapproved GM ingredients (of which the latter may be reported through the European rapid alert system).

As regards the trade flows in feed ingredients, there is a great volume of feed commodities being imported into the European Union given its dependency on protein sources (besides the feed ingredients produced within the European Union). Data are available on the quantities of the various crops produced, imported and exported, while CN numbers appear to be the most widely used harmonized registration system for the various types of ingredients, although food and feed uses of the same ingredient cannot be distinguished. Neither can it be inferred from the data available how much of each particular crop ingredient will be included in a typical diet fed to a certain livestock species in the various countries of the European Union. Besides the legal obligation to

label for the presence of GM ingredients in food and feed products irrespective of whether GMO-related DNA or protein is still present within the product, the European Union has also imposed a traceability requirement in the form of documentation that has to accompany shipments exchanged between links (suppliers and clients) within the production chains and be archived, except for the retail stage (only labelled).

With regard to the possibilities of separating GM and non-GM ingredients, it was noted that many of the regions exporting feed commodities such as soya- and maize-derived products into the European Union do practice GM crop cultivation. Particularly for soybean, it was estimated that on average 90% of the imported ingredients are GM, while this figure amounts to 30% for maize. Generally, there are no practices of separation of GM and non-GM feed ingredients and traces of GM materials may also show up in non-GM batches due to adventitious presence.

For cultivation, the example of maize cultivation in Catalonia was considered. Most of the maize grown there (~80%) is used for feed purposes. In case of GM maize cultivation, co-existence measures apply, including the keeping of buffer zones between fields with GM and non-GM crops. In more general terms, farmers are obliged to inform the authorities of the crops they grow on their land, including seed variety, area cultivated, and yield. It is noted that while it would thus still be possible to distinguish GM from non-GM maize at the farm level, this information may already be lost at the post-harvest dryer facilities when GM and non-GM maize actually may become mixed and no particular care may be taken to separate these two from each other. General feed documentation requirements apply also to the post-harvest stages of transport and feed production. It is not the feed producers but the authorities that carry out inspections to check for the content of GM ingredients.

For organic farming, additional rules apply on top of the general regulations on feed safety and GMOs, which are actually prohibited to be used in organic farming. The latter consideration also raised the question whether organic farms might serve as comparator with non-GM feed being fed to the animal, for non-organic farms where feeds potentially containing GM-crop-derived ingredients are fed. Interestingly, among the key requirements for organic livestock is that pigs, poultry and dairy cattle should be fed 100% organic feed (other livestock at least 85%), while at least 50% of the feed should come from the same farm as where the animals are raised. For dairy cattle, for example, feed should also contain a minimum part (60%) of materials derived from green crop parts

such as roughage, fodder, or silage. The requirements for organic farming actually go beyond the use of non-GMO materials, such as the prohibition to use chemically synthesized pesticides, veterinary medicines, and antibiotics, and the access of livestock to outdoor areas. Moreover, organic and non-organic livestock as well as feed materials should be kept separate, while producers of feed materials are not allowed to use GM materials and, in more general terms, should undertake efforts to avoid cross-contamination with non-organic materials. Besides organic, there are also suppliers of non-GM or "GM-free" feed ingredients yet their quantities are relatively limited compared to the total European market. In various countries, GM-free labels exist, with varying requirements, such as for poultry and milk in Germany.

In conclusion, it was observed that there are possibilities to trace the fate of animals throughout the production chain based on requirements for identification and registration, as well as reporting of health conditions such as infectious diseases. With regard to husbandry systems, considerable variation was found to exist among the various systems in the different European countries, and the same also held true for organic production systems. Certain challenges were observed for the tracing of feed materials to their origins in general and GM feeds in particular given that the feed categories in trade data details do not exactly match those in the European feed catalogue. Neither is there the possibility to know which proportions of feed materials are used for which livestock species. While there appears to be prevalent presence of GM soybean and other GMO-derived materials in the feed production chain, it turned out to be challenging to distinguish between GM and non-GM feed materials being used or imported given that there is no publicly accessible data making this distinction. Moreover, the case of Catalonia showed that all maize feedstuffs were labelled as GM given that conventional and GM are not separated in the production chain. GMO inspections on feed are carried out to a limited extent at national levels. While labelling appears to be correct in most cases, it is not possible to quantify the proportion of GM materials, and specific GM crops in particular, in feed materials.

Feasibility of implementing the epidemiological model

The feasibility of implementing the model was explored for two scenarios:

- Poultry, pig, and cattle production chains in which imported ingredients that may contain GM ingredients of interest are fed to the animals;

- Dairy cattle production chain in Catalonia, Spain. This European region is of particular interest as both GM and non-GM maize are grown there, and the dairy chain is a highly-organized chain with well-documented procedures.

For the case of imported feed ingredients, verification focused on the extent to which background data could be collected from experts and industry as a basis for the calculations to be performed with the new epidemiological tool that had been developed within the MARLON project. With regard to data from industry, it was noted that the accessibility differs from country to country and species to species. For example, milk yield data on dairy cows, and carcass weight data on pigs may be obtained from individual farmers by approaching them directly in Spain and The Netherlands, while respecting certain confidentiality issues.

The aim of the expert survey was to approach experts at different levels in the scenario tree approach, including private clinical veterinarians, veterinarians at the abattoirs, pathologists from veterinary laboratories, and epidemiologists from governmental services. The questions were to elicit answers about baseline levels of occurrence of syndromes, which could then be used for the epidemiological modelling. When the usability of questionnaires was explored, it was observed that the questionnaire asking about the occurrence of syndromes and for estimates of the mean, minimum and maximum prevalence of certain syndromes was perceived as being different from surveys commonly received. It turned out to be useful to approach the surveyed experts directly with the aid of an epidemiologist and to provide explanations to ensure their comprehension and willingness to fill out the questionnaire completely, and also to assure the quality of the data obtained this way.

For the case study on the Catalonian dairy production chain, a preliminary survey among dairy farmers showed that maize grown on a farm would in many instances be fed as forage or silage to the cattle on the same farm, while this was supplemented with concentrates containing, for example, maize and soybean. While the GM content of the maize sown and harvested can be well known, there appears to be little control over ingredients from approved GM crops entering the feed at the feed manufacture stage. Whereas organic dairy farms are not allowed to feed GMO-containing feeds to dairy cattle, the many other differences inherent to the different husbandry practices would not make organic farms a good comparator for GM-feed-feeding farms as there are

other variables that might be confounding factors in the comparison GM *versus* non GM ingredients.

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