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Animal feeding study with SmartStax maize

Analysis of D., 2008, Report Number MSL0021066

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Summary
Monsanto and contractors carried out a feeding study to assess Smartstax. This focussed on creating nutritional data, no health effects were investigated.

In the 42 day nutritional study on broiler chickens, 900 animals were fed diets containing 61-64% of one of eight maize lines. According to the study data, only 100 chicken were fed a mixed diet containing SmartStax maize, while all the others were fed diets containing conventional maize. Parameters tested were characteristics such as weight of the carcass and composition of thigh and breast meat (fat,moisture, protein). According to the applicants, there were no biologically relevant differences in broiler performance, carcass yield or meat composition. In its opinion on SmartStax maize, the EFSA GMO panel agreed with the applicants on the nutritional equivalence of SmartStax. EFSA further declared that no further safety tests (for allergenicity or toxicity) were necessary.

Criticism was voiced by experts from several Member states. Some of the criticism regarding the study was:

- lack of any data on potential health effects
- lack of independent controls for the data that was presented
- insufficient statistical analysis of the data presented
- no investigation of effects on the immune system, despite the fact that Cry toxins are known to show immune reactions.

EFSA rejected the requests made by the Member States. This was justified with reference to their own guidelines and standards set by the Codex Alimentarius. Thus, EFSA hardly deals with the specific scientific arguments, it just makes very general statements.

As comments from experts of many Member States show, SmartStax needs to be tested much more carefully for health risks. The market application for SmartStax is based on a series of insufficient studies that either do not, or only very marginally, address health risks. It is a matter of great concern that EFSA did not reject these inadequate and flawed dossiers that were never subjected to independent quality controls. It is unacceptable that EFSA responds to questions from Member States with more or less rhetorical answers, and thus fails to deal with the substance of concerns.

1. Background of the investigation

As already explained, risk assessment of SmartStax is highly complex: The genetically engineered plants inherit a unique combination of insecticidal toxins that are technically modified and even

artificially synthesised. These proteins are not sufficiently characterised in regard to their toxicity, selectivity, efficacy and their interactivity. Some of them are known to show immunological activity: The toxin Cry1Ac that is one of the Bt proteins used for the production of the synthetic toxin Cry1A.105, is known to be a potent immune stimulator.

To assess their actual risks, the plant's components and other compounds in food and feed products should be taken into consideration because they might display synergistic effects with the insecticidal toxins such as protease inhibitors.

There should also be some discussion on the residues of the herbicide glyphosate and its additives which may have a negative impact on health at very low dosages (e.g. hormone disruption). Because of potential health risks, farmers in Germany are advised not to use certain mixtures of glyphosate for the production of food and feed[1]. The usage of herbicide glufosinate will soon be banned in the EU because of its effects on health. A significant level of residues from these herbicides can be expected in the plants because they were created to be tolerant to these chemicals and they are sprayed as part of agricultural practice.

Additional important issues need to be considered in this context: The continuous ingestion of the combined Bt toxins and the residues from spraying can lead to a change in the composition of the intestinal flora, and thereby indirectly cause severe health hazards in humans and animals. Further, the gene constructs as introduced into the plants and their parts, such as promoters from viral sources, have to be taken into account because these elements might still be biologically active after ingestion. Finally, undesirable components in the plants might emerge because of genetic engineering methods.

Given the complexity of risk assessment for food products made from genetically engineered plants, Testbiotech proposes conducting extensive step by step investigations, starting at the laboratory level and including the usage of *in vitro* systems (for example using human cells). These *in vitro* systems can be used to explore toxicity, including potential synergistic effects as well as hormone disruptive reactions. Once these first steps of risk assessment have been passed, Testbiotech proposes conducting animal feeding studies that include several generations of the animals. In general, the risk analyses should follow a coherent step by step procedure that includes ethical questions and a socio-economic assessment.

As the analysis of the dossiers from Stilwell & Silvanovich, (2007), Phillips, (2008), Levine et al. (2008), MacRae (2008) und Rosenbaum (2008) shows, the relevant risks were not investigated or only explored very poorly. Further, there was no adequate determination of the expression rate of the toxins or potential impact of environmental conditions on the composition of the plants. The amount of residues from spraying with herbicides was not determined. Therefore, feeding studies to investigate effects on health would be of major importance before any usage in the food chain and feed could be considered. In the case of SmartStax, some of the parental lines used to produce the final stacked event were tested in animal feeding studies and showed some signs of toxicity that need further investigation. Laboratory animals fed with maize DAS1507 and DAS59122 showed some significant differences in blood parameters compared to their control groups. Rats fed with MON89034 showed signs that their kidney function might be impacted. (see data from market applications). Furthermore, other genetically engineered crops with similar proteins also showed signs of toxicity that need further investigations.

[1] www.bvl.bund.de/DE/04_Pflanzenschutzmittel/05_Fachmeldungen/2010/psm_anwendungsbestimmungen_tallowamin-Mittel.html

Nutritional studies have almost no relevance to possible health risk assessment. Parameters such as weight or meat composition are determined in nutritional studies. In the case of SmartStax, only one nutritional feeding study was performed by industry, there was no feeding study to investigate the effects on health .

2. Overview of investigations and findings of D., 2008

The nutritional tests for the application of SmartStax maize were performed in 2007. Monsanto, together with contractors carried out the study on broiler chickens. Colorado Quality Research, Global Poultry Consulting and the Experiment Station Chemical Laboratories were involved. Colorado Quality Research, Inc. carried out the most important parts of the study, including feed preparation and housing. Monsanto oversaw the shipping of the test, control and reference articles, statistics and quality assurance. No peer-reviewed article was published.

The broiler chickens were fed different maize diets for 42 days. There were 9 treatments with 900 broiler chickens in total. Male and female chickens received diets containing 61-64% of one of eight maize lines. SmartStax was not the only maize that was tested during this feeding study. There was another maize that was referred to as a "test article" and a special isogenic line was used as comparator for this second type of maize.

Thus, there were eight groups in this study relevant for the assessment of SmartStax. It included maize MON 89034 x 1507 x MON 88017 x 59122, its conventional (isogenic) counterpart and six other commercial maize varieties. Each of these groups had 100 chickens, so 700 chickens received conventional diet and only 100 chickens were fed with SmartStax.

The hundred chickens that were fed with the SmartStax diet were split into two groups with fifty chickens of each gender. These were further divided into smaller groups of ten chickens each for each treatment.

There is some general confusion in the statistical analysis of the data and the various control groups. For example, the report states that the data from the other maize that was tested were not listed in the dossier but were nevertheless used in the statistical analysis:

> *„This report provides statistical analysis results for a single test article (MON 89034 × TC1507 × MON 88017 × DAS59122-7) vs. the 7 control and reference treatments. Results for the second test article evaluated in this study are not included in this report. However, all 9 experiment treatments were included in statistical analyses in order to maximize statistical power."*

According to the study, the transgenic plants used in the feeding study stem from a US field trial with SmartStax maize conducted in 2006. The feed was pellitized, which means that a high temperature was used for the preparation of the diets. This can impact on the availability of the Bt proteins. The study document does not elaborate on the temperature used.

The parameters analysed included weight gain, feed intake, carcass characteristics, the weight of the carcass and carcass parts, the composition of thigh and breast meat (fat, moisture, protein).

After measuring and weighing the dead animals, statistical analyses of the data were made by Monsanto's Statistics Technology Center. Monsanto's Statistics Technology Center also made

statistical analyses on parameters of performance, carcass yield, and meat quality. The study comes to the conclusion that performance parameters as well as carcass measurements did not show significant differences between SmartStax maize and its isogenic control as well as six other conventional varieties. Nevertheless, the document states that there were differences in one parameter (fat pad weight) between chickens fed with SmartStax and the isogenic control.

3. Assessment of the investigations

3.1 Evidence of insufficient testing

There was no independent quality control of the feeding trials and the statistical analysis. The overall design of the study is questionable because many more control groups were involved than groups fed with SmartStax and its conventional counterpart. By choosing this design, relevant findings might be "diluted" by the sheer amount of additional data. Apparently, even data from another "test article" (genetically engineered maize?) was integrated into the statistical analysis – this is highly unusual and could be interpreted as data manipulation.

There are other deficiencies in the quality control of the study. For example, analyses of pesticides or mycotoxins were performed on the grain only and not on the complete diet. In addition, soybeans used for the study were not tested for GM contamination. The pellets were not tested for their Bt toxins content– these might have been destroyed by the high temperature used during the processing of the grains.

For the overall risk assessment of SmartStax it has to be emphasized, that the study is not meant to assess health risk for humans or animals. No other feeding study has been conducted with SmartStax and major uncertainties and gaps in risk assessment are evident in regard to toxicity of the single toxins and their combination in the plants. This feeding trial would not be sufficient to assess the safety of SmartStax, even if its scientific standards fulfilled all requirements.

3.2. Assessment by EFSA and the experts from EU Member States

EFSA (2010 a) mentions the significant difference of a higher fat pad weight of animals fed SmartStax maize, but declares the observed differences in fat pad weight as biologically irrelevant. So in conclusion, EFSA (2010a) agrees with Monsanto on the nutritional equivalence of SmartStax corn (EFSA 2010):

> *„A feeding study on broiler chickens confirmed the nutritional equivalence of maize MON 89034 x 1507 x MON 88017 x 59122 to its conventional counterpart and commercial maize varieties.“*

EFSA referred to this study and was of the opinion that no further feeding studies to assess effects on health were required: (EFSA 2010a):

> *„In addition, the EFSA GMO Panel considers that it is unlikely that the overall toxicity and allergenicity of the whole maize MON 89034 x 1507 x MON 88017 x 59122 has been changed. A feeding study with broiler chickens confirmed that the nutritional properties of grain produced by maize MON 89034 x 1507 x MON 88017 x 59122 are not different from those of its conventional counterpart and commercial maize varieties.“*

Experts from many Member States including Austria, Belgium, France, Germany, Italy, Norway and Spain came up with comments on D. (2008) paper and raised a debate on the nutritional feeding study and the need for further investigation for potential health effects. Some important points are:

- lack of quality control by independent institutions
- possible destruction of the Bt protein in the pelleting process

- deficiencies in the statistical analysis
- statistically significant differences were reported, but not mentioned in the conclusions
- results from feeding studies with some of the parental lines indicate health risks that should be examined further
- there is a very general lack of data concerning health risks.

Many of these arguments were not answered by EFSA in substance. EFSA frequently refers to formalistic standards such as its own Guidance, or to other opinions they have released previously. EFSA only requested information on two issues. The first was a request for statistical analyses on gender related effects from the nutritional study. Further, they asked for reasoning why the synergies between toxins were not tested in mammals but only in pest insects. In the end, the dossier from industry was accepted without substantial amendments.

Table 1 Relevant comments from the experts of Member States and the answer from EFSA 2010b

Member State	Statement	answer from EFSA
Austria	In the technical dossier, the notifier says that the safety of all transproteins, Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, Cry34Ab1, Cry35Ab1, PAT and CP4 EPSPS, expressed in the test material GM maize MON89034x1507xMON88017x59122 have been discussed in detail in other applications for authorisation. This concerns, amongst other things, history of safe use, structural description and digestion in simulated gastric fluid. In contrast to this, we would like to point out that: a) there is no history of safe use of the new recombinant protein expressed by an artificially arranged insert such as Cry1A.105. b) concerning all Bt toxins, a history of safe use cannot be argued on the basis of the safety of Bt sprays applied in organic farming. The inserted genes are truncated and arranged with expression modulating DNA parts originating from different organisms and permanently expressed compared to a tight timely Bt spraying schedule (Lewis et al. 1997; Sexton et al. 2007). c) the simulated gastric fluid is used at a pH of 1.2 only. FAO/WHO recommend using two pH conditions, pH 1.2 and pH 2.0 in order to cover a range of possible stomach conditions (FAO/WHO 2001). d) all eight transproteins used in acute toxicity tests (Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, Cry34Ab1, Cry35Ab1, pat CP4 EPSPS) originated from microbial expression systems. Establishing structural and functional equivalence of this test proteins and the plant derived proteins adds uncertainties to the interpretation of the animal tests (Spök et al. 2008), thus, only limited information about the plant expressed transproteins can be obtained	The safety of the newly expressed proteins was previously evaluated by the EFSA GMO Panel in its opinions on the single parental events for this stacked event (MON 89034, 1507, MON 88017, 59122). Items considered for the safety of these proteins included in vivo toxicity testing with the purified protein (including 28-days study with the Cry34Ab1/Cry35Ab proteins that are also expressed in maize 59122, provided by the applicant at the EFSA GMO Panel‟s request, in vitro resistance to proteolytic degradation, bioinformatics-supported comparisons of the amino acid sequences of the newly expressed proteins with known toxins), and other characteristics of the proteins (e.g. glycosylation). The use of a bacterial analogue of a plant-expressed protein can be acceptable under certain conditions, as explained in section 7.8.1 of EFSA‟s guidance document: “It is essential that the tested protein is equivalent to the newly expressed protein as it is expressed in the GM plant. If, due to the lack of sufficient amount of test materials (e.g. plant proteins), a protein is used which was produced by micro-organisms, the structural, biochemical and functional equivalence of the microbial substitute to the newly expressed plant protein must be demonstrated.” With regard to the 90-days feeding study 59122, the EFSA GMO Panel noted the following on the issue of clinical pathology in its previous opinion on maize 59122, section 4.2.4: “According to the original study report, no adverse diet-related differences were observed with respect to clinical signs of toxicity, ophthalmological observations and neurobehavioral assessments, clinical pathology, organ weights and gross or microscopic findings in rats receiving the maize 59122 diet compared with the four combined

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	. Additionally, a 90-day rat feeding study with GM maize 59122 (Malley 2004) showed alterations of total protein and albumin levels, and we are still of the opinion that this study should be repeated, as recommended and remarked by Austria in the scientific comment on the triple stack GM maize 59122x1507xNK603 transferred to EFSA in September 2007. (page 22/23)	control groups. In addition, there were no adverse, diet-related differences in mean body weight, body weight gain, food consumption or food efficiency. However the EFSA GMO Panel did not consider the statistical analysis as adequate, because the comparisons were made between groups fed maize 59122 and the four combined control groups. Therefore a new statistical analysis was requested. In addition, information regarding the origin of the non-GM control maize with comparable genetic background was requested. The new statistical analysis revealed no significant differences in final body weight, body weight gain, food consumption and food efficiency between rats fed the maize 59122 diet compared with the non-GM control maize. In the clinical pathology examinations, male rats receiving the maize 59122 diet showed statistically significant decreases in absolute reticulocyte count and red cell distribution width as well as increases in mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration. Females showed an increase in platelet count. These differences were small, and the values were generally comparable with those of other control groups in this study and/or fell within the ranges for the historical control means for rats of the same strain in other subchronic feeding studies. Inn addition, there were no statistically significant differences in other parameters which are expected to be affected in case of relevant effects. The EFSA GMO Panel therefore does not consider the observed differences as toxicologically relevant.” (page 22-24)
Austria	Furthermore, according to EFSA, a potential for increased toxicity and/or allergenicity to humans and animals or for modified nutritional value due to the stacked events may arise from additive, synergistic or antagonistic effects of the gene products or by these produced metabolites (EFSA 2007). But the safety of all newly expressed proteins in animal models applied simultaneously and combined was not assessed in the dossier. Insecticidal Cry proteins produced by GM constitute a sum of new plant constituents possibly interacting within the organism. So far, there is absolutely no scientific knowledge about such those new combinations and possibly resulting additive and/or synergistic effects. Therefore, at least one subchronic feeding study (90-days) with rodents with the whole GM maize plant (MON89034x1507xMON88017x59122) should be carried out. Additionally, the introduction of multigeneration studies focussing on reproduction in the risk assessment process should be considered, at least on a case-by-case basis. So far, although GM crops have now been grown for over 20 years, only very few	At the request of the EFSA GMO Panel the applicants provided a risk assessment of potential interactions among the single events with regard to human and animal health, in its response dated 23 June 2009. The EFSA GMO Panel concludes in its opinion, section 5.1.4.1, that “Determination of the levels of the newly expressed proteins in grain produced by maize MON 89034 x 1507 x MON 88017 x 59122 showed comparable levels to those in the respective single maize events (see section 3.1.4). On the basis of the known functions and modes of action, the EFSA GMO Panel considers it unlikely that interactions between these newly expressed proteins (Cry1A.105, Cry2Ab2, Cry1F, PAT, Cry3Bb1, CP4 EPSPS, Cry34Ab1, and Cry35Ab1) would occur that would raise any safety concern.” According to the approach outlined by the EFSA Guidance Document and the Codex alimentarius guidelines (to which also Austria has subscribed), animal safety tests and other tests with GM plant-derived foods are not required per se but on a case

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	life-term and/or multigeneration studies have been carried out (Domingo 2007; Dona and Arvanitoyannis 2009). Moreover, it is suggested to carry out mutagenicity tests on bacteria with the transproteins. (page 24)	by-case basis, based on indications, for example, of certain unintended effects or substantially modified composition. Given the EFSA GMO Panel`s conclusion that interactions that might impact on safety are unlikely, there is no need to carry out such studies. (page 24)
Austria	In the technical dossier, the notifier stresses that a protein is not likely to be an allergen if the protein represents only a very small portion of the total protein in the grain. Anyhow, even though low concentration of introduced proteins in tissues, that may be consumed, and the rapid digestibility in simulated digestive fluids might provide additional safety, it should not be neglected that minimal traces of substances can trigger allergic reactions (Madsen et al. 2009). Furthermore, in the dossier it is remarked, "the Cry proteins by humans on agricultural crops for over 10 years, either as the active ingredients in Bt microbial pesticides and/or in biotechnology derived food and feed crops (maize and cotton). There are no known reports of allergy or toxicity to Bt or to the Cry proteins" (p. 83). Actually, the simple fact that GM corn has been grown for over 10 years on millions of hectars, and that no effects have been transmitted is no proof for safety. The same could have been said about DDT and many other synthetic agricultural supplies that are now banned. Since GM products have not been labelled in the USA and Canada, no epidemiological survey of potential effects has been conducted. Thus, if the GM food may or may not play its part in the increase of nutrition-related health disturbances such as allergies and food intolerances cannot be clarified. Anyway, allergic reactions against Bt toxins have been reported in farm workers exposed to Bt containing pesticides (Bernstein et al. 1999) (page 25)	The allergenicity of the newly expressed proteins has been assessed according to the weight-of-evidence approach devised by the EFSA Guidance Document and Codex alimentarius guidelines (to which Austria also subscribes), as evaluated by the EFSA GMO Panel in its opinions on the single events. This weight-of-evidence approach includes, for example, a consideration of the history of the allergenicity of the source and recipient of the transgene, bioinformatics-supported comparisons of the amino acid sequence of the newly expressed protein with the sequences of known allergens, and resistance of the newly expressed protein to in vitro proteolysis. The quoted publication by Bernstein (1999) concludes that, among others, "it is unlikely that consumers would develop allergic sensitivity after oral exposure to transgenic foods (e.g., tomatoes, potatoes) that currently contain the gene encoding this protein." (page 25)
Austria	As the statistical analyses were conducted by the applicant (Monsanto Statistics Technology Center), the study cannot be considered wholly independent. For instance, according to the European Regulation 428/2008 (assessment and authorisation of feed additives - annex II, section II, 2.6.1.3.), "Performance characteristics of in-house validated methods shall be verified by testing the method in a second, accredited and independent laboratory". This, and other, minimal standards, that are mentioned within the Regulation 428/2008, and that are regarded essential for the characteristics of submitted studies, should also be applied for GMOs. Nine treatments with 100 birds each were investigated, but only the data of 8 treatments or 800	The pertinent paragraph in the quoted regulation pertains to the validation of analytical methods for feed additives. It is noted that analytical methods for detection GMOs are also validated by JRC, which is outside the remit of the EFSA GMO Panel‟s mandate. There is currently no legal requirement for applicants to outsource their research on GMOs. Both issues do not specifically pertain to the application on MON 89034 x 1507 x MON 88017 x 59122. In the evaluation of the chicken broiler study data, the EFSA GMO Panel‟s focus was on the comparison between test and control maize-fed groups. The following extract from section 5.1.6 of the opinion summarizes this as follows: "A 42-day

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	birds are shown in the tables. (1 test, 1 control and six reference groups). What was the ninth treatment (later named treatment 6), which was later named treatment 6, and was not described or defined, used for? We would also like to remark that only 2 of the 9 treatments were directly relevant for the safety of the GMO, the treatment with the GM test corn and that with the close-genetic variant. In other words, only 22% of the birds were fed either with the GM corn or the control. Analyses of pesticides, mycotoxins, amino acids, fatty acids, nutrients and anti-nutrients as well as the verification of presence and absence of the test, control and reference corn were performed on the grain only and not on the complete feed diet. This is not in line with current guidance, which requires description of manufacture and quantitative composition of the diet (EFSA 2008). Furthermore, the soybeans provided by Global Poultry Consulting Inc. and used for the diet formulation were not tested for potential GM contamination. Also, analyses of heavy metals (cadmium, mercury, arsenic, lead) and vitamins were not conducted at all. It is also stated that "the feed was pelleted through a 5-mm die with live steam addition, and starter diets were fed as crumbles and grower/finisher diets were fed as pellets". The application of heat and pressure could inactivate proteins (Spök et al. 2008); therefore, in feeding tests with the aim to investigate the potential effect of recombinant proteins this procedure is not recommended. (page 26-27)	broiler chicken feeding study with adjusted diets containing grain produced by maize MON 89034 x 1507 x MON 88017 x 59122 was evaluated in the frame of the current marketing application1. Both male and female chicken received adjusted diets containing 61-64% of one of eight maize lines, i.e. grain produced by maize MON 89034 x 1507 x MON 88017 x 59122, its conventional counterpart (XE6001), and six commercial maize varieties. “ “No statistically significant differences were observed between the group fed adjusted diets containing grain produced by maize MON 89034 x 1507 x MON 88017 x 59122 and its conventional counterpart, except for a higher absolute and relative (%) fat pad weight in the group fed GM-maize as compared to that fed control maize (47 v. analyses of heavy metals (cadmium, mercury, arsenic, lead) and 43 g; 1.9 v. 1.7% of live weight). However, these differences were not observed in the comparison between the group fed GM-maize and each of the groups fed commercial maize varieties. The observed differences in fat pad weights were also observed in female chicken fed with GM maize compared with non-GM maize when analyzed in a by-gender statistical analysis. In the absence of any other treatment-related effects on performance, the EFSA GMO Panel does not consider the statistically significant difference in fat pad weights to be of biological relevance. The broiler chicken feeding study supported the results of the comparative compositional analysis and confirmed that grains produced by maize MON 89034 x 1507 x MON 88017 x 59122 are nutritionally equivalent to grains of the conventional counterpart and six commercial maize varieties.” It should be remarked here that this chicken feeding study is not regard by the EFSA GMO Panel as a toxicity study for the newly expressed proteins. Moreover, pelleting is a commonly used process for preparing animal feeds. (page 26-27)
Austria	Moreover, it should be remarked that the results of feed intake, which was determined twice during the whole feeding period on day 21 and day 42, showed a number of significant differences between the treatments, but the GM test corn with the overall lowest feed intake was not significantly different from the control. Thus, the feed intake of Golden Harvest 9166 and Dekalb DKC61-50 for instance was significantly higher than of MON89034 x1507xMON88017x59122. The same is true for the feed conversion ratio. It seems to be surprising that some reference corn variants were accepted much more readily than others, keeping in mind that the diet preparation was comparable.	As noted above, the primary focus of the evaluation of the chicken study data in the EFSA GMO Panel‟s opinion on maize MON 89034 x 1507 x MON 88017 x 59122 is on the comparison between the test maize and its non-GM control (not the reference lines), in line with EFSA and Codex alimentarius guidance. If differences are observed in this comparison, the reference groups can then provide further insight into the background variation for the specific parameter showing this difference. With regard to data on males and females separately, the EFSA GMO Panel requested and received from the applicants additional data with a

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	Also significant differences in bird weight on day 42 were recorded. Not between the GM corn and its control, but the mean for MON89034x 1507xMON88017x59122 was significantly lower than the mean for Golden Harvest H9166. Another point is that no separate information on the weight development of males and females is given. Furthermore, the feed intake was determined as the amount consumed per pen. This calculation is not on an individual basis and might mask differences within individuals by just investigating the group average. In this regard, we would like to point to the fact that protocols must be sufficiently sensitive to detect any effects at the lowest recommended dose (EFSA 2008). What can be concluded is, however, that although, the GM corn, using the mixed model, showed no significantly different impact on broiler performance, other corn varieties enhanced feed intake and weight development significantly better than the GM corn variant did.	by-gender (male or female) statistical analysis of the outcomes of the study. The point on the feed intake per pen is taken, whilst it is noted that it is common practice to measure feed intake per pen, which has previously also been done in the chicken feeding studies for other dossier (the replication is then the number of pens). (page 28)
Austria	In the two models different numbers of treatments were analysed. In model 1, data from nine treatments were analysed, of which one treatment (no. 6) was never introduced in the study. Model 1 had two factors, diet and sex. If the interaction of diet and sex was not significant ($p \geq 0.15$), diet comparison was not done for each sex separately. If the interaction was significant ($p < 0.15$), diet comparison for each sex was performed. Why is the level of significance here 0.15 and not 0.05? And, why was no gender specific analysis performed? Model 2 used data from eight treatments only; the analysis compared the test group with a sample of the population of the control and the reference groups. No gender analysis was performed, unless there was a significant diet-by-sex interaction. No separate comparison of the test group and the control group and no gender analysis were performed. This statistical analysis has to be considered insufficient and the applicant is requested to provide a new statistical analysis or a scientific rationale on the discrepancies mentioned above. (page 28/29)	A gender-specific analysis was requested from – and provided by – the applicants to the EFSA GMO Panel. The only statistically significant differences thus observed between test and control was for fat pad weights in female animals (same as for the overall comparison combining both genders). This is summarized as follows in section 5.1.6 of the opinion: “No statistically significant differences were observed between the group fed adjusted diets containing grain produced by maize MON 89034 x 1507 x MON 88017 x 59122 and its conventional counterpart, except for a higher absolute and relative (%) fat pad weight in the group fed GM-maize as compared to that fed control maize (47 v. 43 g; 1.9 v. 1.7% of live weight). However, these differences were not observed in the comparison between the group fed GM-maize and each of the groups fed commercial maize varieties. The observed differences in fat pad weights were also observed in female chicken fed with GM maize compared with non-GM maize when analyzed in a by-gender statistical analysis. In the absence of any other treatment-related effects on performance, the EFSA GMO Panel does not consider the statistically significant difference in fat pad weights to be of biological relevance. ” (page 28/ 29)
Belgium	1) Assessment of the allergenicity of the newly expressed proteins: It must be emphasized that Cry1A.105 displays high aminoacid sequence identity with Cry1Ac and that Cry1Ac has been	To comment 1: The EFSA GMO Panel is of the opinion that the adjuvant effect of Cry proteins, observed after high dosage intragastric or intranasal administration will

Member State	Statement	answer from EFSA
	proposed as an adjuvant for vaccines (Vasquez et al, 1999, Vasquez-Padron et al. 1999, Moreno-Fieros et al. 2003, Esquivel-Perez et al. 2005), which means that this protein is able to enhance the immune responses against antigens that are co-administered, which is not uncommon for a bacterial protein. Other proteins of the Cry family are also suspected of showing adjuvant properties (Calderon et al. 2007). Therefore, doubt may arise about Cry2Ab2, Cry1F, Cry3Bb1 and Cry34/35 Ab1. The consequence of the presence of such immuno-stimulant in a plant destined to human consumption is not known. Particularly the adjuvant effect via intestinal route is poorly documented. The single concentration of Cry1A.105 in maize grains is compatible with the possibility of an adjuvant effect in the context of normal maize grain consumption (but the concentration after processing of the maize or after cooking is not known). If all Cry proteins also have such adjuvant capacity, the adjuvant effect may be multiplied in MON89034x 1507x MON88017 x59122 maize. It is not known whether the presence of these Cry proteins in maize may elicit sensitization against the other maize proteins upon ingestion (and which type of sensitization?). This point needs to be clarified. Therefore, at least study in mice the immune responses against maize proteins when the animals are fed MON89034 x1507xMON88017x59122 maize. 2) Assessment of the allergenicity of the whole GM plant or crop. The applicant did not assess the allergenicity of the whole GM plant. Care should be taken not to underestimate maize food allergy. Indeed, some maize allergens have been described in the literature (Pasini et al. 2002, Pastorello et al. 2003, Weichel et al. 2006, Fasoli et al. 2009) and, recently, patients showed maize-induced anaphylaxis in double-blind placebo-controlled food challenge, with reactions to as little as 100 mg of maize (Scibilia et al. 2008). This reinforces the need to evaluate the allergenicity of the whole GM plant, as care must be taken that no increase in maize allergy incidence appears due to excessive allergenn expression levels in modified maize. It is relevant to analyze whether the expression levels of known maize allergens is increased in the genetically modified maize grains or to analyze whether the overall allergenicity of the modified maize has increased, as compared to a natural counterpart. This is relevant as, theoretically, the introduction of all these new traits, through multiple cascade interactions, might have modified the expression level of some endogenous maize proteins. Patient IgE binding to modified maize grain extract or titration of known major allergens of maize should be carried out.	not raise any concerns regarding allergenicity caused by maize consumption or contact. Furthermore, maize is not a common allergenic food, and only a rare cause of occupational allergy may occur. The EFSA GMO Panel has considered the “weight of evidence” regarding potential allergenicity of MON89034 x 1507 x MON 88017 x 59122 and its transgenic proteins, in line with its guidance and the internationally harmonized approach as described in Codex alimentarius guidelines. This weight of evidence also includes, besides the outcomes of the updated bioinformatics-supported comparisons and the issues previously considered in the evaluations of the single parental events (MON 89034, 1507, MON 88017, 59122), including the history of allergenicity, if any, of the sources of the transgenic proteins and the in vitro resistance of the transgenic proteins towards proteolytic enzymes. Also the potential unintended change in intrinsic allergenicity has been considered in these opinions. To comment 2: Maize has not been officially classified as a major allergen (e.g. “the big eight”). Some of the considerations raised here are more general and do not specifically pertain to maize MON 89034 x 1507 x MON 88017 x 59122. Moreover, these considerations criticize the internationally harmonized approach recommended by Codex alimentarius, to which Belgium has also subscribed. (page 40)

Member State	Statement	answer from EFSA
	The classical evaluation methods have been used and do not demonstrate the GMO to be a product which might be associated with allergy development. However, since the methods used are not completely predictive for allergy development long term follow up is warranted, e.g. the rapid digestibility in simulated digestive fluids is not a guarantee for safety. Bannon et al. (2003) and Herman et al. (2006) concluded that the use of the SGF technique to predict the allergenic status of the proteins remains uncertain and Spök et al (2005) have shown that digestibility studies can not be considered as suitable tools to address the allergenic potential of a protein. (page 40-41)	
Belgium	A broiler chicken feeding experiment with grain of the MON 89034x1507xMON 88017x59122 was performed. No negative effects were noted. The reported data were on pen level so that 10 replications, i.e. 5 pens per sex, were available. Based on the reported variability within treatments, the statistical power is not sufficient to find significant differences. Nevertheless based on the interpretation of the reported intervals of confidence, statistical significant differences were reported, but were not mentioned in the conclusions, because these were not considered as biological significant. However, it is worthwhile to mention that the SEM of some parameters was on average 3 times larger in the reported treatment groups than in the control, and that mortality rate was rather high. The calculation of the feed conversion ratio was not as exact as being possible. (…) page 42	The experimental setup of the chicken feeding study (D., 2008) was similar as that for many chicken feeding studies previously provided. Mortality was highest in the first days of the experiment, which apparently correlated with bacterial infection, dehydration and cervical dislocation (before group sizes were reduced from twelve to ten chicks per pen). Mortality in the second and longest part of the experiment was, on average, 1.9%. The EFSA GMO Panel describes the observed statistically significant differences in its opinion. In addition, it has requested from the applicants additional statistical data, i.e. a by-gender statistical analysis of the comparison between test and control (non-GM) dietary regimes. Both feed gain and adjusted feed gain have been provided in the results of the study. (…) page 42
France	(…) The toxicological evaluation was conducted on maize containing each single transformation event. However, in the absence of convincing explanations as to the origin of the incidence of bladder calculi raised on examination of MON89034 maize or a sub-chronic toxicity study on the hybrid maize MON89034x1507x MON88017x59122, AFSSA cannot comment on the health safety of the maize grain MON89034x1507x MON88017x59122 and its derived products. (...) page 43	The issue of bladder calculi in the 90-days study with maize MON 89034 is discussed in the EFSA GMO Panel‟s opinion on this maize event, which was published in December 2008, as follows (taken from section 4.2.4): “Microscopic findings in organs and tissues were almost equally distributed and no statistically significant differences between males and females of the high dose group and the controls were noted. A numerically higher incidence of kidney alterations in females of the high dose group was attributable to two rats (one died at day 14 of unknown cause, the other survived to the end of the study). The alterations in these two rats included minimal chronic progressive nephropathy, minimal/moderate transitional cell hyperplasia, minimal sub-acute inflammation and moderate hydronephrosis. The animal that died on day 14 additionally showed mild papillary necrosis and minimal tubular necrosis. Both rats had urinary bladder calculi and the study pathologist concluded that the lesions observed most likely were linked to

Member State	Statement	answer from EFSA
		these calculi. It seems unlikely that the urinary bladder calculi and associated kidney alterations could have been induced by the tested maize in 14 days. A low incidence of urinary bladder calculi is known to occur in this rat strain and may be considered a spontaneous finding in sub-chronic studies. According to historical control data supplied in the application, the incidence of urinary bladder calculi in high dose females in this study was also found in female control rats in previous studies conducted with CD rats in the same testing laboratory. The EFSA GMO Panel therefore considers the urinary bladder calculi as well as the associated kidney alterations as incidental findings which were not related to administration of maize MON 89034. The same applies to the nephroblastomas, a very rare tumour of the kidney, which were observed in two female animals of the control group." With regard to a 90-days study with the topical maize event MON 89034 x 1507 MON 88017 x 59122, no indications for unintended effects or substantial compositional changes have been observed that could warrant such a study (in accordance with the approach recommended by EFSA and Codex alimentarius guidance) (page 43/44)
Germany	Testing of the whole GM food/feed is crucial to obtain the necessary information about any adverse unintended effects of the stacked event MON89034x1507xMON88017x59122 maize on human or animal health. In this regard whole plant studies with the stacked GMO are specially important to test for unintended synergistic effects between the different Bt proteins and to account for the high absolute amount Bt protein in food/feed derived from MON89034x1507xMON88017x59122 maize. However, the applicant`s assessment of potential toxic effects of MON89034x1507x MON88017x 59122 maize is mainly reduced to the risk assessment of the single events. Only one 42 day broiler chicken study has been carried out with the stacked event (D., 2008; MSL-0021066). However, this study was not designed to show possible toxicological effects but to show the effect of the genetic modification on broiler performance. The measured parameters are mainly of agricultural and economic relevance. In the broiler feeding study no pathological or histopathological examinations are performed. Parameters of haematology and clinical biochemistry are not investigated. Hence this broiler feeding study cannot be regarded as a sufficient basis for toxicological risk	At the request of the EFSA GMO Panel. The applicants provided a risk assessment of potential interactions among the single events with regard to human and animal health, in its response dated 23 June 2009. The EFSA GMO Panel concludes in its opinion, section 5.1.4.3, that "The EFSA GMO Panel considered all the data available for maize MON 89034 x 1507 x MON 88017 x 59122 and the newly expressed proteins (Cry1A.105, Cry1F, Cry2Ab2, Cry3Bb1, Cry34/35Ab1, CP4 EPSPS, and PAT) and is of the opinion that interactions between the single maize events that might impact on the food and feed safety of maize MON 89034 x 1507 x MON 88017 x 59122 are unlikely. Therefore, the EFSA GMO Panel does not consider additional animal safety studies with the whole GM food/feed necessary." It should be noted that the broiler feeding study is a nutritional study and not a toxicity study. In addition, no indications were identified that would warrant the performance of an animal toxicity study with the whole product. According to the approach outlined by the EFSA Guidance Document and the Codex alimentarius guidelines (to which also Germany has subscribed), animal safety tests and other tests with GM plant-

Member State	Statement	answer from EFSA
	assessment. As a consequence the safety of MON89034 x1507x MON88017x59122 maize for human or animal health cannot be deduced from this study. To complete the risk assessment we recommend at least a 90-day oral toxicity study with rodents. In addition, we advise to carry out supplemental studies with ruminants and swine which differ with respect to their digestive systems and which will be substantially exposed to feed derived from MON89034x1507xMON88017x59122 maize (57-58).	derived foods are not required per se but on a case-by-case basis, based on indications, for example, of certain unintended effects or substantially modified composition. Given the EFSA GMO Panel‟s conclusion that interactions that might impact on safety are unlikely, there is no need to carry out such studies. (57-58)
Italy	Notifier should complete the documentation supplied regarding: - The information on the genetic stability and the toxicity of each single event; - The risk assessment of potential interactions among the 8 newly proteins expressed in the event, taking into account that the aspects related to the possible effects on human and animal health has not been addresse d. (…) Page 72	The molecular data supplied by the applicants do not suggest a structural modification due to the conventional crossing of the single events in the stacked lines. The stability of the single events was demonstrated over several generations, stability of the stacked event over one generation. This is considered to be sufficient from a safety point of view. At the request of the EFSA GMO Panel, the applicants provided a risk assessment of potential interactions among the single events with regard to human and animal health, in its response dated 23 June 2009. The EFSA GMO Panel concludes in its opinion (section 5.1.4.1) that “Determination of the levels of the newly expressed proteins in grain produced by maize MON 89034 x 1507 x MON 88017 x 59122 showed comparable levels to those in the respective single maize events (see section 3.1.4). On the basis of the known functions and modes of action, the EFSA GMO Panel considers it unlikely that interactions between these newly expressed proteins (Cry1A.105, Cry2Ab2, Cry1F, PAT, Cry3Bb1, CP4 EPSPS, Cry34Ab1, and Cry35Ab1) would occur that would raise any safety concern.”
Norway	Assessment of allergenicity of the whole GM plant or crop. Scientific studies, also very recent ones, have shown that the Cry1Ac protein is a potent systemic and mucosal adjuvant, which is an enhancer of immune responses. The GMO Panel of the Norwegian Food Safety Scientific Committee for Food Safety find it difficult, based on the available data, to assess whether kernels from maize MON 89034 x 1507 x MON 88017 x 59122 may cause more allergenic reactions than food from unmodified kernels. As the different Cry proteins are closely related, and in view of the experimental studies in mice, the GMO Panel finds that the likelihood of an increase in allergenic activity due to Cry1A.105, Cry1F, Cry2Ab2, Cry3Bb1, Cry34Ab1 and Cry35Ab1 proteins in food and feed from maize MON 89034 x 1507 x MON 88017 x 59122 cannot be excluded. Thus, the Panel's view is that as the	The EFSA GMO Panel is of the opinion that the adjuvant effect of Cry proteins, observed after high dosage intragastric or intranasal administration will not raise any concerns regarding allergenicity caused by maize consumption or contact. Furthermore, maize is not a common allergenic only a rare cause of occupational allergy may occur. The EFSA GMO Panel has considered the “weight evidence” regarding potential allergenicity of MON 89034 x 1507 x MON 88017 89034 x 1507 x MON 88017 x 59122 and its transgenic proteins, in line with its guidance and the internationally harmonized approach as described in Codex alimentarius guidelines. This weight evidence also includes, besides the outcomes of the updated bioinformatics-supported comparisons and the issues previously considered in the evaluations of the single parental events

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	adjuvant effect of Cry1A.105, Cry1F, Cry2Ab2, Cry3Bb1, Cry34Ab1 and Cry35Ab1 with reasonable certainty cannot be excluded, the applicant in relation to a possible adjuvant effect of Cry1A.105, Cry1F, Cry2Ab2, Cry3Bb1, Cry34Ab1 and Cry35Ab1 must comment upon the mouse studies showing humoral antibody response of Cry1A proteins. Further, although Cry1A.105, Cry1F, Cry2Ab2, Cry3Bb1, Cry34Ab1 and Cry35Ab1 proteins is rapidly degraded in gastric fluid after oral uptake, there is also the possibility that the protein can enter the respiratory tract after exposure to e.g. mill dust. Finally, rapid degradation is no absolute guarantee against allergenicity or adjuvanticity. (page 76)	(MON 89034, 1507, MON 88017, 59122), including the history of allergenicity, if any, of the sources of the transgenic proteins towards proteolytic enzymes. Also the potential unintended change in intrinsic allergenicity of the host maize has been considered in these opinions. The EFSA GMO Panel is of the opinion that discussion on this issue should be closed. Cry proteins have been already assessed GMO Panel. In previous opinions, the EFSA GMO Panel assessed the allerginicity of Cry proteins and the allergenicity of the whole GM plant (i.e. 59122 maize), and took into consideration the potentional adjuvanticity of Cry proteins that is mentioned in the comment. The EFSA GMO Panel confirms its previous opinion and still considers that since maize is not a common allergenic food, even if the presence of a newly expressed Cry protein might enhance an immune response to endogenous maize protein(s), it is very unlikely that this would modify the allergenicity of the whole GM crop. (page 76/77)
Spain	Toxicological and allergenic studies should be provided with the expressed proteins in a combined way into the hybrid. Only repeat dose studies about two proteins have been submitted; the rest of the investigations only include one dose tests. (page 78)	The safety of the newly expressed proteins was previously evaluated by the EFSA GMO Panel in its opinions on the single parental events for this stacked event (MON 89034, 1507, MON 88017, 59122). Items considered for the safety of these proteins included in vivo toxicity testing with the purified protein (including 28-days study with the Cry34Ab1/Cry35Ab proteins that are also expressed in maize 59122, provided by the applicants at the EFSA GMO Panel‟s request, in vitro resistance to proteolytic degradation, bioinformatics-supported comparisons of the amino acid sequences of the newly expressed proteins with known toxins), and other characteristics of the proteins (e.g. glycosylation). At the request of the EFSA GMO Panel. The applicants provided a risk assessment of potential interactions among the single events with regard to human and animal health, in its response dated 23 June 2009. The EFSA GMO Panel concludes in its opinion, section 5.1.4.1, that “Determination of the levels of the newly expressed proteins in grain produced by maize MON 89034 x 1507 x MON 88017 x 59122 showed comparable levels to those in the respective single maize events (see section 3.1.4). On the basis of the known functions and modes of action, the EFSA GMO Panel considers it unlikely that interactions between these newly expressed proteins (Cry1A.105, Cry2Ab2, Cry1F, PAT, Cry3Bb1, CP4 EPSPS, Cry34Ab1, and Cry35Ab1) would occur that would raise any safety

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		concern.” (page 78)

Conclusion

The quality of the results from D. 2008 is highly questionable. There are severe doubts about the reliability of the data concerning study design, validity of data and statistical analyses.

As comments made by experts from many Member States show, SmartStax needs to be tested much more carefully for potential health risks. The market application of SmartStax is based on series of insufficient studies that either do not, or only marginally, address health risks. It is a matter of great concern that EFSA did not reject these inadequate and flawed dossiers that were never subjected to the scrutiny of independent quality controls. EFSA's way of dealing with questions from the Member States was to give more or less rhetorical answers. A policy of not dealing with the substance of concerns is unacceptable.

Sources

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