TESTBIOTECH Background 07 - 04 - 2022

Testbiotech comment on EFSA assessment of genetically engineered maize DP4114 x MON810 x MIR604 x NK603 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2018-150) from Pioneer

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Introduction

The EFSA GMO panel assessed the stacked maize DP4114 x MON810 x MIR604 x NK603 derived from crossing three genetically engineered maize events (EFSA, 2022a). The parental plants were assessed by EFSA in previous opinions. The maize contains genes conferring resistance to two herbicides: glufosinate and glyphosate, and produces five insecticidal cry proteins:

- DP4114 expresses Cry1F, Cry34Ab1, Cry35Ab1 and PAT enzymes which confer resistance to glufosinate-containing herbicides;
- MON810 expresses Cry1Ab;
- MIR604 expresses mCry3A and PMI (marker gene for selection);
- NK603 expressing two variants of CP4 EPSPS protein for tolerance to glyphosate-containing herbicides.

Implementing Regulation 503/2013 was applied in the EFSA risk assessment. However, data on most of the subcombinations are missing.

1. Systematic literature review

A systematic review as requested in Regulation (EU) No 503/2013 was not provided by the applicant. Based on preliminary information, the GMO Panel stated that there is at present only limited value in undertaking a systematic review of maize DP4114 x MON810 x MIR604 x NK603 (EFSA, 2022a). This not acceptable. Even if no papers on the stacked maize have been published, there is plenty of relevant material on the parental plants (e.g. Steinberg et al., 2019 and 2020; Mesnage et al., 2016 and 2017; Zdziarski et al., 2018; Carman et al., 2013, and Ibrahim & Okasha, 2016).

2. Molecular characterisation and gene expression

Annex II of Implementing Regulation 503/2013 specifies that

- "*Protein expression data, including the raw data, obtained from field trials and related to the conditions in which the crop is grown (in regard to the newly expressed proteins).*" (Scientific requirements 1.2.2.3)
- "*In the case of herbicide tolerant genetically modified plants and in order to assess whether the expected agricultural practices influence the expression of the studied endpoints, three*

test materials shall be compared: the genetically modified plant exposed to the intended herbicide; the conventional counterpart treated with conventional herbicide management regimes; and the genetically modified plant treated with the same conventional herbicide management regimes." (Scientific requirements 1.3.1)

- "*The different sites selected for the field trials shall reflect the different meteorological and agronomic conditions under which the crop is to be grown; the choice shall be explicitly justified. The choice of non-genetically modified reference varieties shall be appropriate for the chosen sites and shall be justified explicitly."* (Scientific requirements 1.3.2.1)

Open reading frames and gene insertion
The genetic engineering process led to the emergence of many new open reading frames in the genome of the maize. In order to assess the sequences encoding the newly expressed proteins, or any other open reading frames (ORFs) present within the insert and spanning the junction sites, it was assumed that proteins that may emerge from these DNA sequences would not raise safety concerns. Other gene products, such as ncRNAs (non-coding RNA) from additional open reading frames, were not assessed. Thus, uncertainties remain about other biologically active substances arising from the method of genetic engineering and the newly introduced gene constructs (see also Davalos et al., 2019).

Impact of environmental factors, agricultural practice and genetic backgrounds
The data presented by Pioneer do not meet the requirements of Implementing Regulation 503/2013: (1) the field trials were not conducted in all relevant regions where the GE maize may be cultivated, and extreme weather conditions were not systematically taken into account; (2) the field trials did not take all relevant agricultural management practices into account; (3) nor was a sufficiently broad range of relevant genetic backgrounds (belonging to several maturity groups) taken into account.

Data on environmental factors, stress conditions and their impact on gene expression
Data was only presented from field trials carried out at four sites in the US for just one year. Environmental stress can cause unexpected patterns of expression in the newly introduced DNA (see, for example, Trtikova et al., 2015; Benevenuto et al., 2021). However, the data from the field trials do not allow conclusions to be drawn on how gene expression will, for example, be affected by climate stress due to drought, watering or high temperatures. Therefore, to assess gene expression, the plants should have been grown in different environmental conditions and exposed to defined environmental stress conditions.

It should also not be overlooked that, for example, Brazil is among the most important countries for maize imports into the EU: Brazil is a major producer of genetically engineered maize and is one of the largest importers of maize into the EU (Commission Committee for the Common Organisation of Agricultural Markets, 2021).

In light of the information available for the consultation, we assume that the application and the data provided do not sufficiently represent the agricultural practices and bio-regional conditions under which these plants are likely to be grown.

The need for more a detailed and independent assessment is also backed by a comparison of data for the expression of CP4 EPSPS in maize NK603 in various stacked events, such as DP4114 x MON 810 x MIR604 x NK603, MON89034 x 1507 x NK603 x DAS-40278-9; NK603 x T25; 59122 x NK603; MON89034 x NK603; MON89034 x 1507 x NK603; 1507 x MON810 x MIR162 x NK603 and 1507 x 59122 x MON810 x NK603. These data (see also tabled overview) show a

wide range of means (dry weight) especially for

- pollen: 187-460 µg/g;
- leaf (V2-V4): 162 – 310 µg/g
- leaf (R1): 98-220 µg/g
- root (R1): 40-93 µg/g
- forage: 53-130 µg/g
- grain: 8-15 µg/g.

It is not clear whether this wider range of expression, which in many cases exceeds 100%, is caused by errors in measuring, stacking, agronomic practices, environmental factors or varietal backgrounds.

These observations show that experiments under defined stress conditions, e.g. in a growth chamber, are needed and should take several varietal backgrounds into account.

This is also relevant in the light of the findings of Fang et al. (2018), Wang et al. (2014), Yang et al. (2017), Beres et al. (2018) and Beres (2019), which show that the level of EPSPS expression can impact a wide range of biological plant characteristics.

Tabled overview: Mean concentration and standard deviation (if available) of protein levels CP4 EPSPS (µg/g dry weight) from maize NK603 as expressed in various stacked events (dry weight). Source: EFSA opinions

Event	Leaf (V2-V4)	Leaf (V9)	Leaf (R1)	Pollen (R1)	Root (R1)	Forage (R4)	Grain (R6)
DP4114 x MON 810 x MIR604 x NK603 (unsprayed)				220 +/-32		110 +/- 28	9,4 +/-3.,2
DP4114 x MON 810 x MIR604 x NK603 (sprayed)				330		130	15/12
NK603 x T25 x DAS-40278-9	310 +/- 87	200 +/- 34	220 +/- 29	280 +/- 43	81 +/- 19	98 +/- 32	8.6 +/- 2.5
MON89034 x 1507 x NK603 xDAS-40278-9	162.19 +/- 43.39	108.66 +/- 25.44	97.54 +/-15.25	186.91 +/-41.96	40.26 +/-10.29	57.85 +/-16.56	7.94 +/-1.97
NK603 x T25						53 +/- 17	8.1 +/-1.1
59122x NK603							12
MON89034 x NK603	240			390		74	8.1
MON89034 x 1507 x NK603							8.5
1507 x MON810 x MIR162 x NK603		160 +/- 36	170 +/- 19	460 +/-78	93 +/- 31	120 +/-24	11 +/-3.7
1507 x 59122 x MON810 x NK603			190 +/- 34				14 +/-3.2

In conclusion, the GE maize plants tested in field trials may not sufficiently represent the products intended for import. The data presented by the applicant are insufficient to conclude on the impact of environmental factors or stress conditions on gene expression as specified in EU Regulation 503/2013.

Data on herbicide application rates and their impact on gene expression

From the available information, it seems that the complementary herbicides were only applied in combination and sprayed twice (the number of sprayings post-emergence is unclear from the information provided). Nevertheless, EFSA is of the opinion that the design of the field trials is in accordance with expected agricultural practices (see EFSA, 2022b). EFSA should have presented more detailed reasoning in order to justify this opinion. Furthermore, data for both the treated and untreated plants should be presented in the Annex.

Due to current EFSA practices, it is not possible to access the original data submitted by the companies within the period of consultation. Therefore, the opinion must provide all the data necessary to allow other experts to conclude on whether the provisions of GMO regulation are fulfilled.

In light of the information available, we assume that the application and the data provided do not sufficiently represent the agricultural practices, which could include the use of each of the herbicides alone and higher dosages.

Comparing Table 8 of the EFSA opinion (EFSA, 2022a) with Appendix B (of the EFSA opinion, 2022a) appears to show that at minimum the expression of the EPSPS gene constructs is impacted by application of the complementary herbicides, causing a higher concentration of the EPSPS enzymes in grains, pollen and forage (see also table above). This finding should have led the EFSA to request more data, including different dosages, rates of spraying and combinations of the complementary herbicides.

EFSA should have requested the applicant to submit data from field trials that include all the relevant agricultural practices, all active ingredients, all dosages and all combinations of the complementary herbicides that might be used in agricultural practice of the GE maize producing countries. Without these data, no reliable conclusion can be drawn as requested in Implementing Regulation 503/2013 (in particular for herbicide tolerant GE plants) to assess whether anticipated agricultural practices influence the expression of the studied endpoints (see also Miyazaki et al., 2019).

Consequently, the GE maize plants tested in field trials are likely to not sufficiently represent the products intended for import. The data presented by the applicant are insufficient to conclude on the impact of the herbicide applications on gene expression, plant composition or biological characteristics of the plant as requested in EU Regulation 503/2013.

Impact of genetic backgrounds on gene expression

It is known that the genomic background of the varieties can influence both the expression of the inserted genes and plant metabolism (see, for example, Lohn et al., 2020; Trtikova et al., 2015). Therefore, EFSA should have also requested additional data from transgenic maize varieties, e.g. those cultivated in South America, including those belonging to other maturity groups.

However, EFSA has not taken these issues into consideration. Consequently, the GE maize plants tested in field trials do not sufficiently represent the products intended for import. The data presented by the applicant are therefore insufficient to conclude on the impact of the genetic backgrounds on gene expression as requested in EU Regulation 503/2013.

3. Comparative assessment of plant composition and agronomic and phenotypic characteristics

Implementing Regulation 503/2013 specifies that:
"*In the case of herbicide tolerant genetically modified plants and in order to assess whether the expected agricultural practices influence the expression of the studied endpoints, three test materials shall be compared: the genetically modified plant exposed to the intended herbicide; the conventional counterpart treated with conventional herbicide management regimes; and the genetically modified plant treated with the same conventional herbicide management regimes.*"

"*The different sites selected for the field trials shall reflect the different meteorological and agronomic conditions under which the crop is to be grown; the choice shall be explicitly justified. The choice of non-genetically modified reference varieties shall be appropriate for the chosen sites and shall be justified explicitly.*"

The data presented by Pioneer do not meet the requirements of Implementing Regulation 503/2013: (1) the field trials were not conducted in all relevant regions where the GE maize will be cultivated, and no sufficiently defined extreme weather conditions were taken into account; (2) the field trials did not take all relevant agricultural management practices into account; (3) nor was a sufficiently broad range of relevant genetic backgrounds (belonging to several maturity groups) taken into account.

Data on environmental factors and stress conditions - and their impact on plant composition and phenotype

Field trials to assess plant composition as well as agronomic and phenotypic characteristics of the GE maize were only conducted in the US (only for one year). A smaller number of field trials (eight sites in the US) were carried out to assess plant composition than the number of field trials carried out to assess agronomic and phenotypic characteristics (10 sites in the US). Some extreme weather conditions were reported from the field trials. These, however, remain arbitrary and not well defined, and do not allow any conclusions to be drawn on how gene expression will be affected by more severe climate stress due to drought, watering or high temperatures. In order to assess changes in gene expression, the plants should have been grown in various environmental conditions and exposed to well-defined environmental stress conditions. This requirement is especially relevant in this case, since it is known that the additional epsps genes may show pleiotropic effects, thus affecting seed dormancy, growth and stress responses of the plants (see, for example, Fang et al., 2018; Wang et al., 2014; Yang et al., 2017; Beres et al., 2018, Beres, 2019).

It should not be overlooked that, for example, Brazil is among the most important countries for maize imports into the EU: Brazil is a major producer of genetically engineered maize and is one of the largest export countries of maize to the EU (Commission Committee for the Common Organisation of Agricultural Markets, 2021).

Nevertheless, EFSA is of the opinion that the design of the field trials is in accordance with the expected agricultural practices. To justify this opinion, EFSA should have provided a much more detailed reasoning. Due to current EFSA practices, it is not possible to access the original data from

the companies within the period of consultation. Therefore, the opinion must provide all the necessary data to allow other experts to conclude on whether the provisions of GMO regulation are fulfilled. In light of the information available, we assume that the application and the data provided do not sufficiently represent the agricultural practices and bio-regional conditions under which these plants are likely to be grown.

No experiments were requested to show the extent to which specific environmental conditions influence plant composition and agronomic characteristics. Hence, no data were made available, as requested in Implementing regulation 503/2013, to assess whether the expected environmental conditions under which the plants are likely to be cultivated will influence the expression of the studied endpoints.

Data on herbicide application rates and their impact on plant composition as well as agronomic and phenotypic characteristics

Due to the mode of action of the active ingredients in the complementary herbicides, it is plausible that complementary herbicide applications will cause stress responses in the plants, and thus impact gene expression and plant composition. These effects may vary with the amount of herbicide sprayed onto the crop and the various active ingredients which can be used.

From the available information, it appears that the complementary herbicides were only applied in combination and sprayed twice (the number of spraying post-emergence is unclear from the information provided). Nevertheless, EFSA is of the opinion that the design of the field trials is in accordance with the expected agricultural practices (see EFSA, 2022b). To justify this opinion, EFSA should have presented more detailed reasoning. Furthermore, data for both the treated and untreated plants should be presented in the Annex.

Due to current EFSA practices, it is not possible to access the original data submitted by the companies within the period of consultation. Therefore, the opinion must provide all the data necessary to allow other experts to conclude on whether the provisions in GMO regulation are fulfilled.

In light of the information available, we assume that the application and the data provided do not sufficiently represent the agricultural practices, which could include the use of the each of the herbicides alone and higher dosages.

Furthermore, the available data indicate that applications of the complementary herbicides have a strong impact: only eight criteria were considered for agronomic and phenotypic analysis. Five of those criteria were significantly different to the data from the comparator not treated with the complementary herbicide, six after treatment.

In regard to compositional analysis, the effects were much greater: 29 out of 70 criteria were significantly different to the data from comparator if treated with the complementary herbicide, 51 significant differences were found after treatment.

Several of these findings fell into the highest category of significant differences (compositional analysis as well as phenotypic analysis). Several of the endpoints were consistently found to be different, with and without spraying.

Despite these surprisingly strong effects and the high number of significant differences, EFSA only considered some of these findings in isolation, and did not request further data on the observed

differences or the impact of the herbicide applications. Whatever the case, these findings should have led EFSA to require more data, including different dosages, rates of spraying and combinations of the complementary herbicides.

EFSA should have requested the applicant to submit data from the field trials, including all relevant agricultural practices, all active ingredients, all dosages and all combinations of the complementary herbicides that might be used in agricultural practice of the GE maize producing countries. Without these data, no reliable conclusion can be drawn as requested in Implementing Regulation 503/2013 (in particular for herbicide-tolerant GE plants) to assess whether anticipated agricultural practices influence the outcome of the studied endpoints (see also Miyazaki et al., 2019).

Consequently, the GE maize plants tested in field trials do not sufficiently represent the products intended for import. The data presented by the applicant are insufficient to conclude on the impact of the herbicide applications on gene expression, plant composition or biological characteristics of the plant as requested in EU Regulation 503/2013.

Impact of genetic backgrounds on plant composition as well as on agronomic and phenotypic characteristics

It is known that the genomic background of the varieties can influence both the expression of the inserted genes and plant metabolism (see, for example, Lohn et al., 2020; Trtikova et al., 2015). Therefore, EFSA should have also requested additional data from transgenic maize varieties that are, for example, cultivated in South America, including those belonging to other maturity groups.

However, EFSA has not taken these issues into consideration. Consequently, the GE maize plants tested in field trials do not sufficiently represent the products intended for import. The data presented by the applicant are, therefore, insufficient to conclude on the impact of the genetic backgrounds on gene expression as requested in EU Regulation 503/2013.

Data from compositional and phenotypical analysis show the need for further investigations

Only eight criteria were considered for agronomic and phenotypic analysis. Five of those criteria were significantly different to the data from the comparator not treated with the complementary herbicide, six after treatment. Some of the endpoints were consistently found, both with and without spraying.

In regard to compositional analysis, 29 out of 70 criteria were significantly different to the data from the comparator not treated with the complementary herbicide, 51 significant differences were found after treatment.

Several of these findings fell into the highest category of significant differences (compositional analysis as well as phenotypic analysis). Several of the endpoints were consistently found to be different, both with and without spraying.

Given the above findings on the impact of environmental factors, herbicide applications and genetic backgrounds as well as the higher number of significant findings in fields treated with the complementary herbicides, EFSA should have requested more data: data on agronomic and phenotypic endpoints need to be generated from a wider range of clearly defined stress factors, including all relevant agricultural practices and genetic backgrounds. Such a requirement is especially relevant in this case since it is known that the additional epsps genes may show pleiotropic effects, which also affect seed dormancy, growth and stress responses of the plants (see, for example, Fang et al., 2018; Wang et al., 2014; Yang et al., 2017; Beres et al., 2018, Beres, 2019).

Further, Mesnage et al. (2016) demonstrated alterations in stress-related metabolic pathways for NK603, which were, amongst others, accompanied by increased levels of polyamines. The authors stated that polyamines can provoke toxicological effects on their own or potentiate adverse effects of histamine.

Furthermore, as mentioned by several experts of Member States (EFSA, 2022b), findings by Christ et al. (2017) showing that the PAT/BAR enzyme may also acetylate endogenous amino acids, should have been the starting point for further investigations. As, for example, the German Federal Office of Consumer Protection and Food Safety (BVL) notes: "*Combining metabolomics, plant genetics and biochemical approaches, Christ et al. (2017) have demonstrated non-specific activities of the PAT/bar enzyme in various genetically modified recipient plants (Arabidopsis, soybean, canola, mustard and wheat). In addition to the actual substrate glufosinate-ammonium, PAT/bar was shown to also acetylate two endogenous amino acids (tryptophan and aminoadipate) at low velocity. This results in an ectopic accumulation of acetyl-tryptophan and acetyl-aminoadipate in the genetically modified plant. While acetyl-tryptophan is a naturally occurring metabolite found in many plant species, acetyl-aminoadipate has not yet been described as an endogenous plant metabolite. In general, it is not unexpected that an acetyltransferase also acetylates other than the actual substrate at low velocity. Nevertheless, the applicant should be requested to address the above-mentioned finding within the risk assessment of maize DP4114 x MON810 x MIR604 x NK603 by evaluating a possible relevance for the safety of maize DP4114 x MON810 x MIR604 x NK603 as food and feed.*"

A more detailed analysis would have been necessary to investigate changes in plant composition and phenotype, and also to investigate potential unintended changes in metabolic pathways and the emergence of unintended biologically active gene products.

The material derived from the plants should have been assessed using omics techniques to investigate changes in the activity of the transgene and the plant genome, and also to investigate changes in metabolic pathways and the emergence of unintended biologically active gene products (see Benevenuto et al., 2022). Such in-depth investigations should not depend on findings indicating potential adverse effects, they should always be necessary to draw sufficiently robust conclusions to inform the next steps in risk assessment.

In addition, in awareness of the absence of any independent data on this maize (see literature review, EFSA, 2022a), we strongly recommend establishing a system with independent controls to repeat the trials and double check the data on plant composition and agronomic characteristics.

Conclusion on the comparative assessment of plant composition as well as on phenotypic and agronomic characteristics

The data provided by the applicant and accepted by EFSA are insufficient to conclude on the impact of environmental factors and herbicide applications, or genetic backgrounds on gene expression, plant metabolism and plant composition, or on agronomic and phenotypic characteristics.

To gather reliable data on compositional analysis and agronomic characteristics, the plants should have been subjected to a much broader range of defined environmental conditions and stressors. Furthermore, EFSA should have requested the applicant to submit data from field trials which reflect current agricultural practices, including all relevant complementary herbicides and all relevant genetic backgrounds.

However, only samples from field sites located in the US (and one in Canada) were used to generate the data, and the impacts of environmental factors and agricultural practices were not assessed in detail. Herbicide applications in the field trials did not represent all the relevant agricultural practices. Only one transgenic stacked variety was grown in the field trials.

Consequently, the data presented by the applicant and accepted by EFSA are insufficient to conclude on the impact of environmental factors and herbicide applications, or different genetic backgrounds on plant composition and agronomic characteristics.

Based on the available data, no final conclusions can be drawn on the safety of the plants. Therefore, the data neither fulfill the requirements of Implementing Regulation 503/2013 nor Regulation 1829/2003. This is also underlined in several statements made by experts from Member States (EFSA, 2022b).

In summary, the GE maize plants tested in the field trials do not sufficiently represent the products intended for import.

4. Toxicity

- Implementing Regulation 503/2013 specifies that:
- "*Toxicological assessment shall be performed in order to:*
- *(a) demonstrate that the intended effect(s) of the genetic modification has no adverse effects on human and animal health;*
- *(b) demonstrate that unintended effect(s) of the genetic modification(s) identified or assumed to have occurred based on the preceding comparative molecular, compositional or phenotypic analyses, have no adverse effects on human and animal health;"*
- "*In accordance with the requirements of Articles 4 and 16 of Regulation (EC) No 1829/2003, the applicant shall ensure that the final risk characterisation clearly demonstrates that:*
- *(a) the genetically modified food and feed has no adverse effects on human and animal health;"*

Toxicity of the Bt toxins

In regard to toxicology and potential synergistic or other combinatorial effects, the negative impacts of Bt toxins on human and animal health cannot be excluded a priori. Bt toxins have several modes of action. They are produced in the plants but their biological characteristics are altered and not identical to their natural templates (Hilbeck & Otto, 2015).

Several publications describe the effects of Bt toxins in mammals: some Cry toxins are known to bind to epithelial cells in the intestines of mice (Vázquez-Padrón et al., 1999, Vásquez-Padrón et al., 2000). As far as potential effects on health are concerned, Thomas and Ellar (1983), Shimada et al. (2003) Huffmann et al. (2004), Ito et al. (2004), Mesnage et al. (2013) and Bondzio et al. (2013) show that Cry proteins could potentially have an impact on the health of mammals. Further publications (de Souza Freire et al., 2014; Mezzomo et al., 2014) confirm hematotoxicity of several Cry toxins, including those used in genetically engineered plants, such as Cry1Ab and Cry1Ac. These effects appear to occur with high concentrations and tend to become stronger after several days. Such observations call for the study of effects after long-term exposure to various dosages, including in combination with material sprayed together with the complementary herbicides. In this

context, it is important to consider that the stacked maize is also resistant to the herbicides glyphosate and glufosinate, and the resulting residues should be seen as potential co-stressors at the stage of consumption (see also Then & Bauer-Panskus, 2017).

It has to be considered that the concentration of the insecticidal proteins is much higher in gluten meal produced from the maize, and it can reach much higher concentrations than in the kernels. Therefore, the food and feed products derived from the stacked maize need to be much more carefully risk assessed in regard to their toxicity compared to genetically engineered plants producing just one Bt toxin.

Relevant findings show that the selectivity and efficacy of Bt toxins produced in GE plants can be influenced by many co-factors (see, for example, Then, 2010; Hilbeck & Otto, 2015). Higher toxicity can also cause lower selectivity (Then, 2010): if synergistic or additive effects occur that increase efficacy of the Bt toxin, its selectivity may be decreased and a wider range of non-target organisms may become susceptible.

One crucial impact factor in this context are protease inhibitors (PIs), which show synergistic effects with Bt toxins, strongly enhancing their toxicity. It is likely that PIs delay the degradation of Bt proteins, and thus also enhance their toxicity. Testbiotech has highlighted these effects in many of its comments on EFSA opinions by referring, for example, to Pardo-López et al. (2009). However, EFSA has never provided a detailed response.

Testbiotech is aware of several publications confirming this gap in risk assessment which EFSA has constantly ignored or denied: as Monsanto already showed in the 1990s, maize, cotton and soybeans produce protease inhibitors (PIs) which considerably enhance the toxicity of Bt proteins in plants. In the presence of PIs, Bt toxin will degrade much more slowly than in isolation. This results in a much higher toxicity of the Bt toxin (if it is taken up together with the plant tissue) compared to the isolated toxin (MacIntosh et al., 1990; Zhao et al., 1999; Zhang et al., 2000; Gujar et al., 2004; Zhu et al., 2007; Pardo-López et al., 2009; Ma et al., 2013; Mesén-Porras et al., 2020). The effects described indicate, for example, a 20-fold higher toxicity of Bt proteins if produced in the plants and taken up with PIs (MacIntosh et al., 1990).

It also should be taken into account that the toxicity of Bt toxins can not only be enhanced through interaction with plant enzymes such as PIs, but also by Bt toxins (Sharma et al., 2004; Sharma et al., 2010; Tabashnik et al., 2013; Bøhn et al. 2016; Bøhn, 2018), gut bacteria (Broderick et al., 2009), residues from spraying with herbicides (Bøhn et al. 2016; Bøhn, 2018) and other co-stressors (Kramarz et al., 2007; Kramarz et al., 2009; Khalique and Ahmed, 2005; Singh et al., 2007; Zhu et al., 2005; Mason et al., 2011; Reardon et al., 2004).

Therefore, any risk assessment that does not take synergistic effects caused by the combination of plant material or other stressors with the Bt toxin into account is not reliable and systematically underestimates the risks (see also Testbiotech, 2021).

These issues are especially relevant for the stacked events since the overall concentration of Bt toxins is higher and combinatorial effects with other stressors (such as residues from spraying) more likely.

In summary, the evidence for enhanced toxicity of Bt proteins produced in maize, cotton and soybeans was published by Monsanto 30 years ago (MacIntosh et al., 1990) and has since then been confirmed in multiple studies. Crucially, EFSA has never assessed this aspect in any of its opinions.

Instead, the toxicity of the Bt toxins was assessed on the basis of feeding studies, using only isolated Bt proteins produced by bacteria for gavage experiments in mice. The data from these experiments were then used to calculate NOAEL (No-Observed-Adverse-Effect Level) and to assess the impact of exposure at the stage of consumption. Therefore, considering the above findings, the basic data for toxicity assessment of the stacked maize are neither valid nor reliable. In addition, incorrect assumptions were made on the degradation of the Bt toxins at the stage of consumption and on similarity to known toxins (see below). Therefore, the Monsanto risk assessment depends entirely on incorrect assumptions in regard to toxicity and exposure.

Immunogenicity of the Bt toxins

There are several studies indicating that immune responses in mammals can be triggered by Bt toxins and have to be considered in this context. Studies with the Cry1Ac toxin (Moreno-Fierros et al., 2000; Vázquez-Padrón et al. 1999; Legorreta-Herrera et al., 2010; Jarillo-Luna et al. 2008; E. González-González et al., 2015; Ibarra-Moreno et al., 2014; Guerrero et al. 2007; Guerrero et al., 2004; Moreno-Fierros et al. 2013; Rubio-Infante et al. 2018) are especially relevant (for review also see Rubio-Infante et al. 2016). Since Cry1Ac is also used as an adjuvant in vaccines, the risks to food consumption can be promoted through synergistic effects, this needs to be addressed and carefully examined.

The synergistic effects described by MacIntosh et al. (1990), Zhao et al. (1999), Zhang et al. (2000) Gujar et al. (2004), Zhu et al. (2007), Pardo-López et al. (2009), Ma et al. (2013), Mesén-Porras et al. (2020) causing higher toxicity of the Bt toxins are also relevant in risk assessment in regard to the immune system: combination with protease inhibitors is likely to be associated with a delay in the degradation of the Bt toxins after consumption. This delay in degradation extends the exposure of the intestinal immune system to Bt toxins and may trigger or enhance chronic inflammation and other immune responses (see also Testbiotech, 2021).

There are also findings from several whole food and feed studies indicating a risk of inflammatory processes caused by maize expressing Bt toxins: a study testing corn with a combination of Bt toxins (Cry1Ab and Cry34Ab1) indicates inflammation in rats (Zdziarski et al., 2018), as does a study by Carman et al. (2013) using a triple stack of NK603, MON863 and MON810 maize. In addition, Ibrahim & Okasha (2016) found indications of inflammatory processes in the jejunum of rats fed with MON810 maize.

In this context, it is relevant that Bt toxins produced by plants can survive digestion to a much higher degree than has been assumed by EFSA or shown by the data of the applicant. Chowdhury et al. (2003) and Walsh et al. (2011) showed that when pigs were fed with Bt maize, Cry1A proteins could frequently and successfully still be found in the colon of pigs at the end of the digestion process. This means that Bt toxins are not degraded quickly in the gut and can persist in larger amounts until digestion is completed; therefore, there is enough time for interaction between various food compounds.

It has to be considered that the concentration of the insecticidal proteins is much higher in gluten meal produced from the maize, and that it can reach a much higher concentrations compared to the kernels.

These issues are especially relevant for the stacked events since the overall concentration of Bt toxins is higher compared to the parental plants.

Not only is the concentration of Bt toxins higher in the stacked Maize, there is also a higher likelihood of combinatorial effects with other stressors (such as residues from spraying). However, neither EFSA nor the applicant considered the potential enhancement of toxic or immunogenic effects caused by interaction with plant components such as PIs. In this context, potential impacts on the microbiome also have to be taken into account (see below).

Effects of residues from spraying with complementary herbicide specific to GE plants and their mixed toxicity

The residues from spraying were considered to be outside the remit of the GMO Panel. However, without detailed assessment of these residues, no conclusion can be drawn on the safety of the imported products: due to specific agricultural management practices in the cultivation of the herbicide-resistant plants, there are, for example, specific patterns of spraying, exposure, occurrence of specific metabolites and emergence of combinatorial effects that require special attention.

Both EU pesticide regulation and GMO regulation require a high level of protection for health and the environment. Thus, in regard to herbicide-resistant plants, specific assessment of residues from spraying with complementary herbicides must be considered a prerequisite for granting authorisation.

EU legal provisions such as Regulation 1829/2003 (and Implementing Regulation 503/2013) state that "*any risks which they present for human and animal health and, as the case may be, for the environment*" have to be avoided. Therefore, potential adverse effects resulting from combinatorial exposure of various potential stressors need to be tested for mixed toxicity (EFSA, 2019).

Both glufosinate and glyphosate have been shown to impact or disturb the microbiome, and thus have a substantial impact on the long-term toxicity (mixed toxicity) of whole food and feed derived from the stacked event. Dong et al. (2020) show that glufosinate can severely impact the microbiome. This is especially relevant in regard to combinatorial (accumulated) effects caused by the residues from spraying with glyphosate, which is known to cause shifts in the microbial composition and associated microbiomes of plants and animals. Glyphosate has been shown to cause shifts not only in soil organisms (van Bruggen et al., 2018, 2021, Chávez-Ortiz et al., 2022) and rhizosphere microbiome (Cesco et al., 2021) but also in the composition of the intestinal flora of humans (Mesnage et al., 2021a), cattle (Reuter et al., 2007), poultry (Shehata et al., 2013; Ruuskanen et al, 2020), amphibians (Boccioni et al., 2021), earthworms (Owagboriaye et al., 2021) and rodents (Hu et al., 2021; Liu et al., 2022; Mao et al., 2018; Mesnage et al., 2021b, c; Tang et al., 2020) as well as honey bees (Motta et al., 2020) and daphnia (Suppa et al., 2020). Therefore, antibiotic effects caused by chronic exposure to food and feed derived from glyphosate-resistant GE plants, including this GE maize, are not unlikely to trigger significant changes in intestinal bacteria (see also Testbiotech, 2021).

In general, the microbiome can be seen as a common network of life, encompassing and closely interacting with plants, animals and humans. Microbial networks are thought to have co-evolved with their hosts and have developed a mutualistic relationship that benefits both the host and microorganisms. They act at the interphase and communicate between the organisms and their wider environment while at the same time being part of an organism's closer environment. Microbiomes are considered to be vital for the health of higher organisms, i.e. human, animals and plants.

In regard to food and feed safety, EFSA (2020) considers microbiomes to be highly relevant to the health status of their hosts. Therefore, it is desirable to understand the importance of their role in

risk assessment. EFSA expects that gut microbiome research (not only in the case of GE plants) will play a relevant role in regulatory science with potential implications for future risk assessments and predictive risk models. As EFSA states: "*considering that the gut microbiome is a biological component directly and indirectly involved in the metabolism of food/feed components and chemicals and in the protection of the host against adverse environmental exposure, it would be useful to establish criteria on how to evaluate the potential adverse impacts of perturbators on this defensive barrier, and consequently, on human/animal health.*"

A 2019 study commissioned by EFSA on adjuvanticity / immunogenicity assessment of proteins included the role of the microbiome. Parenti et al. (2019) state that "*one of the most important drivers of immune response is the gut microbiota and other microbial constituent of the human body which are able to regulate host-pathogen balance and to produce systemic pro-inflammatory stimuli. The lifelong antigenic load represented by foods and bacteria/bacterial products leads to a profound remodeling of the gut microbiota and these changes are emerging as a driving force of the functional homeostasis of the immune system. As a matter of fact, a perturbation of the gut microbiota homeostasis due to irregular lifestyles, stress and age may lead to gut microbiota dysbiosis. This condition may predispose the host to metabolic disorders and inflammation*."

These findings are highly relevant for the risk assessment of the GE maize, which inherits combinations of herbicide resistance to glyphosate and glufosinate with Cry toxins. The residues may cause gut microbiome perturbation, depending on exposure and combinatorial effects. It has to be considered a plausible hypothesis that the effects on the microbiome can trigger effects on the immune system, food uptake and body weight. This hypothesis and mixed toxicity need to be tested before any conclusion can be drawn on the health safety of food and feed. Since no such data can be derived from pesticide risk assessment, experimental data on mixed toxicity of the stacked maize have to be requested from the applicant.

In general, antibiotic effects and other adverse health effects might occur from exposure to a diet containing these plants that were not assessed under pesticide regulation. These adverse effects on health might be triggered by the residues from spraying with the complementary herbicide (see also van Bruggen et al., 2021). Further attention should be paid to the specific toxicity of the metabolites of the pesticide active ingredients that might occur specifically in the stacked event.

However, no attempts have been made to integrate the microbiome into the risk assessment of food and feed derived from the GE maize. This is in direct contradiction to Regulation 1829/2003 which requests "*genetically modified food and feed should only be authorised for placing on the Community market after a scientific evaluation of the highest possible standard, to be undertaken under the responsibility of the European Food Safety Authority (Authority), of any risks which they present for human and animal health and, as the case may be, for the environment.*" (Recital 9).

Testbiotech is also aware that the findings from feeding studies with NK603 (Steinberg et al, 2019; Steinberg et al., 2020; Mesnage et al., 2017; Seralini et al., 2014) show the need for further in-depth analysis. For example, Steinberg et al. (2019 and 2020) found a correlation, i.e. a higher uptake of NK603 in rats was associated with higher mortality. They try to explain these findings, but cannot remove uncertainty in regard to adverse effects. The corrected version of these findings (Steinberg et al., 2020) which is similar to the original publication (Steinberg et al., 2019) reads:

"*In weeks 52–78, the male rats fed the NK603 + Roundup diet at an inclusion rate of 33% showed a higher mean body weight and feed consumption when compared to the corresponding control group, while the mean body weight and feed consumption of the female rats were similar in all five experimental groups (Figs. 5 and 6)*".

"*The increased mortality observed between the 12th and 24th month of the feeding trial in male rats fed the 33% NK603 + Roundup diet coincided with an increase in the body weight and feed consumption*".

These effects, which were related to a specific diet, a specific period of time and male animals, can, for example, be caused or facilitated by a disturbance in the microbiome of the animals. Whatever the case, these effects should have been referenced and discussed by both the applicant and EFSA. It should be taken into account that these effects seem to escape the duration of a subchronic feeding study (90 days).

Therefore, potential adverse effects resulting from combinatorial exposure of various potential stressors need specification, and their assessment needs to be prioritised. We conclude that the EFSA health risk assessment for the stacked maize is unacceptable. We propose testing these plants following the whole mixture approach, thus considering them to be "*insufficiently chemically defined to apply a component-based approach"* (EFSA, 2019).

In addition, we request targeted investigation of changes in the microbiome after chronic exposure to a diet with the stacked maize treated with the complementary herbicides. Furthermore, immunotoxicity has to be examined in detail, by taking into account the findings of Zdziarski et al. (2018), Carman et al. (2013) and Ibrahim & Okasha (2016).

It is remarkable, that even the company considered a subchronic feeding study to be relevant. The study highlighted many significant findings (see comments from Member States, especially Hungary, EFSA, 2022b), but these were not analysed in the EFSA opinion (EFSA, 2022a). While it is true that 90 day feeding studies will not be sufficient to assess all relevant hazards, the data can support the development of further hypotheses which can be used for more targeted experiments.

Currently, feeding studies with whole food and feed seem to be the only established methodology that can be used for the investigation of mixed toxicity, changes in the microbiome and systemic effects on health effects at the stage of consumption. It is thus important to develop more targeted methods, if possible without using animals, to draw sufficiently reliable conclusions.

In conclusion, the EFSA opinion on the application for authorisation of the stacked maize (EFSA, 2022a), cannot be said to fulfill the requirements for assessment of potential synergistic or antagonistic effects resulting from the combination of the transformation events in regard to toxicology.

For this purpose, EFSA should have requested the company to submit data from field trials with the highest dosage of complementary herbicides that can be tolerated by the plants, including repeated spraying. The material derived from the plants should have been assessed in regard to organ toxicity, immune responses and reproductive toxicity, also taking combinatorial effects on other plant components into account.

As a result, the toxicological assessment carried out by EFSA is not acceptable.

Allergenicity

The EFSA assessment of allergenic risks is not based on sufficiently realistic exposure to newly introduced proteins and their interactions. Different routes of exposure, the timing of the exposure, microbial exposure, oral and gut microbiota composition, epithelial barrier integrity and/or non-allergenic components of the food matrix, such as immune-modulating components (adjuvants) of allergenic sources that facilitate immune responses, all have to be considered. In particular, the high number of proteins additionally expressed in the plants make it essential for appropriate data to be made available (EFSA, 2022c).

However, the necessary methodology is neither provided nor requested by EFSA. Therefore, the outcome of allergenicity assessment cannot be regarded as sufficient.

Environmental risk assessment

The appearance of teosinte in Spain and France (see Testbiotech, 2016; Trtikova et al., 2017) has to be considered in more detail. Maize volunteers are being found in the EU on a regular basis. This has been reported by Palaudelmàs et al. (2009) in Spain and by Pascher (2016) in Austria. Further, in awareness of the biological characteristics of the GE maize and the findings of Fang et al. (2018), the stacked maize needs to be examined in detail regarding next generation effects, volunteer potential (persistence) and gene flow. Under these circumstances, even a rare single outcrossing that goes unnoticed can have a huge long-term impact on agro-ecosystems.

The EFSA opinion (2022a) is incorrect for several reasons:

- Without more data on the teosinte species growing in the EU, the likelihood of gene flow from the maize to teosinte cannot be assessed (Trtikova et al., 2017). The same is true for gene flow from teosinte to genetically engineered plants.
- Furthermore, the characteristics of potential hybrids and next generations have to be investigated and cannot be predicted simply from the data of the original event. It is well known that there can be next generation effects and interference from genetic background that cannot be predicted from the assessment of the original event (Bauer-Panskus et al., 2020). This issue is relevant for gene flow from maize to and from teosinte.

EFSA should have requested data from the applicant to show that no adverse effects can occur through gene flow from the maize to teosinte and / or from teosinte to the maize volunteers. In the absence of such data, the risk assessment and the authorisation have to be regarded as not valid.

Without detailed consideration of the hazards associated with the potential gene flow from maize to teosinte and from teosinte to maize, no conclusion can be drawn on the environmental risks of spillage from the stacked maize.

Consequently, environmental risk assessment carried out by EFSA is not acceptable.

Others

In regard to monitoring and methods to identify the specific event, Implementing Regulation 503/2013 specifies that:

> *The method(s) shall be specific to the transformation event (hereafter referred to as 'event-specific') and thus shall only be functional with the genetically modified organism or genetically modified based product considered and shall not be functional if applied to other*

transformation events already authorised; otherwise the method cannot be applied for unequivocal detection/identification/quantification. This shall be demonstrated with a selection of non-target transgenic authorised transformation events and conventional counterparts. This testing shall include closely related transformation events.

However, no such method for identification was made available. Based on the information available, it will not be possible to distinguish the stacked event from a mixture of single parental events or stacked events that overlap with the actual stack.

If approval for import is given, the applicant has to ensure that post-market monitoring (PMM) is developed to collect reliable information on the detection of indications showing whether any (adverse) effects on health may be related to GM food or feed consumption. Thus, the monitoring report should at very least contain detailed information on: i) actual volumes of the GE products imported into the EU, ii) the ports and silos where shipments of the GE products were unloaded, iii) the processing plants where the GE products was transferred to, iv) the amount of the GE products used on farms for feed, and v) transport routes of the GE products. Environmental monitoring should be run in regions where viable material of the GE products, such as kernels, are transported, stored, packaged, processed or used for food/feed. In case of losses and spread of viable material (such as kernels) all receiving environments need to be monitored. Furthermore, environmental exposure through organic waste material, by-products, sewage or faeces containing GE products during or after the production process, and during or after human or animal consumption, should be part of the monitoring procedure (see also comments from Member States experts, EFSA, 2022b).

In addition, the example of the stacked maize highlights some general problems. These are:

(1) Due to current EFSA practices it is not possible to access the original data from the companies within the period of consultation. Therefore, the opinion must provide all the necessary data to allow other experts to conclude on whether the provisions of GMO regulation (esp. 503/2013) are fulfilled. We are making this comment after our recent experiences in requesting access to documents, which in many instances took months to achieve. The Commission should advise EFSA to improve transparency.

(2) A Testbiotech report published in 2021 (Testbiotech, 2021), shows how the European Food Safety Authority (EFSA), which is responsible for risk assessment of GE plants, intentionally puts crucial issues aside. This careless approach exemplifies the overall decrease in general food safety standards that has been ongoing since the introduction of GE plants. The number of events authorised for import has, at the same time, steadily increased. In fact, the uncertainties about safety of EU food and feed production have been increasing since the first GE plants were introduced. In addition, environmental damage is being caused in the producing countries. In light of these findings, the Commission should try to avoid ‘rubber stamping’ all applications for import of GE plants, and thus reduce the overall number of products entering the market, while ensuring that these products undergo much more thorough risk assessment.

References

Bauer-Panskus, A., Miyazaki, J., Kawall, K., Then, C. (2020) Risk assessment of genetically engineered plants that can persist and propagate in the environment. Environ Sci Eur, 32(1): 1-15. https://doi.org/10.1186/s12302-020-00301-0

Benevenuto, R.F., Zanatta, C.B., Guerra, M.P., Nodari, R.O., Agapito-Tenfen, S.Z. (2021) Proteomic profile of glyphosate-resistant soybean under combined herbicide and drought stress conditions. Plants, *10*(11): 2381. https://doi.org/10.3390/plants10112381

Benevenuto, R.F., Venter, H.J., Zanatta, C.B., Nodari, R.O., Agapito-Tenfen, S.Z. (2022) Alterations in genetically modified crops assessed by omics studies: Systematic review and meta-analysis. Trends in Food Science & Technology, 120: 325-337. https://doi.org/10.1016/j.tifs.2022.01.002

Beres, Z.T. (2019) Ecological and evolutionary implications of glyphosate resistance in *Conyza canadensis* and *Arabidopsis thaliana.* Dissertation presented in partial fulfillment of the requirements for the degree Doctor of Philosophy in the graduate school of the Ohio State University. http://rave.ohiolink.edu/etdc/view?acc_num=osu1555600547328876

Beres, Z.T., Yang, X., Jin, L., Zhao, W., Mackey, D.M., Snow, A.A. (2018) Overexpression of a native gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) may enhance fecundity in *Arabidopsis thaliana* in the absence of glyphosate. Int J Plant Sci, 179(5): 390-401. https://doi.org/10.1086/696701

Boccioni, A.P.P.C., García-Effron, G., Peltzer, P.M., Lajmanovich, R.C. (2021) The effect of glyphosate and ciprofloxacin exposure on the gut bacterial microbiota diversity of *Rhinella Arenarum* (Anura: Bufonidae) tadpoles. [Preprint]. https://www.researchsquare.com/article/rs-492368/v1

Bondzio, A., Lodemann, U., Weise, C., Einspanier, R. (2013) Cry1Ab treatment has no effects on viability of cultured porcine intestinal cells, but triggers hsp70 expression. PloS One, 8: e67079. https://doi.org/10.1371/journal.pone.0067079

Carman, J.A., Vlieger, H.R., Ver Steeg, L.J., Sneller, V.E., Robinson, G.W., Clinch-Jones, C.A., Haynes, J.I., Edwards, J. W. (2013) A long-term toxicology study on pigs fed a combined genetically modified (GM) soy and GM maize diet. J Org Syst, 8(1): 38-54. http://www.organic-systems.org/journal/81/abstracts/8106.html

Cesco, S., Lucini, L., Miras-Moreno, B., Borruso, L., Mimmo, T., Pii, Y., ... & Trevisan, M. (2021) The hidden effects of agrochemicals on plant metabolism and root-associated microorganisms. Plant Science, 311: 111012. https://doi.org/10.1016/j.plantsci.2021.111012

Chávez-Ortiz, P., Tapia-Torres, Y., Larsen, J., & García-Oliva, F. (2022) Glyphosate-based herbicides alter soil carbon and phosphorus dynamics and microbial activity. Applied Soil Ecology, 169: 104256. https://doi.org/10.1016/j.apsoil.2021.104256

Chowdhury, E.H., Kuribara, H., Hino, A., Sultana, P., Mikami, O., Shimada, N., Guruge, K.S., Saito, S., Nakajima, Y. (2003) Detection of corn intrinsic and recombinant DNA fragments and Cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11. J Anim Sci, 81(10): 2546-2551. https://doi.org/10.2527/2003.81102546x

Christ, B., Hochstrasser, R., Guyer, L., Francisco, R., Aubry, S., Hörtensteiner, S., Weng, J.K. (2017) Non-specific activities of the major herbicide-resistance gene BAR. Nat Plants 3(12): 937-945. https://doi.org/10.1038/s41477-017-0061-1

Commission Committee for the Common Organisation of Agricultural Markets (2021) EU Cereals Trade 2021/22, Marketing Year July – September, 26 November 2021). https://circabc.europa.eu/sd/a/a1135630-e8e9-4531-a522-23670f75e2c5/cereals-trade-2017-18-marketing-year-july-december.pdf

Dávalos, A., Henriques, R., Latasa, M.J., Laparra, M., Coca, M. (2019) Literature review of baseline information on non-coding RNA (ncRNA) to support the risk assessment of ncRNA-based genetically modified plants for food and feed. EFSA Supporting Publication, 16(8): EN-1688. https://doi.org/10.2903/sp.efsa.2019.EN-1688

de Souza Freire, I., Miranda-Vilela, A.L., Barbosa, L.C.P., Martins, E.S., Monnerat, R.G., Grisolia, C.K. (2014) Evaluation of cytotoxicity, genotoxicity and hematotoxicity of the recombinant sporecrystal complexes Cry1Ia, Cry10Aa and Cry1Ba6 from Bacillus thuringiensis in Swiss mice. Toxins, 6: 2872-2885. https://doi.org/10.3390/toxins6102872

Dong, T., Guan, Q., Hu, W., Zhang, M., Zhang, Y., Chen, M., ... & Xia, Y. (2020). Prenatal exposure to glufosinate ammonium disturbs gut microbiome and induces behavioral abnormalities in mice. Journal of Hazardous Materials, 389: 122152. https://doi.org/10.1016/j.jhazmat.2020.122152

EFSA (2019) Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. EFSA J, 17(3): 5634. https://doi.org/10.2903/j.efsa.2019.5634

EFSA (2022a) Scientific Opinion on the assessment of genetically modified maize DP4114 × MON 810 × MIR604 × NK603 and subcombinations, for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2018-150). EFSA J; 20(3): 7134 https://doi.org/10.2903/j.efsa.2022.7134

EFSA (2022b) Comments and opinions submitted by Member States during the three-month consultation period. OpenEFSA portal. https://open.efsa.europa.eu/

EFSA (2022c) Scientific Opinion on development needs for the allergenicity and protein safety assessment of food and feed products derived from biotechnology. EFSA J, 20(1): 7044. https://doi.org/10.2903/j.efsa.2022.7044

Fang, J., Nan, P., Gu, Z., Ge, X., Feng, Y.-Q., Lu, B.-R. (2018) Overexpressing exogenous 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) genes increases fecundity and auxin content of transgenic Arabidopsis plants. Front Plant Sci, 9: 233. https://doi.org/10.3389/fpls.2018.00233

González-González, E., García-Hernández ,A.L., Flores-Mejía, R., López-Santiago, R., Moreno-Fierros, L. (2015) The protoxin Cry1Ac of Bacillus thuringiensis improves the protection conferred by intranasal immunization with Brucella abortus RB51 in a mouse model. Veterinary Microbiology, 175(2-4): 382-388. https://doi.org/10.1016/j.vetmic.2014.11.021

Guerrero, G.G., Dean, D.H., Moreno-Fierros, L. (2004) Structural implication of the induced immune response by Bacillus thuringiensis cry proteins: role of the N-terminal region. Mol Immunol, 41(12): 1177-1183. https://doi.org/10.1016/j.molimm.2004.06.026

Guerrero, G.G. & Moreno-Fierros, L. (2007) Carrier potential properties of Bacillus thuringiensis Cry1A toxins for a diphtheria toxin epitope. Scandinavian Journal of Immunology, 66(6): 610-618. https://doi.org/10.1111/j.1365-3083.2007.01992.x

Gujar, T., Kalia, V., Kumari, A., Prasad, T.V. (2004) Potentiation of insecticidal activity of Bacillus thuringiensis subsp. kurstaki HD-1 by proteinase inhibitors in the American bollworm, *Helicoverpa armigera* (Hübner). Indian J Exp Biol, 42: 157-163. http://hdl.handle.net/123456789/23352

Hilbeck, A. & Otto, M. (2015) Specificity and combinatorial effects of Bacillus thuringiensis Cry toxins in the context of GMO risk assessment. Front Environ Sci, 3: 71. https://doi.org/10.3389/fenvs.2015.00071

Hu, J., Lesseur, C., Miao, Y., Manservisi, F., Panzacchi, S., Mandrioli, D., ... & Petrick, L. (2021) Low-dose exposure of glyphosate-based herbicides disrupt the urine metabolome and its interaction with gut microbiota. Scientific Reports, 11(1): 1-10. https://doi.org/10.1038/s41598-021-82552-2

Huffmann, D.L., Abrami, L., Sasik, R., Corbeil, J., van der Goot, G., Aroian, R.V. (2004) Mitogenactivated protein kinase pathways defend against bacterial pore-forming toxins. PNAS USA, 101: 10995-11000. https://doi.org/10.1073/pnas.0404073101

Ibarra-Moreno, S., García-Hernández, A.L., Moreno-Fierros L. (2014) Coadministration of protoxin Cry1Ac from *Bacillus thuringiensis* with metacestode extract confers protective immunity to murine cysticercosis. Parasite Immunology, 36(6): 266-270. https://doi.org/10.1111/pim.12103

Ibrahim, M.A., & Okasha, E.F. (2016) Effect of genetically modified corn on the jejunal mucosa of adult male albino rat. Exp Toxicol Pathol, 68(10): 579-588. https://doi.org/10.1016/j.etp.2016.10.001

Ito, A., Sasaguri, Y., Kitada, S., Kusaka, Y., Kuwano, K., Masutomi, K., Mizuki, E., Akao, T., Ohba, M. (2004) *Bacillus thuringiensis* crystal protein with selective cytocidal action on human cells. J Biol Chem, 279: 21282-21286. https://doi.org/10.1074/jbc.M401881200

Jarillo-Luna, A., Moreno-Fierros L., Campos-Rodríguez R., Rodríguez-Monroy, M.A., Lara-Padilla, E., Rojas-Hernández, S. (2008) Intranasal immunization with Naegleria fowleri lysates and Cry1Ac induces metaplasia in the olfactory epithelium and increases IgA secretion. Parasite Immunology, 30(1): 31-38. https://doi.org/10.1111/j.1365-3024.2007.00999.x

Legorreta-Herrera, M., Oviedo Meza, R., Moreno-Fierros L. (2010) Pretreatment with Cry1Ac protoxin modulates the immune response, and increases the survival of plasmodium -infected CBA/Ca mice. BioMed Research International: 198921. https://doi.org/10.1155/2010/198921

Liu, J. B., Chen, K., Li, Z. F., Wang, Z. Y., Wang, L. (2022) Glyphosate-induced gut microbiota dysbiosis facilitates male reproductive toxicity in rats. Science of The Total Environment, 805: 150368. https://doi.org/10.1016/j.scitotenv.2021.150368

Lohn, A.F., Trtikova, M., Chapela, I., Van den Berg, J., du Plessis, H., Hilbeck, A. (2020) Transgene

behavior in *Zea mays* L. crosses across different genetic backgrounds: Segregation patterns, cry1Ab transgene expression, insecticidal protein concentration and bioactivity against insect pests. PLoS ONE, 15(9): e0238523. https://doi.org/10.1371/journal.pone.0238523

Ma, Y., Zhang, Y., Chen, R.-R., Ren, X.-L., Wan, P.-J., Mu, L.-L., Li, G.-Q. (2013) Combined effects of three crystalline toxins from *Bacillus thuringiensis* with seven proteinase inhibitors on beet armyworm, *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae). Pestic Biochem Physiol, 105: 169-176. https://doi.org/10.1016/j.pestbp.2013.01.007

MacIntosh, S.C., Kishore, G.M., Perlak, F.J., Marrone, P.G., Stone, T.B., Sims, S.R., Fuchs, R.L. (1990) Potentiation of *Bacillus thuringiensis* insecticidal activity by serine protease inhibitors. J Agric Food Chem, 38: 1145-1152. https://doi.org/10.1021/jf00094a051

Mao, Q., Manservisi, F., Panzacchi, S., Mandrioli, D., Menghetti, I., Vornoli, A., Bua, L., Falcioni, L., Lesseur, C., Chen, J., Belpoggi, F., Hu, J. (2018) The Ramazzini Institute 13-week pilot study on glyphosate and Roundup administered at human-equivalent dose to Sprague Dawley rats: effects on the microbiome. Environmental Health, 17: 5. https://doi.org/10.1186/s12940-018-0394-x

Mesén-Porras, E., Dahdouh-Cabia, S., Jimenez-Quiros, C., Mora-Castro, R., Rodríguez, C., Pinto-Tomás, A. (2020) Soybean protease inhibitors increase Bacillus thuringiensis subs. Israelensis toxicity against Hypothenemus hampei. Agronomía Mesoamericana, 31: 461-478. https://doi.org/10.15517/am.v31i2.36573

Mesnage, R., Clair, E., Gress, S., Then, C., Székács, A., Séralini, G.-E. (2013) Cytotoxicity on human cells of Cry1Ab and Cry1Ac Bt insecticidal toxins alone or with a glyphosate-based herbicide. J Appl Toxicol, 33: 695-699. https://doi.org/10.1002/jat.2712

Mesnage, R., Agapito-Tenfen, S.Z., Vilperte, V., Renney, G., Ward, M., Séralini, G.E, Nodari, R.O., Antoniou, M.N. (2016) An integrated multi-omics analysis of the NK603 Roundup-tolerant GM maize reveals metabolism disturbances caused by the transformation process. Sci Rep, 6: 37855. https://doi.org/10.1038/srep37855

Mesnage, R., Arno, M., Séralini, G.E., Antoniou, M.N. (2017) Transcriptome and metabolome analysis of liver and kidneys of rats chronically fed NK603 Roundup-tolerant genetically modified maize. Environ Sci Eur, 29(1): 1-9. https://doi.org/10.1186/s12302-017-0105-1

Mesnage, R., Calatayud, M., Duysburgh, C., Marzorati, M., Antoniou, M. (2021a) Alterations in human gut microbiome composition and metabolism after exposure to glyphosate and Roundup and/or a spore-based formulation using the SHIME® technology. BioRxiv [Preprint]. https://www.biorxiv.org/content/10.1101/2021.12.16.472928v1

Mesnage, R., Panzacchi, S., Bourne, E., Mein, C.A., Perry, M.J., Hu, J., ... & Antoniou, M.N. (2021b) Glyphosate and its formulations Roundup Bioflow and RangerPro alter bacterial and fungal community composition in the rat caecum microbiome. BioRxiv [Preprint]. https://www.biorxiv.org/content/10.1101/2021.11.19.468976v1

Mezzomo, B.P. (2013) Hematotoxicity of Bacillus thuringiensis as spore-crystal strains Cry1Aa, Cry1Ab, Cry1Ac or Cry2Aa in Swiss albino mice. J Hematol Thromb Dis, 1(1): 1-9. http://repositorio.unb.br/handle/10482/18532

Miyazaki, J., Bauer-Panskus, A., Bøhn, T., Reichenbecher, W., Then, C. (2019) Insufficient risk assessment of herbicide-tolerant genetically engineered soybeans intended for import into the EU. Environ Sci Eur, 31(1): 1-21. https://doi.org/10.1186/s12302-019-0274-1

Moreno-Fierros, L., García, N., Gutiérrez, R., López-Revilla, R., Vázquez-Padrón, R.I. (2000) Intranasal, rectal and intraperitoneal immunization with protoxin Cry1Ac from *Bacillus thuringiensis* induces compartmentalized serum, intestinal, vaginal and pulmonary immune responses in Balb/c mice. Microbes and Infection, 2(8): 885-890. https://doi.org/10.1016/S1286-4579(00)00398-1

Motta, E.V., Mak, M., De Jong, T.K., Powell, J.E., O'Donnell, A., Suhr, K.J., Riddington, I.M., Moran, N.A. (2020) Oral and topical exposure to glyphosate in herbicide formulation impact the gut microbiota and survival rates of honey bees. Appl Environ Microbiol, 6: e01150-20. https://doi.org/10.1128/AEM.01150-20

Owagboriaye, F., Mesnage, R., Dedeke, G., Adegboyega, T., Aladesida, A., Adeleke, M., ... & Antoniou, M.N. (2021) Impacts of a glyphosate-based herbicide on the gut microbiome of three earthworm species (*Alma millsoni*, *Eudrilus eugeniae* and *Libyodrilus violaceus*): A pilot study. Toxicology Reports, 8: 753-758. https://doi.org/10.1016/j.toxrep.2021.03.021

Pardo-Lopez, L., Munoz-Garay, C., Porta, H., Rodriguez-Almazan, C., Soberon, M., Bravo, A. (2009) Strategies to improve the insecticidal activity of Cry toxins from *Bacillus thuringiensis*. Peptides, 30(3): 589-595. https://www.sciencedirect.com/science/article/pii/S0196978108003264

Parenti, M.D., Santoro, A., Del Rio, A., Franceschi, C. (2019) Literature review in support of adjuvanticity/immunogenicity assessment of proteins. EFSA Supporting Publications, 16(1): 1551E. https://doi.org/10.2903/sp.efsa.2019.EN-1551

Pascher, K. (2016) Spread of volunteer and feral maize plants in Central Europe: recent data from Austria. Environmental Sciences Europe, 28(1):28-30. https://doi.org/10.1186/s12302-016-0098-1

Palaudelmàs, M., Peñas, G., Melé, E., Serra, J., Salvia, J., Pla, M., Nadal, A., Messeguer, J. (2009) Effect of volunteers on maize gene flow. Transgenic Research, 18(4): 583-594. https://doi.org/10.1007/s11248-009-9250-7

Reuter, T., Alexander, T.W., Martínez, T.F., McAllister, T.A. (2007) The effect of glyphosate on digestion and horizontal gene transfer during in vitro ruminal fermentation of genetically modified canola. J Sci Food Agric, 87(15): 2837–2843. https://doi.org/10.1002/jsfa.3038

Rubio-Infante, N. & Moreno-Fierros, L. (2016) An overview of the safety and biological effects of Bacillus thuringiensis Cry toxins in mammals. J Appl Toxicol, 36(5): 630-648. https://doi.org/10.1002/jat.3252

Rubio-Infante, N., Ilhuicatzi-Alvarado, D., Torres-Martínez, M., Reyes-Grajeda, J.P., Nava-Acosta, R., González-González, E., Moreno-Fierros, L. (2018) The macrophage activation induced by Bacillus thuringiensis Cry1Ac protoxin involves ERK1/2 and p38 pathways and the interaction with cell-Surface-HSP70. J Cell Biochem, 119(1): 580-598. https://doi.org/10.1002/jcb.26216

Ruuskanen, S., Rainio, M.J., Gomez-Gallego, C., Selenius, O., Salminen, S., Collado, M.C., Saikkonena, K., Saloniemia, I., Helander, M. (2020) Glyphosate-based herbicides influence

antioxidants, reproductive hormones and gut microbiome but not reproduction: A long-term experiment in an avian model. Environ Pollut, 266(1): 115108. https://doi.org/10.1016/j.envpol.2020.115108

Séralini, G.E., Clair, E., Mesnage, R., Gress, S., Defarge, N., Malatesta, M., ... & de Vendômois, J.S. (2014) Republished study: Long-term toxicity of a Roundup herbicide and a Roundup-tolerantgenetically modified maize. Environmental Sciences Europe, 26(1): 1-17. https://doi.org/10.1186/s12302-014-0014-5

Shehata, A.A., Schrödl, W., Aldin, A.A., et al. (2013) The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. Curr Microbiol, 66(4): 350-358. https://doi.org/10.1007/s00284-012-0277-2

Shimada, N., Kim, Y.S., Miyamoto, K., Yoshioka, M., Murata, H. (2003) Effects of *Bacillus thuringiensis* Cry1Ab toxin on mammalian cells. J Vet Med Sci, 65(2): 187-191. https://doi.org/10.1292/jvms.65.187

Steinberg, P., van der Voet, H., Goedhart, P. W., Kleter, G., Kok, E. J., Pla, M., ... & Wilhelm, R. (2019) Lack of adverse effects in subchronic and chronic toxicity/carcinogenicity studies on the glyphosate-resistant genetically modified maize NK603 in Wistar Han RCC rats. Archives of Toxicology, 93(4): 1095-1139. https://doi.org/10.1007/s00204-019-02400-1

Steinberg, P., van der Voet, H., Goedhart, P. W., Kleter, G., Kok, E. J., Pla, M., ... & Wilhelm, R. (2020) Correction to: Lack of adverse effects in subchronic and chronic toxicity/carcinogenicity studies on the glyphosate-resistant genetically modified maize NK603 in Wistar Han RCC rats. Archives of Toxicology, 94: 1779-1781. https://doi.org/10.1007/s00204-020-02751-0

Suppa, A., Kvist, J., Li, X., Dhandapani, V., Almulla, H., Tian, A.Y., Kissane, S., Zhou, J., Perotti, A., Mangelson, H., Langford, K., Rossi, V., BrownJ.B., Orsini, L. (2020) Roundup causes embryonic development failure and alters metabolic pathways and gut microbiota functionality in non-target species. Microbiome, 8(1): 1-15. https://doi.org/10.1186/s40168-020-00943-5

Tang, Q., Tang, J., Ren, X., Li, C. (2020) Glyphosate exposure induces inflammatory responses in the small intestine and alters gut microbial composition in rats. Environ Poll, 261: 114129. https://doi.org/10.1016/j.envpol.2020.114129

Testbiotech (2016) Cultivation of genetically engineered maize: Risks not under control - Overview: Why the EU should not allow the cultivation of transgenic maize engineered to produce insecticidal toxins. Testbiotech Background, https://www.testbiotech.org/node/1759

Testbiotech (2021) Risk assessment of GE plants in the EU: Taking a look at the ‘dark side of the moon’. https://www.testbiotech.org/content/risk-assessment-ge-plants-eu-taking-look-dark-side-moon

Then, C. (2010) Risk assessment of toxins derived from *Bacillus thuringiensis*: synergism, efficacy, and selectivity. Environ Sci Pollut Res Int, 17: 791-797. https://doi.org/10.1007/s11356-009-0208-3

Then, C., & Bauer-Panskus, A. (2017) Possible health impacts of Bt toxins and residues from spraying with complementary herbicides in genetically engineered soybeans and risk assessment as performed by the European Food Safety Authority EFSA. Environ Sci Eur, 29(1):1.

https://enveurope.springeropen.com/articles/10.1186/s12302-016-0099-0

Thomas, W.E. & Ellar, D.J. (1983) *Bacillus thuringiensis* var israelensis crystal delta-endotoxin: effects on insect and mammalian cells in vitro and in vivo. J Cell Sci, 60(1): 181-197. https://jcs.biologists.org/content/60/1/181.short

Trtikova, M., Wikmark, O.G., Zemp, N., et al. (2015) Transgene expression and Bt protein content in transgenic Bt maize (MON810) under optimal and stressful environmental conditions. PLoS ONE 10(4): e0123011. https://doi.org/10.1371/journal.pone.0123011

Trtikova, M., Lohn, A., Binimelis, R., Chapela, I., Oehen, B., Zemp, N., Widmer, A., Hilbeck, A. (2017) Teosinte in Europe – searching for the origin of a novel weed. Scientific Reports, 7:1560. https://www.nature.com/articles/s41598-017-01478-w

Van Bruggen, A.H.C., He, M.M., Shin, K., Mai, V., Jeong, K. C., Finckh, M.R., Morris, J.G. (2018) Environmental and health effects of the herbicide glyphosate. Science of The Total Environment, 616: 255-268. https://www.sciencedirect.com/science/article/pii/S0048969717330279

Van Bruggen, A.H.C., Finckh, M.R., He, M., Ritsema, C.J., Harkes, P., Knuth, D., Geissen, V. (2021) Indirect effects of the herbicide glyphosate on plant, animal and human health through its effects on microbial communities. Frontiers in Environmental Science, 9: 763917. https://doi.org/10.3389/fenvs.2021.763917

Vazquez-Padron, R.I., Moreno-Fierros, L., Neri-Bazan, L., de la Riva, G.A., Lopez-Revilla, R. (1999) Intragastric and intraperitoneal administration of Cry1Ac protoxin from *Bacillus thuringiensis* induces systemic and mucosal antibody responses in mice. Life Sciences, 64: 1897-1912. https://doi.org/10.1016/S0024-3205(99)00136-8

Vazquez-Padron, R.I., Gonzales-Cabrera, J., Garcia-Tovar, C., Neri-Bazan, L., Lopez-Revillac, L., Hernandez, M., Moreno-Fierro, L., de la Riva, G.A. (2000) Cry1Ac protoxin from *Bacillus thuringiensis* sp. kurstaki HD73 binds to surface proteins in the mouse small intestine. Biochem Biophys Res Commun, 271(1): 54-58. https://doi.org/10.1006/bbrc.2000.2584

Walsh, M.C., Buzoianu, S.G., Gardiner, G.E., Rea, M.C., Gelencsér, E., Jánosi, A., Jánosi, A., Epstein, M.M., Lawlor, P.G. (2011) Fate of transgenic DNA from orally administered Bt MON810 maize and effects on immune response and growth in pigs. PLoS ONE, 6(11): e27177. https://doi.org/10.1371/journal.pone.0027177

Wang, W., Xia, H., Yang, X., Xu, T., Si, H.J., Cai, X.X., Wang, F., Su, J., Snow, A.A., Lu, B.-R. (2014) A novel 5-enolpyruvoylshikimate-3-phosphate (EPSP) synthase transgene for glyphosate resistance stimulates growth and fecundity in weedy rice (*Oryza sativa*) without herbicide. New Phytol, 202(2): 679-688. https://doi.org/10.1111/nph.12428

Yang, X., Li, L., Jiang, X., Wang, W., Cai, X., Su, J., Wang, F., Lu, B.-R. (2017) Genetically engineered rice endogenous 5-enolpyruvoylshikimate-3-phosphate synthase (epsps) transgene alters phenology and fitness of crop-wild hybrid offspring. Sci Rep, 7(1): 1-12. https://doi.org/10.1038/s41598-017-07089-9

Zdziarski, I.M., Carman, J.A., Edwards, J.W. (2018) Histopathological investigation of the stomach of rats fed a 60% genetically modified corn diet. Food Sci Nutr, 9: 763-796.

https://doi.org/10.4236/fns.2018.96058

Zhang, J., Wang, C., Qin, J. (2000) The interactions between soybean trypsin inhibitor and δ-endotoxin of Bacillus thuringiensis in *Helicoverpa armigera* larva. J Invertebr Pathol, 74(5): 259-266. https://doi.org/10.1006/jipa.2000.4936

Zhao, J.Z., Fan, Y.L., Fan, X.L., Shi, X.P., Lu, M.G. (1999) Evaluation of transgenic tobacco expressing two insecticidal genes to delay resistance development of *Helicoverpa armigera*. Chin Sci Bull, 44: 1871-1874. https://doi.org/10.1007/BF02886343

Zhu, Y.C., Abel, C.A., Chen, M.S. (2007) Interaction of Cry1Ac toxin (*Bacillus thuringiensis*) and proteinase inhibitors on the growth, development, and midgut proteinase activities of the bollworm, *Helicoverpa zea*. Pestic Biochem Physiol, 87(1): 39-46. https://doi.org/10.1016/j.pestbp.2006.05.004

Zhu, Y.C., Adamczyk, J.J., West, S. (2005) Avidin, a potential biopesticide and synergist to *Bacillus thuringiensis* toxins against field crop insects. J Econ Entomol, 98: 1566-1571. https://doi.org/10.1093/jee/98.5.1566