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Assessment of genetically modified maize MON 89034 × 1507 × MIR162 × NK603 × DAS-40278-9 for food and feed uses, under regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2018-151) by Dow AgroSciences

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Introduction

The GMO Panel assessed the five-event stacked maize MON 89034 x 1507 x MIR162 x NK603 x DAS-40278-9, which is derived from crossing four genetically engineered maize events (EFSA, 2022a). The maize contains genes conferring resistance to four herbicides and produces six insecticidal proteins.

- MON 89034 expressing Cry1A.105 and Cry2Ab2 insecticidal proteins;
- 1507 expressing the Cry1F insecticidal protein and phosphinothricin acetyl transferase (PAT) protein for tolerance to glufosinate-containing herbicides;
- MIR162 expressing Vip3Aa20 (for protection against certain lepidopteran pests) and PMI (selectable marker);
- NK603 expressing the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) and its variant CP4 EPSPS L214P protein
- DAS-40278-9 expressing the aryloxyalkanoate dioxygenase 1 (AAD-1) protein for tolerance to 2,4-D and quizalofop.

Consequently, the stacked maize produces four insecticidal toxins (Cry1A.105, Cry2Ab2, Cry1F and VIP3A20) that target *lepidoptera* insects). Further, the maize is resistant to four groups of complementary of herbicides (glyphosate, glufosinate, quizalofop- and 2,4-D-containing herbicides).

1. Systematic literature review

A systematic review as referred to in Regulation (EU) No 503/2013 was not provided by the applicant. Based on preliminary information, the GMO Panel agreed that there is only limited value in undertaking a systematic review of maize MON 89034 x 1507 x MIR162 x NK603 x DAS-40278-9 at present (EFSA, 2022a). The applicant identified 12 papers relevant for risk assessment. However, the choice of studies remains dubious, as plenty of relevant papers on parental plants, e.g. Steinberg et al. (2019 and 2020) or Mesnage et al. (2016 and 2017) was not considered. Further, new evidence concerning vip3 genes in maize (see chapter 2) is missing in the literature review.

2. Molecular characterisation

The process of genetic engineering involved several deletions and insertions in the parental maize plants. 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 the proteins that might emerge from these DNA sequences would raise no safety issues; and therefore no detailed investigations were carried out in this regard. Furthermore, other gene products, such as miRNA 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.

Previous research indicated that expression of Cry1A.105, Cry2Ab2 and EPSPS proteins in genetically engineered maize can induce changes in the overall proteome of the respective GE maize line, with impacts on associated endogenous metabolic pathways (Agapito-Tenfen et al., 2014). These transgenes are also present in the stacked maize. Thus, robust data should have been presented to assess whether metabolic changes with relevance to biosafety occur in the stacked maize. Further, Mesnage et al (2016) demonstrated alteration 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 effect on their own or potentiate adverse effects of histamine.

Environmental stress can cause unexpected patterns of expression in the newly introduced DNA (see, for example, Trtikova et al., 2015). More specifically, Fang et al. (2018) showed that stress responses especially can lead to unexpected changes in plant metabolism, if they inherit additional EPSPS enzymes. However, the expression of the additional enzymes was only measured under field conditions in Argentina for one year. It is unclear, to which extent specific environmental conditions will influence the overall concentration of the enzymes in the plants. The plants should have been subjected to a much broader range of defined environmental conditions and stressors to gather reliable data on gene expression and functional genetic stability.

Due to increased weed pressure, it has to be expected that these plants can and will be exposed to high and also repeated dosages of glyphosate alone and / or in combination with the other complementary herbicides. Higher applications of herbicides will not only lead to a higher burden of residues in the harvest, but may also influence the expression of the transgenes or other genome activities in the plants. This aspect was completely ignored in risk assessment even though compositional changes were most noticeable after treatment with complementary herbicides; this supports the premise that a potential unexpected aggregation of herbicides increases the impact of genetic modification on plant metabolism (see also comments form the Experts of Member States, EFSA, 2022b).

Industry in its own recommendations suggests dosages on herbicide resistant maize up to

- approx. 90 g a.i./ha quizalofop (postemergence)[1],
- approx. 0,7 kg a.i./ha glufosinate (postemergence), approx. 1,5 kg per season[2],
- approx. 2,7 kg a.i. /ha 2,4-D (postemergence), approx.4 kg per season[3],

[1] https://www.greenbook.net/dupont-crop-protection/dupont-assure-ii
[2] https://www.greenbook.net/bayer-cropscience/liberty-280-sl
[3] https://www.greenbook.net/dow-agrosciences/enlist-duo

- approx. 2,5 kg a.i./ha glyphosate (postemergence), approx. 3,5 kg per season in pesticide mix product containing glyphosate and 2,4-D (Enlist Duo)[4];
- approx. 3,5 kg a.i./ha glyphosate postemergence, 9 kg per season[5], and even higher rates[6] in glyphosate single formulations.

EFSA should have requested that the applicant submit data from field trials with the highest dosage of the complementary herbicides that can be tolerated by the plants, also including repeated spraying and the application of each of the relevant herbicides alone and in combination. The material derived from those plants should have been assessed by using 'Omics' techniques to investigate changes in the gene activity of the transgene, as well as the natural genome of the plants.

It should also be noted that there is new evidence that vip3 gene seem to induce reduced male fertility in maize. In a patent (EP 3632202 B1, https://data.epo.org/publication-server/pdf-document?pn=3632202&ki=B1&cc=EP&pd=20220720) recently issued by the European Patent Office (EPO), the patent holder (Syngenta) claims that maize containing vip3 genes (like maize MIR162) tends to show decreased fertility. In the patent, the trait is therefore considered as part of hybrid breeding programs.

"However, Vip3 has been observed to cause decreased male fertility in certain inbred maize plants under normal growing conditions. This phenomenon is more prominent in inbred maize plants that are homozygous for a vip3A transgene. The degree to which male fertility is decreased is inbred specific - some inbreds exhibit little or no reduction in male fertility when homozygous for a vip3 gene, other inbreds are somewhat sensitive to Vip3 and exhibit a significant reduction in male fertility when homozygous for a vip3 gene, and other inbreds are highly sensitive to Vip3 and exhibit extremely low or no male fertility when homozygous for a vip3 gene. The degree to which male fertility is decreased is also affected by environmental factors, such as water availability and temperature. In Vip3-induced reductions in male fertility, drought and high temperature conditions exacerbate the reduction in male fertility; however, cooler growth conditions have been shown to mitigate the negative effects of Vip3 expression on male fertility."(EP 3632202 B1)

This surprising effect and its underlying causes should have been reported to EFSA by the applicant and assessed by the agency. There is a number of questions arising from this new scientific evidence, for example:

- As the applicant most probably knew about the decreased male fertility in vip3 containing maize plants, why did he not not report this fact to EFSA?
- What molecular mechanism is behind the decreased male fertility?
- What other traits of the maize plants may be compromised and how is this related to plant composition?
- To what extent is the genetic background of maize MON 89034 x 1507 x MIR162 x NK603 x DAS-40278-9 concerned by this phenomenon?
- Are there possible metabolic interferences between Vip3 and the other Bt toxins present in maize MON 89034 x 1507 x MIR162 x NK603 x DAS-40278-9?

[4] https://www.greenbook.net/dow-agrosciences/enlist-duo
[5] https://www.greenbook.net/monsanto-company/roundup-ultra
[6] https://www.greenbook.net/monsanto-company/roundup-weathermax

- Under which climatic conditions may the said effect occur in maize MON 89034 x 1507 x MIR162 x NK603 x DAS-40278-9?

In any case, studies should be conducted to investigate climatic conditions that might have an effect on maize MON 89034 x 1507 x MIR162 x NK603 x DAS-40278-9. As it is stated in the patent*: "It was observed that an increase in environmental stress exacerbated the Vip3A-induced ructions in male fertility*." Therefore, as the observed fertility reduction seems to be more pronounced under drought and high temperatures, it is a plausible hypothesis that climate warming has to be considered as a relevant factor the risk assessments of all events containing vip3 genes (such as MIR162). Therefore, gene expression studies have to be conducted under a broad variety of environmental conditions and the results analysed using 'Omics' techniques.

Data on herbicide application rates and their impact on gene expression

From the available information, it looks like the complementary herbicides were only applied in combination, and only sprayed once. 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 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 has to provide all the data necessary to allow other experts to conclude 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 single herbicide applications, higher dosages and repeated spraying.

Therefore, EFSA should have requested the applicant to submit data from field trials that included 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 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 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). However, it appears that the data on gene expression were confined to a single variety. Therefore, EFSA should also have requested additional data from transgenic maize varieties, e.g. those cultivated in South America.

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.

Further, potential male fertility decrease triggered by the vip3 gene should be assessed in the genetic background of maize MON 89034 x 1507 x MIR162 x NK603 x DAS-40278-9 (see above).

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

Implementing Regulation 503/2013 requests:
"*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 Dow AgroSciences 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 defined extreme weather conditions were taken into account; (2) the field trials did not take all relevant agricultural management practices into account; (3) not all relevant genetic backgrounds were 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 for one year. 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 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 gene may show pleiotropic effects, also 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 exporters 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 has to provide all the necessary data to allow other experts to conclude 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.

However, no experiments were requested to show to which extent 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 looks like that the complementary herbicides were only applied in combination, with only one post-emergent (during the growth of the plants) spraying. 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 has to provide all necessary data to allow other experts to conclude 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, i.e. single herbicide use, higher dosages and repeated spraying.

EFSA should have requested the applicant to submit data from field trials on all the relevant active ingredients used in agricultural practice, including all dosages and combinations of the complementary herbicides which might be used in agricultural practice in GE maize producing countries. Without these data, no reliable conclusions 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 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). However, it appears that the data on gene expression were confined to a single variety. Therefore, EFSA should also have requested additional data from transgenic maize varieties that are, for example, cultivated in South America.

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 analysis show the need for further investigations

71 constituents were subjected to statistical analysis (10 in forage and 61 in grain).

- Statistically significant differences between the five-event stack maize (not treated) and the non-GE comparator were identified for 32 out of 61 endpoints (in grain), with several endpoints in category III/IV (category III: histidine; category IV: ash, behenic acid (C22:0), arginine, glycine, lysine, phosphorus, potassium and phytic acid).
- Statistically significant differences between the five-event stack maize (treated with complementary herbicides) and the non-GE comparator were identified for 38 of 61 endpoints (in grain), with several endpoints falling into category III/IV (same endpoints as in untreated maize: category III: histidine; category IV: ash, behenic acid (C22:0), arginine, glycine, lysine, phosphorus, potassium and phytic acid).

According to EFSA, further assessment regarding food and feed safety was necessary for the levels in grain of: ash, behenic acid (C22:0), arginine, glycine, histidine, phosphorus, potassium, phytic acid (both treated and not treated), lysine (not treated) and pyridoxine (treated).

Given the above reasoning on the impact of environmental factors, herbicide applications and genetic backgrounds as well as a higher number of significant findings in fields treated with the complementary herbicides, EFSA should have requested more data: data on agronomic and phenotypic endpoints should be generated from a wider range of clearly defined stress factors, including all relevant agricultural practices and genetic backgrounds. This 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.

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 by using 'Omics' techniques to investigate changes in the gene 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, herbicide applications and genetic backgrounds on gene expression, plant metabolism, 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 were used to generate the data, and the impact 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 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, herbicide applications or different genetic backgrounds on plant composition and agronomic characteristics.

Further, in the light of new evidence regarding male fertility decrease triggered by the presence of vip3 genes in maize (see chapter 2), the huge number of compositional differences between maize MON 89034 x 1507 x MIR162 x NK603 x DAS-40278-9 and its comparator should also be considered as possible effects of metabolic imbalances caused by VIP3Aa20.

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 by 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 requests:
"*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;"

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, 2019b).

2,4-D, glufosinate and glyphosate, have been shown to impact or disturb the microbiome, which can have substantial impact on the long-term toxicity (mixed toxicity) of whole food and feed derived from the stacked event, whereas data on quizalofop seems to be scarce. Dong et al. (2020) show that glufosinate can severely impact the microbiome; Tu et al. (2019) provide evidence on the adverse effects of 2,4-D. 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, 2021c; 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, animal 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, quizalofop, glufosinate and 2,4-D. These 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 the 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., 2018). 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).

EU legal provisions such as Regulation 1829/2003 (as well as 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 that result from combinatorial exposure of various potential stressors need specification, and their assessment needs to be prioritised. We conclude that the health risk assessment currently performed by EFSA for the stacked maize is unacceptable. We propose testing these plants following the whole mixture approach, considering them to be "*insufficiently chemically defined to apply a component-based approach"* (EFSA, 2019).

Despite all these open questions regarding potential health impacts, we are not aware of a single sub-chronic or chronic feeding study performed with whole food and feed derived from the stacked maize. This observation is supported by the literature review carried out by the company which did not yield any peer reviewed publication. In this context, it is relevant to consider that the outcome of the feeding studies with the parental plants raised several questions concerning their results, methodology and reliability.

Testbiotech is also aware that the findings from feeding studies with NK603 of (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 between higher uptake of NK603 in rats and higher mortality. They they try to explain these findings, however, they can not 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 fostered by a disturbance in the microbiome of the animals. Whatever the case, these should have been mentioned and discussed by 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).

In conclusion, the EFSA opinion on the application for authorisation of the stacked maize (EFSA, 2021a) cannot be said to fulfil 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 with other plants components into account.

Further, possible health effects related to the new scientific evidence regarding vip3 genes in maize can not be ruled out on the basis of EFSA's risk assessment.

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

5. 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 can be found in the EU on a regular basis as has been reported from Palaudelmàs et al. (2009) in Spain or from 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 the agro-ecosystems.

Furthermore, the EFSA (2022a) opinion is also wrong 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 as well from teosinte to maize.

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.

Testbiotech is aware of a recent statement by EFSA (2022c) regarding the teosinte situation in France and Spain. Here, EFSA comes to the conclusion:
"The new evidence retrieved confirms that where maize and EU teosinte plants co-occur and flower synchronously, maize alleles (transgenic or not), can move into teosinte populations at rates that depend on different factors. Hence, the possible introgression of transgenes from maize MON810, Bt11, 1507 and GA21 into EU teosinte may only provide a selective advantage to GM teosinte hybrid progeny under high infestation of target pests and/or when glufosinate-ammonium- and/or glyphosate-based herbicides are applied. However, this fitness advantage will not allow GM teosinte hybrid progeny to overcome other biological and abiotic factors limiting their persistence and invasiveness. Therefore, EFSA considers that the growth habits of EU teosinte plants and teosinte hybrid progeny are such that the acquisition of insect resistance and/or herbicide tolerance is unlikely to change their relative persistence and invasive characteristics under EU conditions."

However, in the updated risk assessment, EFSA still does not consider next generation effects such as possible fitness advantages (as noted, for example, by Fang et al., 2018; Wang et al., 2014; Yang et al., 2017; further literature compiled in Bauer-Panskus et al., 2020). The updated teosinte risk assessment is therefore too narrow to conclude on possible environmental effects and provides no answers to relevant risk related questions.

6. Others

For monitoring and methods to identify the specific event, Implementing Regulation 503/2013 requests:

> *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 GE 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).

We agree with comments made by experts from Member States (EFSA, 2022b) that monitoring requires more improvements, such as those suggested by German authority, BVL:

"The monitoring plan does not relate the monitoring activities to relevant protection goals. Even more, it is not described which routine observations (including parameters or monitoring characters) are carried out in relation to the protection goals. Only reporting on 'any unanticipated effect' is solely not an appropriate parameter, because it already anticipates an evaluation. This evaluation process should be based on a distinct set of parameters and a scientific sound data analysis. It is requested that the applicant specifies in detail, how and which information will be pro-actively queried, gathered, and how they will be evaluated. In addition, it might be useful to integrate information about the use of the product in food and feed to deliver supplementary helpful data (of exposure to consumers and animals) for general surveillance. Therefore, the applicant should specify monitoring activities in the field of human and animal health. He should describe in detail how animal and human health surveillance is integrated in the monitoring plan."

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 has to provide all the necessary data to allow other experts to conclude 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 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. Environmental Sciences Europe, 32(1): 1-15. https://doi.org/10.1186/s12302-020-00301-0

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

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

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

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 Journal, 17(3): 5634. https://doi.org/10.2903/j.efsa.2019.5634

EFSA (2022a) Scientific Opinion on the assessment of genetically modified maize MON 89034 × 1507 × MIR162 × NK603 × DAS-40278-9 for food and feed uses, under regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2018-151). EFSA J, 20(8): 7451. https://doi.org/10.2903/j.efsa.2022.7451

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

EFSA (2022c) Statement on the update of environmental risk assessment conclusions and risk management recommendations of EFSA (2016) on EU teosinte. EFSA J, 20(4): 7228. https://doi.org/10.2903/j.efsa.2022.7228

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

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

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

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: 50. https://doi.org/10.1186/s12940-018-0394-x

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. Environmental Sciences Europe, 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

Mesnage, R., Teixeira, M., Mandrioli, D., Falcioni, L., Ducarmon, Q.R., Zwittink, R.D., ... & Antoniou, M.N. (2021c) Use of shotgun metagenomics and metabolomics to evaluate the impact of glyphosate or Roundup MON 52276 on the gut microbiota and serum metabolome of Sprague-Dawley rats. Environmental Health Perspectives, 129(1): 017005. https://doi.org/10.1289/EHP6990

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. Environmental Sciences Europe, 31(1): 1-21. https://doi.org/10.1186/s12302-019-0274-1

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

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

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

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

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

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

Tu, P., Gao, B., Chi, L., Lai, Y., Bian, X., Ru, H., & Lu, K. (2019) Subchronic low-dose 2, 4-D exposure changed plasma acylcarnitine levels and induced gut microbiome perturbations in mice. Scientific reports, 9(1), 1-11. https://www.nature.com/articles/s41598-019-40776-3

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

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