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Testbiotech comment on Scientific Opinion on the assessment of genetically modified soybean GMB151 for food and feed uses, under Regulation (EC) No 1829/2003 (application EFSA-GMO-NL-2018-153) of BASF

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Introduction

The GMO panel assessed the soybean event GMB151. The soybean contains genes conferring resistance to a group of herbicides known as HPPD-inhibitors, such as isoxaflutole, mesotrionine and tembotrionine. Furthermore, it expresses the insecticidal protein Cry14Ab-1.

Implementing Regulation 503/2013 was applied in this case.

Molecular characterisation and gene expression

Annex II of Implementing Regulation 503/2013 requests 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 soybean. 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 raise no safety concerns. Other gene products, such as ncsRNA 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.

The insertion of the additional gene led to disruption in an endogenous plant gene (BAP1 gene), which is known to cause a constitutively active defence response in *Arabidopsis*, and which results in a dwarf phenotype (Yang et al., 2007). However, this was not observed in soybean GMB151. The explanation provided by EFSA (2021) is that there might be additional endogenous gene copies which can compensate the loss of the disrupted gene function. Still the question arises of whether the gene function will also be stable in other varieties and under stress conditions. However, no such data were requested or provided to assess these questions. Furthermore, -omics should have been applied to assess the impact of gene disruption on plant metabolism.

Impact of environmental factors, agricultural practice and genetic backgrounds

The data presented by BASF do not meet the requirements of Implementing Regulation 503/2013: (1) the field trials were not conducted in all relevant regions where the soybean will be cultivated, and no specific extreme weather conditions were taken into account; (2) the field trials did not take all relevant agricultural management practices into account; (3) only one transgenic variety was included in the field trials.

Data on environmental factors, stress conditions and their impact on gene expression

Environmental stress can cause unexpected patterns of expression in the newly introduced DNA (see, for example, Trtikova et al., 2015). There is strong evidence that climate conditions can significantly impact the content of Bt in the plant tissue (Adamczyk & Meredith, 2004; Adamczyk et al., 2009; Chen et al., 2005; Dong & Li, 2006; Luo et al., 2008; Then & Lorch, 2008; Trtikova et al., 2015; Jiang et al., 2018; Girón-Calva et al., 2020). Therefore, to assess gene expression, the plants should have been grown in various environmental conditions and exposed to defined environmental stress conditions.

Data was only presented from field trials carried out in the US, but not from any other soy producing country (such as Argentina, Brazil, Paraguay or Uruguay). Exceptional weather conditions were reported in some field trials carried out in 2017 (used for compositional analysis). However, only the data from 2016 were taken into account for gene expression, with no reports on specific stress factors.

The striking differences between the climatic conditions in the major US soybean growing regions and those in soybean growing regions in Brazil, another major producer of GE soybean, were not taken into account. Data show there was much more precipitation in soybean growing states, e.g. Paraná or Mato Grosso, compared to the US.[1] There is also a much higher average and maximum temperature in Brazilian soybean growing regions, such as Mato Grosso, compared to US soybean growing regions.[2]

In conclusion, the soybean plants tested in field trials do not sufficiently represent the imported soybeans. The data presented by the applicant are insufficient to conclude on the impact of environmental factors and stress conditions on gene expression as requested by the EU Regulation 503/2013.

Data on herbicide application rates and their impact on gene expression

Of the relevant groups of HPPD inhibitors (one group is known as benzoylisoxazoles bleaching herbicides, such as isoxaflutole, the other as β-triketones, such as mesotrionine), only one active substance (isoxaflutole) was tested in field trials. Other HPPD inhibitors that might be used in the cultivation of soybeans were ignored. According to available publications, at least one further active substance, mesotrione, could also be used in the near future (Schultz et al., 2015). Furthermore, the BASF patent application WO2018119336 describes the application of mesotrione and tembotrionine at differing dosages on the soybean plants.

Data on gene expression of the Bt gene show a tendency towards lower gene expression in plants treated with the herbicide. As a consequence, it is plausible that the application of the complementary herbicides will have an impact on gene expression, which also may depend on the specific active ingredients. However, no such data were requested or provided to assess these questions in more detail. Furthermore, 'Omics' should have been applied to assess the impact of the complementary herbicides applications. 'Omics' were, however, not used.

EFSA should have requested the applicant to submit data from field trials that included all the relevant active ingredients, and all dosages of the complementary herbicides which might be used in agricultural practice of the soy 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.

Consequently, the soybean plants tested in field trials do not sufficiently represent the imported soybeans. The data presented by the applicant are insufficient to conclude on the impact of the herbicide applications on gene expression, plant composition and 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 variety can influence both the expression of the inserted genes and plant metabolism (see for example Barbosa et al., 2012; Zanatta et al., 2020; de Campos et al., 2020). Therefore, EFSA should have requested additional data from several varieties, including those cultivated in South America.

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

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[1] https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=2222000&regionid=br&nationalGraph=False&cntryid=BRA&sel_year=2021&startRow=1&fctypeid=23&fcattributeid=1

[2] https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=2222000&regionid=br&nationalGraph=False&cntryid=BRA&sel_year=2021&startRow=1&fctypeid=24&fcattributeid=1
https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=2222000&regionid=br&nationalGraph=False&cntryid=BRA&sel_year=2021&startRow=1&fctypeid=24&fcattributeid=5

Conclusion - molecular characterisation and gene expression

To gather reliable data on gene expression and functional genetic stability, the plants should have been subjected to a much broader range of defined environmental conditions and stressors. They should have, in addition, been tested in the soybean producing countries in South America. EFSA should also have requested the applicant to submit data from field trials which represent current agricultural practices, including all relevant active ingredients of complementary herbicides.

However, only samples from field sites located in the US were used to generate the data on gene expression, and only one variety of the GE soybean was used in the trials. The impact of environmental factors and agricultural practices was assessed without taking into account more extreme climate conditions as might be expected from climate change. Herbicide applications in the field trials did not represent all relevant agricultural practices.

In summary, the soybeans tested in field trials do not sufficiently represent the imported soybeans. 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 gene expression and plant metabolism.

Based on the available data, no final conclusions can be drawn on the safety of the plants. Therefore, the data do not fulfill the requirements of Implementing Regulation 503/2013 or Regulation 1829/2003.

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 provided by BASF neither reflect anticipated agricultural management practices nor the different meteorological and agronomic conditions under which the crop is to be grown. There are three reasons: (i) the field trials were not conducted in all relevant regions where the Soybean will be cultivated and impact of stress factors were not sufficiently taken into account; (ii) the field trials did not sufficiently take current agricultural management practices into account; (iii) only one transgenic variety was included in the field trials.

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

Field trials for the assessment of plant composition and agronomic and phenotypic characteristics of the soybean were only conducted in the US (for one year), and not in any other relevant soybean production countries, e.g. Brazil, Argentina, Paraguay or Uruguay. According to the EFSA opinion, some exceptional weather conditions (such as rain and drought). However, the impact of environmental stress factors on plant composition and agronomic characteristics were not investigated in any detail.

There are striking differences between the climate conditions in the major US soybean growing regions and those in soybean growing regions in Brazil, another major producer of GE soybean. Data show much more precipitation in soybean growing states, e.g. Paraná or Mato Grosso, compared to the US.[3] There is also a much higher average and

[3] https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=2222000&regionid=br&nationalGraph=False&cntryid=BRA&sel_year=2021&startRow=1&fctypeid=23&fcattributeid=1

maximum temperature in Brazilian soybean growing regions, such as Mato Grosso, compared to US soybean growing regions.[4]

However, no experiments were requested to show to which extent specific environmental conditions will 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 and agronomic and phenotypic characteristics

Due to the mode of action of the active ingredients in the complementary herbicides, it is plausible that complementary herbicides applications will cause stress responses in the plants and impact gene expression and plant composition. These effects may vary with the amount of herbicide sprayed on the crop and the various active ingredients which can be used on this soybean event. It has to be assumed that the differences in complementary herbicide applications will not only lead to a differing burden of residues in the harvest, but will impact the composition of the plants and agronomic characteristics. This assumption is supported by a higher number of significant differences in agronomic characteristics and plant composition in plants sprayed with the complementary herbicide compared to those not sprayed with isoxaflutol.

Therefore, all relevant agricultural management practices need to be considered to assess whether the expected agricultural practices will influence the expression of the studied endpoints. EFSA should have requested the applicant to submit data from field trials with all relevant active ingredients, and all dosages of the complementary herbicides that can be expected in practice in all of the relevant soy producing countries. However, only one active ingredient (isoxaflutole) was used and only one of the spraying regimes was tested in the field trials.

Consequently, the soybean plants tested in field trials do not sufficiently represent the imported soybean. The data presented by the applicant are insufficient to conclude on the impact of the herbicide applications on plant composition or agronomic and phenotypic characteristics of the plant as requested in EU Regulation 503/2013.

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

It is known that the genomic background of the variety can influence the expression of the inserted genes and plant metabolism (see, for example, Barbosa et al., 2012; Zanatta et al., 2020; de Campos et al., 2020). Therefore, EFSA should have requested additional data from several GE varieties, including those to be cultivated in South America.

However, EFSA risk assessment has not yet taken these into account. Consequently, the soybean plants tested in field trials do not sufficiently represent the imported soybean. The data presented by the applicant are insufficient to conclude on the impact of genetic backgrounds on plant composition and the biological characteristics of the plant as requested in EU Regulation 503/2013.

Data from compositional analysis show the need for further investigations

Eleven agronomic characteristics criteria were subjected to statistical analysis. Two of them were significantly different in untreated soybean GMB151, compared to five criteria after spraying with the complementary herbicide.

[4] https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=2222000&regionid=br&nationalGraph=False&cntryid=BRA&sel_year=2021&startRow=1&fctypeid=24&fcattributeid=1
https://ipad.fas.usda.gov/cropexplorer/cropview/comm_chartview.aspx?cropid=2222000&regionid=br&nationalGraph=False&cntryid=BRA&sel_year=2021&startRow=1&fctypeid=24&fcattributeid=5

Of 89 constituents which were subjected to statistical analysis to assess changes in plant composition, 31 were significantly different in plants not sprayed with the complementary herbicide compared to 34 in those sprayed with isoxaflutole.

As mentioned above, the data showed a lower number of significant findings in plant composition and phenotypic characteristics when the plants were not sprayed with the complementary herbicides. This indicates that application of the complementary herbicide might have impacted metabolic pathways. This should have been investigated in more detail.

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.

Furthermore, the data presented did not take into account cultivation of the GE soybean in all relevant major soybean producing countries, or in more extreme drought conditions, such as those occurring due to climate change. The range of differences and their significance are likely to be substantially increased in regional and environmental conditions.

As explained above, EFSA should have requested the applicant to submit data from field trials that , included all relevant active ingredients, and all dosages of the complementary herbicides that can be expected in practice in all of the relevant soy producing countries.

In conclusion, based on the available data, no final conclusions can be drawn on the safety of the plants.

Conclusion on comparative assessment of plant composition and 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 background on gene expression, plant metabolism, plant composition and 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. Whatever the case, they should have been tested in the soybean producing countries in South America. Furthermore, EFSA should have requested the applicant to submit data from field trials which represent current agricultural practices, including all relevant complementary herbicides.

However, only samples from field sites located in the US were used to generate the data on gene expression, and only one variety of the GE soybean was used in the trials. The impact of environmental factors and agricultural practices was not assessed in detail. Herbicide applications in the field trials did not represent all the relevant agricultural practices.

In summary, the soybean plants tested in field trials do not sufficiently represent the imported soybeans. Consequently, the data presented by the applicant and accepted by EFSA are insufficient to conclude on the impact of environmental factors, of herbicide applications and of 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.

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;"

Toxicity of the Bt toxins

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

In this case, the toxin is effective in nematodes, but there seems to be lack of understanding of which mechanisms determine its selectivity in comparison to those Bt toxins which are effective in insects. More generally, specificity in regard to Cry14Ab-1 for nematodes is assumed but not sufficiently demonstrated. This also has implications in regard to demonstrating safety for the food chain.

There are several publications describing the effects of Bt toxins in mammals: some Cry toxins are known to bind to epithelial cells in the intestine 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 Cry 1Ab and Cry1Ac. These effects seem to occur after 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 with the complementary herbicides. In this context, it is important that the soybean is also resistant to HPPD inhibitors, and the resulting residues should be seen as potential co-stressors at the stage of consumption (see also Then & Bauer-Panskus, 2017).

It is known that 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 thereby enhance their toxicity.

Testbiotech is aware of several publications confirming this gap in risk assessment that EFSA has consistently 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 the plants (MacIntosh et al., 1990). 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 (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 described effects 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 PI, but also by interaction with other Bt toxins (Sharma et al., 2004; 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).

In this case, specific residues from isoxaflutole applications can be expected. Isoxaflutol is classified as a "suspected human carcinogen", its specific residues (metabolites) left from spraying transgenic soybeans are still awaiting full risk assessment (EFSA, 2016). Safety of the products cannot be demonstrated as long as the toxicity of these residues and their impact as co-stressors are not fully investigated.

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).

However, the toxicity of the Bt toxins was assessed on the basis of isolated Cry14Ab-1 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 findings shown above, the basic data for toxicity assessment of the soybean are neither valid nor reliable.

In addition, it is likely that incorrect assumptions were made on the degradation of the Bt toxins due to processing and after consumption (see below).

In summary, the risk assessment on toxicity and exposure to Bt toxins are based upon incorrect assumptions. Cry14Ab-1 is a toxin which has not so far ever been present in the food chain and more data on its potential toxicity should have been requested.

Immmunogenicity 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; Vázquez-Padrón et al., 2000; Legorreta-Herrera et al., 2010; Jarillo-Luna et al. 2008; 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 in this context (for review see Rubio-Infante et al. 2016). Since Cry1Ac is also used as an adjuvant in vaccines, risks inherent to food consumption, which can be intensified by synergistic effects, need 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: the 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).

In this context, it is relevant that Bt toxins produced in the plants are known to survive digestion to a much higher degree than has been assumed by EFSA or shown in data provided by 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.

However, neither EFSA nor the applicant considered the potential enhancement of toxic or immunogenic effects caused by interaction with plant components such as PI. Potential impacts on the microbiome also have to be taken into account (see below). EFSA refers to potential adjuvanticity, but it was not investigated in any detail. Cry14Ab-1 is a toxin which has not so far ever been present in the food chain, therefore more data on potential immune responses should have been requested.

Effects from residues of 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 these 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.

In this case, specific residues from applications of isoxaflutole have to be expected. Isoxaflutole is classified as a “suspected human carcinogen”, its specific residues (metabolites) from spraying transgenic soybeans still await full risk assessment (EFSA, 2016). Safety of the products cannot be demonstrated as long as the toxicity of these residues and their impact as co-stressors are not fully investigated.

Nonetheless, 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).

HPPD enzymes are not only found in plants but in almost all living organisms, including microorganisms, where they are involved in the tyrosine degradation pathway (Moran, 2005). The hppd gene coding the targeted enzyme is described in about 2000 bacterial species (Thiour-Mauprivez et al., 2020).

Therefore, the potential impact on the gut microbiome from chronic exposure to food and feed derived from GE plants resistant to HPPD inhibitors, should be considered a relevant issue for risk assessment of the soybean since they may trigger significant changes in intestinal bacteria (see also Testbiotech, 2021).

This issue is also of potential relevance for the risk assessment of the soybean, as it also produces a Bt toxin which may trigger effects on the immune system directly or via the microbiome. This hypothesis needs to be tested before any conclusion can be drawn on the health safety of food and feed.

However, no attempts have been made to integrate the microbiome into the risk assessment of food and feed derived from the soybean. This is in marked 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).

In conclusion, the EFSA opinion on the application for authorisation of the soybean (EFSA, 2021) cannot be said to fulfill assessment requirements for potential synergistic, or antagonistic effects, resulting from the combination of the traits in the soybean event in regard to toxicity. Data on potential combinatorial effects should have been requested, as the combination of residues from spraying with HPPD inhibitors in combination with Cry14Ab-1 toxin has not so far been present in the food chain.

Results from the subchronic feeding study

The results from the feeding study leave room for further discussion and more detailed examinations.

In the light of the analysis provided above, we believe that a relevant hypothesis should be tested to assess immune system responses after chronic exposure from consumption of whole food and feed. However, this hypothesis is not covered by the design of the feeding study.

It appears that the material used in the feeding study was not sufficiently defined in regard to the concentration of the Bt toxins and amount of residues from spraying with complementary herbicides. However, these data are essential pre-conditions for the design of any feeding study with the soybean.

Furthermore, the question arises of whether roasted and defatted kernels are the best material to investigate potential health impacts, as soybeans are used in many processed foods.

In conclusion, the subchronic feeding study does not appear to be adequate to demonstrate the safety of food and feed derived from the GE soybeans.

Conclusions on toxicity

Safety of the soybeans at the stage of consumption was not sufficiently demonstrated.

Allergenicity

Implementing Regulation 503/2013 requests:

"*In cases when known functional aspects of the newly expressed protein or structural similarity to known strong adjuvants may indicate possible adjuvant activity, the applicant shall assess the possible role of these proteins as adjuvants. As for allergens, interactions with other constituents of the food matrix and/or processing may alter the structure and bioavailability of an adjuvant and thus modify its biological activity.*"

"*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;"

Potential effects on the immune system

The synergistic effects between PIs and Bt toxins described above are also relevant for risk assessment in regard to adjuvanticity: the 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 relevant effects.

There are several studies indicating that immune responses, such as adjuvanticity in mammals, are triggered by Bt toxins and have to be considered in this context. Studies with the Cry1Ac toxin (Moreno-Fierros et al., 2000; Vázquez-Padron et al. 1999; Vázquez-Padron et al. 2000; 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 in this context (for review also see Rubio-Infante et al. 2016). Cry1Ac is also used as adjuvant in vaccines and, therefore, risks inherent to food consumption, which can be enhanced by synergistic effects, need to be addressed and carefully examined.

In previous opinions (see EFSA, 2019a), EFSA admits only that "limited experimental evidence" is available to conclude the safety of Bt toxins in regard to immune system responses. Nevertheless, they do acknowledge the need for more detailed testing:
"EFSA has previously highlighted that the testing of adjuvant and allergenic potential of proteins requires stronger and fit-for-purpose standardised study design, and that future studies should consider limitations of current models, using relevant routes and methods of administration, doses, appropriate control proteins, and realistic exposure regimes. These aspects will require a broader discussion with the involvement of the international scientific community and its stakeholders to define a consensus on a fit-for-purpose study design for this assessment.

Given the fact that potential effects of Bt toxins on the immune system have meanwhile been discussed over the course of many years (for overview see, for example, Then & Bauer-Panskus, 2017), and already 45 GE crop events producing Bt toxins have been approved for the EU market, this explanation cannot be accepted. In accordance with EU Regulation 1829/2003, safety of whole food and feed has to be demonstrated before approval for import can be issued. Since this is not the case for the soybean, the risk assessment is not conclusive and no market authorisation can be granted.

These issues are especially relevant for the soybean event since combinatorial effects with other stressors (such as residues from spraying) cannot be excluded. This is further relevant for immune responses exerted via the microbiome (see above). However, neither EFSA nor the applicant considered the potential enhancement of toxic or immunogenic effects caused by interaction with plant components.

Conclusion on potential impact on the immune system

Considering these uncertainties, EFSA should have requested empirical testing of allergenic or adjuvant effects.

Conclusions

The EFSA risk assessment cannot be accepted since it is not sufficient to demonstrate safety as requested by EU law.

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