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Background: Safety of newly expressed protein in genetically engineered plants

Comments to a draft opinion of EFSA

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Introduction

In February 2025, EFSA published a draft „Scientific Opinion on current practice, challenges, and future opportunities in the safety assessment of newly expressed proteins in genetically modified plants“. (EFSA, 2025) This draft resulted from a self tasking activity of EFSA which also provided the following terms of reference (TOR):

„The European Food Safety Authority (EFSA) asked its GMO Panel to issue a scientific opinion on protein safety assessment. This statement should encompass:
1. Lessons learnt from experiences in the assessment of NEPs in the last 20 years, including more recent complex cases.
2. Building on experience above and issues identified, a critical appraisal of new methodologies available with the potential to be used as complementary/alternative testing strategies to current methodologies described in legal frameworks. This point could also contribute to the principles of the 3Rs (replacement, reduction and refinement of animal testing).
3. Road map for future implementation of such complementary/alternative methods in risk assessment strategies.
4. Recommendations for further research or for addressing methodological development needs.“

It is suggested to improve the current standards of protein safety assessment by the following strategy: „An improved strategy for the protein safety assessment could include: 1) history of safe use (HoSU), familiarity, and phylogeny defining the type of data required and waive the need for specific in vitro or in vivo studies; 2) advanced in silico tools, including predictive computational models and improved phylogenetic analysis to enable more accurate comparisons with known allergens, toxins or 'safe' proteins; 3) standardised in vitro gastrointestinal models that replicate physiological conditions; 4) development of targeted in vivo studies; 5) evaluating the role of exposure in the safety assessment; and, where necessary, 6) considering post-market monitoring for risk characterisation."

General comments

The approach of EFSA is largely driven by the idea that experimental and empirical data could be (at least partially) replaced by a ‚weight of evidence' approach. For this purpose, databases could be used (in combination with bioinformatic tools) to compare the newly expressed proteins (NEPs) with existing data in regard to previous usage and health safety. In addition, the development of more targeted and realistic requirements for *in vitro* and *in vivo* tests are suggested. This approach is intended to "*offering the highest level of protection possible for the consumers and the environment*."

Testbiotech agrees with EFSA that the current approaches for risk assessment of NEPs should be improved. There are several reasons:

- Market introduction of an increasing number of transgenic plants producing a range of additional proteins for providing herbicide resistance and insect toxicity. This trend is likely to continue, involving new proteins that were never present in the food chain or even new to nature (see Testbiotech 2024a-d).

- The expectation of a new wave of plants resulting from new genetic engineering techniques (NGT) may produce NEPs without the introduction of transgenes. These new proteins (or new versions of existing proteins) may primarily be caused by unintended frame shift mutations in the attempt to knock out endogeneous plant genes (such as wheat with reduced content in gluten, see EFSA, 2022).

In general, the recent development in genetically engineered plants makes it likely that an increasing number of NEPs will enter the food chain and the environment that have no equivalence in nature and/or are newly introduced into specific ecosystems and foodwebs and/or are newly introduced into food production. EFSA (correctly) assumes that this development will need new approaches in risk assessment methodology.

We agree with EFSA that some of the proposed measures may contribute to protein safety assessment in future. However, we are also of the opinion that additional measures are needed to safeguard "*the highest level of protection possible for the consumers and the environment*." In this context, we are aware of substantial deficiencies in the draft opinion of EFSA in regard to
- the likelihood of combinatorial exposure,
- long term, cumulative, indirect and delayed effects, and
- environmental risks.

Furthermore, cut-off criteria to reject market applications in case of too many unknowns and uncertainties are absent.

Finally, EFSA should consider measures to control or limit the overall number of NEPs into the food chain and the environment.

Likelihood of combinatorial exposure

Most of the *in silico* or *in vitro* measures as proposed by EFSA are meant to gather more information about the characteristics of the NEPs in isolation. This approach (as a stand alone) is not adequate. In most cases, the consumers (animals or humans) and the environment will not be exposed to these proteins in isolation (as it may be case with chemicals or pharmaceuticals) but embedded in the matrix of the plants and in combination with other biological active compounds. Consequently, the effects of NEPs to health and the environment will largely depend on these co-factors.

Also EFSA is mentioning that the food matrix, the plant's constituents and other factors may impact the effects of the NEPs at the stage of consumption. However, EFSA does not develop an adequate combined approach for risk assessment of NEPs with the most relevant co-factors. Consequently, the approach of EFSA, by default, is likely to produce blind spots in risk assessment that can lead to wrong conclusions on health safety of the NEPs. Furthermore, environmental risks have to be considered in the context in much more detail (also see below).

The example of Bt toxins

Coming from existing experience, the risk assessment of insecticidal Bt toxins as produced in transgenic plants can be used as an example to show substantial gaps in current practice of risk assessment as well as gaps in the approach as provided in the draft opinion.

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

Impact of plant constituents and other co-factors

Crucial impact factors in this context are protease inhibitors (PI) which show synergistic effects with Bt toxins, that may strongly enhance their toxicity. It was shown that Bt toxins produced in the plants can indeed survive digestion to a much higher degree than has been previously expected from in-vitro tests with the proteins in isolation. Chowdhury et al. (2003) and Walsh et al. (2011) found that when pigs were fed with Bt maize, Cry1A proteins could frequently still be found intact in the colon of pigs at the end of the digestion process.

As Monsanto scientists already published in the 1990s, maize, cotton and soybeans produce PIs, which considerably can delay the degradation of Bt toxin after consumption (MacIntosh et al., 1990). Thereby, the activity of PIs can result in a much higher toxicity of the Bt toxin(s) (if consumed as genetically engineered plant material) compared to the isolated Bt toxin alone (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 of PIs indicate, for example, a 20-fold higher toxicity of Bt proteins when consumed as genetically engineered plant material (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).

Effects on the immune system

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 also see Rubio-Infante et al. 2016). Since Cry1Ac is also regarded as a promising adjuvant in vaccines (Mezzomo et al., 2015, and overview in Rubio-Infante et al., 2018), 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 PIs 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, adjuvantic effects and other immune responses.

For example, adjuvanticity (that may foster allergenic reactions to other food ingredients) is highly relevant in this context: The delay in degradation extends the exposure of the intestinal immune system to Bt toxins and may trigger or enhance allergenic effects involving a not limited number of other food ingredients.

Already in a reply to experts from Member States (EFSA, 2019), EFSA admitted only that "*limited experimental evidence*" is available to conclude the safety of Bt toxins in regard to immune system responses. Therefore, at that time, EFSA acknowledged 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.*"

Therefore, this issue must not be overlooked in the future approach of risk assessment of NEPs.

The way forward

Any risk assessment of NEPs 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).

To overcome this problem, targeted *in silico* and *in vitro* methods have to be developed to come to correct hypothesis that then could be tested *in vivo*.

Such methods should imply to create databases on plant constitutents such as PI that are known to influence the toxicity, immunogenicity and allergenicity of proteins. These databases should also include other factors such as additional stressors (like herbicide residues from spraying with complementary herbicides in stacked events) or potential interactions with other NEPs that might be produced in the plants (such as other Bt toxins) or mixed within diets or may be released into joint environments.

These databases could then help to develop targeted hypotheses for empirical testing (in-vitro and in-vivo) of combined exposure that has to be expected either in some, many, most or even all cases.

Besides health safety (toxicity, immunogenicity, allergenicity and potential impacts on the reproduction system) this approach has to be extended to assess the environmental risks of NEPs in combined exposure (with plant constituents, other stressors) to food webs, soil organisms, pollinators, protected species and biodiversity (etc).

Long term and cumulative, delayed and indirect effects

EU GMO regulation requires to take into account cumulative and long term effects as well as indirect and delayed effects in mandatory risk assessment.

However, the current and future approach of EFSA would mostly concern immediate and direct effects caused by NEPs in isolation. It is important not to introduce a guidance of risk assessment which is below the general standards as legally required.

Especially chronic diseases, inflammatory processes and multi generation effects will need largely improved methodology and overall approach. The same holds true for the assessment of long term and cumulative effects in regard to the environment and biodiversity.

Indirect, delayed and cumulative effects via the microbiome
Again, risk assessment of insecticidal Bt toxins as produced in transgenic plants and stacked with herbicide resistance can be used as a example to show gaps in EFSA's current practice and the new approach as proposed.

Especially, combined exposure of Bt toxins with co-stressors such as with residues from spraying with the complementary herbicides (in stacked events) can also play a crucial role in regard to delayed and indirect effects: For example, glyphosate is known to cause shifts in the microbial composition and associated microbiomes of plants and animals, thus leading to a specific situation in regard to chronic exposure from food consumption: glyphosate has indeed been shown to cause shifts not only in soil organisms (van Bruggen et al., 2018) but also in the composition of the intestinal flora of cattle (Reuter et al., 2007), poultry (Shehata et al., 2013; Ruuskanen et al, 2020) and rodents (Mao et al., 2018; Mesnage et al., 2021; 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 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. humans, animals and plants. Therefore, changes in the microbiome can trigger a wide range of delayed and indirect effects and also long term risks (even across generations) in regard to health and the environment.

Already in 2020, a document published by EFSA (EFSA, 2020) called attention to the role of the microbiome in environmental risk assessment and food and feed safety. As EFSA stated: "*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.*"

Furthermore, 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.*"

In summary, it has to be considered a relevant hazard that for example the combination of Bt toxins with residues from spraying, can for example trigger effects on the immune system directly or via the microbiome. However, no attempts have been made to integrate such findings into the present draft opinion of EFSA (EFSA, 2025).

The way forward

To overcome these problems, targeted *in silico* and *in vitro* methods have to be developed to come to correct hypothesis that then could be tested in *in vivo* or monitored in a targeted way. Such methods should imply databases on potential cumulative and long term as well as indirect and delayed effects that are likely to be emerge. These databases should also include other factors such as additional stressors (like herbicide residues from spraying with complementary herbicides in stacked events) or potential interactions with other NEPs that are produced in the plants (such as other Bt toxins) or mixed within diets or be released into joint environments that may cause cumulative, long term or indirect and delayed effects.

These databases could then help to develop targeted hypotheses for empirical testing (*in vitro* and *in vivo*) of cumulative, long term, delayed and indirect effects that have to be expected either in some, many, most or even all cases.

Besides the assessment of health safety (chronic toxicity, immunogenicity and potential multi-generation effects), the approach has to be extended to environmental risks including food webs, soil organisms, pollinators, protected species, biodiversity etc.

Since uncertainties may prevail in many cases, cut-off criteria need to be established that allow to in case reject market applications.

Environmental risks

Thhe introduction of NEPs into the environment, the foodwebs and the ecosystems raises complex risks that cannot be fully assessed by the current risk assessment. However, the EFSA draft opinion is mostly silent on environmental risk assessment (ERA).

To perform adequate ERA, data in regard to non-target organisms, on effects and interactions with a changing environment, degradation in soil and water, effects on soil organisms, pollinators and protected species etc. are needed. Existing experience with Bt proteins shows that EFSA's current approach is not sufficient to deal with respective hazards and risks:

For example, EFSA developed several models to quantify risks of Bt toxins for non-target (NT) Lepidoptera associated with the ingestion of Bt maize by pollen deposited on their host plants through estimates of larval mortality based on mathematical models developed by Perry et al. (2010, 2012).

However, as EFSA admitted some years ago, uncertainties were pertaining to the structure of the Perry et al. models, mostly caused by (1) the lack of data from bioassays estimating the sensitivity of a wider range of 'real' NT Lepidoptera for most of the assessed Bt maize events; and (2) uncertainties contributing to the variability in exposure of NT Lepidoptera to Bt maize pollen (see Testbiotech, 2021). Indeed, using the example of *Aglais io* in Catalonia (Baudrot et al., 2020), it has been shown that the EFSA model underestimates the risks in particular for susceptible Lepidoptera subpopulations.
"*When looking at the average lethal effect, Bt-pollen seems to have negligible impact on Non-target Lepidopteras, but when looking at the most Bt-sensitive individuals (i.e. combining highest exposure and lowest survival), we observed a dramatic change in their survival probability.*" The authors also stated that "*the high complexity of the physiological mechanisms of ingestion, solubilisation, activation, binding, storage, depuration, bio-transformation of Cry protein are not enough described and quantified*" to use a standard mechanistic modelling. Therefore, they "*cannot estimate a quantitatively accurate lethal damage effect from a given exposure profile.*"
Further, the researchers explain "*that also other factors such as sub-lethal effects combined with other stressors (e.g., parasitism, chemical compounds, resource depletion) should be taken into account.*"

The lessons learned from this experience were not integrated into the present draft EFSA opinion (EFSA, 2025). In addition, the issues of enhanced toxicity due to combinatorial effects with plant constituents, co-stressors as well as cumulative, long term, delayed and indirect effects are also a challenge to ERA. As mentioned, selectivity and efficacy of Bt toxins can largely be influenced by many co-factors (see, for example, Then, 2010; Hilbeck & Otto, 2015). Crucial impact factors are protease inhibitors (PI), which delay the degradation of Bt proteins and so enhance their toxicity. As Monsanto already showed in the 1990s, maize, cotton and soybeans produce PIs, which considerably enhance the toxicity of Bt proteins in plants. There are also a few publications available which indicate effects on non-target insects from PIs combined with Bt toxins (Babendreier et al., 2005; Liu et al., 2005a; Liu et al., 2005b; Han et al., 2010). Other research was conducted to explore the impact of chemicals in combination with Bt toxins on parasites in the gut of honey bees (Testbiotech, 2009)

In consequence, EFSA should explain how their planned new approach may contribute to answer these complex questions and which other measures will be needed. In addition, cut-off criteria should be introduced to reject market application in the case of too many unknowns and uncertainties.

Conclusion

The introduction of NEPs as produced in genetically engineered plants raise complex questions in regard to the health safety and environmental risks. The strategy proposed by EFSA (2025) "*is to reinforce the weight-of-evidence approach following it in a stepwise manner considering new methodologies as complementary and/or alternative studies and using animal studies only when there is a hypothesis or when key information to conclude on safety of a NEP is missing.*"

However, the weight-of-evidence approach remains limited if adequate data needed for comparison is absent. This is for example relevant in case of combinatorial exposure, long term, cumulative, indirect and delayed effects and the risks for the environment and biodiversity. In this regard, many further experimental (empirical) data will be needed. One way to move forward is to establish adequate databases and to trigger research to generate the relevant data to feed the bioinformatic tools.

However, major uncertainties and unknowns will remain. These uncertainties and related risks may increase in parallel to the number of NEPs introduced and the level of exposure to these proteins via food chain and the environment. Therefore, cut-off criteria will be needed to reject market application in case of to many uncertainties and unknowns.

These cut-off criteria may also help to scale and limit the overall exposure to NEPs in the food chain and to the ecosystems. In addition, these measures will not only be useful to reduce the overall risks and uncertainties but also to lower the number of i*n vivo* experiments and to limit costs and the regulatory efforts.

References

Babendreier D., Kalberer N.M., Romeis J., Fluri P., Mulligan E., Bigler F. (2005) Influence of Bt-transgenic pollen, Bt-toxin and protease inhibitor (SBTI) ingestion on development of the hypopharyngeal glands in honeybees. Apidologie, 36: 585-594. https://doi.org/10.1051/apido:2005049

Baudrot V., Lang A., Stefanescu C., Soubeyrand S., Messéan A. (2021) Extension of the spatially- and temporally-explicit "briskaR-NTL" model to assess potential adverse effects of Bt-maize pollen on non-target Lepidoptera at landscape level. EFSA Supp Publ, 18(4): 6443E. https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/sp.efsa.2021.EN-6443

Bøhn T. (2018) Criticism of EFSA's scientific opinion on combinatorial effects of 'stacked'GM plants. Food Chem Toxicol, 111: 268-274. https://doi.org/10.1016/j.fct.2017.11.023

Bøhn T., Rover C.M., Semenchuk P.R. (2016) *Daphnia magna* negatively affected by chronic exposure to purified Cry-toxins. Food Chem Toxicol, 91: 130-140. https://doi.org/10.1016/j.fct.2016.03.009

Broderick N.A., Robinson C.J., McMahon M.D., Holt J., Handelsman J., Raffa K.F. (2009) Contributions of gut bacteria to *Bacillus thuringiensis*-induced mortality vary across a range of Lepidoptera. BMC Biol, 7: 11. https://doi.org/10.1186/1741-7007-7-11

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

EFSA (2019) Application EFSA-GMO-NL-2016-134, Comments and opinions submitted by Member States during the three-month consultation period, https://open.efsa.europa.eu/questions/EFSA-Q-2016-00686

EFSA (2020) Editorial: Exploring the need to include microbiomes into EFSA's scientific assessments. EFSA J, 18(6): e18061. https://doi.org/10.2903/j.efsa.2020.e18061

EFSA (2022) Scientific Opinion on the evaluation of existing guidelines for their adequacy for the food and feed risk assessment of genetically modified plants obtained through synthetic biology. EFSA J, 20(7): 7410. https://doi.org/10.2903/j.efsa.2022.7410

EFSA (2025) Draft scientific opinion on current practice, challenges, and future opportunities in the safety assessment of newly expressed proteins in genetically modified plants, https://connect.efsa.europa.eu/RM/s/consultations/publicconsultation2/a0lTk000003SPsz/pc1278

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

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

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

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

Han P., Niu C.-Y., Lei C.-L., Cui J.-J., Desneux N. (2010) Quantification of toxins in a Cry1Ac + CpTI cotton cultivar and its potential effects on the honey bee Apis mellifera L.. Ecotoxicol, 19: 1452-1459. https://doi.org/10.1007/s10646-010-0530-z

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

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

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

Khalique F. & Ahmed K. (2005) Compatibility of bio-insecticide with chemical insecticide for management of *Helicoverpa armigera* Huebner. Pak J Biol Sci, 8: 475-478. https://dx.doi.org/10.3923/pjbs.2005.475.478

Kramarz P., de Vaufleury A., Gimbert F., Cortet J., Tabone E., Andersen M.N., Krogh P.H. (2009) Effects of Bt-maize material on the life cycle of the land snail *Cantareus aspersus*. Appl Soil Ecol, 42: 236-242. https://doi.org/10.1016/j.apsoil.2009.04.007

Kramarz P.E., de Vaufleury A., Zygmunt P.M.S., Verdun C. (2007) Increased response to cadmium and *Bacillus thuringiensis* maize toxicity in the snail *Helix aspersa* infected by the nematode *Phasmarhabditis hermaphrodita.* Environ Toxicol Chem, 26: 73-79. https://doi.org/10.1897/06-095R.1

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

Liu X., Zhang Q., Zhao J.-Z., Li J., Xu B., Ma X. (2005a) Effects of Bt transgenic cotton lines on the cotton bollworm parasitoid Microplitis mediator in the laboratory. Biol Control, 35: 134-141. https://doi.org/10.1016/j.biocontrol.2005.08.006

Liu X.D., Zhai B.P., Zhang X.X., Zong J.M. (2005b) Impact of transgenic cotton plants on a non-target pest, *Aphis gossypii* Glover. Ecol Entomol, 30: 307-315. https://doi.org/10.1111/j.0307-6946.2005.00690.x

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

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

Mao Q., Manservisi F., Panzacchi S., Mandrioli D., Menghetti I., Vornoli A., Bua L., Falcioni L., Lesseur C., Chen J., Belpoggi F., Hu J. (2018) The Ramazzini Institute 13-week pilot study on glyphosate and Roundup administered at human-equivalent dose to Sprague Dawley rats: effects on the microbiome. Environ Health, 17: 50. https://doi.org/10.1186/s12940-018-0394-x

Mason K.L., Stepien T.A., Blum J.E., Holt J.F., Labbe N.H., Rush J.S., Raffa K.F., Handelsman J. (2011) From commensal to pathogen: translocation of Enterococcus faecalis from the midgut to the hemocoel of *Manduca sexta*. mBio 2: e00065-00011. https://doi.org/10.1128/mBio.00065-11

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

Mesnage R., Teixeira M., Mandrioli D., Falcioni L., Ducarmon Q.R., Zwittink R.D., Mazzacuva F., Caldwell A., Halket J., Amiel C., Panoff J.-M., Belpoggi F. Antoniou M.N. (2021) 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. Environ Health Perspect, 129(1): 017005. https://doi.org/10.1289/EHP6990

Mezzomo B.P., Miranda-Vilela A.L., Grisolia C.K. (2015) Toxicological Evaluation of a Potential Immunosensitizer for Use as a Mucosal Adjuvant-*Bacillus thuringiensis* Cry1Ac Spore-Crystals: A Possible Inverse Agonist that Deserves Further Investigation. Toxins, 7(12), 5348-5358. https://doi.org/10.3390/toxins7124881

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

Moreno-Fierros L., García-Hernández A.L., Ilhuicatzi-Alvarado D., Rivera-Santiago L., Torres-Martínez, M. Rubio-Infante N., Legorreta-Herrera M. (2013) Cry1Ac protoxin from *Bacillus thuringiensis* promotes macrophage activation by upregulating CD80 and CD86 and by inducing IL-6, MCP-1 and TNF-α cytokines. Int Immunopharmacol, 17(4): 1051-1066. https://doi.org/10.1016/j.intimp.2013.10.005

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

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

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

Perry J.N., Devos Y., Arpaia S., Bartsch D., Gathmann A., Hails R.S., Kiss J., Lheureux K., Manachini B., Mestdagh S., Neemann G. (2010) A mathematical model of exposure of non-target Lepidoptera to Bt-maize pollen expressing Cry1Ab within Europe. Proc Royal Soc B, 277(1686): 1417-1425. https://doi.org/10.1098/rspb.2009.2091

Perry J.N., Devos Y., Arpaia S., Bartsch D., Gathmann A., Hails R.S., Kiss J., Lheureux K., Manachini, B., Mestdagh, S., Neemann, G., Ortego, F., Schiemann, J., Sweet, J.B. (2011) The usefulness of a mathematical model of exposure for environmental risk assessment. Proc Royal Soc B, 278(1708): 982-984. https://doi.org/10.1098/rspb.2010.2667

Perry J.N., Devos Y., Arpaia S., Bartsch D., Ehlert C., Gathmann A., Hails R.S., Hendriksen N.B., Kiss J., Messéan A., Mestdagh S., Neemann G., Nuti M., Sweet J.B., Tebbe C.C. (2012) Estimating the effects of Cry1F Bt-maize pollen on non-target Lepidoptera using a mathematical model of exposure. J Appl Ecol, 49(1): 29-37. https://doi.org/10.1111/j.1365-2664.2011.02083.x

Reardon B.J., Hellmich R.L., Sumerford D.V., Lewis L.C. (2004) Growth, development, and survival of *Nosema pyrausta*-infected European corn borers (Lepidoptera: Crambidae) reared on meridic diet and Cry1Ab. J Econ Entomol, 97: 1198-1201. https://doi.org/10.1093/jee/97.4.1198

Reuter W. (2015) Toxicology of glyphosate, isoxaflutole, dicamba and possible combination effects. Available via Testbiotech. https://www.testbiotech.org/content/toxicology-glyphosate-isoxaflutole-dicamba-and-possible-combination-effects

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

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

Ruuskanen S., Rainio M.J., Gomez-Gallego C., Selenius O., Salminen S., Collado M.C., Saikkonena K., Saloniemia I., Helander M. (2020) Glyphosate-based herbicides influence antioxidants, reproductive hormones and gut microbiome but not reproduction: A long-term experiment in an avian model. Environ Pollut, 266(1): 115108. https://doi.org/10.1016/j.envpol.2020.115108

Sharma H.C., Sharma K.K., Crouch J.H. (2004) Genetic transformation of crops for insect resistance: potential and limitations. Crit Rev Plant Sci, 23(1): 47-72. https://doi.org/10.1080/07352680490273400

Shehata A.A., Schrödl W., Aldin A.A., Hafez H.M., Krüger M. (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

Singh G., Rup P.J., Koul O. (2007) Acute, sublethal and combination effects of azadirachtin and Bacillus thuringiensis toxins on Helicoverpa armigera (Lepidoptera: Noctuidae) larvae. Bull Entomol Res, 97: 351-357. https://doi.org/10.1017/S0007485307005019

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

Tabashnik B.E., Fabrick J.A., Unnithan G.C., Yelich A.J., Masson L., Zhang J., Bravo A., Soberón M. (2013) Efficacy of genetically modified Bt toxins alone and in combinations against pink bollworm resistant to Cry1Ac and Cry2Ab. PLoS ONE, 8(11): e80496. https://doi.org/10.1371/journal.pone.0080496

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

Testbiotech (2021) Risk assessment of GE plants in the EU: Taking a look at the 'dark side of the moon', www.testbiotech.org/node/2692

Testbiotech (2024a) Assessment of genetically engineered maize DP 915635 for food and feed uses (Pioneer). Testbiotech Background, 17-02-2024, https://www.testbiotech.org/publikation/assessment-genetically-engineered-maize-dp-915635-food-and-feed-uses-pioneer/

Testbiotech (2024b)Assessment of genetically modified maize DP23211 for food and feed uses (Corteva Agriscience). Testbiotech Background, 17-02-2024, https://www.testbiotech.org/publikation/assessment-genetically-modified-maize-dp23211-food-and-feed-uses-corteva-agriscience/

Testbiotech (2024c) Assessment of genetically modified maize MON 95275 (application GMFF-2022-5890) by Bayer CropScience. Testbiotech Background, 19-09-2024, https://www.testbiotech.org/publikation/assessment-of-genetically-modified-maize-mon-95275-application-gmff-2022-5890-by-bayer-cropscience/

Testbiotech (2024d) Assessment of genetically modified maize DP51291 (application GMFF-2021-0071) by Corteva Agriscience. Testbiotech Background 12-12-2024, https://www.testbiotech.org/publikation/assessment-of-genetically-modified-maize-dp51291-application-gmff-2021-0071-by-corteva-agriscience/

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

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. Sci Total Environ 616–617: 255–268. https://doi.org/10.1016/j.scitotenv.2017.10.309

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

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

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

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

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

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

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