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Submission on APPLICATION A1097 FOOD DERIVED FROM HERBICIDE-TOLERANT AND INSECT-PROTECTED CORN LINE MON87411 ASSESSMENT REPORT

Submitted to Food Standards Australia/New Zealand (FSANZ)

by

Submitter: Centre for Integrated Research in Biosafety

15 January 2015

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Introduction

The Centre for Integrated Research in Biosafety (INBI) is a research centre hosted by the University of Canterbury and is dedicated to public good research and engagement.

This submission from INBI is in response to an invitation from Food Standards Australia/New Zealand (FSANZ) to comment on application A1097[1]. A1097 is an application to amend the Australia New Zealand Food Standards Code to allow foods derived from corn line MON87411 into the human food supply. The product comprises three significant and stacked genetic modifications: 1. the incorporation of a DNA sequence that when transcribed is intended to produce a 240 nucleotide long RNA which folds to become double-stranded (ds)RNA and is called DvSnf7; 2. a DNA sequence that includes the open reading frame for a modified version of the *cry3Bb1* gene and which is intended to produce the Cry3Bd1 protein sourced from mobile genetic elements isolated from *Bacillus thuringiensis*; and 3. a DNA sequence that includes the open reading frame for a modified version of the *cp4epsps* gene and which is intended to produce the 5-enolpyruvyl-3-shikimatephosphate synthetase protein from *Agrobacterium* strain CP4.

In addition to the three DNA sequences that correspond with the commercial traits, the product has >20 other recombinant DNA sequences from other contexts in corn or other species.

As part of the submission, FSANZ has requested submitters to indicate one of two preferred options.

Option 1 – Prepare a draft variation to Standard 1.5.2

Option 2 – Reject application

We conclude that the preferred action is Option 2: reject, unless further information from both FSANZ and the Applicant can satisfactorily answer the questions or resolve the concerns raised in this submission.

Our area of focus is the insecticide DvSnf7, a dsRNA construct expressed in MON87411. Our focus on the dsRNA insecticide to the exclusion of comment on other aspects of the risk assessment should not be considered an endorsement of the remainder of the studies provided by the Applicant. Rather than provide a comprehensive review of the Applicant's dossier covering issues that we have provided advice on in the past, our intention here is to work with the most novel aspects of the product, the dsRNA insecticide. We have published a suggested risk assessment framework for evaluating products that include dsRNA constructs as active ingredients (Heinemann et al., 2013). We are in agreement with several other expert evaluations (FIFRA, 2014; Lundgren and Duan, 2013).

DvSnf7 is intended as a substrate for the corn rootworm RNAi biochemistry. The first product of transcription is a long RNA molecule of ~1.2kb. It is further modified to a shorter long dsRNA molecule of 240bp. The long dsRNA is ingested along with plant material by the rootworm. It survives digestion and may be further modified to shorter siRNA molecules. The RNAi pathway directs the ingested dsRNA molecules to messenger RNA produced in the worm. The intended target is the worm's *snf7* gene, which encodes an essential vacuolar sorting protein.

[1] This submission is the opinion of the authors and is not necessarily the opinion of the

Review of information on DvSnf7

We received 18 studies from Food Standards Australia/New Zealand (FSANZ) in response to our request to participate in the risk assessment of MON87411. The documents correspond with the list of documents the Applicant indicates were sent to FSANZ as part of the dossier (Table 1). We could identify only two studies that have any relevance to the assessment of the safety of DvSnf7. They are highlighted in grey in Table 1.

Table 1: List of studies provided in support of the safety of MON87411

Study number	Provided to FSANZ
MSL0024586	Assessment of Cry3Bb1 and CP4 EPSPS Protein Levels in Corn Tissues Collected from MON 87411 Produced in Argentina Field Trials during 2011-2012.
MSL0018662	Assessment of the *In Vitro* Digestibility in Simulated Gastric and Intestinal Fluids of the Cry3Bb1.pvzmir39 Protein.
MSL0025314	Amended Report for MSL0025048: Molecular Characterization of MON 87411.
RAR-2013-0213	Northern Blot Analysis of DvSnf7 RNA Expression in MON 87411.
MSL0023307	Amended Report for MSL0022432: Effect of Temperature Treatment on the Functional Activity of CP4 EPSPS.
MSL0024872	Characterization of the Cry3Bb1 Protein Purified from the Maize Grain of MON 87411 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *E. coli*-Produced Cry 3Bb1 Proteins.
MSL0023328	The Effect of Heat Treatment on Cry3Bb1 Functional Activity.
MSL0018711	An Acute Oral Toxicity Study in Mice with *E. coli*-Produced Cry3Bb1.pvzmir39 Protein
MSL0024883	Bioinformatics Evaluation of the Transfer DNA Insert in MON 87411 Utilizing the AD_2013, TOX_2013 and PRT_2013 Databases.
MSL0024900	Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of Inserted DNA in MON 87411: Assessment of Putative Polypeptides
MSL0024870	Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of Inserted DNA in MON 87411: Assessment of Putative Polypeptides.
MSL0024715	Updated Bioinformatics Evaluation of the CP4 EPSPS Protein Utilizing the AD_2013, TOX_2013, and PRT_2013 Databases.
MSL0024658	Composition Analyses of Maize Forage and Grain from Glyphosate Treated MON 87411 Grown in Argentina during the 2011/2012.
MSL0017566	Assessment of the *In Vitro* Digestibility of Purified *E. coli*-produced CP4 EPSPS Protein in Simulated Gastric Fluid.
MSL0024834	Characterization of the CP4 EPSPS Protein Purified from the Maize Grain of MON 87411 and

	Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *E. coli*-Produced CP4 EPSPS Proteins.
MSL0013077	Acute Oral Toxicity Study of CP4 EPSPS Protein in Albino Mice.
MSL0012949	Assessment of the *In Vitro* Digestive Fate of CP4 EPSP Synthase.
MSL0024728	Segregation of the T-DNA Insert in MON 87411 Across Three Generations.

RAR-2013-0213

This study confirmed the presence of the expected long initial transcript of ~1.2kb produced in MON87411.

MSL0024883

This study was a bioinformatics analysis of the insert in MON87411 with the intention of identifying potential *proteins* that might have adverse effects.

On closer inspection we found that neither of these studies had data relevant to establishing the safety of DvSnf7 RNA as a component of food or feed. *We conclude that the Applicant has provided no data in support of the safety of the insecticide DvSnf7.*

Recommendations

It is our recommendation that FSANZ request data sufficient to conclude that MON87411 is as safe as corn not modified to produce both the two insecticides and herbicide-tolerance traits.

We recommend that FSANZ request from the Applicant data that would comply with the protocol outlined in Figure 1. If the Applicant follows this protocol and then FSANZ were to determine based on that information that MON87411 had either no adverse effects or no unmanageable risks when used as food or feed, INBI would no longer advise against Option 1. Our present conclusion is based on absence of tests that demonstrate the safety of DvSnf7 RNA.

Further considerations

FSANZ is on record denying that dsRNA active ingredients require case-specific studies to support a risk assessment, and that is the position it has again taken here[2]. Those arguments have been challenged in the international peer-reviewed literature (see below) and counter evidence has not been forthcoming from FSANZ. Unfortunately, the scientific basis for this argument is not validated, nor is it a consensus argument in the

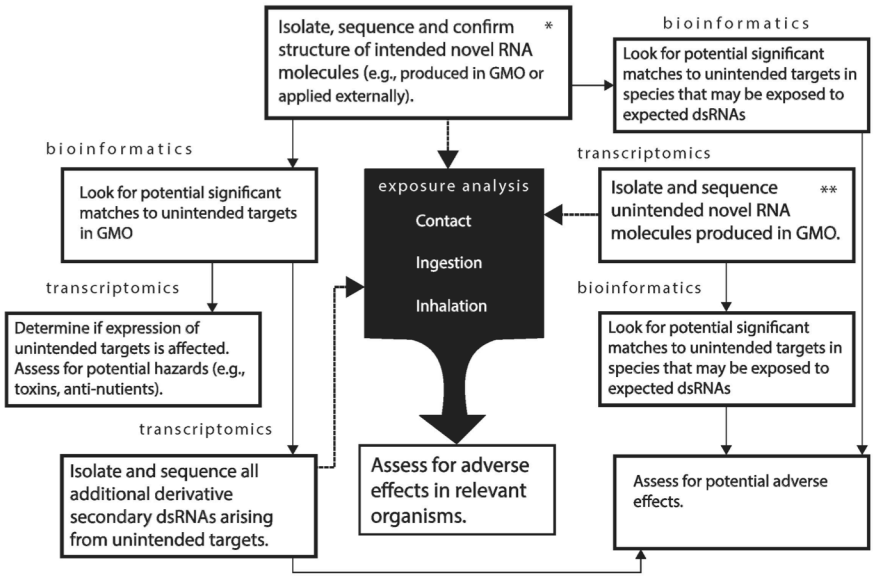
[2] "There are no concerns regarding the safety of the DvSnf7 dsRNA in MON87411. The data provided do not indicate this dsRNA possesses different characteristics, or is likely to pose a greater risk, than other RNAi mediators naturally present in corn. A history of safe human consumption of RNAi mediators exists, including those with homology to human genes. The evidence published to date also does not indicate that dietary uptake of these RNAs from plant food is a widespread phenomenon in vertebrates (including humans) or, if it occurs, that sufficient quantities are taken up to exert a biologically relevant effect. In addition, the level of the DvSnf7 dsRNA present in grain from MON87411 is extremely low, and the anti-DvSnf7 effect observed in corn rootworm is also highly specific to only a very small number of closely-related beetles. Grain containing the DvSnf7 dsRNA is therefore considered to be as safe for human consumption as grain derived from conventional corn varieties" Supporting Document One, A1097.

regulatory community. We encourage FSANZ to review both the non-transparent process by which it has come to its opinion on dsRNA active ingredients and its opinion.

As models to which FSANZ could aspire, we point to the process conducted by the United States Environmental Protection Agency (FIFRA, 2014). The US EPA's Scientific Advisory Panel drafted a review of current risk assessment frameworks and invited comment by submissions to an online portal. The submission period was followed by a large meeting where both the public and technical experts could ask to be heard by the Panel. The submissions and report, as well as the names of those who wrote the report, were public information.

The European Food Safety Authority conducted a similar, if not equally inclusive and transparent process. EFSA invited some technical experts to present on the topic, and others were allowed to attend space permitting. The conclusion of the EFSA workshop was published as a report, with the identities of authors of source information made public (EFSA, 2014).

Figure 1. Reproduced from Heinemann et al. (2013). Sequential approach to assessing the potential for adverse effects arising from dsRNA-initiated modifications to organisms. Bioinformatics is used to capture known hypothetical targets of both intended and unintended dsRNAs so that potential adverse effects can be assessed. Transcriptomics is used to verify and characterise all relevant changes at the transcriptome level. Exposure analysis is used to design the appropriate kinds of organism-level tests for adverse effects. (*) Starting point for intentional introduction of dsRNAs; (**) starting point for unintended changes to the transcriptome. Bioinformatics assessments are inferences or judgments made based on predictions. Assessments made following exposure are based on data from experiments.



These two processes stand in contrast to the process used by FSANZ to so far reject advice on the need to formally consider dsRNA in risk assessments. That process has been non-transparent, with even the name and qualifications of authors kept secret.

Why FSANZ should consider dietary dsRNA to be of relevance to risk assessment

- Other regulators disagree with FSANZ.
- FSANZ has not adequately considered non-ingestion pathways for dietary dsRNA.

- New science has emerged.

Since we published our analysis of dsRNA risk assessments (Heinemann et al., 2013), which included two vignettes based on FSANZ as a case study, more evidence has come to light that is worth mentioning.

The US EPA has since concluded that existing risk assessment frameworks are not sufficient to evaluate dsRNA for safety.

Overall, the Panel agreed with the concerns raised by the EPA regarding the inadequacies of the current environmental fate and non-target effects testing frameworks for dsRNA PIPs [plant incorporated protectant] *and exogenously applied dsRNA products. Uncertainties in the potential modes of action in non-target species, potential for chronic and sublethal effects, and potential unintended consequences in the various life stages of non-target organisms are sufficient justification to question whether the current Agency framework for ecological effects testing is applicable to dsRNA PIPs or exogenously applied non-PIP end-use products. Due to the modes of action of RNAi, no one set of test species will serve as an adequate representation of non-target species for all pesticidal products using RNAi technology. The classic approach of developing and assembling effects data for a standard set of test species will likely not work well for this technology. - (FIFRA, 2014)*

The EPA said that despite the lack of definitive proof of biological activity from dsRNA molecules ingested by mammals, considerable uncertainty remains. This is because it is unlikely that dsRNA in realistic food substrate will be pure, the form used in laboratory studies, but will instead be protected by other kinds of molecules, like those that form 'exosomes'. They therefore say that degradation of dietary dsRNA cannot be assumed. They "recommended experimental testing of the mammalian blood and exposed tissues be done to ensure that the siRNAs processed from the PIP dsRNAs are not present," to confirm that they have been degraded "*since these could have off-target effects after human consumption*" (emphasis added).

Importantly, *ingestion is not the only exposure pathway*. The EPA also recommended that other exposure pathways such as via the lungs through inhalation, or through contact with skin or mucosa, be tested. These pathways could produce very different exposure potentials. The EPA highlighted that other exposure pathways remain unexplored and may at times be more relevant than ingestion.

Moreover, the argument that RNA would not survive digestion is hypothetical because there are few studies on dsRNA stability through digestion, and none that prove complete removal of dsRNA at the stomach acidity levels typical of different kinds of consumers. Consequently, the EPA Panel "recommended that the stability of dsRNA in individuals that manifest diseases, immune compromised, elderly, or children be investigated."

The EPA also noted that bioinformatics and use of long dsRNAs does not guarantee absence of risk. "While 'long' dsRNA" which may be produced by the genetic engineering of the plant "may have no similarity to mammalian genes, processing of dsRNA into shorter siRNAs may present additional issues if these siRNAs have a high degree of similarity to sequences in non-target species including mammals." The short active form may have many more targets than predicted from the intended longer form. "Chances of off-target binding increase as the siRNA becomes shorter and if sequences mismatches between target and off-target sites occur," the EPA Panel said. Off-target effects can result in unintended silencing of other genes in animals or humans, potentially causing unanticipated adverse effects.

It is no longer tenable to use assumptions of RNA instablity to dismiss the potential for even ingestion exposure of dietary dsRNA. Two new publications add to the weight of

evidence that dsRNA active ingredients require testing for the purposes of conducting a formal risk assessment. The first demonstrates that dsRNAs (miRNAs) found in cow milk are biologically active in humans. The research, published in the *Journal of Nutrition*, found that the miRNA in the cow milk survived digestion and could alter gene expression. The authors said: "We conclude that miRNAs in milk are bioactive food compounds that regulate human genes" (Baier et al., 2014).

The first study (above) showed that dsRNA in breast milk could be transferred via ingestion, survive digestion and cause changes in gene expression, invalidating claims that it could not. This second study (Lukaski and Zielenkiewicz, 2014) provides the latest evidence that dsRNAs derived from plants are found in human and pig breast milk, packaged in exosomes. "Our study shows that plant miRNA molecules are abundant in human and porcine breast milk exosomes," the authors said. The obvious recipients of these dsRNAs would be nursing babies. This study establishes that novel dsRNAs introduced into plants or animals by genetic engineering, or sprayed onto plants as a pesticide, may very well survive digestion and accumulate in some tissues.

In contrast to the two studies discussed immediately above, the Monsanto Company has published a new 28-day study where mice were fed dsRNA (Petrick et al., 2015). The claim made by the study authors was that this study is strong evidence that dietary dsRNA has no effect on mammals via ingestion. While it superficially supports the pre-existing view of FSANZ, closer inspection is warranted.

Most notably, the study does not provide evidence that replaces the need for case-specific testing of DvSnf7 in MON87411. We found that—

- The study was a welcome start to the normalisation of dsRNA sequence-determined-risk assessments.
- The findings revealed no adverse effects in mice from five different 'dietary' dsRNAs that perfectly match a target mouse gene.
- The study used dsRNAs chemically modified to mimic those from plants and thus be more realistic tools.

However—

- Exposure was not a realistic reconstruction of ingestion of plants.
- Only ingestion (and not inhalation or contact) exposure was tested.
- The study was far too short to reveal all relevant effects. General well-being measurements were not validated as appropriate in this timeframe.
- The study inappropriately only used *vATPase* mRNA levels as surrogate for all possible sequence-determined effects.
- It lacked robust demonstration that the experimental constructs could have caused an effect on gene expression.

In summary, the study failed in its stated objectives:

"This study uses toxicity and target gene expression as surrogate measures of any biologically meaningful absorption/biodistribution or activity of ingested dsRNA. This is because any relevant impact of an absorbed nucleic acid on gene expression (e.g., ability to reach a target and trigger on-target or off-target gene suppression) would manifest itself as a physiological impact."

The chosen surrogates (weight, blood parameters, etc.) were compromised by the short duration of the study, the choice to monitor mRNA rather than V-ATPase protein levels, and the apparent bias in pursuing only suppression of *vATPase* mRNA levels. The study would have completely missed RNAi manifest as translation inhibition rather than RNA degradation. Moreover, the expectation that there would be an observable physiological impact in only 4 weeks is speculative. The expression of hundreds of different genes

may have been tweaked by the dsRNA, but the cumulative effect might take months to demonstrate a statistically significant physiological impact.

The chosen materials, the siRNAs used in the study, may have no biological effect. The single test in mouse tissue culture cells lacked information on replication or variance. The control experiments with mice kidney cells did not include a test of whether protein levels were changed by uptake of the siRNAs. Critically, the long dsRNA of 218 bp was not even tested for changing *vATPase* mRNA levels.

Another flaw was to use only ingestion as the route of exposure, and to not use dsRNA sourced from plants.

The intention to use siRNAs that should have been powerful suppressors of V-ATPase protein production was appropriate. In theory, if dietary dsRNAs could have an effect, using those with the strongest effect should increase chances of it being detected. But unfortunately the authors did not demonstrate that their siRNAs have a significant effect on mRNA levels as the results present what appears to be a single replication without reporting variance. *The critical flaw was that the authors never demonstrated an effect on target V-ATPase protein levels.* No physiological consequences could occur if the siRNAs had no effect on translation. Coupled with losses from digestion, these dietary dsRNAs could never have been expected to cause a physiological effect in just 4 weeks.

Finally, there was no attempt to use modern molecular techniques to see if the dsRNA caused unintended changes to the expression of other genes (EFSA, 2014; Heinemann et al., 2011). So called 'off-target' effects are common (Hanning et al., 2013; Heinemann et al., 2013). Transcriptomic or proteomic data is needed in this special case to resolve uncertainties about off-target effects.

Our conclusion

The weight of evidence suggests that potential adverse effects from novel dietary dsRNAs cannot be reasonably dismissed without specific testing. The literature has reaffirmed that dietary dsRNA plausibly survives digestion. Leading safety regulators cannot discount non-ingestion exposure pathways for dietary dsRNA (such as inhalation of flour when used in baking). Studies that argue against either the potential for exposure or potential to cause harm fail to meet standards sufficient to dismiss dsRNA active ingredients from case-by-case risk assessment.

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