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Detection of dietary DNA, protein, and glyphosate in meat, milk, and eggs[1]

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ABSTRACT: Products such as meat, milk, and eggs from animals that have consumed genetically engineered (GE) feed are not currently subject to mandatory GE labeling requirements. Some voluntary "non-genetically modified organism" labeling has been associated with such products, indicating that the animals were not fed GE crops, as there are no commercialized GE food animals. This review summarizes the available scientific literature on the detection of dietary DNA and protein in animal products and briefly discusses the implications of mandatory GE labeling for products from animals that have consumed GE feed. Because glyphosate is used on some GE crops, the available studies on glyphosate residues in animal products are also reviewed. In GE crops, recombinant DNA (rDNA) makes up a small percentage of the plant's total DNA. The final amount of DNA in food/feed depends on many factors including the variable number and density of cells in the edible parts, the DNA-containing matrix, environmental conditions, and the specific transgenic event. Processing treatments and animals' digestive systems degrade DNA into small fragments. Available reports conclude that endogenous DNA and rDNA are processed in exactly the same way in the gastrointestinal tract and that they account for a very small proportion of food intake by weight. Small pieces of high copy number endogenous plant genes have occasionally been detected in meat and milk. Similarly sized pieces of rDNA have also been identified in meat, primarily fish, although detection is inconsistent. Dietary rDNA fragments have not been detected in chicken or quail eggs or in fresh milk from cows or goats. Collectively, studies have failed to identify full-length endogenous or rDNA transcripts or recombinant proteins in meat, milk, or eggs. Similarly, because mammals do not bioaccumulate glyphosate and it is rapidly excreted, negligible levels of glyphosate in cattle, pig and poultry meat, milk, and eggs have been reported. Despite consumer concern about the presence of trace concentrations of glyphosate that might have been applied to feed crops and/or the presence of rDNA or recombinant proteins in meat, milk, and eggs, the available data do not provide evidence to suggest that products from animals that have consumed approved GE feed crops differ in any distinguishable way from those derived from animals fed conventional feed or that products from animals fed GE feedstuffs pose novel health risks.

Key words: animal products, deoxyribonucleic acid detection, genetic engineering, glyphosate, recombinant deoxyribonucleic acid

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INTRODUCTION

In many countries, meat, milk, and eggs are produced by animals that consume feed containing genetically engineered (**GE**) ingredients (Van Eenennaam and Young, 2014). Food/feed contains DNA and proteins from plants, animals, microbes, and viruses. Humans and animals consumed DNA, RNA, nucleotides, and proteins long before GE crops were introduced. Biotechnology does not change the chemical characteristics or general amount of DNA in an organism (Jonas et al., 2001) and DNA digestion is not affected by the DNA's origin. The United Nations Food and Agriculture Organization (**FAO**) and the U.S. Food and Drug Administration (**USFDA**) concluded that DNA consumption does

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not present health or safety concerns (FAO/WHO, 1991; USFDA, 1992).

However, public fears persist that animals that consume GE crops could pass on altered DNA in products, potentially adversely affecting human health. In a 2015 survey, 80% of respondents supported "mandatory labels on foods containing DNA," only 2% fewer than those that supported "mandatory labels on foods produced with genetic engineering" (Lusk and Murray, 2015). This highlights a lack of understanding that most food contains DNA.

This review summarizes the literature on the detection of DNA and protein in meat, milk, and eggs, with particular emphasis on detection of recombinant DNA (**rDNA**) and recombinant protein, and reviews evidence for horizontal gene transfer from food/feed. Due to recent concern about glyphosate and that several GE crops are tolerant to this herbicide, available literature on glyphosate levels in feed and animal products is examined. Lastly, given recent calls for mandatory labeling of products derived from animals that might have consumed GE feed and the USDA Food Safety Inspection Service's allowance of absence labeling claims for meat, poultry, and egg products from livestock that have not consumed GE feed (FSIS, 2016), potential implications of such labeling are briefly discussed.

SOURCES OF DIETARY DNA AND PROTEINS

Livestock diets may contain forages such as pasture, hay and silage, crop residues including corn and rice straw, cereal grains, and food and fiber coproducts such as soybeans, canola and cottonseed meals, cottonseed hulls, and corn distiller's dried grains. The United States is the world's largest producer and exporter of corn and the second largest exporter of soybeans after Brazil, whereas China, Japan, and some developing countries are leading importers (Fig 1). Since their approval in 1996, the global area planted to GE crops has steadily increased to approximately 180 million ha in 2015, as has their importance in global feed trade (Van Eenennaam and Young, 2014). Both ruminant and nonruminant livestock production systems worldwide use feedstuffs derived from GE varieties of corn, soybean, cotton, canola (CAST, 2006), and alfalfa. As a result, billions of animals globally have consumed rDNA and corresponding recombinant proteins (Alexander et al., 2007).

It is estimated that 70 to 90% of all GE plants and their biomass are used in farm animal feed (Flachowsky et al., 2012). The traits commonly associated with first-generation GE crops are insect resistance and herbicide tolerance (Sieradzki et al., 2006; James, 2015). The approved varieties of these crops have been deemed compositionally equivalent to their conventional counterparts, and no adverse effects have been demonstrated in animals that consume GE crops (Van Eenennaam and Young, 2014). In fact, there is evidence of some indirect feed safety benefits from insect-resistant crops due to decreased levels of mycotoxins (Wu, 2006). One feed crop that is not compositionally equivalent is low-lignin GE alfalfa, which was developed to have improved digestibility for livestock, especially dairy cattle. It was first commercialized in 2014 but is not yet widely grown for livestock feed because geographically limited commercial production only began in 2016 (Newman and Justen, 2016). Virus-resistant GE crops, including squash and papaya, do not represent typical livestock feedstuffs, and any feed concerns are considered negligible because these crops are modified with target viral DNA sequences to give them immunity to the target virus and these same viral DNA sequences and proteins are routinely consumed when animals are fed plants that have been naturally infected by the target viruses (Faust, 2002).

Deoxyribonucleic acid is found in the nuclei of cells; the amount of DNA in a particular food depends on the number and density of cells present in the edible parts, which naturally varies by food source. For example, animal muscle tissues generally have higher DNA and RNA content than plant storage tissues such as grains, which have a lower number of cell nuclei. Fast growing organisms such as bacteria, yeasts, and mushrooms contain a high amount of nucleic acids (Jonas et al., 2001; Rizzi et al., 2012), and amounts of nucleic acids in milk and eggs vary depending on lactation and developmental stages, respectively (Table 1).

In addition to the variation observed based on cell type and content, DNA begins to degrade as soon as a plant or animal is harvested; the rate of degradation depends on many factors (e.g., temperature, microbial, and enzymatic activity). The matrix in which the DNA is contained can affect its stability. Many of the foodstuffs that make up human diets are processed to some degree prior to consumption, which can affect the amount and integrity of the nucleic acids (Jonas et al., 2001). Although processing is known to significantly degrade DNA, some processed products for human consumption, including polenta, tofu, and summer sausages, have been shown to contain large (>1 kb) DNA fragments (Rizzi et al., 2012). Other processed foods, including tomato products and corn and potato chips (Bauer et al., 2004; Rizzi et al., 2012), have also been shown to retain DNA fragments of 200 to 400 bp. Highly refined products such as sugar and vegetable oil are devoid of DNA (Klein et al., 1998; Gryson et al., 2002), but some traces have been found in cold-pressed vegetable oil and maize starch (Vaïtilingom et al., 1999).

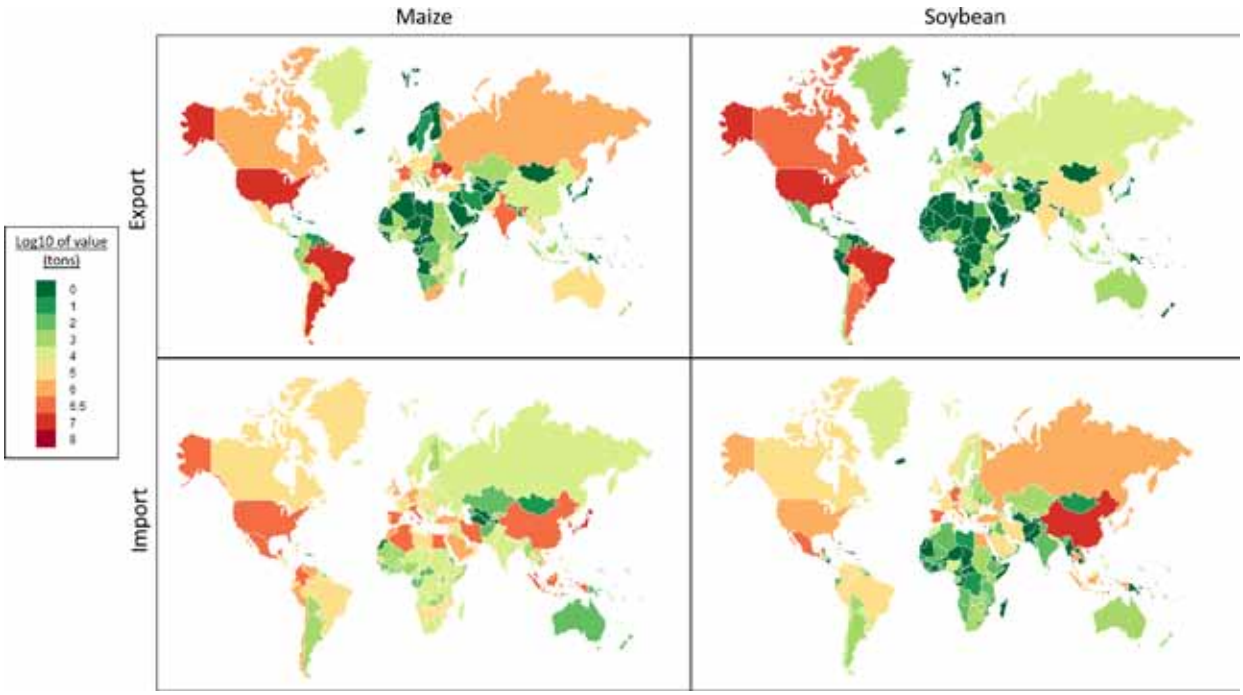

Figure 1. Global export and import of maize and soybean in 2013. Data were obtained from FAOSTAT (FAOSTAT, 2017) and include both non-genetically engineered and genetically engineered varieties and represented as $\log_{10}$ of the value in tonnes. Vector world map from allfreedesigns.com/vector-world-map-files-free-download/ (Allfreedesigns.com, 2017).

Most feed consumed by food-producing animals also undergoes some form of processing prior to consumption. Processing treatments including milling, extruding, ensiling, grinding, wet or dry heating, mixing, and steam pelleting are generally used to preserve feed, improve palatability, or increase nutritional value. A number of methods produce byproduct feeds, which are the accompanying result of processing plant material for another application (e.g., fermentation of distiller's grains, pressing for oil extraction, etc.). All of these processes rely on various combinations of heat, pressure, added ingredients, and mechanical manipulations. This is important because factors such as high temperatures and acidic pH levels (<5.0) are known to degrade DNA (Hupfer et al., 1999; Bauer et al., 2003).

Several studies have investigated the amount of DNA degradation caused by processing treatments on common livestock feed crops. In a study of various canola substrates (whole seed, cracked seed, meal, and mixed diet), processing fragmented DNA of both parental and transgenic varieties, but endogenous and recombinant plant genes could still be detected (Alexander et al., 2002). Temperature and pressure were found to affect DNA degradation in cottonseed meal that was processed using heat and extrusion, but water content of the processing treatment did not have much of an effect (Guan et al., 2013). Studies of insect-resistant *Bacillus thuringiensis* (*Bt*) corn found that the average DNA fragment size that could be detected was inversely related to the duration of the ensiling process and that the process of ensiling in general resulted in considerable DNA fragmentation, with no differences observed between the rate of DNA degradation in conventional vs. transgenic corn (Hupfer et al., 1999; Chiter et al., 2000; Einspanier et al., 2004; Lutz et al., 2006; Flachowsky et al., 2007). Air drying and low-temperature aqueous extraction processes conserve crop DNA and protein, which can be detected throughout the normal storage period. In contrast, chemical extraction processes used in oilseed meals result in the detection of only highly fragmented DNA (Chiter et al., 2000). Mechanical treatments such as crushing and pressing do not appear to fragment DNA, whereas extraction and toasting processes (desolventizing) cause DNA to become highly fragmented (Flachowsky et al., 2007). Grinding and milling similarly did not cause significant disruption of the DNA, but heat treatment resulted in DNA fragmentation (Forbes et al., 2000). Studies have also reported that analysis of mixtures of processed foods and feeds that have different particle size distributions as a result of processing treatments can result in a significant underestimation or overestimation of GE content in that product due to the heterogeneous distribution of rDNA in the different sized particles and the resultant variability in any given sample (Paoletti et al., 2003; Moreano et al., 2005).

Additional factors that affect the concentration of DNA and protein in a particular GE feedstuff prior to consumption by livestock include environmental conditions, gene copy number, and the transgenic event itself.

Different events (i.e., approved crop/GE construct combinations) vary in their expression levels of targeted recombinant proteins and possibly in which plant tissues and at which plant developmental stages they are expressed. The overall plant biomass can change in response to environmental conditions, such as lignification as a result of aging. The distribution of cells in different parts of a plant is also variable, meaning that rDNA content varies across the plant and with number of cells per gram DM in feedstuffs (Alexander et al., 2007). Additionally, the genome size of crops is not constant, with individual plant varieties showing up to 25% variation (Stave, 2002; van den Eede et al., 2002; Alexander et al., 2007), adding another potential source of variability in DNA content. Lastly, some endogenous genes are found at very high copy numbers in an organism, which makes them easier to identify in feedstuffs than single-copy genes, such as most transgenes (Alexander et al., 2007).

Nucleic acid intake can vary widely by individual. In humans, it is thought to be in the range of 0.1 to 1 g/d (Doerfler, 2000; Jonas et al., 2001). In cows, it is approximately 0.6 g/d (Beever and Kemp, 2000). It has been estimated that the amount of intact DNA in feed crops is less than 0.02% on a DM basis (Beever and Kemp, 2000). In a given GE crop, a proportionately smaller amount of DNA is recombinant (e.g., a few thousand basepairs) in comparison with the total amount of DNA (tens of billions of basepairs) in the plant (Jonas et al., 2001; Rizzi et al., 2012). Different species of livestock also have distinct nutritional requirements, meaning that the composition of their diets, and hence feed intake, vary considerably. Even within species, diets may contain different ratios of particular feed ingredients based on breed, location, age, season, health status, and other factors. Overall, it was estimated that for cows consuming a diet containing 40% silage and 20% grain made from GE corn varieties, approximately 0.00042% of the animal's total daily DNA intake would consist of rDNA (Beever and Kemp, 2000).

Some plant proteins are deficient in vitamins and essential AA, such as L-lysine and L-tryptophan, so feed additives are regularly included in animal feedstuffs to provide essential nutrients. These AA are synthesized by microorganisms, but wild strains of microorganisms often do not produce large enough quantities, so overproduction is achieved through the use of GE strains of microorganisms (von Wright and Bruce, 2003). Nonessential feed additives, including enzymes such as phytase (Düngelhoef et al., 1994), are also used in animal nutrition. Many of these additives are produced from GE microorganisms (Flachowsky et al., 2005a); however, the actual additives themselves often do not contain the GE production microorganism or its DNA (von Wright and Bruce, 2003).

Table 1. Average RNA and DNA contents of selected foods (data from Herbel and Montag [1987], Lassek and Montag [1990], and Gil and Uauy [1995])

Organism	Food	RNA, g/kg DM	DNA, g/kg DM
Yeast	Baker's yeast	66.2	6.0
Mushrooms	Champignon	20.5	0.9
	Chanterelle	6.0	1.0
	Oyster	24.1	1.4
Cattle/beef	Kidney	13.5	16.1
	Liver	22.1	19.5
	Lymph nodes	33.0	100.9
	Muscle	3.2	1.7
	Pancreas	87.9	16.2
	Spleen	17.9	32.6
Pigs/pork	Kidney	15.5	17.6
	Liver	32.1	14.8
	Lymph nodes	26.5	68.5
	Muscle	4.1	1.9
	Pancreas	71.4	21.2
	Spleen	21.7	40.4
Fish	Cod	4.7	0.3
	Mussels	10.8	9.2
	Salmon fillet	2.5	0.6
	Tuna	1.7	0.8
Grains	Corn	4.1	1.1
	Wheat	1.1	0.6
	White millet	1.5	0.7
Beans	Kidney beans	4.7	1.0
	Wax beans	4.2	1.0
	White beans	3.4	1.8
Vegetables	Broccoli	20.6	5.1
	Chives	9.1	3.3
	Kale	8.4	1.8
	Onion	2.6	0.7
	Spinach	14	2.6
	Species	RNA, mg/dL	DNA, mg/dL
Milk	Cattle	8 to 19	11 to 39
	Human	11 to 60	0.8 to 12

DIGESTION

In addition to treatment prior to consumption, feed is broken down through a series of processes, both mechanical and enzymatic, in the digestive tract to a soluble state that is conducive to absorption and nutrient uptake. Herbivores have longer, more complex digestive tracts than carnivores and omnivores because they are designed to break down tough plant cell walls before nutrients can be absorbed. Nonruminant animals, such as pigs and humans, have single-compartment stomachs. Ruminants, such as cows, have stomachs with 4 compartments: the rumen, reticulum, omasum, and abomasum. They use these compartments to house bacteria, protozoa, and fungi that digest plant carbohydrates, starch, and proteins (Beever, 1993). Poultry

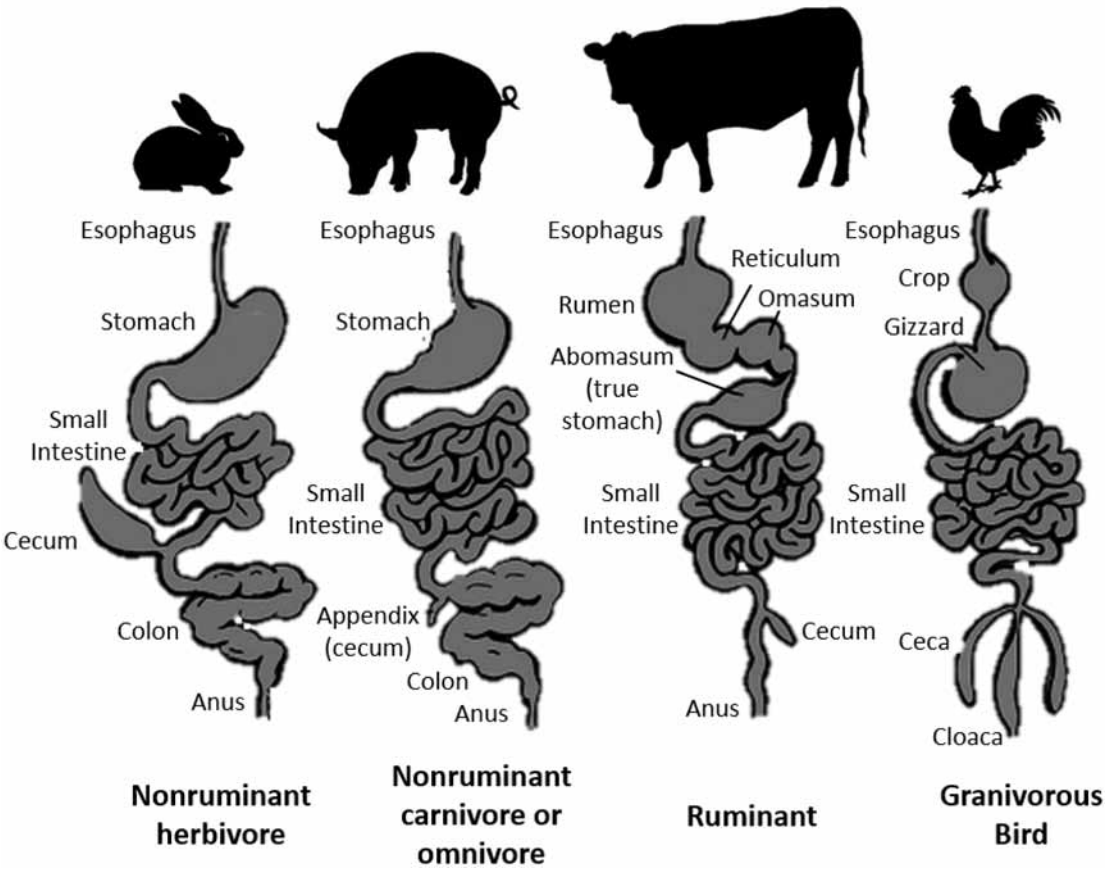

Figure 2. Examples of animal digestive systems: rabbit (monogastric herbivore), pig (monogastric omnivore), cow (ruminant), and chicken (granivorous bird). Figure modified from one published by Vimr (2013). (Animal silhouette vectors from All-free-download.com, 2017)

species, including chickens, turkeys, and ducks, have single-chambered stomachs but have additional organs—the crop, proventriculus, and gizzard—which aid in the breakdown of food (Fig 2).

In nonruminants, nucleic acid digestion occurs in the mouth, stomach, and intestines. Upon ingestion, enzymes in saliva, including RNA and DNA nucleases, begin to act on the food (Beever and Phipps, 2001). Acids in the stomach are responsible for further breakdown of the digesta before it passes into the intestines, where it encounters additional enzymes such as pepsin, α-amylase, proteases, lipases, and nucleases that are secreted by the pancreas (Beever et al., 2003). These enzymes break down nucleotides into phosphoric acid and nucleosides, which are further cleaved into sugars and purine and pyrimidine bases (Jonas et al., 2001).

At the level of the small intestine, nucleic acid digestion is comparable in both nonruminants and ruminants. However, the bacterial, protozoal, and fungal populations present in the rumen have a direct effect on digestion. In order to digest the plant fiber, feed spends varying amounts of time in the rumen, dependent upon both the feed composition and level of feeding (Beever et al., 2003). As the digesta moves throughout the rumen, DNA and RNA are further broken down (Fig 3).

From the rumen, digested and any remaining undigested feed enter the small intestine. As much as 85% of the nucleic acids that enter the small intestine at this point in the digestive process are reduced to the nucleotide level or smaller and any larger nucleic acid fragments are primarily of microbial origin (McAllan, 1982). Collectively, these data suggest that DNA and RNA from feed are rapidly degraded by nucleases in the rumen fluid (McAllan and Smith, 1973; Duggan et al., 2000), and an in vitro study demonstrated rapid DNA degradation of both parental and GE herbicide-tolerant (glyphosate tolerant, also known as "Roundup Ready") canola seeds upon release into rumen fluid cultures (Alexander et al., 2002).

DEOXYRIBONUCLEIC ACID AND PROTEIN DETECTION METHODS

Plant material from commercialized GE crops can be identified by testing for either rDNA or protein (Fig. 4). Each approach has its relative strengths and weaknesses (Table 2). Deoxyribonucleic acid-based methods tend to be comparatively inexpensive, rapid, and specific. Protein-based methods are generally more expensive and cannot be used to unequivocally determine which specific GE crop event produced the recombinant protein.

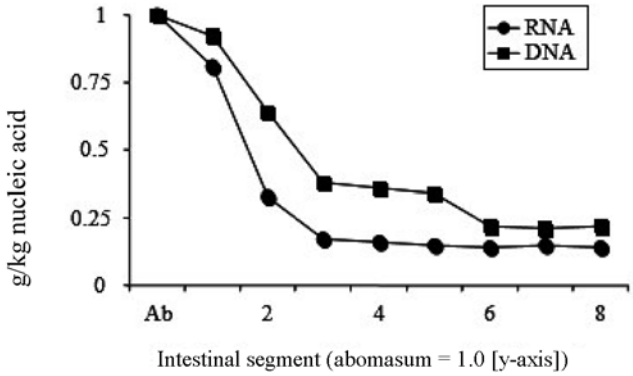

Figure 3. Fate of RNA and DNA in intestines of cattle (McAllan, 1982; Beever et al., 2003).

Furthermore, some proteins are unstable and most are nearly impossible to reliably detect in processed products.

Detection methods must be validated before application to routine regulation and novel matrices. Specificity, sensitivity, linearity, limit of detection, and limit of quantification of GE detection methods are tested with intra- and interlaboratory analysis of certified reference materials. Several recent peer-reviewed papers and reviews discuss the many nuances associated with detection of GE content in food and feed (Holst-Jensen, 2009; Holst-Jensen et al., 2016; Grohmann, 2010; Fraiture et al., 2015; Xu, 2016; Li et al., 2017; Mano et al., 2017).

DEOXYRIBONUCLEIC ACID AND PROTEIN DETECTION IN MEAT

A number of different researchers have investigated DNA detection in meat and meat products from livestock species (Table 3). Einspanier and colleagues fed conventional and GE *Bt* maize to cattle and chickens. In cattle, *Bt* maize and endogenous plant genes were not observed in muscle, liver, spleen, and kidney. Endogenous plant DNA fragments, but not *Bt* maize–specific gene fragments, were identified in broiler muscle, liver, spleen, and kidney. The authors suggested that the abundance of plant DNA fragments could have resulted from a combination of unprocessed corn in the diet and the short digestion path in the chicken gastrointestinal tract, which would allow less processing time for feed than the cattle ruminant digestive system (Einspanier et al., 2001).

Sharma and colleagues fed GE herbicide-tolerant and conventional canola to sheep and pigs and evaluated DNA detection. They identified high and low copy plant gene fragments in some visceral organ samples from both species. Transgene fragments were not observed in ovine liver or kidney samples but were detected in 1 sample of liver and kidney collected from 2 different pigs (2 samples out of 108 visceral tissues tested). The authors suggested possible reasons for the nonuniformity of the results but determined that the data supported previous studies suggesting that the likelihood of the uptake of rDNA into organ tissues is low and that both native and rDNA are similarly processed in the gastrointestinal tract (Sharma et al., 2006).

In *Bt* corn–fed piglets, fragments of endogenous plant genes were detected with varying frequencies in blood, spleen, liver, and kidney but not muscle. In contrast to other studies, a small fragment of the *cry1A(b)* transgene was detected in blood, liver, spleen, and kidney from test group animals. However, PCR results were inconsistent either in the replicates performed for each gene or across the DNA isolations for each tissue. It was concluded that *cry1A(b)* behaved similarly to the single-copy endogenous plant gene that was tested, except in blood, where it was found at higher concentrations. The authors hypothesized that DNA fragments in the blood are progressively degraded or diluted before reaching peripheral positions, which is why they detected DNA in blood but not in muscle. They concluded that DNA transfer to muscle tissues is unlikely, that the uptake of foreign DNA is a normal process, and that there are no higher risks of DNA transfer from food containing GE crops to an organism than there are for feeds containing the comparable conventional crops (Mazza et al., 2005). A study using weanling pigs did not observe rDNA or recombinant protein in kidney, liver, spleen, muscle, heart, or blood (Walsh et al., 2011). A study that fed high concentrations of *Bt* corn to pigs for long periods did not detect any rDNA or recombinant proteins in the blood of sows or the blood, heart, kidney, spleen, or muscle of their offspring at birth (Buzoianu et al., 2012a).

To date, no studies have identified rDNA fragments in meat or visceral organs from chickens. However, similar to the aforementioned species, some studies have detected fragments of endogenous plant genes (Table 2). Because endogenous plant-specific DNA sequences are not found in chicken embryos, it is likely that any endogenous plant gene fragments that are detected are transferred from feed to the animal (Klotz et al., 2002). Studies in quail have also failed to identify rDNA fragments in samples from muscle and internal organs (Flachowsky et al., 2005b; Korwin-Kossakowska et al., 2013).

A study that aimed to investigate the presence of DNA fragments in tissues from rabbits fed GE soybean meal identified only chloroplast DNA and was not able to detect the endogenous lectin gene or the cauliflower mosaic virus 35S (*CaMV 35S*) promoter present in the GE soybean (Tudisco et al., 2006). Another study similarly failed to detect GE-feed-derived genes in leg muscle samples from rabbits fed transgenic poplar leaf pellets (Yang et al., 2014).

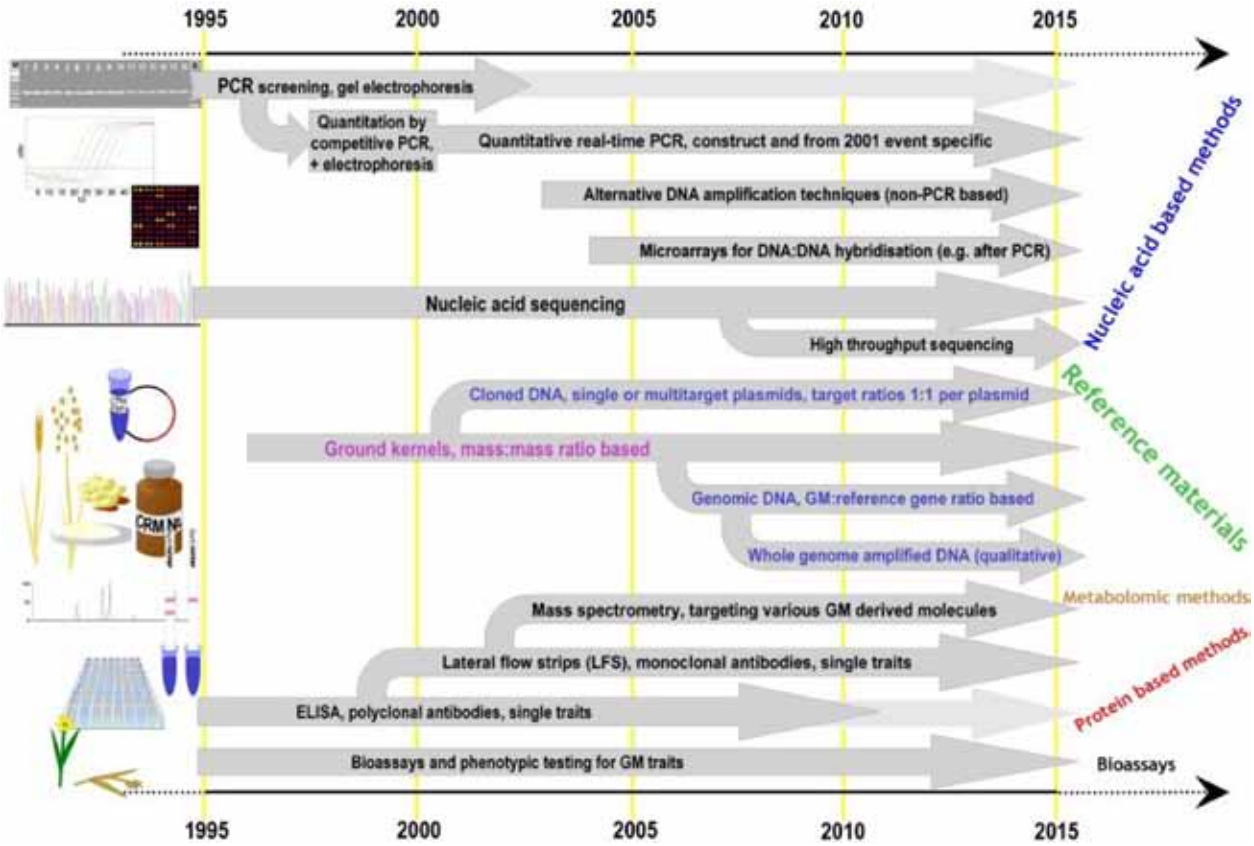

Figure 4. Evolution of genetically engineered detection methods and associated reference materials (reproduced with permission from Holst-Jensen [2009]). Ab = abomasum.

Similarly, dietary DNA fragments detected in fish have not been large enough to code for functional proteins (Table 2). A study investigating the fate of transgenic sequences from GE soybean during Atlantic salmon feeding experiments failed to amplify any soy DNA fragments (conventional or GE) in liver or muscle (Sanden et al., 2004). In Atlantic salmon force-fed feed containing spiked high copy number GE maize and soybean, short DNA targets (<300 bp) from GE ingredients were detected in some samples from kidney and liver, but this varied widely among individuals and the presence of endogenous plant DNA fragments was not investigated for comparison (Nielsen et al., 2005). A later study in Atlantic salmon conversely identified endogenous *rubisco* DNA fragments of soybean and maize origin in various tissue samples but did not detect any rDNA (Wiik-Nielsen et al., 2011). Studies assessing the use of GE soybean meal in feed for rainbow trout (Chainark et al., 2006, 2008) and tilapia (Ran et al., 2009; Suharman et al., 2009) have detected fragments of the *CaMV 35S* promoter in some samples. Conversely, *CaMV 35S* promoter DNA fragments were not detected in muscle from common carp fed 1 of 2 diets containing GE soybean meal (Suharman et al., 2010). Collectively, these studies have failed to identify full-length endogenous or rDNA transcripts or recombinant proteins in meat.

DEOXYRIBONUCLEIC ACID AND PROTEIN DETECTION IN MILK

Milk is an important and popular food due to its nutritional value and is a subject of food safety studies due to the vulnerability of its primary consumers, children. Several studies have investigated the potential transfer of DNA from feed to milk. To date, whole transgenes have not been detected in milk from cows or goats (Table 4), although endogenous plant DNA fragments have been detected in some instances (Einspanier et al., 2001; Phipps et al., 2003; Nemeth et al., 2004; Ponzoni et al., 2009). One report from Italian market samples found very small fragments of rDNA due to environmental contamination (Agodi et al., 2006).

Phipps et al. (2003) did not detect any DNA from single-copy genes, including transgenes, in milk from cows fed total mixed rations that included both GE soybean meal and GE corn grain. However, small fragments of the high copy endogenous corn gene *rubisco* were detected in the majority of milk samples (Phipps et al., 2003). Similarly, Einspanier et al. (2001) reported faint signals near the detection limit for highly abundant endogenous plant DNA in milk. Castillo et al. (2004) did not detect any transgenic or plant DNA fragments in milk from Argentinean Holstein dairy cows that consumed

Table 2. Examples of DNA- and protein-based analytical methods and their strengths and weaknesses

DNA-based analytical methods	Pros and cons of using DNA-based analytical methods
• Molecular hybridization • PCR/quantitative PCR (qPCR) • Digital PCR • Loop-mediated isothermal amplification • PCR capillary gel electrophoresis • Luminex • Microarray • DNA biosensors • Sequencing	• Need to know DNA sequence of interest • Inexpensive, rapid • DNA not present in some processed plant products (e.g., oil) • PCR primers are often based on elements originated from natural organisms, such as p35S from CaMV and tNOS from Agrobacterium. For this reason, the qPCR system provides merely an indirect proof of the presence of genetically modified organisms in a food/feed matrix because it can be confirmed only by the sequence of their transgene flanking regions. • Inhibitors, such as polysaccharides, polyphenols, pectin, xylan, or fat, can alter the efficiency of the PCR reaction.
Protein-based analytical methods	**Pros and cons of using protein-based analytical methods**
• ELISA • Lateral flow strip • Mass spectrometry • Monoclonal antibodies • Polyclonal antibodies • Flow cytometry • Two-dimensional gel electrophoresis • Immunomagnetic electrochemical sensors	• Protein-based methods depend on the expression level of targeted proteins, which is variable according to the plant tissues and the plant developmental status. • Relatively expensive • Some methods need specific antibody for each protein so hard to scale up • Proteins often degraded during feed processing • Proteins not present in some processed plant products (e.g., oil) • Any modification in the targeted proteins could alter the specificity and sensitivity of the assay. • This strategy is not applicable if the genetic modification has no impact at the protein level.

feed containing sources of GE whole cottonseed despite using assays with extremely high sensitivity. Singhal et al. (2011) also did not detect recombinant proteins from transgenes in milk from lactating cows in India that had consumed GE cotton. Yonemochi et al. (2003) reported a lack of Cry9C recombinant protein and *cry9c* rDNA fragments in milk from cows fed diets containing insect-resistance event CBH 351 (trade name StarLink; Bayer CropScience, Kansas City, MO)-derived hybrid corn. A study using a small number of Simmental cows failed to identify DNA transfer from feed containing soy and maize (non-GE) into milk (Poms et al., 2003).

A study of conventional and organic milk (1 sample was raw sheep's milk) from the Italian market that used highly sensitive methods identified GE corn DNA fragments and GE soybean DNA fragments (both <150 bp in size) in 25 and 11.7% of samples in conventional and organic samples, respectively. Endogenous maize genes were also identified in 40% of samples and endogenous soybean genes in 60% of samples. It was concluded that the presence of the transgenic sequences was likely due to fecal or airborne contamination of feed or feed particles or other environmental contamination. This is supported by results from a 2003 study in which raw milk was left exposed in the cow shed, and airborne soy and maize feed contamination was detectable when placed up to 10 m away from the feeding site (Poms et al., 2003). In their conclusions, the authors support the assertion that there is no inherent risk in consuming DNA, including DNA from transgenic crops (Agodi et al., 2006). A later study of plant diet contents in raw cow milk samples from the Italian market and stock farms detected endogenous chloroplast genes in total milk as well as skimmed and cream fractions (Ponzoni et al., 2009). A 2015 study testing for GE plants in milk and dairy products commercially available in Greece, including yogurt and cheese, failed to identify any rDNA fragments (Paramithiotis et al., 2015).

A 2012 study (Hassan and Ali, 2012) reported the detection of a 184-bp fragment of the NOS-terminator sequence in 3 of 7 imported dried milk powder samples purchased from a market in Iraq. Unfortunately, there are few details on the origin of these milk samples except that 1 of the positive samples, Dielac, was identified as being from New Zealand. That sample had a positive band when amplified with primers designed to the NOS-terminator. New Zealand tends to have a pastoral dairy production system and does not grow GE crops. They do import a small amount of maize and soy (Fig. 1), which could have been fed to the cows and could explain the presence of this sequence in dried milk powder from New Zealand. However, the authors of this paper did not follow the good laboratory practice of including the amplification of abundant endogenous plant DNA sequences (e.g., soy and maize housekeeping genes) in their analyses as positive controls to show that plant DNA can survive milk processing or negative controls of milk powder samples from cows that did not consume GE feed. Given that the results of this paper disagree with the many published studies that show no presence of rDNA fragments in milk, these controls would have helped support the validity of the results presented. This report appeared in the *Journal of American Science*, published by Marsland Press, which has appeared on Beall's List of Predatory Publishers (Beall, 2016) since 2012, casting further doubt that this paper underwent rigorous peer review.

In 2010, a controversial and subsequently retracted paper claimed to have identified rDNA fragments in milk from goats fed GE soybean in Italy. The report also claimed that small fragments of rDNA were detected in tissues and organs of goat kids whose dams had consumed GE soybeans. The authors noted that the results

Table 3. Deoxyribonucleic acid and protein detection in meat commonly consumed from different livestock species[1]

Species	Crop	GM trait[1]	Event	Transgene	GM content in feed	Material analyzed[2]	rDNA[3] fragment size, bp	DNA/protein detection	Reference
Cattle	Maize	IR	Bt[4]-Cesar	*cry1Ab*	N/A[5]	B, M, L, S, and K	189	No rDNA; chloroplast DNA in all	Einspanier et al. (2001)
			Bt11		43.30%	B, M, L, S, and K	110	No rDNA; chloroplast DNA in all	Chowdhury et al. (2004)
			MON810		N/A	M	123 and 149	No rDNA, chloroplast DNA in 5%	Nemeth et al. (2004)
			CBH 351 (StarLink[6])	*cry9C*	35%	B, M, and L	379	No rDNA or recombinant protein; did not look for endogenous plant DNA	Yonemochi et al. (2003)
	Maize and soybean	IR and HT	MON810 GTS40-30-2	*cry1Ab* and *cp4 epsps*	56 and 25%	M, L, S, and K	170, 172, 123, and 118	No rDNA or endogenous plant DNA	Furgał-Dierżuk et al. (2014)
					N/A	B, M, L, S, and K	123 and 118	No rDNA or endogenous plant DNA	Sieradzki et al. (2013)
	Soybean	HT	GTS40-3-2	*cp4 epsps*	24% grower, 19% early finisher, or 4% late finisher	M	272	No rDNA or endogenous plant DNA	Jennings et al. (2003c)
Sheep	Canola	HT	N/A	*cp4 epsps*	6.50%	L and K	179–1,363	No rDNA; chloroplast DNA at low frequencies	Sharma et al. (2006)
	Maize	IR	Bt176	*cry1Ab*	Varied	B, L, and S	211	No rDNA; did not look for endogenous plant DNA	Trabalza-Marinucci et al. (2008)
Pigs	Canola	HT	N/A	*cp4 epsps*	7.5% grower and 15% finisher	L, S, and K	179–1,363	rDNA in 1 L and 1 K sample; chloroplast DNA in all at varying frequencies	Sharma et al. (2006)
	Maize	IR	CBH 351 (StarLink)	*cry9C*	70%	B, M, and L	379	No rDNA; did not look for endogenous plant DNA	Yonemochi et al. (2010)
			N/A	*cry1Ab*	Varied	M	N/A	No rDNA or protein or endogenous DNA	Weber and Richert (2001)
					70%	M, L, S, and K	211	No rDNA or maize-specific DNA; chloroplast DNA at varying frequencies in all	Reuter and Aulrich (2003)
			MON810		N/A	M	123 and 149	No rDNA; chloroplast DNA in 43% of samples	Nemeth et al. (2004)
					50%	B, M, L, S, and K	519	rDNA in B, L, S, and K; maize-specific DNA in all but M	Mazza et al. (2005)
					39%	B, M, L, S, and K, H	211	No rDNA or protein; no endogenous plant DNA	Walsh et al. (2011)
					39% starter, 65% weaner, 73% finisher1, and 79% finisher2	B, M, L, and K	149	No rDNA; single copy endogenous DNA; multiple copy chloroplast DNA detected in 0–20% B, 20–50% M, 40–60% L, and 30–50% K	Walsh et al. (2012)
			Pactol Bt	*cry1a*	22.10%	B, M, L, and S	211 and 251	No rDNA or chloroplast DNA	Klotz et al. (2002)
		Nitrogen assimilation	Noncommercial variety	*gdhA*	58%	B, M, and L	490	No rDNA or endogenous plant DNA	Beagle et al. (2006)
	Maize and soybean	IR and HT	GTS40-3-2 and MON810	*cp4 epsps* and *cry1Ab*	Varied for growing and finishing	B, M, L, and S	170 and 172	No rDNA; did not look for endogenous plant DNA	Swiatkiewicz et al. (2011a)
						B, M, L, and S	123 and 118	No rDNA or endogenous plant DNA	Sieradzki et al. (2013)
	Soybean	HT	GTS40-3-2	*cp4 epsps*	24% grower, 29% early finisher, and 14% late finisher	M	272	No rDNA or endogenous plant DNA	Jennings et al. (2003b)
	Potato	Inulin production	N/A	*1-SST* + *1-FFT*	40%	M, L, S, and K	104	No rDNA or endogenous plant DNA	Broll et al. (2005)

Continued

Table 3. (cont.)

Species	Crop	GM trait[1]	Event	Transgene	GM content in feed	Material analyzed[2]	rDNA[3] fragment size, bp	DNA/protein detection	Reference
Chickens	Maize	IR	Bt-Cesar	*cry1Ab*	50%	M, L, S, and K	189	No rDNA; chloroplast DNA in all	Einspanier et al. (2001)
			Bt176		N/A	B, M, L, S, K, and H	211 and 129	No rDNA; chloroplast DNA in B, M, L, S, and K	Tony et al. (2003)
					60%	M, L, and S	479	No rDNA; chloroplast DNA in all	Aeschbacher et al. (2005)
			MON810		55–60%	M	211	No rDNA, recombinant protein, or endogenous plant DNA	Jennings et al. (2003a)
					N/A	M	123 and 149	No rDNA fragments; chloroplast DNA in 15% of M samples	Nemeth et al. (2004)
			MON863	*cry3Bb1*	51%	B, M, and L	N/A	No recombinant protein; results from B inconclusive	Scheideler et al. (2008)
			CBH 351 (StarLink)	*cry9C*	70%	B, M, and L	379	No rDNA or recombinant protein	Yonemochi et al. (2002)
		Increased phytase	N/A	*phyA2*	62%	B, M, L, S, K, and H	678	No rDNA or recombinant protein; did not look for endogenous plant DNA	Ma et al. (2013)
					62%	B and M	NA	No rDNA or endogenous DNA	Gao et al. (2014)
					54% d 1 to 21 and 61% d 22 to 42	M, L, K, and H	678	No rDNA or recombinant protein; did not look for endogenous plant DNA	Lu et al. (2015)
	Maize and soybean	IR and HT	MON810 GTS40-3-2	*cry1Ab* and *cp4 epsps*	N/A	B, M, L, S, K, and H	203, 171, and 195	No rDNA or single copy endogenous DNA; variable detection of multicopy chloroplast DNA	Deaville and Maddison (2005)
					55 to 60% corn and 32 to 37% soybean	B, M, L, and S	170 and 172	No rDNA or single-copy endogenous DNA	Swiatkiewicz et al. (2010)
					60 to 62%	B, L, and S	170 and 172	No rDNA or endogenous plant DNA	Swiatkiewicz et al. (2011b)
					Varied for growing and finishing	B, M, L, and S	123 and 118	No rDNA or endogenous plant DNA	Sieradzki et al. (2013)
	Soybean	HT	N/A	*cp4 epsps*	N/A	L	N/A	No recombinant protein; did not look for endogenous plant DNA	Ash et al. (2003)
Quail	Maize	IR	Bt176	*cry1Ab*	40% starter and 50% grower/layer	M, L, S, K, and H	211	No rDNA; did not look for endogenous plant DNA	Flachowsky et al. (2005b)
	Maize and soybean	IR and HT	MON810 GTS40-3-2	*cry1Ab* and *cp4 epsps*	Varied	M, L, S, K, and H	118 and 123	No rDNA; did not look for endogenous plant DNA	Korwin-Kossakowska et al. (2013)
					21/39% grower and 25/30% finisher		118 and 123	No rDNA; did not look for endogenous plant DNA	Korwin-Kossakowska et al. (2016)

Continued

Table 3. (cont.)

Species	Crop	GM trait[1]	Event	Transgene	GM content in feed	Material analyzed[2]	rDNA[3] fragment size, bp	DNA/protein detection	Reference
Fish	Maize and soybean	IR and HT	Bt176 and GTS40-3-2	*cry1Ab* and *cp4 epsps*	Spiked with high copy number	B, L, and K	151 and 81	Spiked rDNA detected at various levels; did not test for endogenous DNA	Nielsen et al. (2005)
			MON810GTS40-3-2	*cry1Ab* and *cp4 epsps*	N/A	B, M, L, S, K, and H	NA	No rDNA; endogenous plant DNA in all	Wiik-Nielsen et al. (2011)
	Soybean	HT	GTS40-3-2	*cp4 epsps*	17%	M and L	120 and 195	No rDNA or endogenous DNA	Sanden et al. (2004)
					16 or 31%	M	220	rDNA promoter fragment detected; did not test for endogenous DNA	Chainark et al. (2006)
					31%	B, M, L, S, and K	220	rDNA promoter fragment detected in B, K, and M; chloroplast DNA in B and S	Chainark et al. (2008)
					34 or 48%	M	205	rDNA promoter fragment in small number of samples; did not look for endogenous plant DNA	Suharman et al. (2009)
					30%	M, L, S, and H	254	rDNA promoter fragment detected in all, did not look for endogenous plant DNA	Ran et al. (2009)
					34 or 48%	B and M	205	No rDNA promoter fragments; did not look for endogenous plant DNA	Suharman et al. (2010)
Rabbits	Soybean	HT	GTS40-3-2	*cp4 epsps*	20%	B, M, L, K, and H	195	No rDNA or single copy endogenous plant DNA; chloroplast DNA in all	Tudisco et al. (2006)
					10%	B, M, L, K, and H	84	No rDNA or endogenous plant DNA	Morera et al. (2016)
	Poplar	IR	N/A	*Chitinase-BmkIT* and *NPTII*	N/A	B and M	1 kb and 280 and 750 bp	No rDNA; did not look for endogenous plant DNA	Yang et al. (2014)

[1]GM = genetically modified; IR = insect resistance; HT = herbicide tolerance.

[2]B = blood; M = muscle; L = liver; S = spleen; K = kidney; H = heart. (Several of these studies also investigated DNA detection in other organs not commonly part of the human diet such as gastrointestinal tract tissues.)

[3]rDNA = recombinant DNA.

[4]Bt = *Bacillus thuringiensis*.

[5]N/A indicates information that was not reported.

[6]Bayer CropScience, Kansas City, MO

Table 4. Deoxyribonucleic acid and protein detection in milk

Species	Crop	GM trait[1]	Event	Transgene	GM content in feed	rDNA[2] fragment size, bp	DNA/rec[3] protein	Endogenous plant DNA/ protein[4]	Reference
Cow	Cotton	IR and HT	N/A[4]	*cp4 epsps*, *cry1Ac*, *cry1Ac* + *cry2Ab*, and *cry1Ac* + *cp4 epsps*	10%	215	–	–	Castillo et al. (2004)
		IR	N/A	N/A	Approximately 40%	N/A	–	N/A	Singhal et al. (2006)
		IR	MLS 9124	*cry1C*	N/A	N/A	–	N/A	Mohanta et al. (2010)
			BGII	*cry1AC* and *cry2Ab*	40%	N/A	–	–	Singhal et al. (2011)
	Maize	IR	CBH251	*cry9C*	35%	379	–	–	Yonemochi et al. (2003)
			Bt[6] Cesar	*cry1Ab*	N/A	189	–	+	Einspanier et al. (2001)
			MON810		N/A	200	–	N/A	Phipps et al. (2001)
					N/A	123 and 149	–	+	Nemeth et al. (2004)
					N/A	N/A	–	N/A	Gürtler et al. (2009)
					N/A	206	–	–	Guertler et al. (2009)
					7.6% (grain), 21.2% (pellets), and 41.9% (silage)	206	–	–	Guertler et al. (2010)
		HT	T25	*pat*	N/A	320	–	–	Phipps et al. (2005)
		IR and HT	N/A	*mepsps* + *cry1Ab*	45% each	123 and 149	–	N/A	Calsamiglia et al. (2007)
	Soybean	HT	N/A	*cp4 epsps*	26% or 14%	172 and 180	–	–	Phipps et al. (2002)
	Maize and soybean	IR and HT	MON810 GTS40-3-2	*cry1Ab* and *cp4 epsps*	41.2% silage, 18.5% grain, and 13% meal	203	–	+	Phipps et al. (2003)
					N/A	113, 121, and 101	–	–	De Giacomo et al. (2016)
		N/A	N/A	N/A	31% maize silage and 53.5% soya slugs	N/A	N/A	–	Poms et al. (2003)
	Cotton and maize	IR	Bollgard, MON810	*cry1Ac* and *cry1Ab*	N/A	272	–	–	Jennings et al. (2003c)
Goat	Maize	IR	E176	*cry1Ab* and bla_{TEM}	54.60%	80	–	–	Rizzi et al. (2008)
Market samples	Maize and soybean	IR and HT	N/A	*cry1Ab* and *cp4 epsps*	N/A	106 and 145	– +	+	Agodi et al. (2006)
	Maize and soybean	IR and HT	NA	*NOS-terminator of bar* and *35S promoter of ESPS*	N/A	184	– +	N/A	Hassan and Ali (2012)

[1]GM = genetically modified; IR = insect resistance; HT = herbicide tolerance.

[2]rDNA = recombinant DNA.

[3]rec = recombinant.

[4]"–" and "+" indicate absence and presence, respectively. Cells with both a "–" and a "+" indicate that presence was detected in some samples but not others.

[5]N/A indicates information that was not reported.

[6]Bt = *Bacillus thuringiensis*.

conflicted with published results from other studies. Their data were called into question, and an investigation concluded that some of the presented results were "likely attributable to digital manipulation, raising serious doubts about the reliability of the findings," and the journal consequently retracted the manuscript (Tudisco et al., 2016, p. 1076). A 2013 study by the same authors also claiming significant detection of transgenic fragments in liver and kidney from goat kids fed milk from dams that consumed GE soybean meal was also retracted for fraud due to data fabrication, and the results were declared to be invalid (Mastellone et al., 2013). A third paper from this group, citing similar issues with GE soybean meal and likewise containing digitally manipulated images (Tudisco et al., 2015), has not been retracted but is under investigation.

Unfortunately, these retracted studies in journals published by predatory publishers continue to be cited by groups opposed to GE, and these falsified data are used to incorrectly suggest there is a distinguishable difference between milk derived from animals that have been fed GE feed compared with those that were not. The weight of evidence from numerous well-controlled, peer-reviewed papers does not support this conclusion, because rDNA fragments cannot be reliably or repeatedly detected in the milk from animals that have consumed GE feed.

Table 5. Deoxyribonucleic acid and protein detection in eggs

Species	Crop	GM trait[1]	Event	Transgene	GM content in feed	rDNA[2] fragment size, bp	DNA/rec[3] protein[4]	Endogenous plant DNA/protein[4]	Reference
Chickens	Maize	IR	Bt[5]-Cesar	*cry1Ab*	50%	189	–	–	Einspanier et al. (2001)
			Bt176		60%	479	–	–	Aeschbacher et al. (2005)
		Increased phytase	N/A[6]	*phyA2*	62.4%	678	–	–	Ma et al. (2013)
	Soybean	HT	N/A	*c4 epsps*	N/A	N/A	–	N/A	Ash et al. (2003)
	Maize and soybean	IR and HT	MON810 and RR (MON 40-3-2)	*cry1Ab* and *c4 epsps*	60%	170 and 172	–	–	Swiatkiewicz et al. (2011b)
					N/A	123 and 118	–	–	Sieradzki et al. (2013)
Quail	Maize	IR	Bt176	*cry1Ab*	40% starter and 50% grower	211	–	N/A	Flachowsky et al. (2005b)
	Maize and soybean	IR and HT	MON810 and MON 4-3-2	*cry1Ab* and *c4 epsps*	N/A	118 and 123	–	N/A	Korwin-Kossakowska et al. (2013)
						118 and 123	–	N/A	Korwin-Kossakowska et al. (2016)

[1]GM = genetically modified; IR = insect resistance; HT = herbicide tolerance.

[2]rDNA = recombinant DNA.

[3]rec = recombinant.

[4]"–" indicates absence.

[5]Bt = *Bacillus thuringiensis*.

[6]N/A indicates information that was not reported.

DEOXYRIBONUCLEIC ACID AND PROTEIN DETECTION IN EGGS

Similar to meat and milk, eggs are primary sources of protein, fats, and micronutrients in the human diet. Because eggs are relatively inexpensive and easy to handle and store, they provide important nutrition for consumers in both developed and developing countries (NRC, 2015). Eggs contain only a small amount of DNA (Aeschbacher et al., 2005), which, coupled with low copy numbers for transgenes, may hinder attempts at endogenous DNA and rDNA detection (Ma et al., 2013).

In 2001, Einspanier and colleagues reported that they did not detect any foreign plant DNA fragments in eggs from chickens fed diets containing conventional or recombinant *Bt* maize, despite that chloroplast DNA was reliably detected in chicken organs (Einspanier et al., 2001). Similarly, a 2005 study also failed to identify any endogenous or rDNA fragments in eggs from hens fed diets containing 60% conventional or *Bt*176 corn (Aeschbacher et al., 2005). A 2011 report detected no transgenic or endogenous plant DNA fragments in eggs from hens fed diets containing both insect-resistant maize (MON810) and GE herbicide-tolerant soybean (Swiatkiewicz et al., 2011b). Ash et al. (2003) tested whole egg, egg albumen, and egg white from commercial layers fed GE herbicide-tolerant soybeans and reported that all samples were negative for recombinant protein.

A long-term feeding study in quail, in which 10 generations of birds consumed diets containing *Bt* corn, did not detect rDNA fragments in eggs after the initial 12-wk feeding period and also not after 1 yr of feeding *Bt* corn. They concluded that "there is no indication of enrichment of rDNA fragments after long-term exposition" (Flachowsky et al., 2005b, p. 451). A subsequent study evaluating 4 generations of quail fed diets containing GE soybean meal and maize grain also did not detect rDNA in eggs from test animals (Korwin-Kossakowska et al., 2013). All studies to date have failed to amplify endogenous or rDNA or recombinant protein from feed in eggs (Table 5), suggesting that eggs from poultry fed GE feed are indistinguishable from those fed non-GE feed, given the available data.

POTENTIAL HORIZONTAL GENE TRANSFER OF DNA

One of the main concerns voiced by the public is that DNA from GE crops could be taken up by bacteria in the gut of food animals or humans. In mammals, the main point of entry of foreign macromolecules is the gastrointestinal tract (Rizzi et al., 2012). The human intestine is estimated to harbor more than 10^{14} microorganisms from thousands of different bacterial species (Aron-Wisnewsky and Clement, 2016). Bacteria are known to exchange genetic information by horizontal gene transfer, defined as "the non-parent-to-offspring exchange of genetic material between donor and recipient cells" (van Elsas and Bailey, 2002, p. 187). Gene transfer from transgenic plants to bacteria is extremely rare, with the probability of such an event estimated at 2×10^{-11} to 1.3×10^{-21} per bacterium (Dröge et al., 1998).

The successful transfer of genetic information by horizontal gene transfer is dependent upon a number of criteria and the completion of several steps. In order to result in a complete gene that could potentially

be expressed, the incorporated DNA would have to be largely intact. As discussed, once food and feed have been processed and are consumed, the DNA is most often fragmented into small pieces, regardless of whether it is GE or not. Overall, lower uptake efficiency has been observed with shorter fragments (van den Eede et al., 2004). In addition, the further species are from one another on the phylogenetic tree, the fewer sites are available for homologous recombination (Jonas et al., 2001).

In addition to the low likelihood that large full-length DNA fragments would be present in the gastrointestinal tract for horizontal gene transfer to occur, any fragments that were present would have to compete with the rest of the digested DNA, potentially from multiple dietary sources, for transfer into a bacterium, because the process is not sequence specific. The risk of integration of rDNA fragments is no different from the integration of any other DNA (Jonas et al., 2001; Thomson, 2001). Expression of transgenes by gut bacteria would also be contingent upon the simultaneous transfer of regulatory elements, such as promoters, in the proper orientation and context (Thomson, 2001). The physiology of the recipient cell would also play a role in whether transferred DNA is expressed, including the compatibility of its transcription and translation machinery with the signals of the incoming DNA (Jonas et al., 2001). In the extremely remote instance an intact gene was taken up, it is highly unlikely that it would be advantageous in the absence of selective pressure for the encoded protein (Thomson, 2001).

A number of animal feeding studies have shown no changes in intestinal bacteria in food animals as a result of consumption of feed with GE ingredients based on DNA sequencing. Buzoianu et al. (2012b) showed no differences in intestinal bacterial taxa in pigs that consumed *Bt* MON810 maize for 110 d aside from a minor increase in the genus *Holdemania*, which was not thought to be of clinical significance because it has not been observed to be pathogenic. A subsequent study by the same group further confirmed the absence of adverse effects of *Bt* maize on porcine intestinal microbiota across generations of sows and their offspring (Buzoianu et al., 2013). Similarly, no adverse effects on intestinal microflora in broilers were observed after consuming diets containing GE herbicide-tolerant soybean meal (Tan et al., 2012). In a study using Holstein-Friesian cows, quantitative changes in ruminal bacterial communities were analyzed by real-time PCR, and the authors concluded that diversity in microbial populations depends more on the individual animal and the sampling day than it does on the type of maize used to produce the silage consumed (Wiedemann et al., 2007). Other studies of the effects of diets containing transgenic corn on cow rumen in vivo similarly revealed no overall impact on rumen microbiota (Einspanier et al., 2004; Brusetti et al., 2011). A 3-yr longitudinal study of consumption of *Bt* maize in sheep likewise showed no differences and revealed no evidence of horizontal gene transfer to ruminal microorganisms or animal tissues (Trabalza-Marinucci et al., 2008).

Additionally, although several bacterial species are capable of acquiring external DNA by natural transformation, bacterial uptake of dietary DNA in vivo in the intestine has not been detected to date (Rizzi et al., 2012). Most of the studies that have investigated horizontal gene transfer of recombinant plant DNA have been under optimized conditions, and all of these studies have yielded negative results unless the recipient bacterial strains were genetically modified to facilitate integration of recombinant plant DNA by homologous recombination (EFSA, 2009).

Horizontal gene transfer from plant to animal genomes is at most very infrequent. It accounts for less than 10% of the few examples proposed as evidence of horizontal gene transfer gain in humans and other primates since their common evolutionary ancestor (Crisp et al., 2015). Gene loss during animal evolution cannot be ruled out as an explanation for the rare examples that have been proposed. In eukaryotes, horizontal gene transfer seems to be associated mainly with single-celled protists, especially those that engulf their food, or in multicellular organisms with parasites in close cellular contact with their hosts. Overall, the available data do not provide evidence for horizontal gene transfer of genomic rDNA between GE feed and eukaryotic animals or their gut bacteria.

GLYPHOSATE RESIDUES IN FOOD AND FEED

Glyphosate (*N*-(phosphonomethyl)glycine) is a widely used, broad-spectrum, systemic herbicide used for weed control in many crops. It inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase, which is not present in mammals, and as a result, the acute toxicity of this pesticide to animals including humans is low (McQueen et al., 2012). The GE glyphosate tolerance trait is most commonly associated with "Roundup Ready" crops including alfalfa, canola, corn, cotton, and soybean crops. Glyphosate is additionally registered for pre- and postemergence applications for non-GE field crops and some varieties of fruits and vegetables and has desiccant applications for several commodities. It is also used for nonagricultural purposes such as to control nondesirable vegetation in residential areas, roadways, and forests. Although it rapidly breaks down in the environment, it is known to persist in low concentrations in food crops, and nonoccupational exposures are most commonly associated with consuming residues in food (Kamrin, 1997). Because animals are indirectly exposed to herbicides through feed consump-

tion, concerns have been raised over possible glyphosate residues in animal-derived products and whether they pose dangers to consumer health.

Given sufficiently sensitive analytical techniques, a large number of chemicals can be detected in any food. In fact, Ames et al. (1990) estimated that 99.99% (by weight) of the pesticides in our food are naturally occurring chemicals that plants produce to defend themselves. The important toxicological concept is that the dose makes the poison. Therefore, models are required to accurately estimate exposures (McQueen et al., 2012) and determine the risk.

Maximum residue limits, which legally establish upper limit concentrations for pesticide residues in or on food and feed (LeDoux, 2011), are internationally established by the Codex Alimentarius Commission and country-specific authorities for pesticide residues in commodities, including residues in animal commodities that are the result of transfer from feed (Ehling and Reddy, 2015). Maximum residue limits vary among food types, meaning that consumers and livestock can be exposed to different glyphosate concentrations depending on the types of foods consumed. As such, maximum residue limits are unlikely to actually directly reflect dietary intake (McQueen et al., 2012).

The Codex Alimentarius currently lists meat, milk, and eggs with a maximum residue limit of 0.05 mg/kg glyphosate, corresponding to the limit of determination (Codex Alimentarius, 2016). Research has shown that physiochemical properties of a pesticide are responsible for any accumulation in fat and milk of animals and their products. Glyphosate is a polar molecule that is incompletely (15 to 36%) orally absorbed, undergoes very little biotransformation, and is rapidly excreted unmetabolized (Williams et al., 2000). Water-soluble compounds, such as glyphosate, are quickly excreted in urine and do not result in significant residues in meat or milk (MacLachlan and Bhula, 2008). Additionally, it has been determined that glyphosate does not bioaccumulate in mammals (EFSA, 2015).

Many factors affect the exposure and uptake of pesticides including crop and livestock management practices, levels of feed processing, species and physiological status of animals, food handling, and the properties of the chemicals themselves. The Joint FAO/WHO Meeting on Pesticide Residues (2005) reported that after dairy cows were intentionally fed diets containing a 9:1 mixture of glyphosate and its major environmental degradate, aminomethylphosphonic acid (**AMPA**), at total combined daily dietary levels from 40 to 400 mg/kg, no residues were detected in milk or fat. Low levels of glyphosate residues were detectable in the livers and kidneys, 0.21 (total residue 0.47) and 3.3 (4.5) mg/kg, respectively, from animals fed 400 mg/kg**]**. Tissue residues in single animals slaughtered 28 d following the cessation of feeding the treated diets were below the minimum threshold for analytical determination (0.05 mg/kg) in all tissues and milk.

Feeding pigs a diet with glyphosate and AMPA in a 9:1 ratio at 400 mg/kg resulted in maximum residues of 0.72 glyphosate (total residue 1.4) mg/kg in liver, 9.1 (11) mg/kg in kidney, and 0.06 (0.06) mg/kg in muscle. Residues in fat throughout the experiment, and all tissues 28 days after access to treated feed was stopped, were <0.05 mg/kg. An analogous experiment in laying hens incorporating glyphosate and AMPA in a 9:1 ratio at total combined daily dietary levels of 40, 120, and 400 mg/kg for periods of up to 28 days resulted in 0.12 glyphosate (total residues 0.16) mg/kg in eggs at the highest feeding level, although none were detected at the lowest feeding level. Residues at the highest feeding level were 0.61 (total residue 1.1) mg/kg for liver and 4.3 (4.8) mg/kg for kidney and were below the limit of detection for poultry fat and muscle.

The Joint FAO/WHO Meeting on Pesticide Residues (2005) report went on to state that at the expected maximum glyphosate residue dietary feed burden (381 mg/kg for beef cattle, 266 mg/kg for dairy cattle, and 23 mg/kg for pigs and chickens), the maximum residues of glyphosate expected in animal products are <0.05 mg/kg in fat, <0.05 mg/kg in muscle, 0.20 mg/kg in the liver, 3.1 to 3.5 mg/kg in kidneys, 0.5 mg/kg in edible pig offal, <0.05 mg/kg in eggs, and <0.05 mg/kg in milk. In other words, meat, milk, and eggs are all expected to be below the limit of determination (<0.05 mg/kg) for both glyphosate and AMPA when animals are fed a typical ration.

In a study using lactating goats, excretion in milk was shown to occur to a minor extent, ≤0.1 mg/L whole milk at a dose level of 120 mg/L in the diet (INCHEM, 1994). Recent peer-reviewed studies have shown no detectable levels of glyphosate or AMPA in human or bovine milk samples (Ehling and Reddy, 2015; Jensen et al., 2016). A non-peer-reviewed biomonitoring report published online by Moms Across America suggesting bioaccumulation of high levels of glyphosate in human breast milk was deemed to be "biologically implausible when compared with systemic and external doses estimated from urine biomonitoring findings, or from blood concentrations resulting from such doses" (Bus, 2015, p. 760).

Studies from a laboratory in Germany purported to show detectable glyphosate residues in animal and human urine and organs (Krüger et al., 2013, 2014a) and associated glyphosate concentrations of 0.87 to 1.13 mg/kg with malformations in piglets (Krüger et al., 2014b). These studies suffered from many shortcomings, including no analysis of the feed that was stated to be "contaminated" with glyphosate, absence of controls, lack

of described clinical signs or quantified pathological changes, poor statistical correlations, no consideration of differential diagnosis, and citing of selected manuscripts that have been shown to be scientifically deficient and do not represent the vast majority of scientific reports in this area. Additionally, these studies made use of ELISA kits that have not been verified across the varied matrices tested in these analyses. Although it is stated in the paper that validation of test results had been done by a comparison with gas chromatography–mass spectrometry, this validation data was not shown in this paper. No information was provided as far as modifications to the standard ELISA protocols and validations for analyzing tissue samples, nor were limits of detection or limits of quantitation reported (Member State Germany, and co-rapparteur Member State Slovakia, 2015). These reports were all published in the same journal managed by a known predatory publisher, OMICS Publishing Group (Beall, 2016). These 3 studies are at odds with the weight of evidence from research conducted in other laboratories and field animal health data on billions of animals that have consumed feed from herbicide-tolerant GE varieties (Van Eenennaam and Young, 2014).

Regulatory agencies review toxicity studies and set allowable limits for consumption of pesticide residues in food. The U.S. Environmental Protection Agency has set the glyphosate reference dose at 1.75 mg·kg $BW^{-1}·d^{-1}$ (USEPA, 2012). The Joint FAO/WHO Meeting on Pesticide Residues reconfirmed the average daily intake at 1 mg·kg $BW^{-1}·d^{-1}$ (JMPR, 2016), and the European Food Safety Authority set an average daily intake of 0.5 mg·kg $BW^{-1}·d^{-1}$ (EFSA, 2015). Public exposures range from 0.00001 to 0.001 mg·kg $BW^{-1}·d^{-1}$; occupational exposures range up to 0.01 mg·kg $BW^{-1}·d^{-1}$ (Williams et al., 2016). A recent review of glyphosate exposures in the general population and applicators showed systemic doses more than 150 times lower than the European Food Safety Authority average daily intake (Solomon, 2016). Therefore, both dietary and occupational exposures to glyphosate are orders of magnitude lower than the most conservative allowable average daily intake of 0.5 mg·kg $BW^{-1}·d^{-1}$.

In a survey-based study to assess maternal and prenatal glyphosate exposure in humans, 75% of the 20 composite food samples analyzed showed quantifiable glyphosate residues across a wide range of low concentrations (<0.005 to 0.5 mg/kg), with a mean concentration of 0.08 mg/kg. The mean estimated maternal daily dietary exposure to glyphosate residue was 0.001 mg·kg $BW^{-1}·d^{-1}$ (range of 2×10^{-5} to 0.005 mg·kg $BW^{-1}·d^{-1}$), which accounted for 0.4% of the allowable average daily intake (range 0.005 to 2%) and considered all food products consumed, including animal-derived products (McQueen et al., 2012).

Concerns around glyphosate residues in food were heightened in 2015 when the World Health Organization's International Agency for Research on Cancer (**IARC**) reclassified glyphosate as "probably carcinogenic to humans (Group 2A)" (IARC, 2015). It is important to note that this hazard classification is not a health risk assessment. International scientists and agencies continue to maintain that glyphosate is unlikely to be genotoxic or to pose a carcinogenic risk to humans through dietary exposure based on the available data (JMPR, 2016). Other regulatory agencies in the United States (USEPA, 2016) as well as Europe (EFSA, 2015; European Chemicals Agency, 2016), Canada (Canadian Pest Management Regulatory Agency, 2015), Japan (Japan Food Safety Commission, 2016), New Zealand (New Zealand Environmental Protection Authority, 2016), and Australia (Australian Pesticides and Veterinary Medicines Authority, 2016) have reaffirmed that data do not suggest that glyphosate is carcinogenic at typical levels of exposure. Furthermore, 4 independent expert panels pertaining to glyphosate exposure, animal carcinogenicity, genotoxicity, and epidemiologic studies that were convened in the wake of the IARC decision did not support IARC's conclusion and, in concordance with previous regulatory assessments, showed that glyphosate is not a carcinogen in laboratory animals and "further concluded that glyphosate is unlikely to pose a carcinogenic risk to humans" (Williams et al., 2016, p. 3).

LABELING

To date, mandatory GE labeling laws have largely excluded products from animals fed GE feed as well as GE processing aids and enzymes (such as rennet for making cheese). Considering that 40% of U.S. corn production and 60% of the soy bean crop is used for animal feed, tracking animals fed GE feed at any point in their lives and their associated products such as meat, milk, and eggs would be complicated and expensive (CAST, 2014). Public law number 114-216, which was signed into law by President Obama in July of 2016, "prohibits a food derived from an animal being considered bioengineered solely because the animal consumed feed produced from, containing, or consisting of bioengineered substance" (Public Law 114-216, 2016).

Voluntary process-based labeling, which is market driven and includes organic, Non-GMO Project, and the Whole Foods labeling initiative, among others, is allowed by the USFDA with the stipulation that it cannot be false or misleading. Some markets have established tolerance levels or thresholds for the presence of GE ingredients in food, both for mandatory disclosure requirements or for absence claims, such as non-genetically modified organism (**GMO**). The Agricultural Marketing

Service of the USDA currently allows a company to label and market a product as "USDA Process Verified" to a claim of meeting a 99.1% threshold of non-GMO/non-GE traits. Claims that a product is "GE-free" are not allowed because a zero GE level cannot be proven (CAST, 2014). As has been discussed in this review, the amount of all DNA in feed crops is <0.02% prior to undergoing breakdown in the gastrointestinal tract of food animals. Because studies have failed to reliably identify rDNA and no study has reported identifying full-length rDNA coding sequences or recombinant proteins in meat, milk, or eggs, all animal products would be far below the non-GE threshold, irrespective of the GE content of the feed. As such, mandatory GE labeling of products from animals fed GE feed would be misleading, because such products are not materially or compositionally distinguishable from those derived from animals fed non-GE feed.

CONCLUSIONS

Researchers have concluded that there is nothing to suggest that DNA from GE crops behaves any differently in the gastrointestinal tract of animals than non-GE counterparts. Absorption of dietary DNA across the intestinal wall appears to be a normal process that does not have adverse effects on food animals, regardless of whether the DNA is transgenic or endogenous. The summary of a 2007 review on the topic remains relevant today: "Studies undertaken to address concerns that transgenic protein or DNA may enter the market by means of animal products have shown that recombinant materials in transgenic feed are unlikely to be incorporated into animal products at significant levels" (Alexander et al., 2007, p. 56).

Traces of dietary DNA and protein cannot be reliably detected in meat, milk, or eggs. Numerous studies have looked for the presence of rDNA and recombinant proteins in animal products, and the bottom line is that meat, milk, and eggs from animals that have consumed GE feed are analytically indistinguishable from those that have eaten non-GE feed. This makes mandatory labeling of such products misleading, because it would require labeling for something that was not discernable in the products. Given the wide trade and usage of GE livestock feeds globally, managing separate supply chains for indistinguishable animal products based on the GE content of the diet they consumed would be inefficient and expensive and would have no public health benefit based on available scientific data.

From a public health perspective, foodborne illnesses are a widespread health risk, with costs in the United States alone estimated at US$77 billion/yr (Scharff, 2012). In addition, toxins such as heavy metals, dioxin, and mycotoxins are of concern in animal feed (Coffey et al., 2016). These potential food contaminants have wide-reaching effects and pose genuine risks to human health. These documented risks are in stark contrast to the available data suggesting that there are no unique human health risks associated with products derived from animals that have consumed approved GE feed crops.

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