# Does transgenic feed cause histopathological changes in rats?

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Dedicated to my Godmother,
Alina Wóycicka (1944 – 2016).
Ciociu, I wish you were still here
to hear that I had finally finished it.

Dedicated also to the friend
who asked for its completion.

## Contents


Abstract ... vi

Declaration ... vii

Acknowledgements ... viii

1. Introduction ... 1

    1.1 History and present status ... 1

        1.1.1 Production of GM crops ... 3

        1.1.2 Insect resistance ... 3

        1.1.3 Herbicide-tolerant crops ... 8

        1.1.4 Safety evaluations and the possible negative influence of GM crops on health ... 10

        1.1.5 Crop of interest: Herbicide-tolerant and insect-resistant triple-stacked corn ... 14

    1.2 Gastrointestinal tract ... 16

        1.2.1 Stomach ... 16

        1.2.2 Small intestine ... 18

        1.2.3 Digestion ... 19

        1.2.4 Histopathological changes in the GIT ... 20

    1.3 Critical Review of Literature – *published* ... 33

        1.3.1 Materials and Methods ... 33

        1.3.2 Results ... 36

        1.3.3 Discussion ... 45

        1.3.4 Conclusion ... 51

    1.4 Update to the published Critical Review ... 52

        1.4.1 Materials and Methods ... 52

        1.4.2 Results ... 54

        1.4.3 Discussion ... 59

        1.4.4 Conclusion ... 61

2. Overall objectives ... 62

3. Study 1: Long-term feeding study of rats fed 60% corn ... 65

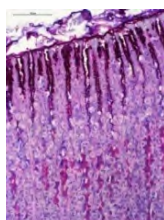
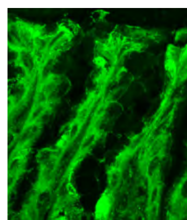
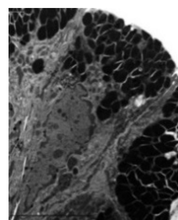
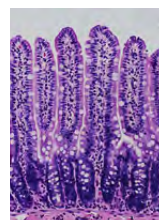
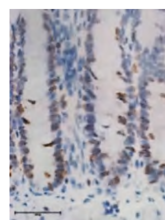
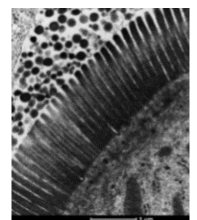
    3.1 Introduction ... 65

    3.2 Materials and Methods ... 65

        3.2.1 GM and non-GM corn ... 65

3.2.2 Diet ........ 66
3.2.3 Animal Feeding ........ 66
3.2.4 Histopathology ........ 67
3.2.5 Statistical analysis ........ 70
3.3 Results ........ 71
3.3.1 Animal feeding ........ 71
3.3.2 Histopathology ........ 71
3.4 Discussion ........ 91
3.4.1 Animal feeding ........ 91
3.4.2 Chemically-induced damage ........ 91
3.4.3 The *Bt* toxin as a source of toxicity ........ 94
3.4.4 Other observed changes ........ 96
3.5 Conclusion ........ 100
Summary table of Study 1 results ........ 101
4. Study 2: Long-term feeding study of rats fed 30% corn ........ 103
4.1 Introduction ........ 103
4.2 Materials and Methods ........ 103
4.2.1 Diet ........ 103
4.2.2 Animal Feeding ........ 103
4.2.3 Histopathology ........ 104
4.2.4 Statistical analysis ........ 105
4.3 Results ........ 105
4.3.1 Animal feeding ........ 105
4.3.2 Histopathology ........ 106
4.4 Discussion ........ 131
4.4.1 Chemically-induced damage ........ 131
4.4.2 The *Bt* toxin as a source of toxicity ........ 133
4.5 Conclusions ........ 134
Summary table of Study 2 results ........ 135
5. Study 3: Expression of tight junction proteins in the stomachs of 60% corn-fed rats ........ 138
5.1 Introduction ........ 138
5.2 Materials and Methods ........ 138

5.2.1 Immunohistochemical light microscopic investigation........139
5.2.2 Immunofluorescence confocal microscopic investigation........141
5.2.3 Statistical analysis........141
5.3 Results........141
5.3.1 Immunohistochemical light microscopic investigation........141
5.3.2 Immunofluorescence confocal microscopic investigation........148
5.4 Discussion........170
5.5 Conclusion........171
Summary table of Study 3 results........172
6. Overall discussion........173
6.1 The use of a non-isogenic variety of corn........176
7. Further research........177
8. Conclusion........179
Appendix A: Feed specifications
A1.1 Composition of 60% corn diet........A1
A1.2 Composition of 30% corn diet........A3
Appendix B: Protocols and microscope settings
B1.1 Haematoxylin and eosin staining procedure........A5
B1. 2 Combined alcian blue and periodic acid Schiff staining procedure (pH2.5)..A6
B1.3 Immunohistochemistry – DAKO automated machine procedure........A8
B1.4 Immunofluorescence method for confocal microscopy........A10
B1.5 Processing samples for TEM........A14
Reference List........R1
Publications........P1

## Abstract

Genetically modified (GM) crops have been commercially available for human and animal consumption since the 1990s. The safety evaluations are based on the concept of substantial equivalence, which assumes that the toxicity of a product can be investigated by assessing the toxicity of individual components of the product and not the product as a whole. In other words, the test for substantial equivalence does not require animal feeding trials unless one or more of the individual components of the crop indicates a need. Such an approach does not take into account the changes, which may have arisen during, or following, the production of the GM crop. Furthermore, the few animal feeding studies that have been performed very rarely report results of any morphometric histological analyses.

The present study, aimed to investigate the effects of feeding a GM-corn diet to rats at two doses (60% and 30%) by studying the morphological features of the mucosa of the stomach and small intestine, both at light and electron microscopic levels. The morphological features were quantified using morphometric methods. In addition, tight junction proteins were investigated using immunohistochemistry and immunofluorescence confocal microscopy.

Both studies (60% and 30% of corn in the diet) showed changes in morphology and cell-counts that indicate that GM crops may have an effect on rat health. These findings support the importance of animal feeding studies and the need for morphometric analyses to evaluate the safety of GM-feed consumption on animal health.

## Declaration

The work described in this thesis, unless otherwise stated, has not been previously submitted for a degree at this or any other institution. No part of this work will, in the future, be used in a submission under the author's name for any other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Irena M. Zdziarski

15th December, 2016

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# 1. Introduction

## 1.1 History and present status

The development of human society and culture has been dependent on, to a large extent, the manipulation of the environment, especially with respect to cultivation of food. Early agriculture focused on opportunities for reliable food supplies and the evolution of food crops was a significant advancement. The further improvement of crops through breeding strategies, based on observation for beneficial plant traits, led to the efficient and productive agricultural and horticultural systems we are familiar with today. More recently, developments in genetic technologies have presented even more opportunities to establish desirable traits. The production of transgenic crops is one such example.

Transgenic or genetically modified (GM) crops are developed through the transfer and incorporation of plant, bacterial, viral, fungal or animal genes. The purpose of producing GM crops is to generate traits that would not be otherwise achievable via normal plant cross-breeding. The technology allows for cross-species alterations. Therefore in theory the opportunities are limitless.

Transgenic crops have been grown for human and animal consumption since the 1990's (Clive and Krattiger, 1996). The GM crops generated/grown in the United States of America (USA) between 1987 and 1998 conferred traits for: herbicide tolerance (29%), insect resistance (24%), altered composition, e.g. high lysine soybeans or modified-oil soybeans (20%), and disease resistance (15%) (Krimsky, 2002). Currently, there are more than 200 different crops with various traits, which have been approved worldwide for human and animal consumption (ISAAA, 2013). The most common traits of GM crops are herbicide tolerance (57%), insect resistance (16%) and combined multiple traits (i.e. stacked traits) (28%) (Figure 1A) (Clive, 2014). The less common traits include altered composition and virus resistance (less than 1%). The most common transgenic crops are soybean and corn/maize (Figure 1B) with 82% of the world's soybean and 30% of the world's corn/maize being GM (Clive, 2014).

Seventy seven per cent of GM crops are grown in the USA, Argentina and Brazil with only 0.01% grown in Australia (Clive, 2014). In Australia, although there are over 85 approved crops for consumption (FSANZ, 2015), only GM cotton and canola are commercially grown (Clive, 2014).

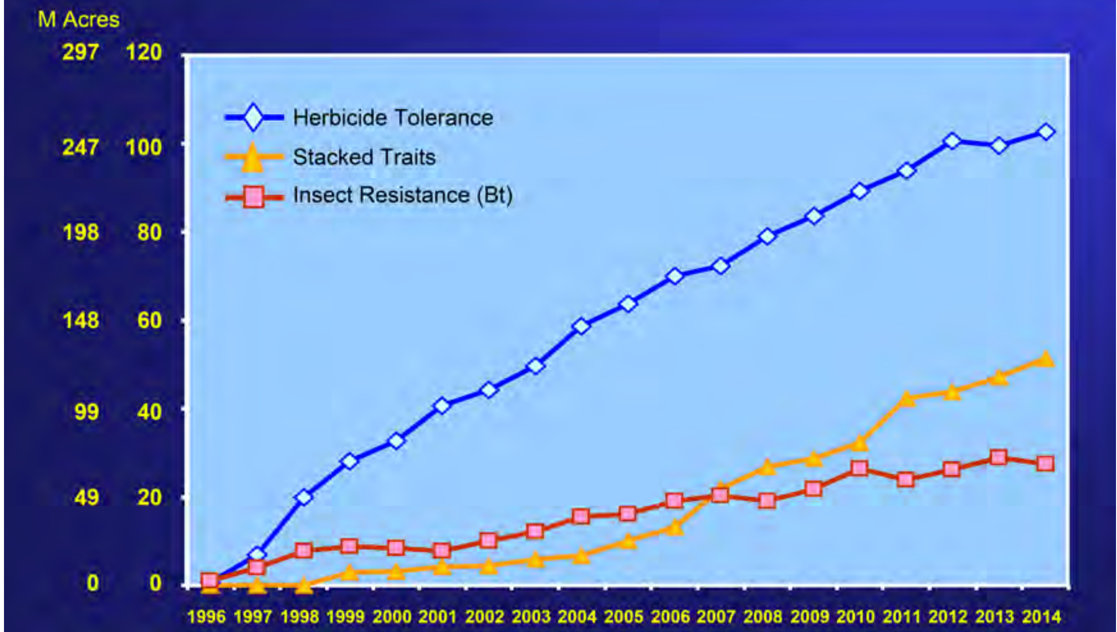


**Figure 1 Global are of GM crop cultivation by trait (A.) and by crop type (B).**

A.) Graph of GM crop cultivation by trait, depicting herbicide tolerance as the most cultivated trait. An increase is also seen in GM crops with stacked traits. These tend to be the combined traits of herbicide tolerance and insect resistance;

B.) Graph of GM crop cultivation by crop type depicting soybean and corn/maize as the most cultivated crops in the world.

(Source: Clive, 2014)

### 1.1.1 Production of GM crops

Transgenic crops are most often produced through the insertion of a gene cassette, which consists of the desired trait genes and several other genes including viral promoter and marker genes. The two most common methods of inserting the gene cassette into the crops are: 1) *Agrobacterium*-mediated transformation, and 2) microparticle bombardment, also known as microparticle acceleration or biolistics (ISAAA, 2013; Wilson *et al.*, 2006). *Agrobacterium*-mediated transformation utilises the ability of the tumour-inducing plasmid of *Agrobacterium* to infect plant cells. In place of the tumour-inducing genes, a gene cassette with the desired trait(s) is introduced. The bacterium then delivers and incorporates the cassette into the plant cell (Gelvin, 2003).

Particle bombardment, involves metal particles coated with the desired DNA fragment (i.e. the gene cassette) being accelerated at plant cells (Christou, 1992). These plant cells can be single cells or part of organised tissues. The only requirements are that these cells must have a regenerative ability (Altpeter *et al.*, 2005) and will allow themselves to be transformed (Christou, 1992).

Following *Agrobacterium*-mediated transformation and particle bombardment, the transformed cells are cultured and seedlings are grown in a specific medium that selects for the transformed seedlings/cells (Personal communication, Davies, 2015). The marker gene(s) present in the inserted gene cassette, give the crop the necessary properties to withstand the selection process. Such genes usually encode for antibiotic resistance or herbicide tolerance (ISAAA, 2013).

### 1.1.2 Insect resistance

There are several different types of insect resistant crops. Most of these crops obtain their resistance through utilising the insecticidal abilities of the bacterium, *Bacillus thuringiensis* (ISAAA, 2013). *Bacillus thuringiensis* is a gram-positive soil bacterium that produces crystalline inclusions during sporulation (Hofte and Whiteley, 1989). The crystalline inclusions, when ingested by certain insects, dissolve in the mid-gut and release insecticidal proteins commonly known as *Bt* toxins (or δ-endotoxins) (Hofte and Whiteley, 1989). The toxins bind to the mid-gut cell membrane, induce cell death (Soberón *et al.*, 2010) and consequently weaken and/or kill the insect (Figure 2) (Pigott and Ellar, 2007).

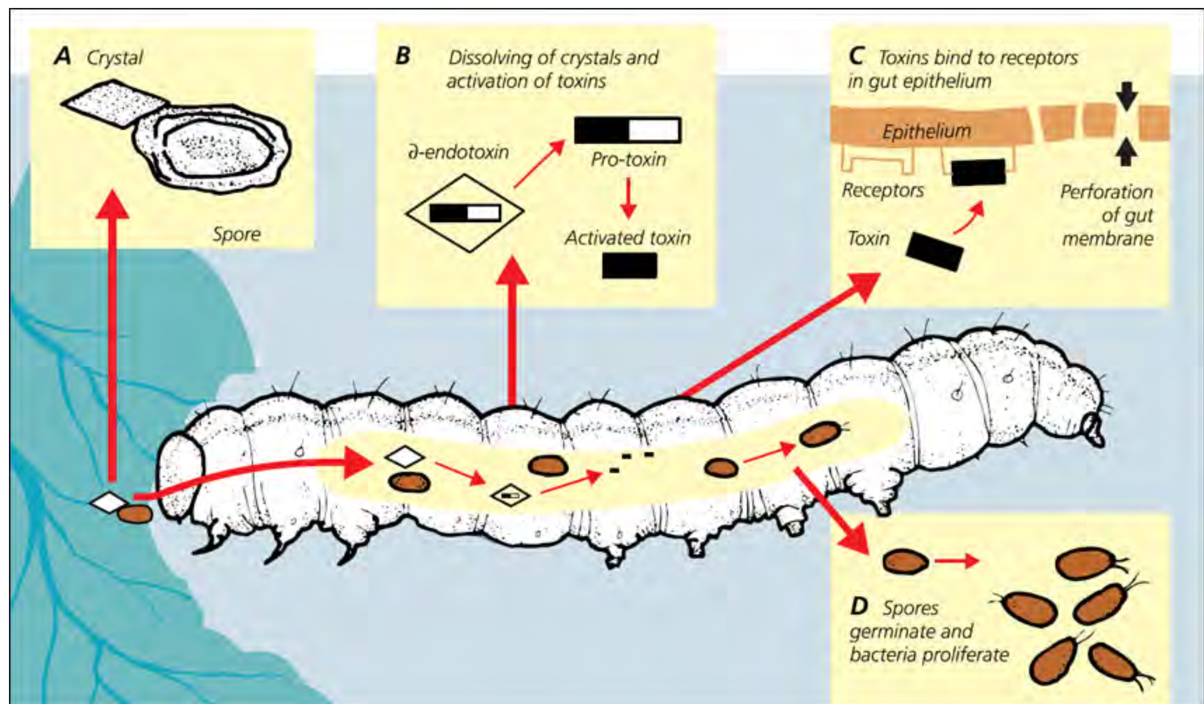



**Figure 2. Mode of action of *Bacillus thuringiensis* in insect gut**

A) The *B. thuringiensis* bacterium forms a crystalline inclusion during sporulation. The insect ingests the spore along with the crystal. In the mid-gut the crystalline inclusion dissolves releasing crystal toxins (*Bt* toxins or δ-endotoxins).

B) The insect's mid-gut proteases activate the toxin by cleaving off the structural fragment (Figure 3).

C) Once activated, the fragment is able to bind to specific receptors in the insect's gut epithelium and induce cell death.

D) *B. thuringiensis* spores have the ability to germinate, thus bringing about bacterial proliferation.

(Source: WHO, 1999)

*Bt* toxins are believed to be highly species-specific and thus *Bacillus thuringiensis* bacteria have been a popular biological insecticide since their introduction in 1938 (Glare and O'Callaghan, 2000). There are currently over 600 identified *Bt* toxins (Crickmore *et al.*, 2012). The majority of which are the so-called *Cry* proteins. Based on their insecticidal specificity and similarities in their molecular/genetic structure, the proteins are categorised into major classes and several subclasses (Hofte and Whiteley, 1989). Major class 1 (*Cry1*) are specific to insects of *Lepidoptera* and some *Coleoptera* species, class 2 (*Cry2*) are specific to *Lepidoptera* and some *Diptera*, class 3 (*Cry3*) are specific to *Coleoptera*, and class 4 (*Cry4*) are specific to *Diptera* (Gill *et al.*, 1992; Soberón *et al.*, 2010).

## *Mode of Action of* Bt *toxin and its Species-specificity*

The *Bt* toxin mode of action is as follows: Once ingested, the *Bt* toxin is cleaved several times by insect mid-gut proteases from the C-terminal towards N-terminal (Gill *et al.*, 1992) leaving an active fragment and an exposed binding site (Figure 3 & 4). The

exposed binding site binds to the cell membrane protein, cadherin (Rausell *et al.*, 2004). Thereafter, *Bt* toxin may act according to two models: 1) the signal transduction model, and 2) the pore-forming model (Soberón *et al.*, 2010). In the signal transduction model, the *Bt* toxin binds to cadherin, which induces a cascade of events, which brings about cell death (Zhang *et al.*, 2006; Zhang *et al.*, 2008). In the pore-forming model the, *Bt* toxin binds to cadherin, which initiates the formation of a pre-pore structure (oligomerization). This structure then binds to a cell membrane receptor and pushes its way into the cell forming an active pore (Figure 4) (Rausell *et al.*, 2004; Soberón *et al.*, 2010). The cell membrane receptor can be aminopeptidase or alkaline phosphatase (Pigott and Ellar, 2007).

In summary, the *Bt* toxin mode of action is thought to be species specific by:

1) the insects' proteases which cleave the structural fragment (Soberón *et al.*, 2010),
2) the *Bt* toxin binding to the insects' cadherin receptor (Pigott and Ellar, 2007), and
3) the *Bt* toxin binding to the insects' cell membrane receptor(s) (Hofte and Whiteley, 1989; Schnepf *et al.*, 1998).

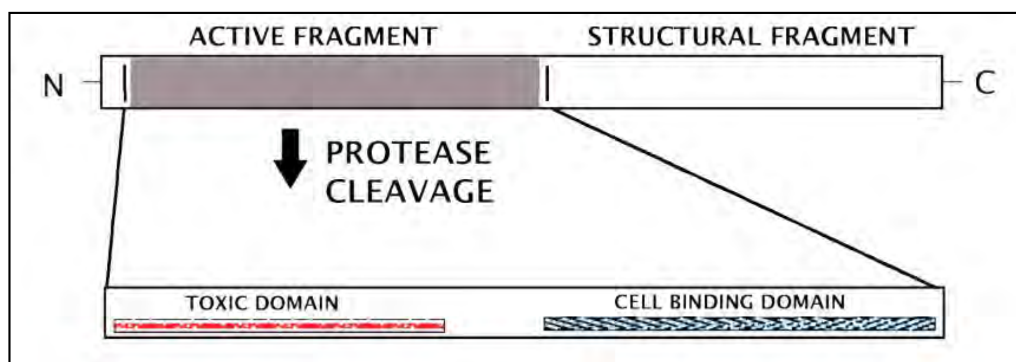



**Figure 3. *Bt* toxin genome.** Insect mid-gut proteases cleave the structural fragment at the C-terminal and part of the initial segment at the N-terminal leaving an active fragment with a toxic domain and cell binding domain. It is believed that this cleavage makes the *Bt* toxin species specific. Part of the structural fragment is absent in the gene fragment that is inserted into GM crops. (Redrawn from Gill *et al.*, 1992)

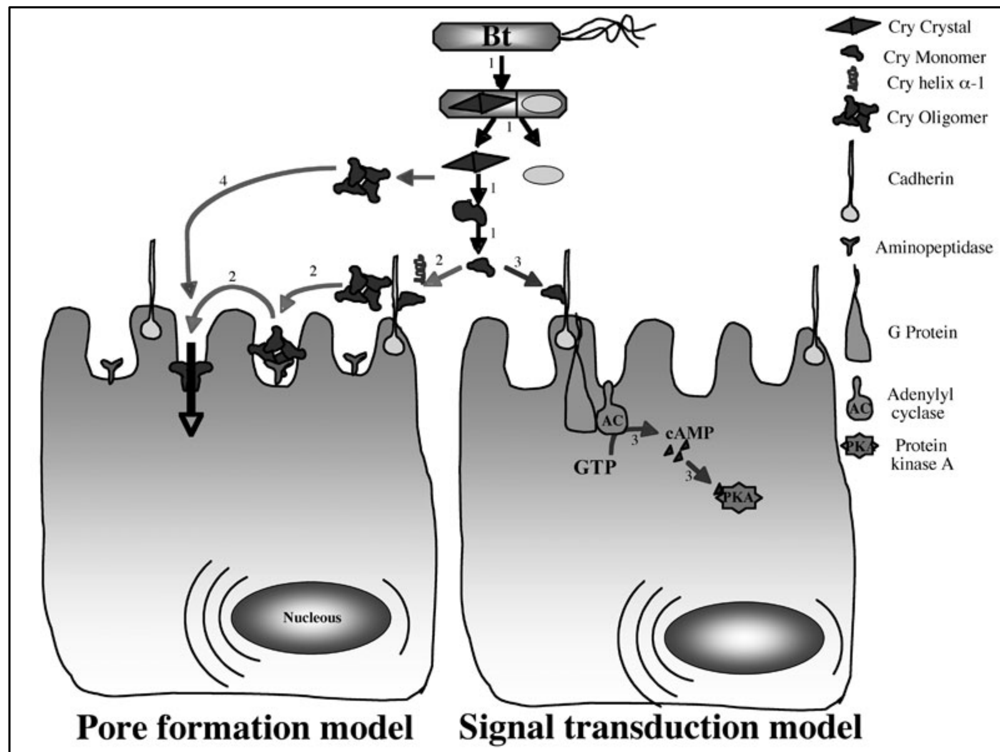



**Figure 4. Two modes of action of *Bt* toxin: pore-formation and signal transduction.**

1) The insect ingests the spore with the crystalline inclusion. In the insect's mid-gut the crystalline inclusion dissolves away releasing crystal (*Cry*) toxins, which are activated by the insect's mid-gut proteases.
2) **The pore-formation model:** The activated *Cry* toxin binds to the cadherin receptor. *Cry helix α-1* is cleaved, which allows for oligomerization of the toxin. The oligomerized toxin binds to a membrane receptor (e.g. aminopeptidase) and inserts itself into the lipid membrane forming a pore. The cell undergoes osmotic shock and dies.
3) **Signal transduction model:** The activated *Cry* toxin binds to cadherin, which activates protein G increasing adenylyl cyclase activity. This results in an increase in intracellular cAMP levels, which activates protein kinase A, resulting in a pathway that leads to cell death.
4) Due to the development of insect gut resistance to *Bt* toxins in GM crops, some recent GM crops have an altered *Bt* toxin genome where the *Cry helix α-1* has been removed. This means that binding to cadherin is not necessary for oligomerization and pore formation.

(Source: Soberón *et al.* 2010)

## *Insecticidal* Bt *crops and their mode of action*

With advancements in technology and knowledge of genetics, scientists are now targeting specific *Bt* toxins and conferring their insecticidal abilities to crops. *Bt* crops in general contain truncated genes which encode for the active toxins rather than the inactivated crystalline protoxins produced by the bacterium, *B. thuringiensis* (ISAAA, 2013). The reason for this is that the genes coding for the full toxin, do not give the plant a strong insecticidal property.

Vaeck *et al.* (1987) published one of the first papers describing the inclusion of *B. thuringiensis* insecticidal properties to a plant. Using *Agrobacterium*-mediated transformation, they inserted a bt2 gene into a tobacco plant, thus generating a plant that would produce a protein toxic to several *Lepidoptera* larvae, including the tobacco hornworm. Vaeck *et al.* (1987), however, found that when they introduced the full length of the bt2 gene, the tobacco plant was still susceptible to insect infestation. They tried several truncated versions of the bt gene and found that the shorter the bt gene sequence, the more toxic it was to the larvae (Figure 5).

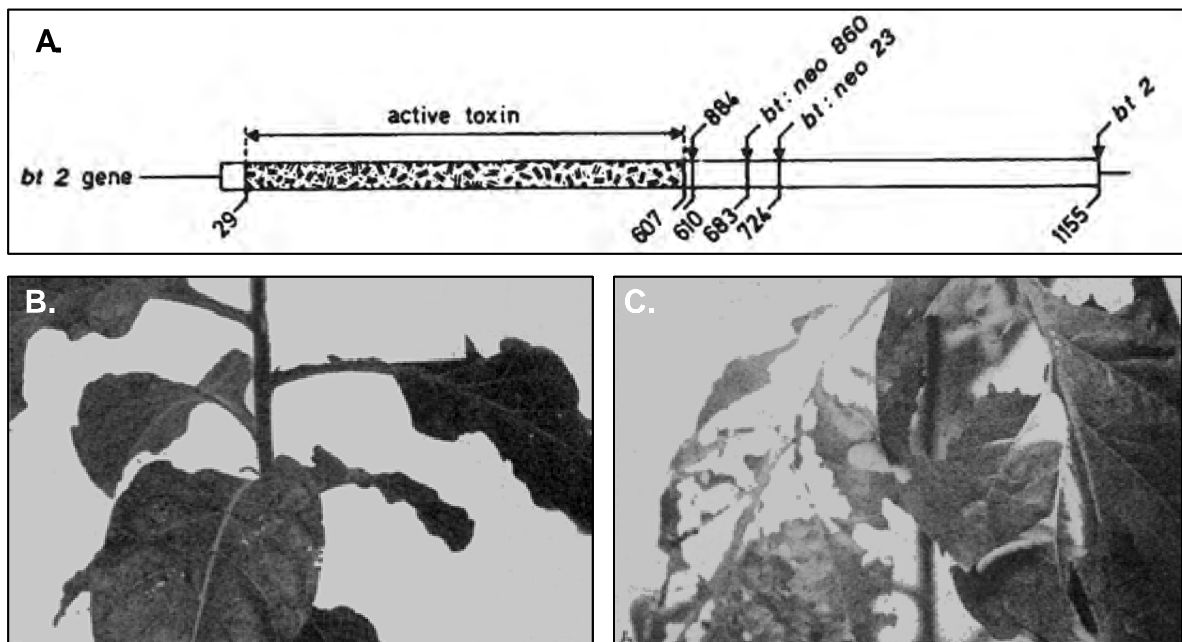



**Figure 5. Structure of the bt2 gene and the results of the transgenic tobacco plant trials.** Alterations were made to the bt toxin gene prior to transfer to make the plant more insecticidal. Figures B and C are of two separate plants taken 11 days after larvae infestation.

A.) Bt2 gene depicting the different truncations.

B.) Plant with truncated bt gene (bt : neo 23) was 100% resistant to larvae infestation at day 3.

C.) Control transgenic plant with only nptII marker gene, which has no insecticidal properties (Source: Vaeck *et al.* 1987).

By altering the structural component, the toxicity of the *Bt* toxin may have been altered. In other words, it may no longer be necessary for organisms ingesting the toxins to have the species-specific properties for activation of the toxin (Saxena and Stotzky, 2000).

More recently, crops are being transformed with a modified *Bt* toxin that does not require binding to cadherin for pore-formation to occur in larvae gut epithelium (Figure

4). This is to counteract the increasing development of resistance amongst insects to the action of the *Bt* toxin in GM crops (Soberon *et al.*, 2007; Soberón *et al.*, 2010).

### 1.1.3 Herbicide-tolerant crops

Herbicide-tolerant crops are most commonly resistant to the action of glyphosate, sulfonylurea, bromoxynil, glufosinate, and bialphos (Krimsky, 2002). The first such crop approved for commercialisation was the glyphosate-tolerant soybean (GTS, line 40-3-2) which is commonly known as Round-up-Ready® soy (or RR soy) (Krimsky, 2002). In the mid-1990s the first Round-up-Ready® (RR) corns, GA21 and MON832, were commercialised and in 2000, a second generation of RR corns, including NK603, were introduced, which had additional modifications (CERA, 2012; ISAAA, 2013).

#### *Glyphosate-tolerant crops and the EPSPS gene*

In the RR corn, glyphosate tolerance is obtained through the expression of the 5-enolpyruvylshikimate-3-phosphate synthase gene (EPSPS, EC 2.5.1.19) from *Agrobacterium* sp. Strain CP4 (CP4 EPSPS) (Ridley *et al.*, 2002).

Glyphosate-based herbicides, such as Round-up®, target the Shikimate pathway, which is responsible for the synthesis of aromatic amino acids, such as, phenylalanine, tyrosine, and tryptophan (Figure 6). This pathway is an integral component of the metabolism of plants and microorganism, but importantly, it is not an integral component of animal metabolism (Mensah *et al.*, 2014). Inhibition of any part of this pathway, results in plant death. Glyphosate-based herbicides inhibit the action of the enzyme, EPSPS, thus, preventing the production of chorismate, a precursor to several aromatic amino acids necessary for plant survival (Mensah *et al.*, 2015) (Figure 6).

Glyphosate tolerant crops, such as RR corn, contain extra copies of the EPSPS gene. This means that the GM plant produces extra amounts of chorismate, enough for the plant to survive when sprayed with a glyphosate-based herbicide (Padgette *et al.*, 1995).

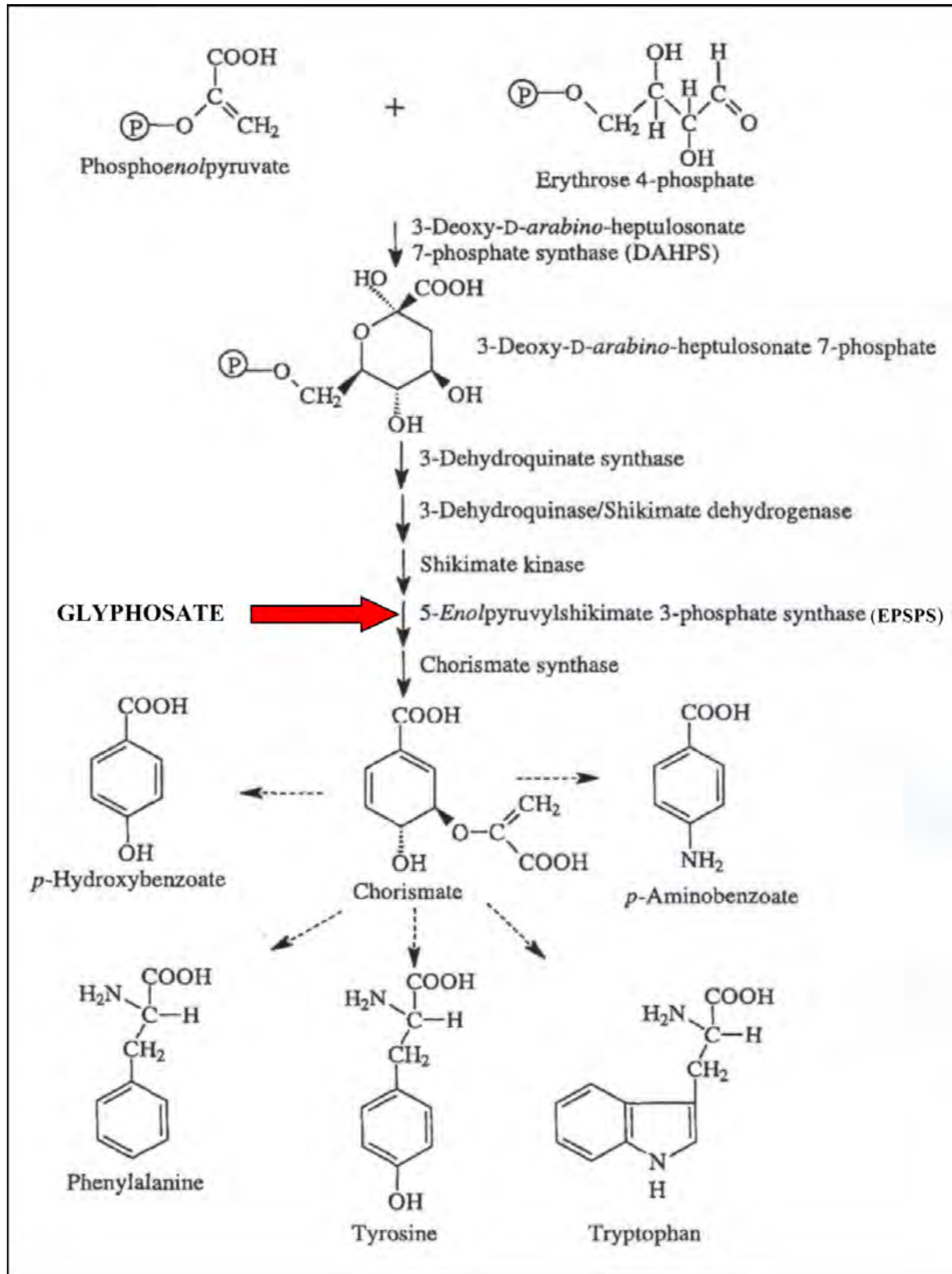



**Figure 6. The Shikimate pathway in the production of amino acids and the action of glyphosate, which terminates this pathway by binding to EPSPS** (Source: Mensah *et al.* 2014)

### 1.1.4 Safety evaluations and the possible negative influence of GM crops on health

Genetically modified crops have been promoted as being equivalent to naturally bred crops; however, the public are still concerned about their safety and their effects on human and animal health (Polya, 1999; Nightingale, 2013). Worldwide approval of GM crops have often been based on the effects of the GM crop on animal production, such as on meat and milk yields (FSANZ, 2011; Hammond *et al.*, 1996). Thus far, there is a limited number of published comprehensive reports on the effect on animal or human health (Domingo and Bordonaba, 2011; Snell *et al.*, 2012; Zdziarski *et al.* 2014).

#### *Safety evaluations of GM crops*

The establishment of substantial equivalence of the GM crop to its isogenic or near-isogenic crop, is the only required safety assessment for many countries including Australia (FAO/WHO, 2000; FSANZ, 2007). However, recently countries of the European Union require additional safety assessments, which include mandatory rat feeding trials (Implementing Regulation (EU), 2013). Countries like the USA or Australia, do not require animal feeding trials prior to crop approval for human and/or animal consumption, unless the test for substantial equivalence proves it necessary (FAO/WHO, 2000; FSANZ, 2007).

Substantial equivalence is based on the concept that the safety of GM foods can be assessed through a comparison with compounds or organisms of known safety. The purpose of the test for substantial equivalence is to identify possible hazardous components, which become the focus of further assessment (FSANZ, 2007). The test for substantial equivalence examines the individual characters and not the GM crop as a whole. The outcome of this test is the classification of the novel crop as being as safe (or unsafe) as the existing non-GM crop.

The test assesses the degree of equivalence of the GM crop to the crop's closest traditional counterpart in genotype, phenotype and nutritional value (Figure 7). In addition, the transformed crop's genome is evaluated and compared to genetic sequences of known allergens, toxins and anti-nutrients. The transfer method is assessed, as well as the stability of the insertion site. The history of safe consumption of the host crop and donor crop must also be evident. In addition, the toxicity of the new protein(s)/compound(s) that the GM crop is designed to produce, such as an insecticidal

protein or a protein conferring herbicide tolerance, is also assessed. These tend to be deemed safe based on the safety of the protein in its wild-type form (Kuiper *et al.*, 2001).

The further toxicological assessments are based on the degree of equivalence. Food regulators may not require further examinations, if the assessment indicates that there are no differences outside what could be obtained through natural variation (Schilter and Constable, 2002).

This type of general safety assessment does not consider that the genes present in the novel food may be additional or different from what is anticipated (Vaeck *et al.*, 1987; Delannay *et al.*, 1995; Wilson *et al.*, 2006). Even with a thorough assessment of the genome, unintended effects may arise that are unpredictable (Kuiper and Kleter, 2003). Hence, GM crops should undergo thorough safety evaluations that do not consider the GM food as being composed of several substances of known safety, but as a novel entity, the safety of which needs to be evaluated as a whole.

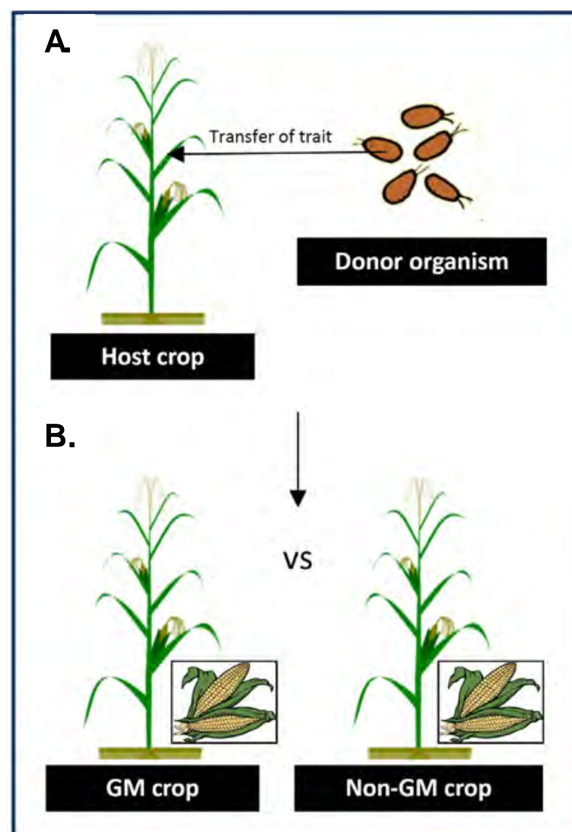



**Figure 7. Concept of substantial equivalence is used to establish the safety of GM crops.**

A.) Establish: 1) the safe history of host and donor organism consumption; 2) the safety of the method used to transfer the desired trait into the host crop; 3) the stability of the transferred trait in the GM crop; 4) similarity between the transferred DNA sequence and the DNA sequences of known toxins, allergens and anti-nutrients (Kuiper and Kleter, 2003).

B.) Establishing the similarities and differences between the GM crop and the non-GM crop (i.e. agronomical, morphological, genetic, and differences in composition) (Kuiper and Kleter, 2003).

## *Unintended effects and the need for animal feeding studies*

The consumption of GM feed or food may have effects on animal or human health that may not be predicted by the test for substantial equivalence (Carman, 2004). It is possible that the transformation process (Altpeter *et al.*, 2005) or the consumption of feed/food containing more than one GM trait may have adverse effects (Schnepf *et al.*, 1998). Animal feeding studies can better evaluate these risks and identify the effects (Carman, 2004).

A common criticism of GM crops is that the methods used in their production are imprecise. In both *Agrobacterium*-mediated transformation and particle bombardment, the insertion site of the new DNA is random (Altpeter *et al.*, 2005; Wilson *et al.*, 2006) and more than one copy of the DNA fragment may be inserted into the target genome (Christou, 1992; Gasson, 2003). This can affect gene expression in a positive or negative manner, for example, by causing gene suppression or gene silencing (Dai *et al.*, 2001; Altpeter *et al.*, 2005). In microparticle bombardment, the extra copies of the inserted DNA can be scrambled, inverted or incomplete (Altpeter *et al.*, 2005). In addition, in microparticle bombardment, the site of insertion may undergo further recombination (Christou *et al.*, 1988; Windels *et al.*, 2001; Altpeter *et al.*, 2005). The incorporation and positioning of the cassette into the plant genome may cause changes to occur in the plants, which are unintentional, but more importantly unpredictable (Pusztai *et al.*, 2003). Because of these reasons, the toxicity or nutritional value of the GM crop should be assessed as a whole. Furthermore, the inserted gene cassette consists of several genes such as the genes encoding the desired-trait, and viral promoter and marker genes. These tend to be truncated or shortened versions and may even have gene sequence changes (Vaeck *et al.*, 1987; Delannay *et al.*, 1995; ISAAA, 2013). The effect of these genes acting together is not often determined or even required (FAO/WHO, 2000; FSANZ, 2007).

In addition, there is some concern over the genes of a cassette being taken up and becoming incorporated into the gut bacteria or epithelial cells. One such example is the possibility of the gut bacteria to endocytose and incorporate the antibiotic resistance genes, which are used as marker genes, thus making the bacteria antibiotic resistant (Yoshida, 2000). However, several publications (Gasson, 2003; Kuiper and Kleter, 2003) state that this would be rare and that if it were to occur, it would be of little importance since the antibiotic resistant genes used in the genetic engineering of crops are only for

the antibiotics that are now rarely used (Francescon, 2001) or are of an already common gastrointestinal (GI) tract bacterial resistant strain (Yoshida, 2000).

Some scientists have concerns for the incorporation of the cauliflower mosaic virus 35S (CaMV35S) promoter gene into epithelial cells, which theoretically causing over-expression of genes in those cells (Ho *et al.*, 1999). Thus, making the cells prone to mutagenesis or carcinogenesis, or even the reactivation of dormant viruses, or generation of new viruses (Aaziz and Tepfer, 1999; Cummins *et al.*, 2000). However, studies have been inconclusive, with some depicting the possibility while others finding no such outcomes (Aaziz and Tepfer, 1999; Paparini and Romano-Spica, 2006).

Another concern is the general effect that the GM crop may have on the microflora of the digestive tract, particularly in association with long-term consumption of the crop (Yuan *et al.*, 2011; Buzoianu *et al.*, 2013). Microflora, such as bacteria, can play an important role in human and animal health. They can be beneficial or detrimental, causing changes to GI tract or indirectly affecting other parts of the body, such as the brain (Samsel and Seneff, 2013). In addition, an imbalance of already existing microflora can cause problems in its resident environment or the microflora can move to another area of the GI tract, where their presence may not be tolerated (Ojetti *et al.*, 2009). Over-colonisation of microflora or infections of the gut by pathogenic bacteria, viruses, or parasites can be cytotoxic to mucosal cells, change mucosal permeability by affecting tight junctions (Coruzzi, 2010), or produce carcinogens which may promote the formation of cancers (Guarner and Malagelada, 2003). In the intestinal mucosa of both the large and small intestine, the increased or displaced microflora can cause focal reactive hyperplasia or intestinal epithelial hypertrophy (Peckham, 2002). In the small intestine, they can also stimulate the secretory cells inducing severe watery diarrhoea (Coruzzi, 2010).

### *Multi-trait stacked crops and the consumption of several GM traits*

With 82% of the world's soybean and 30% of the world's corn being GM (Clive, 2014), animals and humans most probably consume GM material and GM products of various traits in a single meal. In addition, double- or multi-trait stacked crops are becoming increasingly common (Figure 1). These are obtained either through more than one trait being inserted into one crop, or through cross-breeding of two or more GM crops (ISAAA, 2013). Many food regulatory bodies do not require any studies to be done

on crops containing several stacked genes, if all the genes in the stack have been previously approved individually for use in the same kind of plant (EFSA, 2010; FSANZ, 2010). However, the effect of two or more traits acting together is unknown, for example, two insecticidal proteins, when ingested together, may have a potentiating or synergistic effect (Schnepf *et al.*, 1998). In addition, other constituents of the feed may have a potentiating or synergistic effect on the GM component. Therefore, there should be long-term animal feeding studies investigating the toxicity of crops possessing more than one trait or the toxicity of feed containing more than one GM component/crop.

### 1.1.5 Crop of interest: Herbicide-tolerant and insect-resistant triple-stacked corn

In the present PhD study, the GM crop investigated was a triple-stacked corn that was first approved for human and animal consumption in 2004 (CERA, 2012; ISAAA, 2013). More specifically, it is a $F_1$ hybrid *Zea mays L.* maize obtained through the cross-breeding of the transgenic line MON863xMON810 with NK603 (CERA, 2012), thus making it insect resistant to *Coleopterans* (East and West Corn Rootworms) and *Lepidopterans* (European Corn Borer), as well as, herbicide tolerant to Monsanto's glyphosate herbicide, Roundup®. Consequently the crop contains the gene cassettes for CP4 EPSPS (vector PV-ZMGT32), cry1Ab (vector PV-ZMBK07), and cry3Bb1 (vector PV-ZMIR13), as well as nptII, which is a gene conferring antibiotic resistance (vector PV-ZMIR13) (Table 1) (Dong *et al.*, 2008; BCH, 2015). Each of the cassettes was introduced into the parental crops via particle bombardment (CERA, 2012).

**Table 1. List of known inserted genes in the triple-stack corn (MON863xMON810xNK603)** (Dong *et al*., 2008; BCH, 2015)

<table>
<tr><td rowspan="2">Vector PV-ZMCT32*</td><td>cp4 epsps<br>cassette 1</td><td>P-ract1 (0.80Kb) – Rice actin 1 gene promoter from Oryza sativa (rice).<br>I-Ract1 (0.60Kb) – Rice actin 1, intron from Oryza sativa (rice).<br>TP-CTP2-ARATH (0.20Kb) – chloroplast transit peptide from Arabidopsis thaliana (thale cress or mouse-ear cress). TS-CTP2 codes for a N-terminal chloroplast transit peptide.<br>CS-EPSPS-RHIRD (1.40Kb) – Codes for 5-enolpyruvylshikimate-3-phosphate synthase isolated from Agrobacterium tumefaciens CP4 strain.<br>NOS 3' (0.30Kb) – Nopaline synthase gene terminator (3'-polyadenylation) from Agrobacterium tumefaciens.</td></tr>
<tr><td>cp4 epsps<br>cassette 2</td><td>P-e35S (0.60Kb) – CaMV enhanced 35S promoter gene. This gene contains modifications to enhance the activity of the promoter in plants.<br>I-Hsp70 (0.80Kb) – HSP70 intron from Zea mays (corn/maize)<br>TP-CTP2-ARATH (0.20Kb) – chloroplast transit peptide from Arabidopsis thaliana (thale cress or mouse-ear cress). TS-CTP2 codes for a N-terminal chloroplast transit peptide.<br>CS-EPSPS-RHIRD (1.44Kb) – Codes for 5-enolpyruvylshikimate-3-phosphate synthase isolated from Agrobacterium tumefaciens CP4 strain.<br>NOS 3' (0.30Kb) – Nopaline synthase gene terminator (3'-polyadenylation) from Agrobacterium tumefaciens.</td></tr>
<tr><td>Vector PV-ZMBK07**</td><td>cry1Ab<br>cassette</td><td>P-e35S (0.61Kb) – CaMV enhanced 35S promoter gene. This gene contains modifications to enhance the activity of the promoter in plants.<br>I-Hsp70 (0.80Kb) – HSP70 intron from Zea mays (corn/maize)<br>Cry1Ab (3.46Kb) – Synthetically developed and enhanced δ-endotoxin (Btk HD-1)<br>No terminator present. The terminator was lost through 3′truncation during integration.</td></tr>
<tr><td rowspan="2">Vector PV-ZMIR13</td><td>cry3Bb1<br>cassette</td><td>P-35S/AS1 (0.22Kb) – Synthetically produced promoter (CaMV 35S) plus four repeats of activating sequence (4AS1)<br>Lcab (0.06Kb) – 5' untranslated leader sequence from Triticum aestivum (wheat) chlorphyll a/b binding protein<br>I Ract1(0.49Kb) – Rice actin 1, intron from Oryza sativa (rice)<br>Cry3Bb1 (1.96Kb) – Codes for cry3Bb1 δ-endotoxin from B. thuringiensis<br>T-Hsp17 (0.23Kb) – 3' untranslated sequence of Triticum aestivum (wheat) heat shock protein 17.3. It terminates transcription and provides a signal for mRNA polyadenylation (polyA tail)</td></tr>
<tr><td>nptII<br>cassette</td><td>CaMV 35S (0.35Kb) – Cauliflower mosaic virus promoter<br>CS-ntpII-Ecolx (0.97Kb) – Neomycin phosphotransferase II (nptII) from Escherichia coli<br>NOS 3' (0.26Kb) – Nopaline synthase gene terminator from Agrobacterium tumefaciens, (nos) 3'-polyadenylation signal</td></tr>
</table>

* PV-ZMCT32 vector contains two cp4 EPSPS cassettes. The second cassette, containing the CaMV 35S promoter, is fused to the first cassette at the 3′ terminal end.

** The corn was transformed to produce the cry1Ab protein using two vectors (vectors PVZMBK07 and PV-ZMGT10). However, vector PVZMBK07 was not integrated into the plant genome.

## 1.2 Gastrointestinal tract

The digestive tract is the first site of contact for any ingested substance. Therefore, if a compound is toxic, the first signs of toxicity may be observed in the GI tract. The mucosa of the GI tract is the barrier between ingested compounds and the rest of the body. The stomach and the small intestine are the sites of longest residence for any ingested product. Consequently, these are the most important sites for the evaluation of the effects of an ingested compound. It is difficult to assess damage and diagnose diseases in the digestive tract purely on macroscopic appearance (Morini and Grandi, 2010); therefore a histopathological analysis should be part of the investigation.

### 1.2.1 Stomach

The normal rat stomach has two distinct parts – non-glandular (60%) and glandular (40%) (Frantz *et al.*, 1991; Gärtner, 2002) (Figure 8). The non-glandular stomach, also known as the forestomach, has a mucosa comprising of keratinised stratified squamous epithelium (Frantz *et al.*, 1991). It is a compartment were ingested products reside for a longer period for two reasons: 1) until the host's energy need increases, and 2) for further digestion of starch, fat and chitin (a component of insects) (Gärtner, 2002). At the junction between the non-glandular/glandular stomach, is the limiting ridge where the mucosa of the non-glandular region is thickest (Poel, 1963; Frantz *et al.*, 1991) (Figure 8B). The limiting ridge prevents rodents from vomiting by closing the orifice to the oesophagus during retching (DeSesso and Jacobson, 2001) (Figure 8A).

The glandular stomach is structurally and functionally similar to other laboratory animals (Frantz *et al.*, 1991) and is divided into the fundic (corpus) and pyloric (antral) regions. The fundic mucosa is comprised of pits, which branch into relatively straight gastric glands (Hoffmann, 2008) (Figure 8C). The surface of the fundus, as well as the pits, is lined by mucus-producing cells. The gastric glands are lined by chief and parietal cells, with a few enteroendocrine cells. Between the pits and the glands is the isthmus (neck region), where cellular proliferation occurs. Proliferating cells migrate either luminally to form mucus-producing cells, or basally to form cells of the gland (Fenoglio-Preiser, 1998). Between these glands is scant loose connective tissue (lamina propria), which has very low numbers of leukocytes and smooth muscle cells.

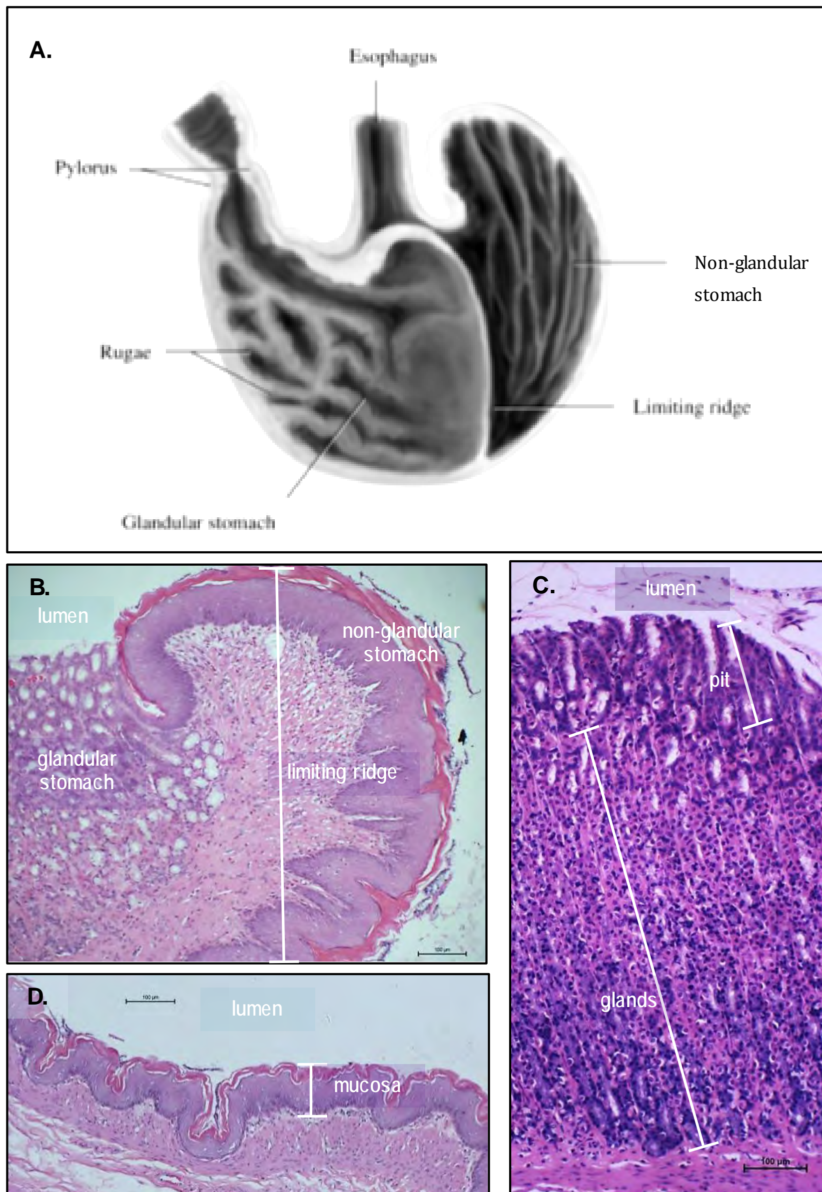



**Figure 8. The rat stomach**

A.) Drawing of a rat stomach showing the two distinct regions: non-glandular (forestomach) and glandular stomach. These two regions are separated by the limiting bridge. (Source: DeSesso and Jacobson (2001).

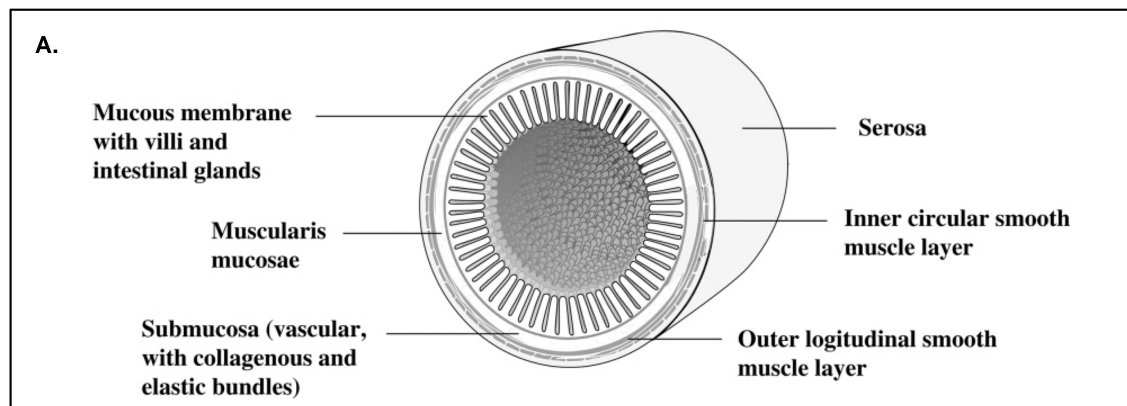
B.) Light micrograph of the non-glandular stomach showing keratinized stratified squamous epithelium (H&E; Scale bar: 100μm).

C.) Light micrograph of the glandular stomach showing gastric pits lined by mucus-producing cells, and the gastric glands lined by chief and parietal cells. (H&E; Scale bar: 100μm).

D.) Light micrograph of the junction between glandular/non-glandular stomach called the limiting ridge, which has a substantially thicker mucosa (keratinized stratified squamous epithelium). (H&E; Scale bar: 100μm).

### 1.2.2 Small intestine

The normal rat small intestine is very similar to humans and other mammals. Along its length, it is divided into three unequal regions: 1) duodenum, 2) jejunum and 3) ileum, which are structurally similar (DeSesso and Jacobson, 2001). The mucosa of these regions is made up of villi lined by absorptive cells (enterocytes) and mucin-secreting goblet cells. The lamina propria is highly cellular and contains many defence cells (Mowat, 2003; Denning *et al.*, 2007; Betton, 2013) (Figure 9B). In between each villus and at its base there are relatively short, straight crypts extending down to the muscularis mucosae (Cuvelier *et al.*, 2001). The crypts are primarily lined by goblet cells with a few enteroendocrine and Paneth cells. Proliferating zones are found near the base of the crypts (Takeda, 2004; Barker *et al.*, 2009). Deeper to the mucosa there is a fibrous submucosa followed by circular and longitudinal muscle layers, the muscularis propria (muscularis externa) (Figure 9). The rat, unlike humans, lacks circular folds (DeSesso and Jacobson, 2001; Hosoyamada and Sakai, 2005).



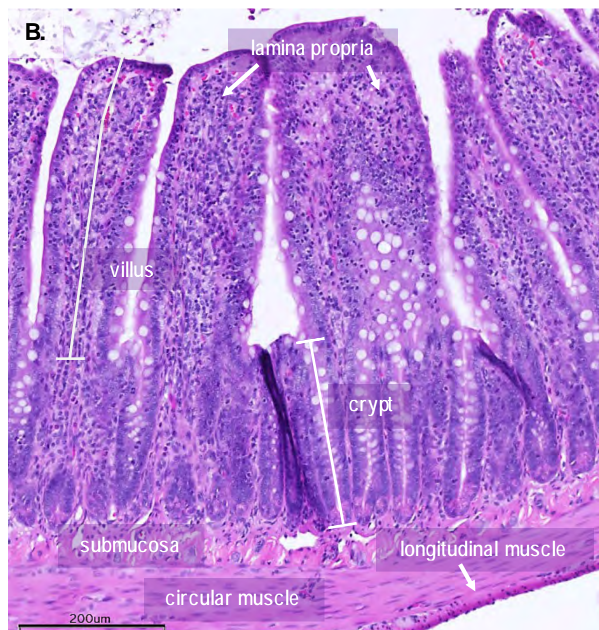




**Figure 9. The rat small intestine.**

A.) Schematic representation of the small intestine wall at cross-section. Inner-most layer is the mucosa, which is comprised of finger-like villi and straight crypts (glands). (Source: DeSesso and Jacobson (2001)).

B.) Light micrograph of small intestine (ileum) at cross-section showing villi, crypts and cellular lamina propria. The fibrous submucosa and the inner circular and outer longitudinal muscle layers are also evident. (H&E; Scale bar: 200µm)

### 1.2.3 Digestion

Digestion is the physical and chemical breakdown of ingested food into smaller compounds that can be absorbed by the intestinal epithelium. Chewing and secretion of saliva in the mouth begin the digestive process. Once the ingested food forms a bolus, it is swallowed and reaches the stomach via the oesophagus. In the rat, the food first resides in the non-glandular stomach for one to three hours or longer, depending on the rat's energy requirements (Gärtner, 2002). Then the food is emptied into the glandular stomach for further physical and chemical breakdown (Cleary *et al.*, 2015). In the stomach, the food is transformed into a semi-liquid chyme (Schulze, 2006). The chemical digestion of starch, fat and chitin begins in the non-glandular stomach, while the chemical digestion of cellulose and proteins begins in the glandular stomach (Gärtner, 2002) with the production of pepsinogen by chief cells and hydrochloric acid by the parietal cells. The hydrochloric acid activates the pepsinogen, thus forming pepsin, a proteolytic enzyme which breaks down the bonds of the amino acid chains (Campbell, 2012a; Campbell, 2012b). The action of pepsin stops when the chyme arrives in the more alkaline duodenum (Campbell, 2012b). In the duodenum, pancreatico-biliary and intestinal secretions further breakdown the amino acid chains into individual amino acids, which can be readily absorbed (Schulze, 2006; Campbell, 2012b). The pancreatic juices also contain enzymes that can break down carbohydrates (amylase), fat (lipase), nucleic acids and phospholipids (Campbell, 2012a).

The digestion of ingested particles relies on the appropriate enzymes being able to act on specific sites. Therefore, if a protein has an abnormally assembled structure, enzymes may not be able to attack the specific sites and thus break the amino acid chains. Likewise, if the body does not produce the appropriate enzymes, the molecules cannot be broken down and thus remain unabsorbed in the intestines. In addition, the breaking down of large particles into smaller ones exposes more sites for the action of enzymes. Therefore, if the breakdown in the stomach, and less so in the duodenum, is incomplete, the particles found in the mid-intestines will be larger and thus not readily absorbed (Schulze, 2006).

### 1.2.4 Histopathological changes in the GIT

#### *Stomach*

The examination of stomach tissue should assess the overall histological architecture including vascular changes (Table 2) (Fenoglio-Preiser, 1998). In the assessment of stomach pathologies in humans, a common starting point are the categories described in the Updated Sydney System (Dixon *et al.*, 1996; Stolte and Meining, 2001). When this System is applied to rats, it assesses the degree of: 1) inflammation, 2) atrophy or hyperplasia, and 3) degree and presence of intestinal metaplasia (Figure 10).

1) *Inflammation:* Normally, the stomach has a scant number of leukocytes, thus an increase in them indicates inflammation (Dixon *et al.*, 1996). An increase in granular leukocytes would indicate ongoing acute inflammation, while an increase in mononucleated cells would signify chronic inflammation (Dixon *et al.*, 1996).

2) *Atrophy or hyperplasia:* Gastric atrophy is characterised by a shortening of the gastric glands. It is usually poorly diagnosed due to the presence of inflammation, which often alters the architecture of the mucosa including the glands (Dixon *et al.*, 1996; Staibano *et al.*, 2002). Hyperplasia in the stomach is most often seen in the pits with an increase in mucus-producing cells and an increase in the proliferative region. In humans, pits can become coiled in severe cases (Dixon *et al.*, 1996). However, this change has not been reported in rats.

3) *Intestinal metaplasia* is characterised by the presence of intestinal-type cells in the gastric mucosa that appear as an adaptational response to changes in the stomach luminal content and gastric mucosa (Dixon *et al.*, 1996; Gutiérrez-González and Wright, 2008).

**Table 2. Assessing damage to glandular stomach** *(adapted from Fenoglio-Preiser, 1998)*

| |
|---|
| 1. Are there any focal differences seen in the section? Do these affect:<br>• the surface<br>• the epithelium<br>• the stroma? |
| 2. Is there inflammation present?<br>• acute<br>• chronic<br>• mixed<br>3. Where is the inflammation present?<br>• surface<br>• pits<br>• mucous neck region<br>• glands<br>• stroma |
| 4. Is there architectural distortion? |
| 5. Is there atrophy or parietal cell loss? |
| 6. Is there metaplasia?<br>• intestinal metaplasia<br>• spasmolytic polypeptide-expressing metaplasia |
| 7. Is the mucosa expanded? if so, is it by<br>• inflammation<br>• pit expansion<br>• glandular expansion<br>• an abnormal cellular infiltrate? |
| 8. Are the blood vessels normal? If not, are they<br>• dilated<br>• thrombosed<br>• thickened<br>• enlarged<br>• dysplastic<br>• neoplastic? |

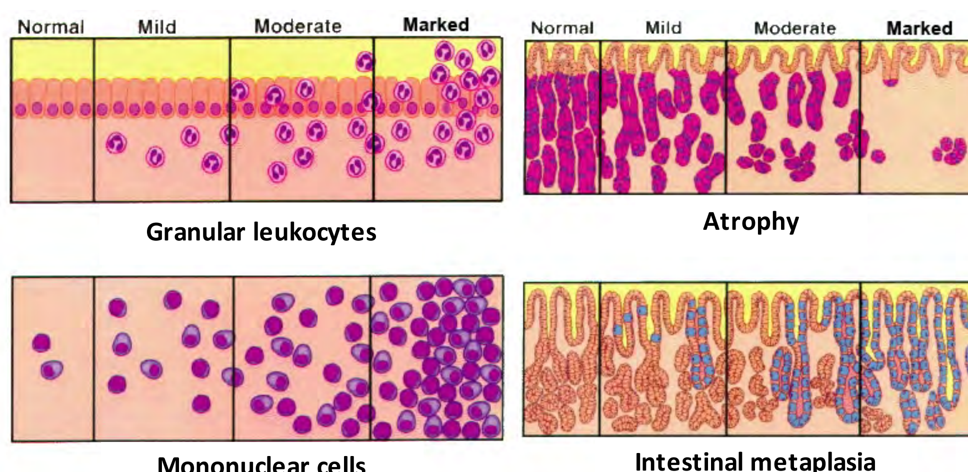



**Figure 10. Modified Updated Sydney System to grade changes in the rat stomach.**

**Granular leukocytes and mononuclear cell infiltrate:** Normal, mild, moderate, and marked.

**Atrophy:** A mild, moderate or marked difference may also include a decrease in mucosa thickness.

**Intestinal metaplasia:** With aid of AB/PAS stain (pH 2.5) the presence and degree of intestinal metaplasia is graded as: Normal mucosa/no intestinal metaplasia (no blue-staining cells), mild metaplasia (a scant presence of blue-staining cells), moderate (metaplasia present in several glands), marked (metaplasia present in most glands) (Redrawn from Dixon *et al.*, 1996)

### *Small intestines*

The examination of the small intestine should assess the overall histological architecture, as well as, changes to cell appearance and number (Table 3) (Howarth *et al.*, 1996). Intraepithelial lymphocytes (IELs) are an important part of the mucosal barrier (Cuvelier *et al.*, 2001). They are known to increase following non-specific intestinal damage (Ewen and Pusztai, 1999), therefore performing an intraepithelial lymphocyte count helps in determining the presence and type of pathological change.

**Table 3. Assessing damage to small intestine** *(adapted from Howarth et al., 1996)*

| |
|---|
| 1. Calculate villi height/crypt depth ratio |
| 2. Are there changes to the lining cells?<br>• disruption of brush border and surface enterocytes<br>• reduction in goblet cell number<br>• disruption or distortion of crypt cells<br>• reduction of mitotic figures<br>• increase in intraepithelial lymphocytes |
| 3. Is there architectural distortion?<br>• villi fusion and stunting (atrophy)<br>• crypt loss or crypt architectural disruption |
| 4. Is there an increase of cells in the lamina propria?<br>• infiltration of lymphocytes and/or polymorphonuclear cells |
| 5. Is there lymphatic or capillary dilatation? |
| 6. Is there thickening and/or oedema in the submucosa and muscularis propria layers? |

In sections where the intestine is severely damaged and the normal intestinal morphology is not apparent, calculating the "surface length" index has been suggested as a method of evaluation (Corazza *et al.*, 1985; Howarth *et al.*, 1996). This is the ratio of surface length to area of the lamina propria (SL/ALP). The surface length (SL) is measured along the epithelial luminal surface, while the area of the lamina propria (ALP) is the area contained within the muscularis mucosae and the basement membrane of the mucosal epithelium. Cross-sectioned crypts are subtracted from this measurement (Figure 11). The ratio of the SL to ALP is obtained at 3 random sites.

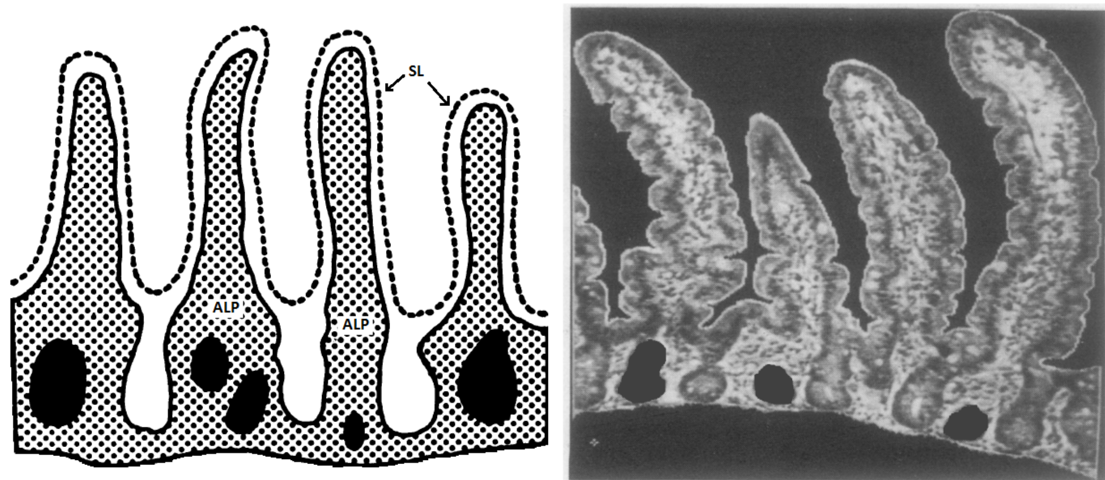



**Figure 11. "Surface length" index: A ratio of surface length (SL) to area of lamina propria (ALP).** SL is obtained through the tracing of the epithelial luminal surface (dashed line). ALP is obtained through tracing the lamina propria area boundaries, which consist of the basement membrane of the mucosal epithelium, the two sides in the plane of view and the superficial aspect of the muscularis mucosae (outlined by a solid line), with the subtraction of the area taken up by the cross-sectional crypts (blackened-out areas). (Redrawn from Corazza *et al.*, 1985.)

## *Mucosal barrier dysfunction*

The mucosa of the GI tract is the barrier between the luminal environment and the body. This barrier can be readily breached, if the mucosa is compromised. The GI tract mucosa is constantly exposed to a variety of noxious agents (Hoffmann, 2008), therefore, it naturally has continuously-operating protective mechanisms. When any part of these mechanisms break down or change considerably, pathological changes can ensue (Su *et al.*, 2009; Morini and Grandi, 2010). The effect of an ingested compound on the mucosa can also be influenced by the interaction of the substance with the GI tract luminal content and the protective mucous layer overlying the mucosa (Bertram *et al.*, 1996). In the stomach, the presence of food in the lumen is proposed to have a buffering effect on acid secretion by the parietal cells (Morini and Grandi, 2010). Therefore, the presence, as well as the constituents of the feed, can be important factors in determining the potential or severity of damage on the gastric mucosa (Morini and Grandi, 2010). These can enhance or reduce the toxic effect of noxious agents.

### The GI tract mucosal barrier

The mucosa protective mechanisms have extrinsic and intrinsic barrier functions (Figure 12) (Powell, 1984). The **extrinsic component** includes: 1) at the epithelial surface - the mucous gel layer (Allen and Flemstrom, 2005; Hirst, 2011), acid secretion

(stomach), the production of antimicrobial peptides (Hoffmann, 2008) and the secretion of IgA (small intestine) (Alverdy et al. 1985); and 2) deeper to the epithelium - the tissue acid-base balance and blood circulation (Powell, 1984), which includes acute inflammatory responses (Martin and Wallace, 2006). The **intrinsic component** of the mucosal barrier involves the epithelial lining, namely the epithelial cells' apical membrane and the tight junctions between the epithelial cells (Powell, 1984; Pitman and Blumberg, 2000). The mucous barrier function is further increased by the continual regeneration of the epithelial lining (Hoffmann, 2008) and the fast repair mechanisms (restitution, reepithelialisation or reconstitution) (Hoffmann, 2008; Hirst, 2011). The tight junctions between the epithelial cells are critically important intrinsic components. They work as a barrier by regulating the paracellular passage of ions and various molecules as well as maintaining cell polarity by keeping the two environments (pre-epithelial and paracellular) separate (Sawada *et al.*, 2003).

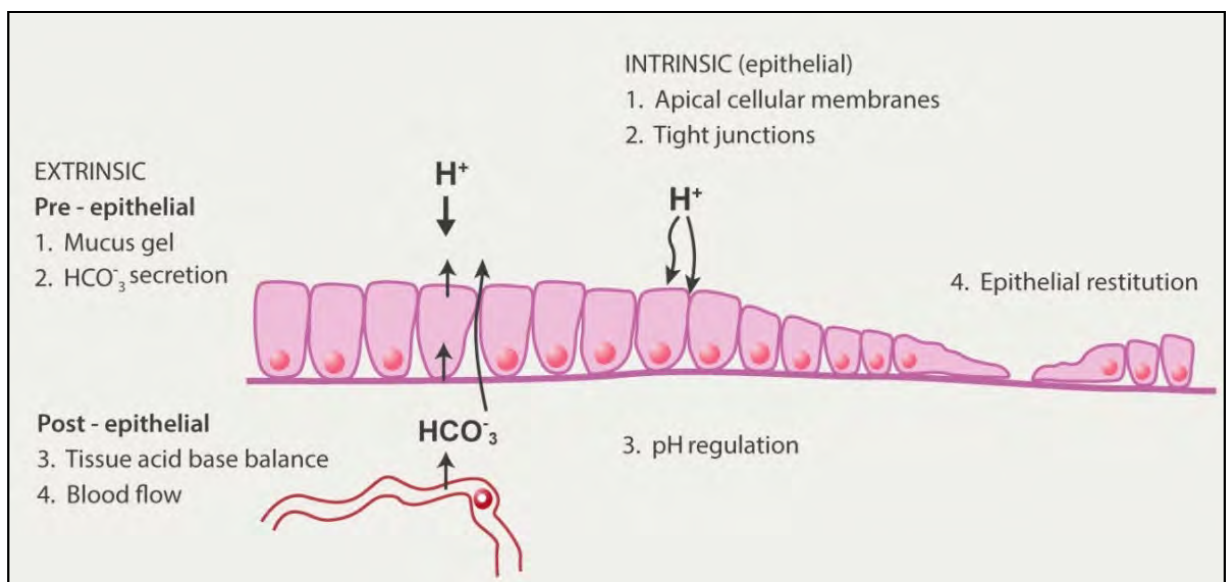



**Figure 12. Gastrointestinal tract mucosal barrier components: Extrinsic and intrinsic.**
**Extrinsic components:** 1) Mucous gel layer that protects the epithelial surface, 2) acid secretion (in stomach) which protects from microbial invasion, 3) tissue acid balance, and 4) blood flow, which brings in defence cells when needed. **Intrinsic components:** 1) apical epithelial cell membrane, 2) tight junctions, 3) regulation of pH by the epithelium, and 4) epithelial restitution – the rapid repair mechanism of the epithelium following epithelial cell death or erosion. (Redrawn by T. Morgenstern from Hirst, 2011)

## Tight junctions in GI tract disease

Tight junctions are the most important components of the mucosal barrier since they prevent the passage of large molecules or agents that could activate an immune response or cause damage to underlying tissue (Edelblum and Turner, 2009; Menozzi

and Ossiprandi, 2010). They are found at the apex of the intercellular space, they regulate the passage of ions and various molecules, and they also maintain tight cell-cell adherence. This tight adherence maintains cell polarity by keeping the two environments (luminal and paracellular) separate (Sawada *et al.*, 2003).

Tight junctions are composed of transmembrane proteins, occludin, claudins, and junctional adhesion molecules (JAMs), which are anchored to the cell cytoskeleton via cytosolic plaque proteins, the zonula occludens proteins (ZO-1, ZO-2, and ZO-3) (Figure 13) (Tsukita *et al.*, 2001; Weber *et al.*, 2008).

Occludin appears to have no isotypes even between species (Morita *et al.*, 1999). It is a paracellular protein that has four transmembrane domains, three cytoplasmic domains, and one short intercellular and two extracellular loops (Tsukita and Furuse, 1999). Occludin colocalises with ZO-1, which results in cell-cell adhesiveness (Van Itallie and Anderson, 1997). Occludin is believed to be responsible for the paracellular "seal" (Tsukita and Furuse, 1999); however in occludin knock-out mice, the structure and resistance of tight junctions were unaffected (Saitou *et al.*, 2000). This suggests that occludin may not be the only molecule involved in the "seal" function of the tight junction. Studies imply that this "seal" function is supported by claudins (Markov *et al.*, 2010).

Occludin may also be implicated in the transepithelial migration of neutrophils (Huber *et al.*, 2000). A cell culture study (Huber *et al.*, 2000), investigated several induced variations of occludin by mutating the epithelial cells. Induced mutation of one of the extracellular loops was found to cause inhibition of neutrophil migration, with no change in the selective paracellular permeability or transepithelial electrical resistance of the epithelium.

Claudin has been identified to have more than 20 isotypes (Samonte *et al.*, 2004) with some exclusively expressed in certain tissue types (Morita *et al.*, 1999). In addition, more than one claudin isotype can be expressed in one tight junction (Furuse *et al.*, 1998). Claudins are paracellular proteins that have four transmembrane domains: a short internal sequence, two extracellular loops, and a longer and variable cytoplasmic tail (Anderson and Van Itallie, 2009). The first extracellular loop is believed to be responsible for the electrostatic pore selectivity and the second for cell-cell adhesiveness. In this way, claudins are responsible for the regulation of permselectivity and electrical resistance (Anderson and Van Itallie, 2009). In the small intestine and

colon, the different claudin isotypes appear to have different functions (Markov *et al.*, 2010). Markov *et al.* (2010) found that the claudins responsible for tight junction permeability were claudins-2, -7 and -12, with most of these increasing in expression along the proximal-distal direction in the small intestine. In addition, they found that the claudins responsible for the barrier property of the tight junction in the small intestine were claudin-1, -3, -4, -5, and -8. This was supported by their transepithelial resistance tests using Ussing chambers. Both transepithelial resistance and the expression of the claudins responsible for the "seal" function of the tight junction were highest in the colon, second highest in the duodenum, but often decreased or absent in the jejunum. These results seem to correlate with the function of the intestines, with the highest resistance of transepithelial permeability being in the duodenum and colon, and lowest in the jejunum and ileum (Markov *et al.*, 2010).

The tight junction transmembrane proteins furthest from the lumen are the JAMs (Figure 11). They are part of the immunoglobulin (Ig) superfamily and are known to have four isotypes, JAM-1, -2, -3 and -4, which are also known as JAM-A, -B, -C and IGSF5 respectively (Shen *et al.*, 2011). The JAMs are a paracellular transmembrane protein with one extracellular region containing two V-type Ig domains, one transmembrane region, and one cytoplasmic tail (Shen *et al.*, 2011). The JAM-1 protein in particular, facilitates the junctional localization of ZO-1 and occludin (Bazzoni *et al.*, 2000) and is believed to promote cell-cell adhesiveness (Martìn-Padura *et al.*, 1998), as well as the regulation of leukocyte migration (Laukoetter *et al.*, 2007, Martìn-Padura *et al.*, 1998) and epithelial cell proliferation (Laukoetter *et al.*, 2007).

The ZO proteins, ZO-1, -2 and -3 are part of the membrane-associated guanylate kinase homologs (MAGUKs) family of proteins (Fanning *et al.*, 1998), which are often referred to as scaffold or plaque proteins (Shen *et al.*, 2011). They are believed to play an important role in organisation and regulation of the intramembranous tight junction proteins (Fanning *et al.*, 1998; Fanning and Anderson, 2009). They also interact with actin and actin-binding proteins, thus linking the tight junction to the cytoskeleton (Fanning *et al.*, 1998; Fink, 2003). In addition, it is suggested that the ZO proteins, particularly ZO-1, act as a link between adherens and tight junctions and through this link the ZO proteins promote the assembly of both junctions (Hartsock and Nelson, 2008; Fanning and Anderson, 2009).

Tight junction structural integrity is usually evaluated using transmission electron microscopy (TEM) (Eastwood and Erdmann, 1978; Keefe *et al.*, 1997; Soler *et al.*, 1999; Edelblum and Turner, 2009), freeze fracture EM (Meyer *et al.*, 1986), Western blot (Markov *et al.*, 2010), light microscopy immunohistochemistry (Nusrat *et al.*, 2001), and confocal microscopy (Bruewer *et al.*, 2005). Tight junction "leakiness" is evaluated by measuring the permeability of the gut to radioactive labels or sugars either through the direct introduction of the labels into the gut of live animals (Samonte *et al.*, 2004) or measuring them post-mortem using Ussing chambers (Markov *et al.*, 2010).

"Leaky" or poorly apposed tight junctions have been found to accompany several pathologies such as, chemical/reactive gastritis (Eastwood and Kirchner, 1974; Eastwood and Erdmann, 1978; Meyer *et al.*, 1986), inflammatory bowel disease (Edelblum and Turner, 2009) and colon cancer (Soler *et al.*, 1999). Compromised tight junctions are also associated with bacterial infections (Nusrat *et al.*, 2001; Fasano and Nataro, 2004; Samonte *et al.*, 2004; Aktories and Barbieri, 2005). In addition, burns victims are known to have compromised tight junctions in the gut (Samonte *et al.*, 2004). It has also been suggested that certain tight junction proteins (occludin and ZO-1) may be involved in the formation of certain gastric carcinomas (Kimura *et al.*, 1997).

The molecular mechanisms regulating the structural changes in "leaky" tight junctions are still poorly understood, (Takeuchi *et al.*, 2002; Fasano and Nataro, 2004; Ma *et al.*, 2004). There is increasing evidence that pro-inflammatory cytokines, hormones, bacterial toxins or bacterial adherence can affect tight junction integrity through altering actin, triggering actomyosin contractility or through down regulation or displacement of tight junction proteins (Figure 13B) (Mankertz *et al.*, 2000; Nusrat *et al.*, 2000; Fasano and Nataro, 2004; Ma *et al.*, 2004; Capaldo and Nusrat, 2009; Groschwitz and Hogan, 2009). In addition, during inflammation the increased leukocyte migration through the junctions, may affect epithelial barrier and tight junction integrity (Nusrat *et al.*, 2000; Samonte *et al.*, 2004). *In vivo* studies have also found evidence that luminal glucose may affect tight junction permeability and cause intrajunctional tight junction dilatations (blebbing) (Atisook and Madara, 1991; Nusrat *et al.*, 2000).

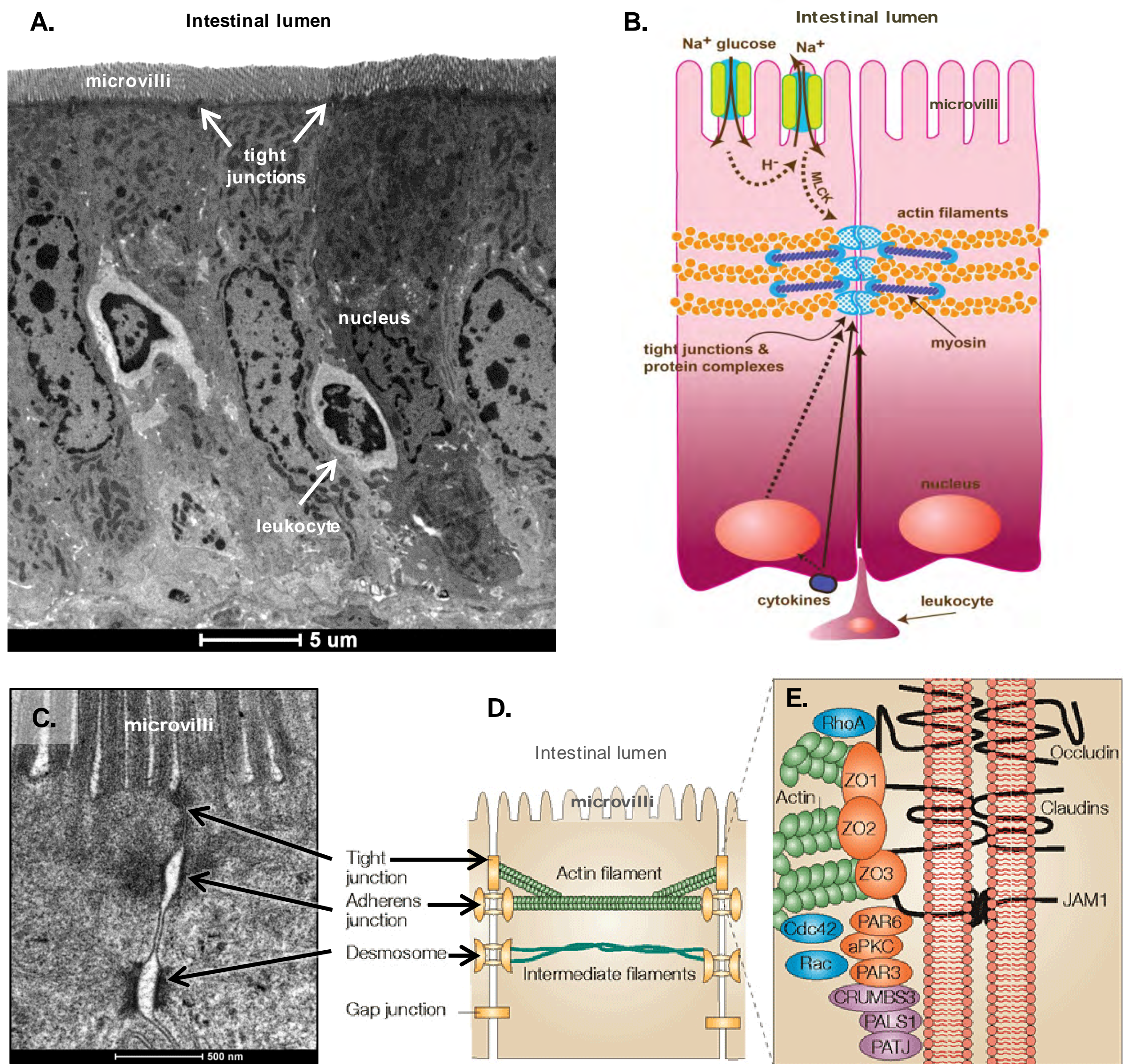



**Figure 13. Tight junctions in gut epithelium.**

A.) Transmission electron micrograph of epithelium in the small intestine of the rat showing the placement of the tight junctions at the apical end of the paracellular space. An intraepithelial lymphocyte (leukocyte) is also present. Scale bar 5μm.

B.) Schematic representation of anchoring of the tight junction to the cytoskeleton via the actin filaments. Tight junctions may become "leaky" due to the action of cytokines, luminal glucose (*in vivo*), or increased leukocyte migration. (Redrawn by T. Morgenstern from Nusrat *et al.*, 2000)

C.) Transmission electron micrograph of epithelial junctions at apical end of the paracellular space: Tight junction, adherens junction, and desmosome. Scale bar 500nm.

D.) Schematic representation of epithelial junctions at the apical end of the paracellular space: Tight junction, adherens junction, desmosome and gap junction.

E.) Schematic representation of tight junction transmembrane proteins, occludin, claudins and JAM1, and anchoring proteins, ZO1, ZO2 and ZO3. Other proteins are also proposed to be part of the tight junction; however their role is still to be elucidated. (Source: Aktories and Barbieri, 2005)

### Erosion or ulceration

Serious damage caused by toxicological agents in the stomach and intestines, primarily leads to erosion or ulceration (Bertram *et al.*, 1996). These arise as a result of an imbalance between the defensive and aggressive mechanisms that are in play in the digestive tract (Morini and Grandi, 2010). Erosion is the damage or necrosis of the superficial mucosa that does not extend down to the muscularis mucosa, while ulceration is when the damage affects the full thickness of the mucosa, penetrating through the muscularis mucosa (Frantz *et al.*, 1991; Betton, 2013).

In the stomach, erosion or ulceration may be accompanied by inflammation (Bertram *et al.*, 1996; Betton, 2013). There may also be atrophy of the gastric glands, sometimes followed by replacement of fibrous tissue or loss of the normal matrix (Dixon *et al.*, 1996). Microscopically, evidence of erosion can be seen through the presence of fibrin deposits (Dixon *et al.*, 1996), as well as regenerative changes such as an upward migration of pit cells, possible reactive hyperplasia (Frantz *et al.*, 1991), and the presence of clustered eosinophilic chief cells in the mucosal glands (Whiteley *et al.*, 1996). In areas adjacent to the erosive damage, epithelial cells may be necrotic, cuboidal or flattened (Bertram *et al.*, 1996). Reactive hyperplasia is an increase in epithelial cell number in reaction to a certain stimulus, such as the onset of ulcerative changes (Frantz *et al.*, 1991). In the stomach it occurs mainly in the pit region with pits becoming tortuous (Dong *et al.*, 2005) and it may further lead to the formation of adenomas (Frantz *et al.*, 1991).

In the small intestine, ulcerative lesions are accompanied by signs of acute and/or chronic inflammation and may be accompanied by a decrease in mitotic activity (Bertram *et al.*, 1996). The inflammation, associated with ulceration, may disrupt the muscularis mucosa causing crypt herniation (Whiteley *et al.*, 1996). In addition, surrounding the areas of inflammation or ulceration, focal reactive hyperplasia or hypertrophy of intestinal epithelium may occur (Peckham, 2002). Villous atrophy may also be associated with ulcerative lesions, and during the stages of repair, goblet cell hyperplasia may occur (Whiteley *et al.*, 1996). In addition, in areas adjacent to the erosive damage, epithelial cells may be necrotic, cuboidal or flattened (Bertram *et al.*, 1996).

## Neoplastic lesions

In healthy rats, neoplastic lesions are rare (less than 0.5%) in the stomach and small and large intestines (Whiteley *et al.*, 1996; Peckham, 2002). However, following long-term ingestion of a noxious agents (such as food allergens, chemicals or pathogens) (Coruzzi, 2010) they can be more prevalent due to the high turn-over of the lining epithelium (Hoffmann, 2008). Neoplastic lesions arise either through metaplasia or directly from the stem-cell/proliferative zone (Hoffmann, 2008). Often these lesions can be distinguished by their appearance – with epithelial dysplasia and loss of neighbouring architecture. However, sometimes special staining is necessary, such as, for neuroendocrine cell tumours in the stomach, which are immuno-reactive for non-specific enolase, chromogranin-A, and histamine (Frantz *et al.*, 1991). In addition, DNA repair markers such as OGG1,2 can help identify pre-cancerous changes (Antushevich *et al.*, 2013).

In cancerous lesions, changes in cell proliferation and/or apoptosis are present (Tudek and Speina, 2012), with the number of apoptotic and proliferative cells often decreased or absent in or around the lesion (Niimi *et al.*, 2002; Kania *et al.*, 2003). Immunolocalisation of Ki67 and activated caspase 3 can identify proliferative cells (Niimi *et al.*, 2002) and apoptotic cells respectively (Kania *et al.*, 2003) around neoplastic lesions. In addition, labelling for the tumour suppressor gene, p53 can further demonstrate the change. In cancers of the digestive tract, p53 labelled cells are increased (Godlewski *et al.*, 2006). In addition, in precancerous lesions, if the p53 is inversely proportional to the caspase 3 labelling, the cells are coping with the change. However, if caspase 3 is greater than p53, the damage of the DNA is irreversible (Godlewski *et al.*, 2006).

## Metaplasia

In the stomach, the two most common types of metaplasia are intestinal (IM) and spasmolytic polypeptide-expressing metaplasia (SPEM). In all cases of metaplasia, the pathogenesis is believed to be caused by an accumulative effect of bacteria, environmental factors and inflammatory cytokines working together to bring about adaptational changes in the gastric mucosa (Gutiérrez-González and Wright, 2008). The aetiology begins with loss of parietal cells (gastric atrophy) followed by the

differentiation of stem cells into either an IM or SPEM lineage (Gutiérrez-González and Wright, 2008; Hoffmann, 2008). Parietal cells are known to be largely involved in signalling cellular growth and differentiation. Their loss leads to an increase in undifferentiated cells and subsequent differentiation of metaplasia lineages (Fox and Wang, 2007).

Intestinal metaplasia is characterised by the appearance of intestinal-type cells with a brush border, goblet cells containing mucins, and Paneth cells with eosinophilic granules in their cytoplasm (Gutiérrez-González and Wright, 2008). This type of metaplasia is best diagnosed using a combined alcian blue-periodic acid Schiff stain (AB/PAS) (pH 2.5), which stains the metaplastic cells blue or purple (Dixon *et al.*, 1996).

In SPEM, the loss of parietal cells leads to antralisation of the glands that express trefoil family factor 2 (TFF2; formerly known as spasmolytic polypeptide) (Hoffmann, 2008). This type of metaplasia is detected through immuno-staining for TFF2 (Halldorsdottir *et al.*, 2003).

Intestinal metaplasia and SPEM predispose malignancy, particularly SPEM (Hoffmann, 2008). Both types of metaplasia are also associated with chronic inflammation (Dixon *et al.*, 1996; Hoffmann, 2008)

## *Chemically-induced damage*

### Stomach

Chemical (reactive) gastritis occurs when there are surface-damaging agents such as chemical irritants or certain medications/drugs in the gastric lumen (Dixon *et al.*, 1996; Fenoglio-Preiser, 1998). In chemical gastritis the pathological changes are subtle, but are characterised by pit hyperplasia, mucus depletion, superficial oedema (Dixon *et al.*, 1996), with the absence of severe inflammation, atrophy, metaplasia, ulcers and polyps (Fenoglio-Preiser, 1998). The glands also have an increase in mitotic activity (Fenoglio-Preiser, 1998). In severe cases, glandular elongation and pit coiling occurs, as well as smooth muscle cell proliferation in the lamina propria (Dixon *et al.*, 1996; Owen, 2003). The absence of severe inflammation is a major diagnostic feature of chemical gastritis, thus, chemical gastropathy is sometimes the preferred term (Dixon *et al.*, 1996).

### Small intestine

Drug-induced enterocolitis is the name given to changes associated with ingestion of medications/drugs, such as antiarrhythmics, antibiotics, magnesium-containing

antacids, lactose- or sorbitol-containing products and non-steroidal anti-inflammatory drugs (NSAIDs) (Geboes *et al.*, 2006). This type of enterocolitis affects both the mucosa and submucosa and in severe cases could extend down to the muscularis propria. The pathological changes include erosion of the mucosa, and formation of ulcers and strictures, which appear mainly in the distal small intestine (the distal jejunum and the ileum) and the colon (Parfitt and Driman, 2007). Erosion and ulceration are predisposed by loss of goblet cells and enterocytes (Faure *et al.*, 2003). The loss of goblet cells leads to a decrease in mucus lining the surface, subsequently making the surface prone to erosive damage and ulceration (Faure *et al.*, 2003). In the small intestine there is also villous atrophy and sometimes crypt hyperplasia (Isaacs *et al.*, 1987). Changes in the mucosa tend to be accompanied by a marked increase of inflammatory cells in the lamina propria (Lang *et al.*, 1988) and epithelial lining (Lee, 1993; Lee, 1994), which are predominantly mononuclear cells (Isaacs *et al.*, 1987) and/or eosinophils (Lee, 1994; Price, 2003; Casella *et al.*, 2009).

Intestinal strictures are a clinical feature of NSAID use in humans (Lang *et al.*, 1988, Parfitt and Driman, 2007); however, the formation of strictures can also be induced in rats (Marlow and Blennerhassett, 2006). Intestinal strictures are protrusions of a fibrotic mucosa and submucosa that can sometimes almost completely occlude the intestinal lumen (Parfitt and Driman, 2007). These strictures occur mainly in the ileum, but have also been reported in the jejunum and colon (Price, 2003; Klein *et al.*, 2011). The cause of intestinal stricture formation is not known, but inflammation is believed to be involved (Marlow and Blennerhassett, 2006). The suggested model of stricture formation involves an increase in collagen fibres in the submucosa (Lang *et al.*, 1988), as well as an increase in smooth muscle cell proliferation and disorganisation (Marlow and Blennerhassett, 2006). The collagen fibres are deposited among the proliferating smooth muscle cells, and in severe cases there is fusion of the muscularis mucosae with the muscularis propria (Marlow and Blennerhassett, 2006). Overlying the mucosal and submucosal fibrotic lesions, there is villus/crypt irregularity, and erosion or ulceration (Lang *et al.*, 1988; Marlow and Blennerhassett, 2006).

## 1.3 Critical Review of Literature – *published*

The critical review was published as: Zdziarski, IM, Edwards, JW, Carman, JA, and Haynes, JI. 2014. GM crops and the rat digestive tract: A critical review. *Environment International,* 73**,** 423-433. The full publication appears at Publications P3. This has been cited in recent publications (Azadi *et al.*, 2015; Benbrook, 2016; Domingo, 2016; Ibrahim and Okasha, 2016; Ishii and Araki, 2016; Trojan et al., 2016; Wong and Chan, 2016).

### 1.3.1 Materials and Methods

The purpose of this literature review was to examine the relationship between GM crops and histopathological observations in rats. The search only included crops possessing one or more of three specific traits which are commonly found in commercialised GM crops: herbicide tolerance via the EPSPS gene, and insect resistance via cry1AB or cry3Bb1 genes. A list of crop event names was first generated (Table 4) based on GM approval databases (Chen *et al.*, 2011; FSANZ, 2011; CERA, 2012; ISAAA, 2013) and publications, such as literature reviews (Pusztai *et al.*, 2003; Domingo, 2007; Domingo and Bordonaba, 2011; Snell *et al.*, 2012). The search used PubMed, Google Scholar and Embase to find studies that were published before April 2013. The search was restricted to published studies. Reports, such as European Food Safety Agency (EFSA) reports, were not included since they do not contain detailed histopathological results. The keywords used were rat, rats*, rattus* and the specific crop event line name (Table 4). To make results comparable with each other, the search was limited to long-term rat feeding studies of no less than 90 days duration. The search excluded multigenerational studies, unless there was a histopathological investigation in the first generation of rats. No language limit was set. For non-English publications, help was obtained with their translation and accurate understanding.

**Table 4. Literature search:** List of GM crop event names that were used in the search for published studies. Year of approval for animal and/or human consumption of each event name and number of publications found per event.

| Crop type: | Event name (other name and/or code name)[a, b] **Roundup Ready (RR) or glyphosate-tolerant crops:** ***Crops containing EPSPS genes*** | Number of published long-term rat feeding studies | Year approved for food and/or feed[c] |
|---|---|---|---|
| *Alfalfa/Lucerne* | J101 (MON-00101-8) | 0 | 2004 |
| | J163 (MON-00163-7) | 0 | 2004 |
| *Canola* | GT200 (RT200, MON89249-2) | 0 | 1997 |
| | GT73 (RT73, MON737) | 0 | 1994 |
| | MON88302 (MON-88302-9) | 0 | 2012 |
| | ZSR500 (ZSR500 x GT73) | 0 | 1997 |
| | ZSR502 (ZSR502 x GT73) | 0 | 1997 |
| | ZSR503 (ZSR503 x GT73) | 0 | 1997 |
| *Corn/Maize* | GA21 (MON00021-9) | 1[d] | 2000 |
| | HCEM485 | 0 | 2012 |
| | NK603 (MON-00603-6) | 2 | 2000 |
| | MON832 | 0 | 1996 |
| | MON87427 (MON-87427-7) | 0 | 2012 |
| *Cotton* | GHB614 (BCS-GH205) | 0 | 2008 |
| | MON1445 (MON1445-2) | 0 | 1995 |
| | MON1698 (MON89383-1) | 0 | 1995 |
| | MON88913 (MON88913-8) | 0 | 2005 |
| *Creeping bentgrass* | ASR368 (SGM-36800-2)[e] | 0 | 2003 |
| *Potato* | RBMT22-082 (RBMT22-82, NMK-89896-6) | 0 | 1998 |
| | RBMT22-186 | 0 | 1998 |
| | RBMT22-238 | 0 | 1998 |
| | RBMT22-262 | 0 | 1998 |
| *Soybean* | 40-3-2 (GTS 40-3-2, MON04032-6) | 4 | 1995 |
| | FG-72 (MST-FG072-2) | 0 | 2012 |
| | MON87705 (MON87705-6) | 0 | 2011 |
| | MON87708 (MON87708-9) | 0 | 2011 |
| | MON87769 | 0 | 2011 |
| | MON89788 (MON89788-1) | 1[f] | 2007 |
| | glyphosate-tolerant [g] | 3[g] | NA |
| | 305423x40-3-2 (DP305423xGTS40-3-2, DP-305423-1xMON-04032-6)[b] | 1 | 2010 |
| *Sugar beet* | GTS B77 (T9100152, SY-GTSB-77-8) | 0 | 1998 |
| | H7-1 (KM71-4) | 0 | 2003 |
| *Wheat* | MON71800 (MON-71800-3) | 0 | 2004 |
| | **Insect resistant or *Bt* crops:** ***Crops containing Cry3Bb1 and EPSPS genes*** | | |
| *Corn/Maize* | MON88017 (MON-88017-3)[h] | 2[h] | 1996 |
| | ***Crops containing Cry3Bb1 genes*** | | |
| *Corn/Maize* | MON863 (MON-00863-5) | 1 | 2001 |

**Table 4. (cont.) Literature search**

| Crop type: | Event name (other name and/or code name)[a, b] Insert resistant or *Bt* crops: *Crops containing Cry1Ab genes* | Number of published long-term rat feeding studies | Year approved for food and/or feed[c] |
|---|---|---|---|
| *Corn/Maize* | 5307 (SYN-05307-1) | 0 | 2012 |
| | Bt10 | 0 | 1995 |
| | Bt11 (x4334CBR, x4634CBR. SYN-Bt011-1) | 0 | 1996 |
| | Bt176 (176, SYN-EV176-9) | 0 | 1995 |
| *Cotton* | COT67B (IR67B, SYN-IR67B-1,) | 0 | 2009 |
| | GFM Cry1A (GTL-GFM311-7) | 0 | cultivation only (2006) |
| | GK12 | 0 | cultivation only (1997) |
| | T303-3 (BCS-GH003-6) | 0 | cultivation only (2012) |
| | T304-40 (BCS-GH004-7) | 0 | 2010 |
| *Rice* | KMD 1 rice (Kemingdao 1, TR30) | 3 | 2009 |
| | GM Shanyou 63 | 0 | 2009 |
| | Hauhui-1/TT51-1 | 0 | cultivation only (2009) |
| | Tarom molaii + cry1ab | 0 | 2004 |
| *Tomato* | RLE13-0009[i] | 1 | no approval info.[i] |
| | RLE6-1000[i] | 0 | no approval info.[i] |
| | *Crops containing Cry1Ab and EPSPS* | | |
| *Corn/Maize* | MON801 (MON80100) | 0 | 1996 |
| | MON802 (MON-80200-7) | 0 | 1997 |
| | MON809 (PH-MON-809) | 0 | 1996 |
| | MON810 (MON-00810-6) | 2[d] | 1996 |
| | *Number of GM crop event lines studied* | 10[f,h] | 9 approved |
| | *Total number of GM crop event lines* | 53[f,h] | 47 approved |
| | *Total number of published studies found* | 21 | 19 approved |

[a] Each line contains one crop. Succeeding names are the other names given to the crop event name and/or the crop's code name.

[b] GM crop hybrids are not listed in the table, unless the crop is listed in databases as a single event name. An exception is the GM soybean line 305423x40-3-2, since a feeding study publication was found during the search for publications.

[c] The year that the crop was first approved somewhere in the world for human and/or animal consumption.

[d] One publication contained results for two feeding studies – one on MON810 corn and the other on GA21 corn. As these are two separate feeding studies they have been counted as two published studies.

[e] ASR368 creeping bentgrass is solely intended for the production of turf grass in golf courses, but it can be used as livestock feed (Chen *et al.*, 2011)

[f] One study generating two published reports. The first, reported results for the analysis of morphological, haematological, biochemical parameters and system biomarkers (Tutel'ian *et al.*, 2010). The second, reported the allergenic potential and immuno reactivity, as well as looked for signs of genotoxicity (Tyshko *et al.*, 2010). Since the reports are of the same study, they have been counted as one published study.

[g] The GM crop or event name was not listed in three publications. The GM crop studied contained the *EPSPS* gene, which confers glyphosate tolerance. This was not counted as a separate event line in the final number of GM crop event lines studied nor in the total number of GM crop event lines.

[h] One study generating two published reports. The first, reported results for the analysis of morphological, haematological, biochemical parameters and system biomarkers (Tutel'ian *et al.*, 2008). The second, reported the allergenic potential and immuno reactivity, as well as looked for signs of genotoxicity (Tyshko *et al.*, 2008). Since the reports are of the same study, they have been counted as one published study.

[i] The *Bt* tomatoes may never have been released. The feeding study by Noteborn *et al.* (1995) looked at the effect of only RLE13-0009 on the rat. The effect of RLE6-10001 was not investigated in the rat, but in other laboratory animals.

### 1.3.2 Results

The search yielded 21 published studies (Table 5) with an additional two re-analyses of raw data of some of these studies (Seralini *et al.*, 2007; de Vendomois *et al.*, 2009). The re-analyses concentrated only on blood, serum and urine test results. (These publications are not counted nor listed in the tables or figures since they are not original feeding studies). Eighteen (86%) of the 21 studies investigated crops that have been approved for human and/or animal consumption somewhere in the world (Table 4). These 18 studies investigated only nine of the 47 approved GM crops (19%) known to possess at least one of the traits of interest. No published rat-feeding studies could be found for the remaining 38 (81%) approved crops. Of all the 21 studies found, 12 (57%) generally assessed the long-term effect of GM feed on rat health (Wang *et al.*, 2002; Hammond *et al.*, 2004; Hammond *et al.*, 2006a; Hammond *et al.*, 2006b; Sakamoto *et al.*, 2007; Schrøder *et al.*, 2007; Healy *et al.*, 2008; Sakamoto *et al.*, 2008; Tutel'ian *et al.*, 2008; Tutel'ian *et al.*, 2010; Qi *et al.*, 2012; Seralini *et al.*, 2012), while seven (33%) examined specific outcomes - signs of allergic or immunological reactions (Teshima *et al.*, 2000; Kroghsbo *et al.*, 2008), effects of GM diet on blood, urine and liver (Tutel'ian *et al.*, 1999; Tutel'ian *et al.*, 2001), fate of the inserted DNA (Zhu *et al.*, 2004), comparison of GM soy versus conventional soy and its nutritional impact (Daleprane *et al.*, 2009), and the impact of a soy diet, be it GM or non-GM, on aortic wall remodelling (Daleprane *et al.*, 2010).

The majority of the studies found were published in the last decade (Figures 11 and 12). The earliest study was published in 1995, which was of a GM tomato that was probably never commercially grown (Noteborn *et al.*, 1995). The study investigated the effect of the insecticidal protein *Cry1Ab*, on its own or in the GM tomato, on various mammalian digestive systems. However, at the time of publication, the researchers had not yet performed an histopathological analysis of the effect of the GM crop on rat health.

The earliest published study on an approved crop was in 1999 (Tutel'ian *et al.*, 1999)(Figure 12), which was four years after that crop had been approved for human and animal consumption. This study only investigated the blood, urea and the liver of animals fed GM soy. The first study that generally assessed the long-term effect of GM feed on rat health was in 2002 (Wang *et al.*, 2002). It investigated a GM rice (KMD1) that is approved for commercial use only in China. This approval was granted seven years

after the Wang *et al.* (2002) study was published (Chen *et al.*, 2011). Two other studies also investigated this crop (Schrøder *et al.*, 2007; Kroghsbo *et al.*, 2008). Both of which were published prior to the approval. The remaining 16 (76%) published studies found in this review were published after the crops had been approved for human and/or animal consumption. Half of these were performed at least nine years after the approval was granted.

Five studies based their methodology on the Organization for Economic Cooperation and Development (OECD) guidelines for the testing of chemicals - OECD Guideline 408: Repeated dose 90 day oral toxicity study (OECD, 1981; Zlatkina *et al.*, 1990). Fourteen studies indicated that the digestive tract was investigated histopathologically, but no details were given as to what analyses were performed. The only details most often provided were that tissue samples were processed, paraffin embedded, and sections were cut and stained with haematoxylin and eosin (H&E). Sections were then assessed using light microscopy (LM). Seralini *et al.* (2012) indicated that sections were stained with HES, but failed to specify whether this abbreviation meant haematoxylin and eosin, haematoxylin eosin safran/saffron or haematoxylin erythrosine saffron stain. Seralini *et al.* (2012) also indicated that if any tumours were observed, they were processed for transmission electron microscopy (TEM). There was no mention if tumours were observed in the GI tract.

Six of the studies indicate that a pathologist or veterinary pathologist performed the histopathological analysis. Five studies provided some form of results of their analyses, whilst most limited their results section to a statement that overall there were no treatment-related or diagnostically-significant observations.

Overall, all the studies examining the GI tract concluded that there were no toxicological or pathological changes observed that could be related to feeding GM crops to rats.

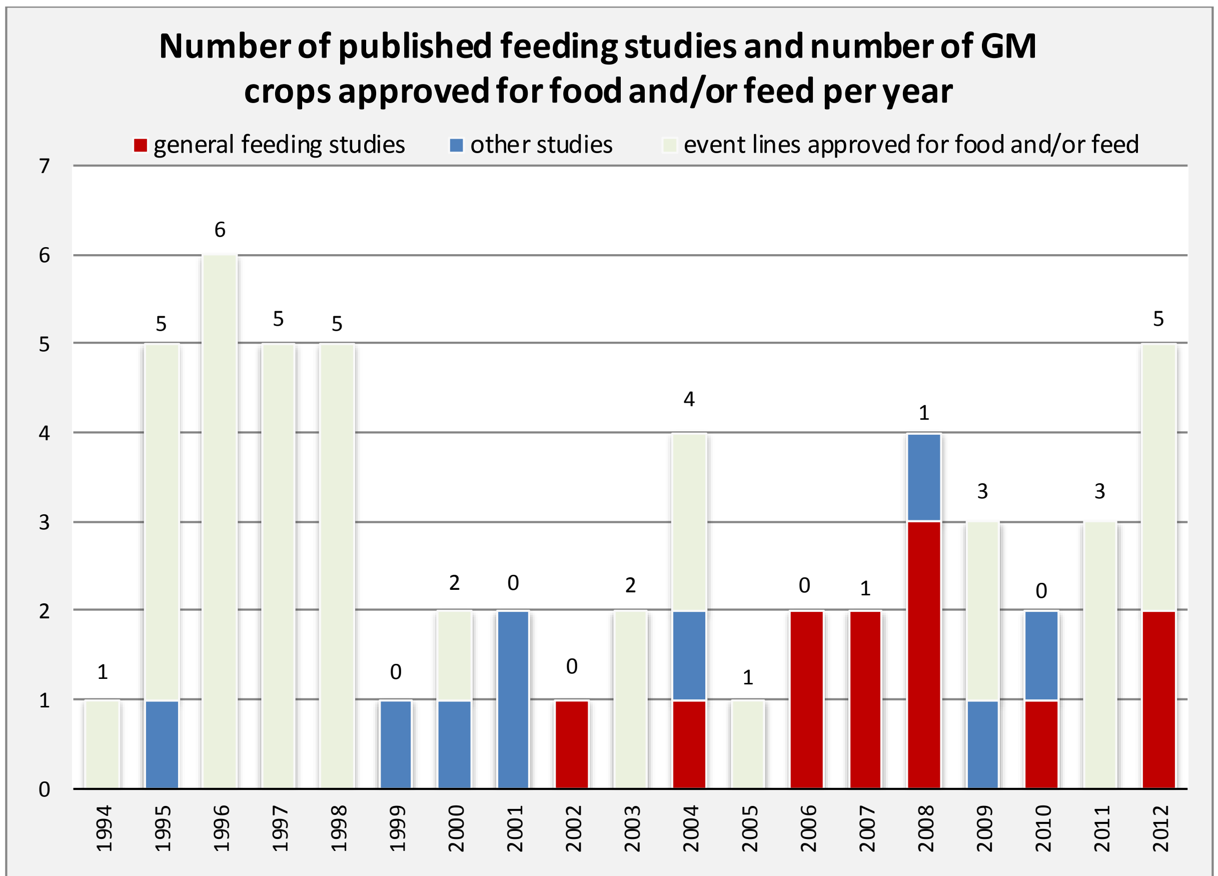



**Figure 14. Number of publications looking at the effects of feeding rats GM crops long-term and number of event lines approved each year for human and/or animal consumption.** Studies researched the effects of ingesting GM crops that possessed the traits for herbicide tolerance (via the EPSPS gene) and insect resistance (via the cry1Ab or cry3Bb1 genes). Studies investigating the general long-term effect of GM feed on rat health are indicated in red. Other studies investigating certain specific effect of GM feed on rats are indicated in blue. Cream-coloured bars indicate the number of GM crop event lines possessing the traits for herbicide tolerance (via the EPSPS gene) and insect resistance (via the cry1Ab or cry3Bb1 genes) that were approved each year somewhere in the world for human and/or animal consumption (number of approvals per year indicated above the bar). The year of approval for each event line is only counted once, being the first time the crop was approved somewhere in the world.

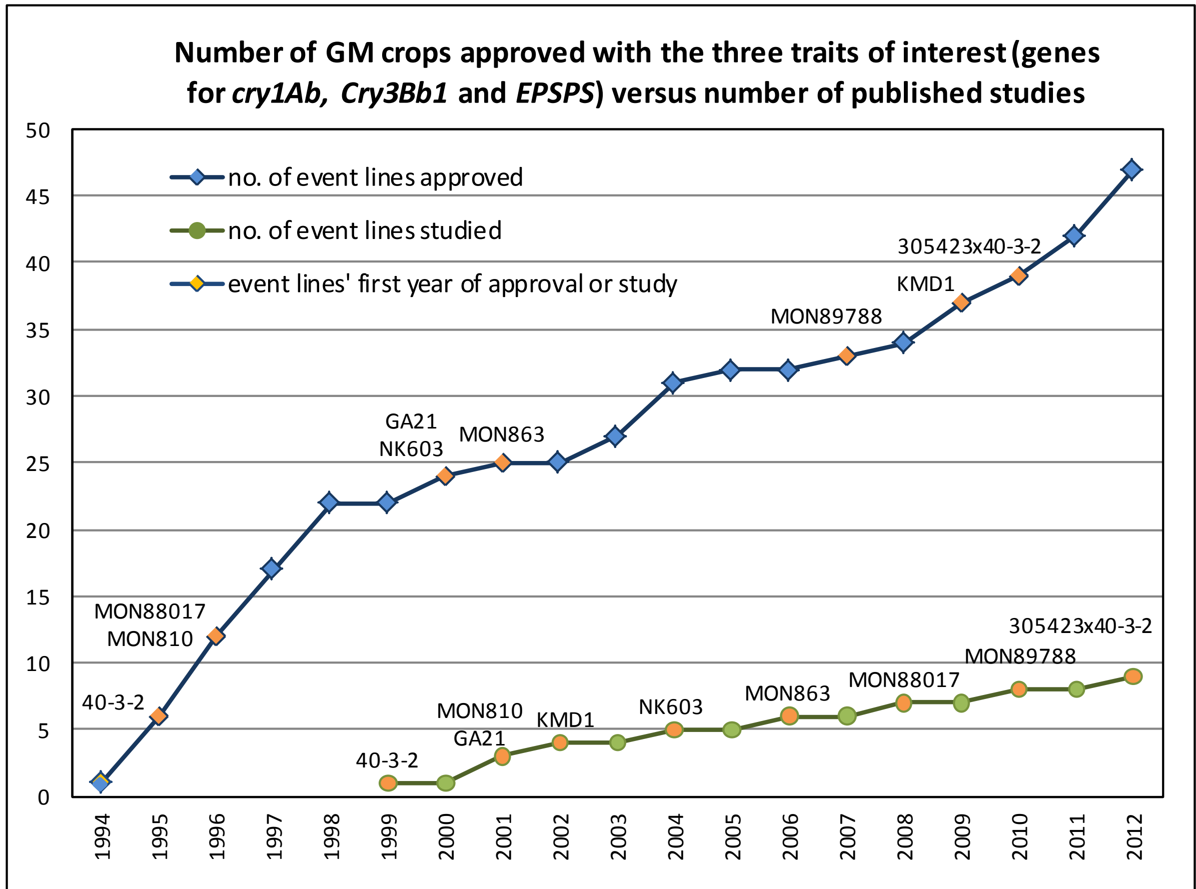



**Figure 15. The number of approved GM crops in the world for human and/or animal consumption (♦) and the number of approved GM crop lines with published studies investigating the effects of long-term feeding of these crops (●).** Each crop was counted once when the first study appeared (●) investigating that crop. The year of approval is the year that the crop was first approved somewhere in the world (♦).

**Table 5. Summary of published studies in order of trait and publication date**

| Study | GM component (event or crop name) | Duration | Purpose of study | No. of rats/ treatment group | Histopathology performed and analysis of GI tract | Histopathological results for GI tract | Feeding study guidelines |
|---|---|---|---|---|---|---|---|
| *EPSPS* | | | | | | | |
| Tutel'ian *et al.* 1999 | 1.25g/rat/day of GM soy (RR soy) | 5 months | To investigate blood, urea and liver of animals fed GM soy | Not stated | No histopathology performed | NA | |
| Teshima *et al.* 2000 | 30% GM soy | 15 weeks | Study of the immune system of rats and mice | 5 | Peyer's patches collected for histopathology, specific area of small intestine not mentioned. Histopathological examination of H&E stained sections assessed structure of crypt and composition of cells (especially goblet cells and intraepithelial lymphocytes) according to Kawabata (1996). However, Kawabata did not contain guidelines as to how this assessment should be performed and what observations would be considered to be abnormal. No other details were provided as to how the histopath. assessment/analysis was performed. | No difference of crypt structure or goblet cell frequency. Results for intraepithelial lymphocytes were not stated. No actual data of any analyses were shown.<br><br>Conclusion: No diagnostically significant abnormalities observed in mucosa of small intestine. | |
| Tutel'ian *et al.* 2001 | 3g/rat/day of GM corn (GA21) | 6 months | To investigate blood, urea and liver of animals fed GM corn GA21 or GM corn MON810 | Not stated | No histopathology performed | NA | |
| Zhu *et al.* 2004 | 30-90% GM soy (RR soy) | 13 weeks | Nutritional assessment and fate of DNA | 10 | Stomach and intestine collected for histopathology. Sections stained with H&E and examined by board-certified pathologist using LM. However, no details given as to what histopath. analyses were performed. | Collapse of jejunum villi was observed, but actual incidence, including incidence in treatment and/or non-treatment group is not reported. No actual data of any analyses were shown.<br><br>Conclusion: No treatment related differences seen. | |

**Table 5. (cont.) Summary of published studies in order of trait and publication date**

| Study | GM component (event or crop name) | Duration | Purpose of study | No. of rats/ treatment group | Histopathology performed and analysis of GI tract | Histopathological results for GI tract | Feeding study guidelines |
|---|---|---|---|---|---|---|---|
| *EPSPS (cont.)* | | | | | | | |
| **Hammond** *et al.* 2004 | 11-33% GM corn (NK603) | 13 weeks | General study to assess the effect of the GM corn on rat health | 20 | Stomach, duodenum, jejunum, ileum, colon, and rectum collected for histopathology. Sections stained with H&E and examined by a board-certified pathologist using LM. However, no details given as to what histopath. analyses were performed. | Incidence of microscopic findings was only listed for those tissues with an incidence of 2 or more findings. No mention of any GI tract observations.<br>Conclusion: According to the examining pathologist, appearance of tissue was within normal limits. Microscopic changes observed were those that are typically seen in rats of this age and strain. | Modified from OECD 408 (1981) |
| **Sakomoto** *et al.* 2007 | 30% GM soy (RR soy) | 26 and 52 weeks | General study to assess the effect of the GM soy on rat health | 10 | Stomach and intestines collected for histopathology. Sections stained with H&E. However, no details given as to what histopath. analyses were performed. The method used for the eosinophil and goblet cell counts in jejunum was described in a table caption. | Pathological findings showed no meaningful differences between rats fed GM or non-GM soybeans. Relatively detailed results given for histological findings including eosinophil and goblet cell counts for jejunum. Gastric gland, dilatation of slight grade was observed in all groups.<br>Conclusion: No obvious differences observed between GM and non-GM fed rats. | |
| **Sakomoto** *et al.* 2008 | 30% GM soy (RR soy) | 52 and 104 weeks | General study to assess the effect of the GM soy on rat health | 50 | Stomach and intestines collected for histopathology. Sections stained with H&E. However, no details given as to what histopath. analyses were performed. | Detailed results given of the incidence of neoplastic and non-neoplastic lesions observed. No incidence or increase in incidence of any specific type of neoplastic or non-neoplastic lesions in GM fed group in both genders and there were no lesions reported in the GI tract.<br>Conclusion: No meaningful differences between rats fed GM and non-GM soy. | |

**Table 5. (cont.) Summary of published studies in order of trait and publication date**

| Study | GM component (event or crop name) | Duration | Purpose of study | No. of rats/ treatment group | Histopathology performed and analysis of GI tract | Histopathological results for GI tract | Feeding study guidelines |
|---|---|---|---|---|---|---|---|
| *EPSPS (cont.)* | | | | | | | |
| **Delaprane *et al.* 2009** | 10% GM soy | 15 months (455 days) | Nutritional study – study of growth and haematology of rats on GM soy, non-GM soy or conventional diet | 10 | No histology performed | NA | |
| **Delaprane *et al.* 2010** | 10% GM soy | 15 months (455 days) | Health of aorta of rats on GM soy, non-GM soy or conventional diet | 10 | Histology of aorta | NA | |
| **Tutel'ian *et al.* 2010** | 38% GM soy (MON 89788) | 30 and 180 days | General study to assess the effect of the GM soy on rat health | 50 | Safety of the GM crop was examined as in (Tutel'ian *et al.* 2008). Review of macro- and microscopic examinations according to textbook guidelines (Lillie 1969). Morphometric analysis of small intestine and colon performed with aid of computer program AxioVision. No other information provided as to what other organs were collected and what histopath. analyses were performed. | Detailed results were given of morphometric analysis of small intestine, but which section of the small intestine these results pertain to, was not mentioned. No results given for morphometric analysis of colon.<br>Conclusion: Morphological analysis did not reveal toxic effect of GM soy. | |
| **Seralini *et al.* 2012** | 11%, 22% and 30% GM corn (NK603) | 2 years | General study to assess the effect of the GM corn on rat health | 10 | Oesophagus, stomach, duodenum, jejunum, ileum, Peyer's patches, and colon collected for histopathology. Sections stained with HES. However, no details of what histopath. analyses were performed. | Results for histopathological analysis of GI tract were not provided. | |
| **Qi *et al.* 2012** | 7.5%, 15% and 30% GM soy (305423x40-3-2) | 90 days | General study to assess the effect of the GM soy on rat health | 10 | Stomach, duodenum, jejunum, and ileum collected for histopathology. Sections stained with H&E and examined by a pathologist from the Chinese Academy of Medical Sciences. However, no details of what histopath. analyses were performed. | No observations or results listed for GI tract.<br>Conclusion: No test-substance related observations. | |

**Table 5. (cont.) Summary of published studies in order of trait and publication date**

| Study | GM component (event or crop name) | Duration | Purpose of study | No. of rats/ treatment group | Histopathology performed and analysis of GI tract | Histopathological results for GI tract | Feeding study guidelines |
|---|---|---|---|---|---|---|---|
| *Cry3Bb1 and EPSPS* | | | | | | | |
| **Healy *et al*. 2008** | 11-33% GM corn (MON88017) | 13 weeks | General study to assess the effect of the GM corn on rat health | 20 | Stomach, duodenum, jejunum, ileum, colon, and rectum collected for histopathology. Sections stained with H&E and examined by board-certified veterinary pathologist using LM. However, no details of what histopath. analyses were performed. | Results table provided with microscopic findings, however findings for tissues that had an incidence of 1/20 were not reported. No mention of any GI tract results/observations.<br>Conclusion: No test-article related lesions. | Modified from OECD 408 (1998) |
| **Tutel'ian *et al*. 2008** | 11g/rat/day of GM corn (MON88017) | 30 and 180 days | General study to assess the effect of the GM corn on rat health | Not stated | Review of macro- and microscopic examinations according to textbook guidelines (Lillie 1969) with the morphometric analyses performed on internal organs according to Avtandilov (1982; 1990) and Stefanov (1985). List of organs collected for hitopath. analysis not provided. | Detailed results are given of morphometric analysis of ileum. No mention of results for any other area of the GI tract.<br>Conclusion: Morphological analysis did not confirm any toxic effect of GM corn. | |
| *Cry3Bb1* | | | | | | | |
| **Hammond *et al*. 2006b** | 11-33% GM corn (MON863) | 90 days | General study to assess the effect of the GM corn on rat health | 20 | Stomach, duodenum, jejunum, ileum, colon, and rectum collected for histopathology. Histopath. examination was performed by a pathologist at Covance laboratories. However, no details provided as to what histopath. methods used including what stains were used and what analyses were performed. | Parasitism was observed in the rectum and glandular dilatation was observed in the stomach of animals in both GM and non-GM groups. The severity/degree of the parasitism or glandular dilatation was not mentioned.<br>Conclusion: Differences if seen were not considered to be test article related. | Modified from OECD 408 (1981) |
| *Cry1Ab* | | | | | | | |
| **Noteborn *et al*. 1995** | 10% GM tomato (RLE13-0009) | 91 days | Study to see if the Cry1Ab protein on its own or in the GM tomato, acts on mammals in a similar way as on target insect guts | 12 | Histological analysis was still in progress at time of publication. | NA | |

**Table 5. (cont.) Summary of published studies in order of trait and publication date**

| Study | GM component (event or crop name) | Duration | Purpose of study | No. of rats/ treatment group | Histopathology performed and analysis of GI tract | Histopathological results for GI tract | Feeding study guidelines |
|---|---|---|---|---|---|---|---|
| *Cry1Ab (cont.)* | | | | | | | |
| Tutel'ian *et al.* 2001 | 3g/rat/day GM corn (MON810) | 6 months | To investigate blood, urea and liver of animals fed GM corn GA21 or GM corn MON 810 | Not stated | No histopathology performed | NA | |
| Wang *et al.* 2002 | 19-64% GM rice (KMD1) | 14 weeks (90 days) | General study to assess the effect of the GM rice on rat health | 20 | Stomach, duodenum, jejunum, ileum, colon, and rectum collected for histopathology. Sections were stained with H&E. However, no details of what histopath. analyses were performed. | No mention of any GI tract observations.<br><br>Conclusion: No toxicologically relevant changes. | |
| Hammond *et al.* 2006a | 11-33% GM corn (MON810) | 90 days | General study to assess the effect of the GM rice on rat health | 20 | Methods section indicates that histopathological examination was performed in the same manner as Hammond *et al.* (2004). No other details provided. | No mention of any GI tract observations.<br><br>Conclusion: No treatment-related observations. | Modified from OECD 408 (1981) |
| Schrøder *et al.* 2007 | 60% GM rice (KMD1) | 90 days | General study to assess the effect of the GM rice on rat health | 16 (10 used in histopath. examination) | Stomach (fore and glandular), duodenum, jejunum, ileum, caecum, colon, and rectum collected for histopathology. Sections stained with H&E. "Main focus of histopathological examination was on intestinal tract and related organs", but no specific details given as to the type of analyses performed. | No mention of any pathological findings in the GI tract.<br><br>Conclusion: No dose-related changes were observed in intestinal tract and related organs. | OECD 408 (1981) with modifications[a] |
| Kroghsbo *et al.* 2008 | 60% GM rice (KMD1) | 28 and 90 day | Immuno study | 10 | No histopathology performed | NA | |

[a] Modifications from Consultation Meeting of Experts on Sub-chronic and Chronic Toxicity Testing (1995)

### 1.3.3 Discussion

The digestive tract is the first site of contact with the body of any ingested food. Therefore, if a novel food is toxic to the body, signs of toxicity may be present in the GI tract. Often these changes may only be detectable by histopathological analysis and not macroscopic observations (Morini and Grandi, 2010).

#### *Review of the methods*

While 14 of the 21 studies reviewed (67%) indicated that organs of the digestive tract were collected for histopathological examination, none of the Methods sections in these publications included any details as to the nature of the histopathological examination. Several of the studies (Hammond *et al.*, 2004; Zhu *et al.*, 2004; Hammond *et al.*, 2006a; Hammond *et al.*, 2006b; Healy *et al.*, 2008; Qi *et al.*, 2012) simply stated that a pathologist or veterinary pathologist performed the analysis, but no mention was given as to what these analyses entailed, for example what pathological parameters were used or what was measured and why. The exception appears to be a study by Teshima *et al.* (2000) who stated that the morphology of the small intestine mucosa was assessed, in particular the composition of goblet cells and intraepithelial lymphocytes. According to the authors, the analysis was based on a chapter in an immunotoxicology textbook (Kawabata, 1996). However, that chapter did not mention the purpose or even how the investigation of the small intestine should appear. In particular, it did not include the definition of what constitutes abnormal or diseased, such as, what changes in goblet cell population would indicate a pathology.

A paper that appears to be well-structured and thorough was the Tutel'ian *et al.* (2008) study published in Russian. The Methods section clearly stated that the morphometric analysis of the internal organs was conducted according to textbook guidelines (Avtandilov, 1982; Avtandilov, 1990) and results were compared according to guidelines set out by Stefanov (1985). The two Russian textbooks (Avtandilov, 1982; Avtandilov, 1990) are manuals on how to conduct quantitative research to obtain a meaningful assessment of morphological changes. In other words, the Tutel'ian *et al.* (2008) study appears to be thorough and well set out, especially since detailed results are provided for the analyses. However, the publication lacks basic information. It does not specify the number of rats used in the study and it does not list which organs were collected for the histopathological analyses. Results seem to imply that the ileum was

the only section of the GI tract to be analysed. A more thorough study would have investigated other sections of the GI tract to more accurately ensure that the GM crop did not have any adverse effects.

Another Russian study (Tutel'ian *et al.*, 2010) also appears to be properly conducted. Its safety assessment is based on the Tutel'ian *et al.* (2008) study, which implies that the same rigorous morphometric analysis was also utilised. However, even this publication lacks key information. For example, the paper indicated that the morphometric analysis was conducted on the small intestine and colon, but results were only reported for the small intestine. In addition, the publication does not specify which section of the small intestine these results pertain to. This lack of detail in both Russian papers makes it difficult to determine the veracity of the results. It also makes it difficult to reproduce and further the study or to compare these studies to others. Indeed, in all the published papers, a lack of uniformity in the analytical approach as well as documentation of the methods and results makes any comparison or assessment of adequacy or inadequacy of the studies difficult.

### *Selection of endpoints*

A major flaw in all the studies reviewed was the lack of any definition of toxicity or signs of pathology. Of all the studies generally assessing rat health on a GM diet, not one explained how the study would adequately show that the crop is safe for human and/or animal consumption. Furthermore, all the studies reviewed failed to justify or give reason for the choice of methods used. Yet, most studies conclude that the investigation did not reveal any meaningful differences between animals fed the GM or non-GM feed. One study even stated that *"since no meaningful differences were observed, no further microscopic examinations were deemed necessary"* (Hammond *et al.*, 2004). However, the absence of meaningful differences in a preliminary investigation does not mean that further analysis would not find meaningful differences. In addition, the authors did not support this statement with proof since they provided few details as to what their microscopic examinations entailed or found. Therefore, they give very little evidence that their study adequately assessed the safety of consuming the GM crop.

Another common remark in these publications was that all changes observed were not diagnostically significant, were within the normal range, or are common to this strain and age of rat. The six studies that made this remark gave little evidence to support this conclusion (Teshima *et al.*, 2000; Hammond *et al.*, 2004; Hammond *et al.*,

2006a; Hammond *et al.*, 2006b; Healy *et al.*, 2008; Qi *et al.*, 2012). Most gave no evidence at all. For example, Qi *et al.* (2012) referenced a study by Tang *et al.* (2012) to support their notion that *"microscopic observations occurred spontaneously in Sprague-Dawley rats of this age."* However, the referenced study made no mention of microscopic observations occurring spontaneously and the study did not even use Sprague-Dawley rats.

A very common statement found in the reviewed studies was that since the lesions or changes were observed in both groups, they were not deemed to be diet-related (Wang *et al.*, 2002; Sakamoto *et al.*, 2007; Healy *et al.*, 2008; Sakamoto *et al.*, 2008). For example, in two studies (Hammond *et al.*, 2006b; Sakamoto *et al.*, 2007), there was a brief mention of gastric gland dilatations being observed in both the GM and non-GM fed groups. Gland dilatations can occur in aged rats (Frantz *et al.*, 1991), but they can also be a pathological occurrence for example in alendronate-induced injury (Şener *et al.*, 2004), ulcer healing (Tarnawski *et al.*, 1991) or underlying neoplastic lesions (Frantz *et al.*, 1991). In these pathologies, the dilatations are large, they may sometimes extend into the submucosa and they may become dysplastic (Kikuchi *et al.*, 2010). In the two publications (Hammond *et al.*, 2006b; Sakamoto *et al.*, 2007), no specific details are mentioned, for example, the size of these dilatations, whether the treatment group had larger dilatations than the other group, whether the affected area was more widespread in one group than the other, or if the cells lining the dilatations had a specific staining property or shape common only to one group. If a pathology is seen, regardless of whether it occurs in both groups, further analysis should be performed to determine the nature of the occurrence and to completely rule-out disease. Furthermore, while the incidence of a pathology may be equal in both groups, the degree or severity may vary. Therefore, it is always important to record and report the severity of a pathology. For example, an animal may be prone to a certain pathology (e.g. Sprague-Dawley rats are known to spontaneously develop certain neoplastic lesions (Chandra *et al.*, 1992; Kaspareit and Rittinghausen, 1999), but it is possible that the GM component may increase the severity or risk of this development. In addition, the type of crop fed may cause a pathology. For example, soy is known to have adverse effects on bone and the digestive tract (Godlewski *et al.*, 2006; Piastowska-Ciesielska and Gralak, 2010). Therefore, feeding soy would naturally cause changes to the gut, but the GM component may increase the severity of these changes. Hence, detailed histopathological and morphometric analyses are required before stating that the incidences of a pathology or

lesion are equal between the GM and non-GM fed groups. Furthermore, isolation of the pathology causing factor of the plant and the relationship of that to the genetic modification should be investigated to confirm the finding. In other words, it is not sufficient to say that the GM food is safe if incidences of a pathology or lesion are equal in both groups. Further testing should be carried out to completely rule out the GM component's involvement in the development of the pathological incidence(s).

Another common conclusion made was that no changes were seen that could be considered treatment, test-article, or test-substance related, or toxicologically relevant. However, the six studies that made this conclusion did not identify treatment-related or toxicologically relevant changes. (Wang *et al.*, 2002; Zhu *et al.*, 2004; Hammond *et al.*, 2006a; Hammond *et al.*, 2006b; Healy *et al.*, 2008; Qi *et al.*, 2012). Therefore, they did not provide clearly defined criteria by which to judge if a given tissue was normal or not, and if abnormal, whether the abnormality was toxicologically relevant and/or treatment-related. Some food regulators, such as Food Standards Australia New Zealand (FSANZ, 2007) describe GM food as novel food. In other words, they recognise that no definition yet exists for toxicologically relevant or test-substance related changes. However, by applying the test for substantial equivalence, food regulators argue that an existing compound or plant of known toxicity can be used to evaluate or predict the action of a novel compound or food such as a GM crop (Kuiper and Kleter, 2003; FSANZ, 2007). Consequently, the published studies should have been able to identify the test-article or toxin, and consequently, the evidence that their results showed no treatment-related or toxicologically relevant changes.

An existing compound may not be able to predict the action of a GM crop on animal health. Further investigation may be necessary. Known toxicity of single components of the GM crop may not define an overall toxicity of the entire crop. It is not clear whether the test for substantial equivalence is sufficient because it does not take into account the changes that could arise from the transformation process: 1) through the random insertion of the genes, 2) through the genetic alterations made to the transferred genes as a result of the transformation process, 3) through the genetic alterations made to the plant as a result of the transformation process (Wilson *et al.*, 2006), 4) through the insertion of several traits or genes into one crop or 5) through the alteration made to the genes encoding the desired trait prior to the transformation.

### *Lack of transparency in results*

Several of the reviewed publications do not adequately report their results. Some do not even provide any results (Table 2). For example, the paper by Zhu *et al.* (2004) not only lacks a detailed methods section, but limits its histopathological results to a simple statement that *"although some slight lesions (such as slightly dilated alveolus cavity, pelvic dilation of the kidneys, slight disconnection of myocardial fibre and collapse of jejunum villi) occurred in rats examined, they were not treatment related*." Such a statement could imply that other changes may have been observed, but are not reported. Furthermore, this study does not mention the incidence or severity of any histopathological changes, including whether they occurred in the treatment or non-treatment group. For example, they do not state how many rats showed collapsed jejunum villi and whether these were more prevalent in one group or whether the collapsed villi were more severe in one group. A lack of transparency in results does not allow other researchers to judge whether a certain finding is pathologically relevant. Another paper (Tutel'ian *et al.*, 2010) indicated that they had performed a morphometric analysis of the small and large intestines, but they did not report the colon results. A lack of transparency is also evident in two other studies: 1) Hammond *et al.* (2004) report the findings from *"only those tissues with an incidence of 2 or more findings"*; 2) Healy *et al.* (2008) state that *"findings in other tissues with an incidence of 1/20 are not reported."* Neither of the papers provide a full account of pathologies present. Furthermore, Hammond *et al.* (2004) do not clearly state whether "incidence" pertains to two incidences per tissue or per rat. Such a lack of information does not ensure that the study and its results are reproducible or even comparable.

### *Relevance of OECD guidelines in the evaluation of the safety of consuming GM crops*

Five of the published studies indicate that OECD 408 guidelines were used to assist in planning the study (Table 5). The guidelines provide details on how such a feeding study should be conducted, including information on sample size, duration etc. However, the guidelines do not specify the histopathological analysis that should be performed. They do not specify what morphometric quantitational analyses should be done. Therefore, there is a question as to whether these OECD guidelines are relevant to investigation of the safety of consuming GM crops. While they may be used as a starting point, it is our view that guidelines should be established specifically for GM crops. Since GM food is considered to be a novel food, the guidelines should list details for a thorough

investigation that includes an histopathological analysis of the gut and other organs. In other models of GI tract damage, such as mucositis (Howarth *et al.*, 1996; Sukhotnik *et al.*, 2008; Logan *et al.*, 2009), neonatal adjustment of piglets to normal diet (Strzalkowski *et al.*, 2007; Godlewski *et al.*, 2009), or in gastric biopsies (Fenoglio-Preiser, 1998; Staibano *et al.*, 2002), the analytical method is detailed and specific, listing the changes that need to be investigated and the microscopic techniques and morphometric analyses that need to be used. For example, mitosis, apoptosis and autophagy are known to be good indicators of mucosal regeneration in the small intestine following injury. Therefore, immunohistochemistry with in-tissue cytometry looking at the expression of markers for mitosis (Ki67), apoptosis (caspase 3) and autophagy (MAP I LC3) can be used to assess mucosal regeneration (Godlewski *et al.* 2009). In mucositis-induced models, the investigation of the degree of damage, not only regularly requires detailed quantitative histological analyses to be conducted (Howarth *et al.*, 1996; Sukhotnik *et al.*, 2008; Logan *et al.*, 2009), but also immunohistochemistry for markers of apoptosis (caspase 3), cell proliferation (BrdU) (Sukhotnik *et al.*, 2008), and pro-inflammatory cytokines (such as TNF, IL-1β and IL-6) (Logan *et al.*, 2009). Such rigorous analyses allow for a more precise assessment of possible pathological changes, while at the same time decreasing the chance of subtle changes being overlooked. Therefore, it is our view that in the investigation of the safety of GM crops on animal and human health, such a rigorous and in-depth approach should also be implemented.

### *Have enough studies been conducted to adequately state that GM crops are safe for human and animal consumption?*

Genetically modified crops have been approved for human and animal consumption for nearly 20 years (Clive and Krattiger, 1996) yet the debate about their safety continues. Fifty-three crops are known to possess at least one of the genes investigated in this review (herbicide tolerance via the EPSPS gene and insect resistance via the cry1Ab or cry3Bb1 genes). Forty-seven of these crops have been approved for animal and/or human consumption and yet only nine of these crops (19%) have published studies investigating their toxicity (Table 4). Of greater concern is that for eight of these crops, publications appeared after the crop had been approved for human and/or animal consumption. We understand that other studies may exist that are commercial in confidence, but these studies are not accessible to the scientific community. Other than the few studies mentioned in the EFSA reports, where histopathological results

were not reported, our review of the published literature wasn't able to identify or locate any reported safety evaluations performed on rats on these eight crops prior to their approval. Our literature review also did not identify or locate published reports on rats for the remaining 38 crops.

The present review limited the search to only include feeding studies done on rats so that the results may be comparable. It is possible that more studies may be found if the search were to be extended to other animals. However, based on what has been found for rat studies, it is unlikely that any additional studies would involve a thorough safety investigation and a detailed report of all of the 47 approved GM crops possessing one or more of the three traits. Moreover, the rat model is the accepted OECD standard for toxicological studies of this type (OECD, 2008b).

While the safety of a GM crop is primarily and sometimes solely evaluated by government food regulators using the test for substantial equivalence, this is likely to be inadequate to fully assess the safety of the crop for reasons stated above. Animal feeding studies provide a more thorough method of investigating unintended effects of the GM process or the unintended effects of ingesting GM crop components. Animal feeding studies can identify target organs as well as predict the chronic toxic effect of an ingested compound (OECD, 2008b).

### 1.3.4 Conclusion

The evidence reviewed here demonstrates an incomplete picture regarding the toxicity (and safety) of GM crops consumed by humans and animals. The majority of studies reviewed lacked a unified approach and transparency in their methodology and results, making it impossible to properly review or repeat these studies. Furthermore, such lack of detail makes it difficult to generate evidence-based guidelines to aid in the delivery of an optimum safety assessment process for GM crops for animal and human consumption.

When considering how a better risk assessment could be done, it is important to consider systems established for other novel substances that may generate unintended effects. For example, the registration of pharmaceutical products requires an examination of both benefits and risks associated with their use and a complete assessment of those benefits and risks to establish whether the products are appropriate for general use at a range of doses. We argue that each GM crop should be

assessed using similar methods, where a GM crop is tested in the form and at the rates it will be consumed by animals and people.

Whilst this provides for an effective general approach, there are additional issues for assessing GM crops that need to be taken into account. For example, the process of developing GM crops may generate unintended effects. Furthermore, the plant developed is a novel entity with genes, regulatory sequences and proteins that interact in complex ways. Therefore, the resultant plant should be assessed as a whole so that any pleiotropic effects can also be assessed. As a result, long-term animal feeding studies should be included in risk assessments of GM crops, together with thorough histopathological investigations using a variety of methods to better detect subtle changes or the beginning or presence of pathologies. Such robust and detailed studies will then make it possible to put evidence-based guidelines in place, which will substantially help to determine the safety of GM crops for human and animal consumption.

## 1.4 Update to the published Critical Review

### 1.4.1 Materials and Methods

As previously described (Section 1.3.1), a search for publications was performed using the crop/event names listed in Table 4 and Table 6. The search used PubMed, Google Scholar and Embase to find studies that were published before May 2015. Crops producing the *Cry1Ab* protein via the insertion of cry1A.105 gene were added to the search (Table 6).

**Table 6. The 2015 literature search.** List of GM crop event names that were included and/or added to the search, since the 2013 search. Year of approval for animal and/or human consumption of each event name and number of publications found per event.

| Crop type: | Event name (other name and/or code name)[a, b]<br>**Roundup Ready (RR) or glyphosate-tolerant crops:**<br>***Crops containing EPSPS genes*** | Number of published long-term rat feeding studies | Year approved for food and/or feed[c] |
|---|---|---|---|
| *Corn/Maize* | VCO-01981-5 | 0 | 2013 |
| *Soybean* | DAS-44406-6 | 0 | 2013 |
| | MON87712 | 0 | 2013 |
| | **Insect resistant or *Bt* crops:**<br>***Crops containing cry3Bb1 and EPSPS genes*** | | |
| *Corn/Maize* | MON87411 | 0 | 2014 |
| | ***Crops containing cry1Ab genes*** | | |
| | mfb-MH86 | 1 | no approval data[d] |
| | ***Crops containing cry1Ab and EPSPS*** | | |
| *Corn/Maize* | Ajeeb YG | 2[e] | approval revoked[f] |
| | ***Crops containing cry1A.105 genes*** | | |
| *Corn/Maize* | MON89034 (MON-89034-3) | 0 | 2007 |
| *Soybean* | MON87751 (MON-887751-7) | 0 | 2014 |
| | *Number of GM crop event lines studied* | 2 | 0 |
| | *Number of GM crop event lines* | 8 | 6 |
| | *Number of publications found* | 3 | 0 |
| | **Crops from 2013 search (Table 4) that have new or additional published studies:** | | |
| *Corn/Maize* | MON810 (MON-00810-6) | 1 | 1996 |
| *Rice* | GM Shanyou 63 | 1[g] | 2009 |
| | Hauhui-1/TT51-1 | 1[g] | cultivation only (2009)[h] |
| | glyphosate-tolerant[i] | 1 | NA |
| **Results for 2015 search:** | | | |
| | *Number of GM crop event lines studied* | 5 | 2 |
| | *Number of GM crop event lines* | 11 | 8 |
| | *Number of publications found* | 7 | 2 |
| **Combined results from 2013 and 2015 searches[j]:** | | | |
| | *Total Number of GM crop event lines studied* | 14 | 13 |
| | *Total number of GM crop event lines* | 61 | 53 |
| | *Total number of published studies found* | 28 | 21 |

[a] Each line contains one crop. Succeeding names are the other names given to the crop event name and/or the crop's code name.

[b] GM crop hybrids are not listed in the table, unless the crop is listed in databases as a single event name.

[c] The year that the crop was first approved somewhere in the world for human and/or animal consumption.

[d] Crop produced in China. No specific approval data has been made public for this crop.

[e] Another publication, Gab-Alla *et al.* 2012 reports the biochemical changes in the same feeding study as described by El-Shamei *et al.* 2012. Therefore both publications were counted as one published study.

[f] First approval was given in 2008; however, the local government revoked the approval (Burnett, 2014). No new approval information was found.

[g] Previously there was no published rat feeding study for this crop.

[h] According to ISAAA (2013), this crop is approved for cultivation only; however, they add that there are no official public documents available. This could mean that the crop is approved for human and/or animal consumption.

[i] The GM crop or event name was not listed in the publication. The GM crop studied contained the EPSPS CP4 gene, which confers glyphosate tolerance. This was not counted as a separate GM crop event line in the final counts.

[j] Results include the results from 2013 study (Table 4).

### 1.4.2 Results

Six crops have been approved since the 2013 search (Table 6). No published studies, investigating these crops, were found. The 2015 search yielded seven published studies (Tables 6 and 7), three of which investigate two newly-developed crops that have yet to be approved (El-Shamei *et al.*, 2012; Abdo *et al.*, 2014; Song *et al.*, 2015). The two newly-developed GM crops were Mfb-MH86 rice from China, and Ajeeb YG, an Egyptian variety of corn cross-bred to contain the MON810 gene cassette. There were four published studies (Wang *et al.*, 2013a; Wang *et al.*, 2013b; Zeljenkova *et al.*, 2014; Oraby *et al.*, 2015) investigating crops that were listed in the previous search (Table 4). Two of the crops (GM Shanyou63 rice and Huahui-1/TT51-1 rice) had no previous publications.

Of the six studies found (Table 7), three were general feeding studies that assessed the long-term effect of GM feed on rat health (El-Shamei *et al.*, 2012; Zeljenkova *et al.*, 2014; Song *et al.*, 2015). Two of these were for two crops that were not part of the 2013 search (Tables 6 and 7) (El-Shamei *et al.*, 2012; Song *et al.*, 2015). The three studies that were not general feeding studies, investigated the effect of GM rice on the haematology and enzyme activity of certain organs in female rats (Wang *et al.*, 2013a), the biochemistry and liver histopathology of two generations of rats on a GM corn diet (Abdo *et al.*, 2014), or investigated the effects of feeding rats a GM diet containing commercially available feed with GM corn and GM soy, both of unknown traits/event lines (Oraby *et al.*, 2015).

One published study (Zeljenkova *et al.*, 2014) based their feeding study on OECD Guidelines 408, for conducting 90 day oral toxicity study in rodents (OECD, 1998), as well as, the EFSA guidance on conducting repeated-dose 90 day oral toxicity study in rodents on whole food/feed (EFSA, 2013). They also indicated that their raw data was available online (www.cadima.info).

Three out of the six studies, indicated that the GI tract was investigated (Table 7). All six studies indicated that tissue sections were stained with H&E and one study (Oraby *et al.*, 2015) had an additional stain, bromophenol for measuring protein content in tissue.

One study (Wang *et al.*, 2013a) indicated that a senior pathologist, with the assistance of a trained team, performed the necropsy and that the National Centre for Food Safety Risk Assessment (Beijing, China) performed the histopathological assessment. This publication provided no results of their analyses, and limited their results section to a statement that no group-related histopathology changes were

observed in several tissues. The stomach and small intestine were collected, but no results were presented.

Three studies out of the six (El-Shamei *et al.*, 2012; Abdo *et al.*, 2014; Oraby *et al.*, 2015), found adverse effects associated with the GM diet. These effects were primarily observed in the blood biochemical analyses, and histopathology of the liver. Two of the studies (El-Shamei *et al.*, 2012; *Abdo et al.*, 2014) also reported changes in the histopathology of the kidney and testis. One of the studies investigated the GI tract and found changes in the small intestine of GM-fed animals (Table 7) (El-Shamei *et al.*, 2012); however, the section of the small intestine was not identified. The El-Shamei *et al.* (2012) and Abdo *et al.* (2014) studies investigated the AjeebxMON810 corn hybrid that contains the cry1Ab genes. This crop had been approved in 2008; however, the government revoked the approval. The El-Shamei *et al.* (2012) study investigated GM soy and GM corn of unknown event-line that contained the CaMVP-35S and EPSPS genes. No approval information could be established because the event line is unknown. Two out of the three studies that reported adverse effects (El-Shamei *et al.,*2012; Oraby *et al.*, 2015) concluded that the GM crop investigated is not safe and needs further study.

Combined results from 2013 and 2015 searches revealed that 13 out of the 53 approved crops have been studied (25%). No published rat-feeding studies could be found for the remaining 40 (75%) approved crops.

**Table 7. Update to Critical Review.** Summary of published studies in order of trait and publication date

| Study | GM component (event or crop name) | Duration | Purpose of study | No. of rats/ treatment group | Histopathology performed and analysis of GI tract | Histopathological results for GI tract | Feeding study guidelines |
|---|---|---|---|---|---|---|---|
| *EPSPS* | | | | | | | |
| Oraby *et al.* 2015 | Specific crop event name unknown. Feed contained GM corn and soy, which was tested for the presence of EPSPS CP4 and CaMV-promoter genes | 30, 60 and 90 days | Post-marketing biosafety assessment of GM food products using chronic toxicity study | 10 | Liver, kidney and testis collected for histopathology. Sections were stained with H&E. Hitopath. examination included measuring protein content in tissue stained with bromophenol.<br>GI tract was not investigated. | NA | |
| *Cry1Ab* | | | | | | | |
| Wang *et al.* 2013a | GM Shanyou63 rice (containing 0.122ng/g of *Cry1Ab* protein) | 30 and 90 days | To evaluate the effect of the GM rice on haematology and enzyme activity of certain organs in female rats | 10 | Brain, kidney spleen, marrow collected for histopathology.<br>GI tract was not investigated. | NA | |
| Wang *et al.* 2013b | 60% TT51 rice | 90 days | To evaluate the effects of the GM rice on the reproductive system of male rats | 8 | Brain, heart, liver, kidney, spleen, thymus, and testes collected for histopathology. Sections stained with H&E. Histopath. examination was conducted at the National Centre for Food Safety Risk Assessment (Beijing, China).<br>GI tract was not investigated. | NA | |

**Table 7. (cont.) Update to Critical Review**

| Study | GM component (event or crop name) | Duration | Purpose of study | No. of rats/ treatment group | Histopathology performed and analysis of GI tract | Histopathological results for GI tract | Feeding study guidelines |
|---|---|---|---|---|---|---|---|
| *EPSPS (cont.)* | | | | | | | |
| Song *et al.* 2015 | 17.5%, 35% and 70% mfb-MH86 rice | 90 days | General feeding study to assess the effect of the GM rice on rat health | 10 | Anatomic pathological analysis was conducted only for high dose animals. Brain, liver, spleen, heart, lungs, thymus, kidneys, adrenals, testes or ovaries, thyroid, stomach and small intestine sections collected for histopathology. Sections were stained with H&E. No details of what histopath. analyses were performed and what section(s) of the small intestine was examined. | No observations or results listed for GI tract.<br><br>Conclusion: No atypical or group-related histopath. observations were present. | |
| *Cry1Ab and EPSPS* | | | | | | | |
| El-Shamei *et al.* 2012 | 30% Ajeeb YG corn (Ajeebx MON810) | 45 and 91 days | General feeding study to assess the effect of the GM corn on rat health | 10 | Liver, kidneys, testes, spleen and small intestine collected for histopathology. Sections were stained with H&E. No details of what histopath. analyses were performed and what section(s) of the small intestine was examined. | At 45 days, hyperplasia and hyperactivation of mucous secretory glands, and necrosis of intestinal villi. At 91 days, shortening of villi and leucocytic cell infiltration in lamina propria.<br><br>Conclusion: The present work demonstrates that GM corn intake has influence on the histopath. features of liver, kidney, testis, spleen and small intestine. | |
| Zeljenkova *et al.* 2014 | 33% corn in feed with GM corn (MON 810) either at 33% or 11%. In the 11% GM corn dose, the remainder 22% of corn was of the parental line. | 90 days | General feeding study to assess the effect of two varieties of the GM corn on rat health | 16 | Stomach, small and large intestines from animals from high-dose group H&E light microscopic examination of the tissue structure. No details of what histopath. analyses were performed and what section(s) of the small intestine was examined. | Study 1. Small intestine of both GM (1/16 rats) and non-GM (2/16 rats) had lymphoepithelial granulomas. Study 2. No observations or results listed for GI tract.<br><br>Conclusion: The results obtained show that the MON810 maize at a level of up to 33 % in the diet did not induce adverse effects in male and female rats after sub-chronic exposure, independently of the two different genetic backgrounds of the event. | EFSA Guidance on conducting repeated dose 90-day oral toxicity study in rodents on whole food/ feed (2011); and OECD 408 Guidelines (1998) |

**Table 7. (cont.) Update to Critical Review**

| Study | GM component (event or crop name) | Duration | Purpose of study | No. of rats/ treatment group | Histopathology performed and analysis of GI tract | Histopathological results for GI tract | Feeding study guidelines |
|---|---|---|---|---|---|---|---|
| *Cry1Ab and EPSPS (cont.)* | | | | | | | |
| **Abdo *et al.* 2014** | 30% Ajeeb YG corn (Ajeeb x MON810) | 1.5 and 3 months | To evaluate the effect of GM corn on biochemistry and liver histopathology in two generations of rats | 3 | Liver, kidney, spleen, and heart collected for histopathology. Sections were stained with H&E. GI tract was not investigated. | NA | |

### 1.4.3 Discussion

The number and quality of the long-term feeding studies investigating the effect of GM crops on rat health have not changed significantly since the publication of the Critical Review (Zdziarski *et al.* 2014). There are still no published long-term rat feeding studies for 40 (75%) of the approved crops. In addition, it is still common to find studies with no explanation of what analyses were performed and with little or no evidence to support their conclusion that diet-related changes were not observed. One study (Wang *et al.*, 2013b), however, confirmed that their study "*cannot exclude the possibility that the Cry1Ab protein may have effects on the other untested factors in haematology and organs of the Swiss rat*." This lack of uniformity and systematic approach to the investigation of GM crop safety, makes it is difficult to compare studies and establish whether the effects are confined to only certain GM crops or GM traits. If all GM crops, prior to approval, were to be assessed via long-term animal feeding trials and the tissues were to be assessed morphometrically in a systematic and reproducible manner, information data bases could be established for comparisons in order to establish regulations for the safety evaluations of GM crops.

#### *Transparency of methods and results*

The original Critical Review (Zdziarski *et al.* 2014) concluded that there was a marked absence in the description of the methodologies used for their histopathological investigations and in the results published. The update to the Critical Review showed little improvement with many publications lacking details in their methodology and/or results (Table 7). Of the three studies that included a GI tract histopathological investigation (El-Shamei *et al.*, 2012; Zeljenkova *et al.*, 2014; Song *et al.*, 2015), none gave any details as to what histopathological analyses were performed. All three publications indicated that the small intestine was investigated, but all three failed to include, which specific region this investigation referred to. In addition, in one of the studies (El-Shamei *et al,.* 2012) the light micrographs that were included as evidence of adverse effects were of poor quality – the magnification was not high enough to see the described features, and/or the tissue sections were striated indicating that a blunt knife was used to cut the sections.

### *New regulation concerning the approval of GM crops in the European Union*

In 2013, the European Union implemented new regulations in the process of approving GM crops for food/feed (Implementing Regulation (EU), 2013). These new changes include, a mandatory 90 day rat feeding trial for the evaluation of the safety of single-stacked GM crops prior to their approval. It also requires the raw data to be made publically available. The feeding study guidelines, however, do not specify the specific histopathological analyses that should be performed. In fact, they closely resemble the OECD guidelines discussed in Section. 1.3.3. The lack of detailed guidelines will lead to the accumulation of data that may not be comparable or reproducible, thus will not help to establish the safety of GM crop consumption.

The first published study implementing the new European Union regulations was that of Zeljenkova *et al.* (2014), which was a general feeding study of MON810 corn. The study lacked any details in its methodology, particularly of the histopathological analysis. The raw data made available online (www.cadima.info) included details of several aspects of the study (e.g. animal weight gain and haematology), but lacked adequate details of the histopatholical findings. However, they have indicated that their histological slides can be examined on request.

### *Post-marketing biosafety and monitoring contamination feed and crops*

Oraby *et al.* (2015) studied the effects of consuming a diet containing commercially available GM corn and GM soy. Their control was a nutritionally comparable diet containing non-GM wheat. While a better control would have been an equivalent non-GM corn and soy diet at the same dose-level as the GM diet, the study is of particular interest because it portrays a dilemma that arises once a crop has been approved for commercial cultivation and consumption. Once approved and released, a GM crop can uncontrollably contaminate non-GM crops and the environment by a number of routes, including movement of pollen and seeds by wind, animals, birds, and leaking transport trucks, as well as mixing in post-harvest storage. Contamination of one GM variety with another GM variety can similarly occur. In addition, many GM crop varieties are now being cultivated that contain several "stacked" GM genes in them. Consequently, food eaten generally contains a mixture of GM genes and their protein products. It can therefore be very difficult to monitor and ascribe a given health outcome to the consumption of a particular GM crop, or a particular GM gene. Furthermore, while

particular GM genes in food may be tested-for using DNA methods, these tests are expensive and rely on using particular probes for particular GM genes. Oraby *et al.* (2015) therefore used a more general test. They tested for the presence of EPSPS CP4 and CaMV promoter sequences since these are the most common sequences inserted into GM crops. They did not test for any other possible gene inserts; hence, they did not identify the GM crops that were being studied. To do so they would have had to have either mapped the full genome of each crop or tested their feed for the presence of every possible GM cassette. The aim of their study was to evaluate the effects of a GM diet per se, irrespective of the specific GM crops present. This kind of study resembles a real-life scenario, where the consumer (animal or human) does not know the amount or the varieties of GM crops, products or proteins they are consuming. Furthermore, in countries where GM labelling laws are in place, the labelling only mentions the presence of a GM component/product, but not the name of the specific GM crop variety or gene sequence. For example, packaged food may list "genetic engineered soy" in their ingredients label, but will not specify if this is Roundup Ready®, Optimum GAT™, Liberty Link™ or any of the other 31 GM soy varieties, each of which contain completely different genetic modifications (ISAAA, 2013). As such, if a specific GM crop or its product is causing health problems, it is difficult to identify it as the cause. Consequently, the monitoring of post-market biosafety is problematic.

### 1.4.4 Conclusion

Published studies investigating the health effects of GM feed consumption are still scares. Of the studies reviewed, the majority lacked a unified approach and transparency in their methodology and results. Since the publication of the Critical Review, 90 day rat feeding trials have been made mandatory in the European Union (Implementing Regulation (EU), 2013). However, there are still no detailed guidelines as to what the histopathological analyses should entail.

## 2. Overall objectives

Scientific literature suggests that GM crops may have negative effects on the health of animals, particularly mammals (Pusztai *et al.*, 2003). Theoretical considerations (such as in the test for substantial equivalence) cannot take into account all possible interactions between the contents of GM foods and mammalian organisms. Animal feeding studies can evaluate this experimentally and, in particular, can identify target organs or organ systems.

This study tested the following null-hypothesis: There are no negative effects of GM crops on the histopathological characteristics of the gastrointestinal tract in rats.

The aims of this project were:

To determine:

A. the effects of a commonly eaten variety of transgenic corn on rat gut morphology using light, electron and confocal microscopy, as well as immunohistochemistry techniques
B. whether there is a dose-dependent response to the consumption of transgenic corn
C. whether the microstructural changes are similar to those seen in chemically-induced damage of the stomach (chemical/reactive gastropathy) or small intestines (chemically-induced enterocolitis)

Specific aims:

*Stomach*

A1. Using the established categories of the Updated Sydney System, to determine whether there are histopathological changes in the stomachs of rats fed a diet containing GM corn at 60% and 30% of the diet.

A2. Using LM and TEM techniques, determine whether the epithelial barrier is compromised in the stomach of rats fed the GM corn diet.

A3. Whether the GM feed causes changes in the stomach resembling chemically-induced changes.

*Small Intestine*

A4. Using LM morphometric analyses, determine whether there are histopathological changes in the small intestine of rats fed a GM corn diet.

A5. Using TEM techniques determine whether the epithelial barrier is compromised in the intestine of rats fed a GM corn diet.

A6. Whether the GM feed causes changes in the intestine resembling drug-induced enterocolitis.

Corresponding hypotheses to be tested:

*Stomach*

*H1 relating to aim A1*

$H1_0$ There will be no changes in the normal histology of the stomach as described by the categories of the Updated Sydney System. This means that either of the following will not be seen: 1) presence/increase of granular leukocyte, 2) increase in mononuclear cells, 3) presence of glandular atrophy, and 4) presence of intestinal metaplasia, as compared with the control group.

$H1_a$ One of the following will be seen: 1) there will be an increase in granular leukocytes, 2) glandular atrophy will progress, 3) number of mononuclear cells will increase, and 4) intestinal metaplasia will be present, as compared with the control group.

*H2 relating to aim A2*

$H2_0$ Epithelial barrier of the stomach will not be compromised due to the GM corn diet – morphology of the epithelium will not differ between experimental and control groups.

$H2_a$ Tight junctions in stomach epithelium will be compromised.

*H3 relating to aim A3*

$H3_0$ There will be no chemically-induced changes in the stomach. Therefore, there will be no change in pit depth and mitotic activity in the rat stomach compared with the control.

$H3_a$ There will be an increase in pit depth and mitotic activity in the rat stomach.

*Small intestine*

*H4 relating to aim A4*

$H4_0$ There will be no changes in the normal histology of the small intestine i.e. there will be no change in either one of the following: 1) villi height, 2) crypt depth, 3) goblet cell or 4) intraepithelial lymphocyte numbers, or 5) an increase in mitotic figures in the crypts of the small intestines, compared with the control.

$H4_a$ One of the following will be seen: 1) villi stunting (atrophy), 2) loss of crypt architecture, 3) a decrease in goblet cells, 4) increase intraepithelial lymphocytes, and 5) a decrease in mitotic figures, compared with the control.

*H5 relating to aim A5*

$H5_0$ Epithelial barrier of the small intestine is not compromised by the GM corn diet – morphology of the epithelium will not differ between experimental and control groups.

$H5_a$ Tight junctions in intestinal epithelium will be compromised.

*H6 relating to aim A6*

$H6_0$ Drug-induced enterocolitis will not be present in the small intestine.

$H6_a$ There will be changes similar to drug-induced enterocolitis, such as, erosion or ulceration or strictures.

# 3. Study 1: Long-term feeding study of rats fed 60% corn

## 3.1 Introduction

The focus of this investigation was to evaluate the health effects of a triple-stacked GM corn using reproducible criteria, including those indicating histopathological changes in tissues and organs. The present study utilised a range of microscopic and morphometric methods, to look for signs signifying a pathological change in the digestive tract, particularly in the stomach and ileum. However, the study did not investigate neoplastic or vascular changes.

Long-term studies should be of minimum 90 days duration (FAO/WHO, 2000). The present feeding study was of 180 days duration, so as to better assess the safety of long-term consumption of a GM crop on rat GI tract health.

## 3.2 Materials and Methods

All procedures were performed under ethics approval (project no. 646/07) from the Animal Welfare Committee of Flinders University, South Australia. The feeding trial commenced November 2010 and ended April 2011. The tissues were collected, processed, embedded and appropriately stored till the commencement of the PhD candidature.

All animal work was performed in accordance the South Australian Prevention of Cruelty to Animals Act (1985) and with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).

All procedures were performed under blinded conditions.

### 3.2.1 GM and non-GM corn

The GM corn had been obtained from a commercial farm in the USA. It was a triple-stacked variety of GM corn containing MON863, MON810 and NK603 genes. This GM variety has been formed through conventional cross-breeding of several GM corn varieties.

The control diet contained a commercially-grown non-GM corn variety grown in Australia. The corn was not sourced from the US due to the difficulties in finding completely uncontaminated non-GM corn from that country, as a previous study by colleagues has shown (Carman *et al.,* 2013). In contrast, Australia does not grow any GM corn, whether commercially or in field trials (ISAAA, 2013; Clive, 2014; OGTR, 2016),

which would imply an essentially zero likelihood that the non-GM corn had been contaminated with any GM corn.

An isogenic or near-isogenic variety of corn would have been ideal as the control; however, such a variety was not available due to the difficulty of commercially obtaining the parental lines for cross-breeding. A cross-bred isogenic line was also not commercially available. According to various government regulators, the GM corn used in this study is compositionally equivalent to non-GM varieties (EFSA, 2008). Changes that were observed in the two feeding groups in this study are comparable due to the theory of substantial equivalence.

### 3.2.2 Diet

The joint FAO/WHO report (2000) has suggested feeding studies investigating GM crop consumption should investigate the effects at a range of dose levels. The highest dose is the maximum achievable dose that would not cause nutritional imbalance in the test animal, and the lowest dose is the dose that closely resembles the anticipated human intake (FAO/WHO, 2000). The maximum achievable dose of corn for semi-purified diets is 60%, according to the feed manufacturer, Specialty Feeds (Glen Forrest, Australia). Therefore, this study used a feed that contained 60% of either a GM or non-GM corn.

The experimental diets, containing either GM or non-GM corn, were semi-purified diets, formulated by Specialty Feeds (Glen Forrest, Australia) to meet the nutritional requirements for growth and well-being of rats (i.e. comparable to the standard rat diet, AIN-93G Growth Purified Diet; Appendix A1.1). Both GM and non-GM feeds were stored separate, but under the same conditions.

### 3.2.3 Animal Feeding

Twenty outbred, male Sprague Dawley (SD) rats weighing 50g ± 15g were obtained after weaning at 3 weeks of age. They were randomly placed into two groups, GM (n=10) and non-GM-fed (n=10) and fed a diet containing either 60% GM corn or 60% non-GM corn for 26 weeks. Rats were housed in pairs with *ad libitum* access to water and feed. Animal rooms were maintained at 21$^{o}$C ± 2$^{o}$C temperature, 40-60% humidity and a 12h light/dark cycle. The rats were monitored daily and weighed weekly. After 26 weeks, the rats were weighed, anaesthetised with isoflurane and euthanized via the removal of the

heart. The stomach and ileum were removed immediately post-mortem by a certified veterinarian.

### Stomach

For stomach samples, an incision was made along the greater curvature, content removed, flushed with phosphate buffer saline (PBS), and weighed. Half of the stomach was fixed in 10% buffered formalin (pH 7.4) for 12-18h (overnight) and routinely processed for light microscopy. One millimetre by 1mm sections of the stomach (fundus) wall were fixed with 2% glutaraldehyde and 3% paraformaldehyde in 0.1M phosphate buffer (pH 7.4), and processed for electron microscopy.

### Ileum

Sections of the ileum (i.e. 2cm from the caecum) were collected, flushed with a PBS filled syringe, and fixed in 10% buffered formalin (pH 7.4) for light microscopy. In addition, 1mm transverse sectioned rings of the ileum were fixed in a solution containing 2% glutaraldehyde, 3% paraformaldehyde and 0.1M phosphate buffer (pH 7.4), then cut into 1mm cubes and processed for electron microscopy.

## 3.2.4 Histopathology

### *Light microscopy*

Fixed samples were processed, and embedded in paraffin wax (ileums were embedded as 1-3 cross-section rings). Five to six micron thick sections were cut using a rotary microtome and mounted on glass slides with two sections per slide (ileum sections were cut at 300μm intervals). Slides were routinely stained with haematoxylin and eosin (H&E; Appendix B1.1) and with alcian blue and periodic acid Schiff (combined AB/PAS pH 2.5; Appendix B1.2). Sections were viewed under light microscope and morphometric analyses were performed using NIS-Elements BR (Nikon) software. All observations and analyses were performed under blinded conditions.

### Stomach

In the non-glandular stomach, the thickness of the keratinized and non-keratinized epithelial layers was measured in areas of thinnest mucosal thickness. In the glandular stomach (fundic region), the pit and gland depth and mucosa thickness was obtained in at least 20 well-orientated areas. Granular leukocytes (GL) were counted in the lamina propria below the gastric glands. This was performed in areas where the muscularis

mucosae and the base of the mucosal glands were well defined. The procedure was as follows: Using NIS-Elements BR (Nikon) software and a light microscope with a 20x objective lens, an area of interest was outlined and the area recorded. Granular leukocytes, found in the marked area, were counted and a percentage of GL per area was obtained. Granular leukocytes were those with a bright pink cytoplasm and a multi-lobed nucleus. This procedure was repeated randomly along the length of the stomach. The total area measured per rat was on average $0.16mm^2$.

### Ileum

Villi height and crypt depth was measured for 5-15 well-orientated villi/crypt units. Well-orientated villi/crypt units were those that had a continuous epithelium from villus tip to crypt base. Enterocytes, goblet cells and intraepithelial leukocytes (IEL) were counted per well-orientated villus that had a simple epithelium present along the whole villus. Results were presented as the number of cells (enterocytes, goblet cells or IELs) per villous height, and as a ratio of total number of goblet cells or IELs per total number of enterocytes. Near Peyer's patches, IELs may be increased therefore measurements and counts were obtained three villi away from the Peyer's patch. The normal values for villous epithelial cell populations for SD rats were obtained from the laboratory of the Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences, Warsaw, Poland. These were the reference values established at this institute. In the current study, they were also used as reference values to determine whether the above parameters were within normal range. A percentage population of 20% of goblet cells to enterocytes, and a percentage population of 0-20% of IELs to enterocytes, were seen as being within normal range. Populations above 20% were considered to be elevated.

## *Immunohistochemistry*

### Ki67 and caspase 3

Sections were cut at 4µm, mounted on DAKO slides, and dried overnight at 30$^{o}$C. Slides were deparaffinised in histolene, rehydrated in graded ethanol solutions to distilled water. Antigen retrieval was performed using high pH antigen retrieval solution (DAKO) and set on 20 min cycle at 97$^{o}$C (DAKO PT Link). Using an automated cycle (DAKO Autostainer Plus), sections were first quenched with 3% hydrogen peroxide (FLEX peroxidase blocker, DAKO) for 5 min and then immersed in Protein Block (DAKO) for 30 min, followed by a 60 min incubation in primary antibody, caspase 3 (1:1000

dilution with actual antibody concentration at 0.003mg/mL, abcam ab4051) or Ki67 (1:1000 dilution with an estimated antibody concentration of 0.01-0.05µg/mL, abcam ab16667). Sections were then labelled with anti-rabbit horse-radish peroxidase (HRP) (30 min, DAKO) and developed with 3, 3 –diaminobenzidine (DAB; DAKO) for 10 min, washed with distilled water, manually counterstained with Harris haematoxylin (10 sec), blued in ammonia solution (1 min), dehydrated in a graded series of ethanol and histolene, and cover slipped with DPEX mounting medium.

Breast tumour tissue from a rat that had received chemotherapy treatment, and stomach or small intestine tissues from a rat known to have been fed a non-GM diet were used as the controls in the following way:

1) Positive control: breast tumour tissue treated with primary antibody (caspase 3 or Ki67). The breast tumour tissue expresses caspase 3 in the centre of the lesion, and Ki67 at the periphery.
2) Negative control: rat breast tumour tissue and non-GM stomach or small intestine treated with buffer solution in place of the primary antibody

*Stomach*

Cell counts were performed in well-orientated areas of the fundus, 3mm from the glandular/non-glandular stomach junction, and 100µm from fundus/pylorus junction (Loogna *et al.*, 2002). Using NIS-Elements BR (Nikon) software, a 100-300µm-wide area, perpendicular to the mucosa and comprising the entire thickness of the mucosa, was outlined. Within this area, all labelled and unlabelled epithelial cells were counted (Li and Helander, 1996; Loogna *et al.*, 2002). This procedure was repeated one to four times per rat. The results were expressed as the ratio of Ki67 labelled nuclei (proliferative index: PI) or caspase 3 labelled nuclei (apoptotic index: AI) to total number of nuclei counted (Li and Helander, 1996; Yu *et al.*, 2005).

*Small intestine (ileum)*

Cell counts were performed in the crypts using an Olympus BH2 light microscope and a 40x objective lens. The number of labelled (Ki67 or caspase3) and unlabelled cells were counted in 8-15 well-orientated crypts. Proliferative index was expressed in two ways: $PI_1$ total no. of labelled nuclei per 10 crypts (Sukhotnik *et al.*, 2008); $PI_2$ ratio of labelled nuclei to total number of nuclei counted (nuclei of 8-15 crypts) (Li and Helander, 1996). Apoptotic index was expressed as the ratio of caspase 3 labelled nuclei to total number of nuclei counted in crypts (Li and Helander, 1996).

### *Electron microscopy*

Fixed sections of glandular stomach and ileum were secondarily fixed in 1% osmium tetroxide and routinely processed for TEM and embedded in epoxy resin (Appendix B1.5). Thin sections (70-90nm) were cut with an ultra-microtome, mounted on Cu/Pd grids and assessed for morphological changes using a Phillips CM100 Transmission Electron Microscope (stomach) or FEI Tecnai G2 Spirit Microscope (ileum).

In stomach and ileum sections, tight junction integrity between epithelium lining the surface or gastric pits (stomach) or intestinal villi (ileum) was assessed using the grading system reported by Keefe et al. (2000). Tight junctions were examined at a final magnification of 43,000. A tight junction was assessed as either: 1) open, 2) closed, or 3) cut obliquely (thus not counted). A tight junction was considered closed if there was no gap between opposing cells at the luminal end and no gap between opposing leaflets of the tight junction (Keefe *et al.*, 2000). In the stomach, the tight junctions were assessed between cells cut in cross-section (luminal surface clearly defined and basal surface sitting on a basement membrane). In the small intestine, tight junction apposition was assessed between enterocytes with luminal microvilli perpendicular, or near perpendicular, to the electron beam (Keefe *et al.*, 2000). A minimum of 20 junctions (stomach) or 100 junctions (ileum) were assessed per rat.

Transmission electron micrographs of the ileum enterocytes from the top ¾ of villi were used to calculate microvilli length and microvilli density. Microvilli length was measured using NIS-Elements BR (Nikon) software. Fifty to 133 measurements were made per rat. Microvilli density was analysed via two methods: 1) the number of microvilli per length of cell membrane, and 2) point-counting method (Weibel, 1990). The point-counting method determined the microvilli volume density per cytoplasm density using Image J software and a 199cm$^2$ point graticule overlaying transmission electron micrographs (9-15 micrographs per rat) of the enterocyte apical surface taken at 16,300 magnification. Points falling on microvilli were counted and divided by the number of points falling in the cytoplasm. Points falling in the lumen were not counted.

## 3.2.5 Statistical analysis

Statistical analyses of continuous data were conducted using SPSS version 20 and 22, and in consultation with a biostatistician. The results were averaged for each rat and then tested for normal distribution using the Shapiro-Wilk test. If data of both groups were normally distributed, a t-test was performed. If data of both groups were not

normally distributed, a Mann Whitney U test was used. In addition to the t-test, Levene's test for equality was performed. The appropriate p value for the t-test was reported according to whether equal variance could be assumed. A 2-tailed t-test was used, unless otherwise stated and a $p \leq 0.050$ was considered significant. In the data set, while SPSS occasionally found outliers, none were extreme, thus were not removed.

Statistical analyses of categorical data were conducted using Epi Info™ version 7 (developed by the US Centres for Disease Control and Prevention), and in consultation with a biostatistician. Results were reported as a relative risk (also known as a risk ratio) and associated 95% confidence interval. Fisher Exact p value was reported since sample sizes were generally low and the expected numbers in the tables' cells were often less than five. A $p \leq 0.050$ was considered significant.

All statistical analyses were performed in accordance with the OECD Environment Directive on working with chemicals, pesticides and biotechnology (OECD, 2012).

## 3.3 Results

### 3.3.1 Animal feeding

The average initial weights of the rats for the GM and non-GM-fed groups were similar (Table 8). In the first seven weeks of the feeding trial, it was noted that the rats in the GM group were not gaining as much weight as would be expected. This was also noted in the non-GM-fed group at a later stage (data not shown at collaborators' request). It was found that the pellets were too hard for the rats to eat. The feed manufacturer (Specialty Feeds, Glen Forrest, Western Australia) resolved this problem by increasing the moisture content in the feed, which resulted in the rats of both groups gaining weight at a normal or expected rate (Siglin and Baker, 2002). Throughout the whole feeding trial, rats of both groups maintained food consumption and body weight (or weight gain) and at no time did the animals stop eating or lose weight (data not shown at collaborators' request).

The final body weight of rats showed a statistically significant increase in the GM-fed group ($p = 0.000$; Table 8). The weight of the stomachs was also significantly higher ($p = 0.038$) in the GM than in the non-GM-fed group. However, the difference in the stomach to body weight ratio was not statistically significant (Table 8).

**Table 8. Final body and organ weights of rats fed a 60% GM or non-GM corn diet.** Values show mean or median and standard deviation (SD) or interquartile range (IQR) depending on whether the data are normally distributed. Statistically significant values are given in bold.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050)[b] | Change (%)[c] |
|---|---|---|---|---|---|---|---|---|
| | Mean or median[a] | SD or IQR[a] | n | Mean or median[a] | SD or IQR[a] | n | | |
| average initial weight of rats (g) | 49.40 | 8.36 | 10 | 48.40 | 7.72 | 10 | NS | -2.02 |
| average final weight of rats (g) | **355.30** | **34.90** | **10** | **443.40** | **28.98** | **10** | **P = 0.000*** ** | **24.80** |
| average final weight of rat stomachs (g) | **1.69** | **0.21** | **10** | **2.08** | **0.48** | **10** | **P = 0.038*** | **23.08** |
| stomach weight/final rat weight (%) | 0.46[a] | 0.44-0.50[a] | 10 | 0.43[a] | 0.40-0.49[a] | 10 | NS | -6.52 |

[a] Not normally distributed data, the median and interquartile range (IQR) are given.

[b] Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001

[c] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

## 3.3.2 Histopathology

### *Non-glandular stomach*

There were no observations that could be deemed histopathological in either GM or non-GM-fed groups. Mucosal thickness measurements revealed no differences between the groups (Table 9; Figures 16 and 17).

**Table 9. Non-glandular stomach morphometric analyses from rats fed a 60% GM or non-GM corn diet.** Values show mean or median and standard deviation (SD) or interquartile range (IQR) depending on whether the data are normally distributed.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Mean or median[a] | SD or IQR[a] | n | Mean or median[a] | SD or IQR[a] | n | | |
| average mucosa thickness (µm) | 59.80 | 8.90 | 5 | 57.41 | 6.15 | 5 | NS | -4.00 |
| average thickness of keratinized layer (µm) | 8.53[a] | 7.65-8.75[a] | 5 | 8.13[a] | 7.40-8.23[a] | 5 | NS | -4.69 |
| average thickness of non-keratinized layer (µm) | 51.57 | 7.90 | 5 | 48.41 | 3.62 | 5 | NS | -6.13 |
| % keratinized/mucosa thickness | 13.79[a] | 12.23-15.12[a] | 5 | 13.83[a] | 13.47-14.85[a] | 5 | NS | 0.29 |
| % non-keratinized/mucosa thickness | 86.71[a] | 84.88-87.77[a] | 5 | 86.17[a] | 85.15-86.53[a] | 5 | NS | -0.62 |

[a] Not normally distributed data, the median and interquartile range (IQR) are given.

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

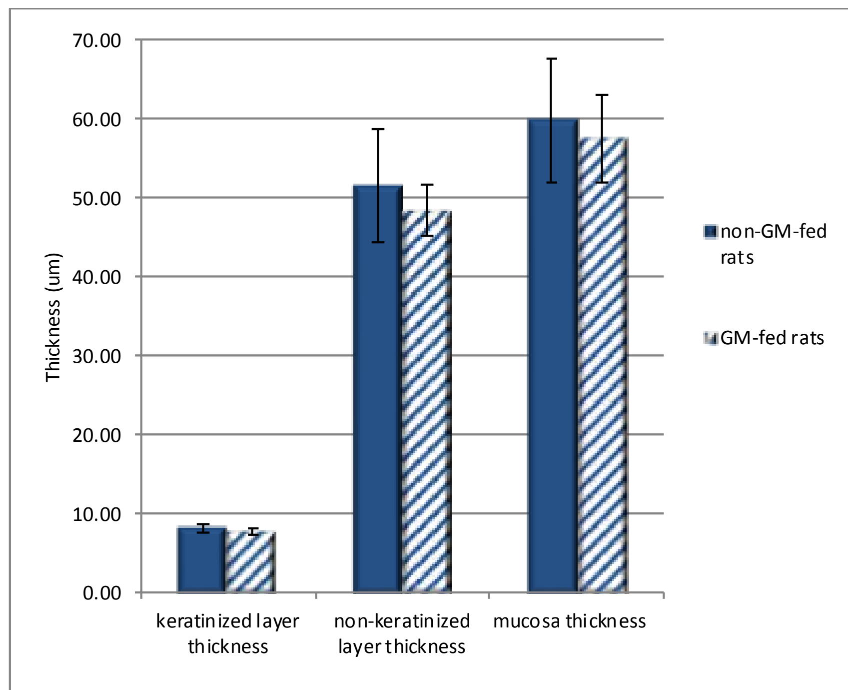



**Figure 16. Graph of mucosal measurements of the non-glandular stomach of rats fed a 60% GM or non-GM corn diet.** Graph shows mean or median and bars indicate standard deviation or interquartile range depending on whether the data are normally distributed.

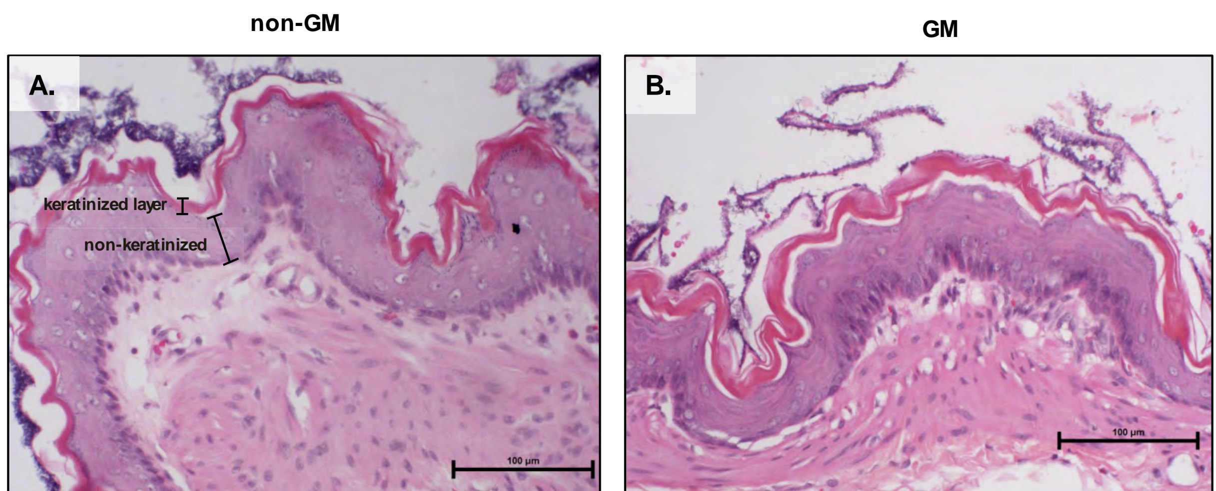



**Figure 17. Sections of the non-glandular stomach, sainted with H&E, from rats fed a 60% GM or non-GM corn diet.** A.) Non-GM-fed rat and B.) GM-fed rat. Scale bar = 100µm

## *Glandular stomach*

### Light microscopy

Morphometric analyses showed an increase in mucosal thickness, pit and gland depth in GM-fed group. However, these results were not statistically significant (Table 10; Figure 18 and 19). Mononuclear inflammatory cells were rarely seen in the pit and glandular region of the mucosa. In most animals of both groups, granular leukocytes infiltration was mild and was primary located in the lamina propria of the deep

glandular region (Figure 20). In two non-GM-fed animals the infiltration was moderate. Granular leukocyte count in the lamina propria below the glandular region showed no significant difference between groups (Table 10).

At the junction between glandular and non-glandular stomach, glandular dilatations were seen in several animals of both groups (6/10 in GM-fed; 4/10 non-GM-fed; Table 11). In the non-GM-fed group, the gland dilatations were small and the lining cells were most often cuboidal or columnar. In the GM-fed group, the dilatations were larger and some of the lining cells were elongated. In addition, isolated groups of the lining cells stained intensely with PAS (dark pink), or AB/PAS (dark purple) suggestive of epithelial dysplasia (Figure 20; Table 11).

**Table 10. Glandular stomach (fundus) morphometric analyses and cell counts from rats fed a 60% GM or non-GM corn diet.** Values show mean or median and standard deviation (SD) or interquartile range (IQR) depending on whether the data are normally distributed.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Mean or median[a] | SD or IQR[a] | n | Mean or median[a] | SD or IQR[a] | n | | |
| average mucosa thickness (µm) | 738.51 | 100.31 | 10 | 774.34 | 89.53 | 10 | NS | 4.85 |
| average pit depth (µm) | 94.21 | 15.58 | 10 | 101.94 | 11.25 | 10 | NS | 8.21 |
| average gland depth (µm) | 606.28 | 95.62 | 10 | 627.18 | 87.52 | 10 | NS | 3.45 |
| average pit + gland depth (µm) | 700.49 | 95.57 | 10 | 729.11 | 85.32 | 10 | NS | 4.09 |
| average thickness of lamina propria below glands (µm) | 38.02 | 9.66 | 10 | 45.22 | 20.20 | 10 | NS | 18.94 |
| % pit/ mucosa thickness | 12.96 | 2.79 | 10 | 13.34 | 2.23 | 10 | NS | 2.93 |
| % gland/ mucosa thickness | 81.88 | 2.92 | 10 | 80.86 | 3.45 | 10 | NS | -1.25 |
| % pit + gland/ mucosa thickness | 94.71[a] | 94.24-95.36[a] | 10 | 95.25[a] | 93.93-95.81[a] | 10 | NS | 0.57 |
| % lamina propria thickness below gland/ mucosa thickness | 5.29[a] | 4.64-5.76[a] | 10 | 4.75[a] | 4.21-6.07[a] | 10 | NS | -10.21 |
| % granular leukocyte count per lamina propria area below gland | 0.16 | 0.10 | 10 | 0.11 | 0.05 | 10 | NS | -31.25 |

[a] Not normally distributed data, the median and interquartile range is given.

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

**Table 11. Number of rats with glandular dilatations in the gastric glands of the fundus from rats fed a 60% GM or non-GM corn diet.**

| | GM | | non-GM | | Relative risk | 95% confidence interval | Statistical significance (P ≤0.050)[a] |
|---|---|---|---|---|---|---|---|
| | Outcome of interest | | Outcome of interest | | | | |
| | present | absent | present | absent | | | |
| Rats with gland dilatations | 6 | 4 | 4 | 6 | 1.50 | 0.60 – 3.74 | NS |
| Rats with gland dilatations exhibiting AB/PAS or PAS positive cells | 4 | 6 | 0 | 10 | 4.40[b] | 0.59-33.07[b] | NS |
| Rats with gland dilatations exhibiting elongated epithelium | 4 | 6 | 0 | 10 | 4.40[b] | 0.59-33.07[b] | NS |
| Gland dilatations with AB/PAS or PAS positive cells | 4 | 2 | 0 | 4 | 3.33[b] | 0.53-21.03[b] | NS |
| Gland dilatations with elongated epithelium | 4 | 2 | 0 | 4 | 3.33[b] | 0.53-21.03[b] | NS |

[a] Statistical analysis performed was the Fisher's exact test

[b] Relative risk and confidence interval was calculated by putting a "1" into the cell with zero. Statistical significance: $p < 0.050$.

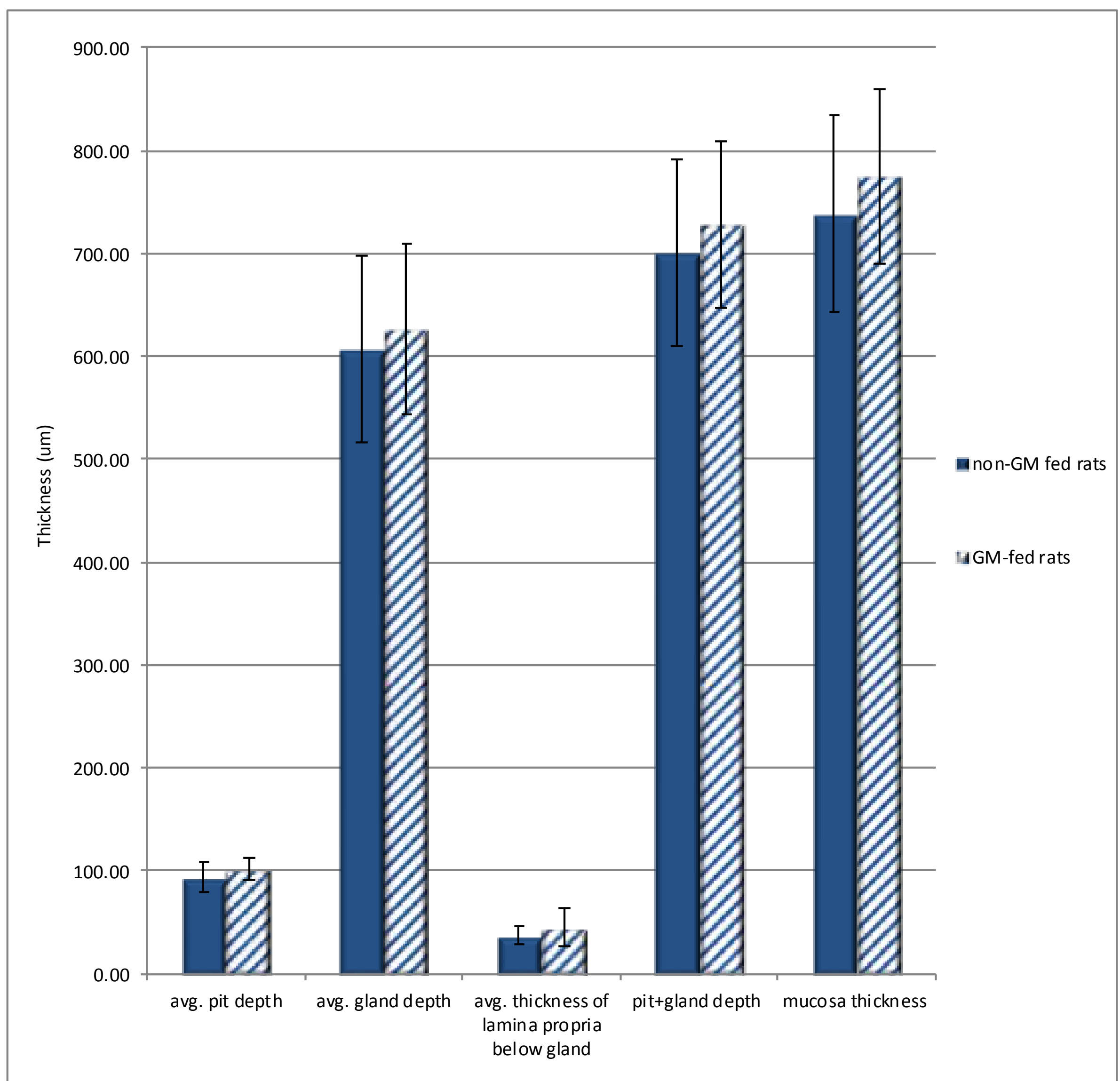



**Figure 18. Average mucosal measurements for glandular stomach (fundus) of rats fed a 60% GM or non-GM corn diet.** Bars indicate standard deviation.

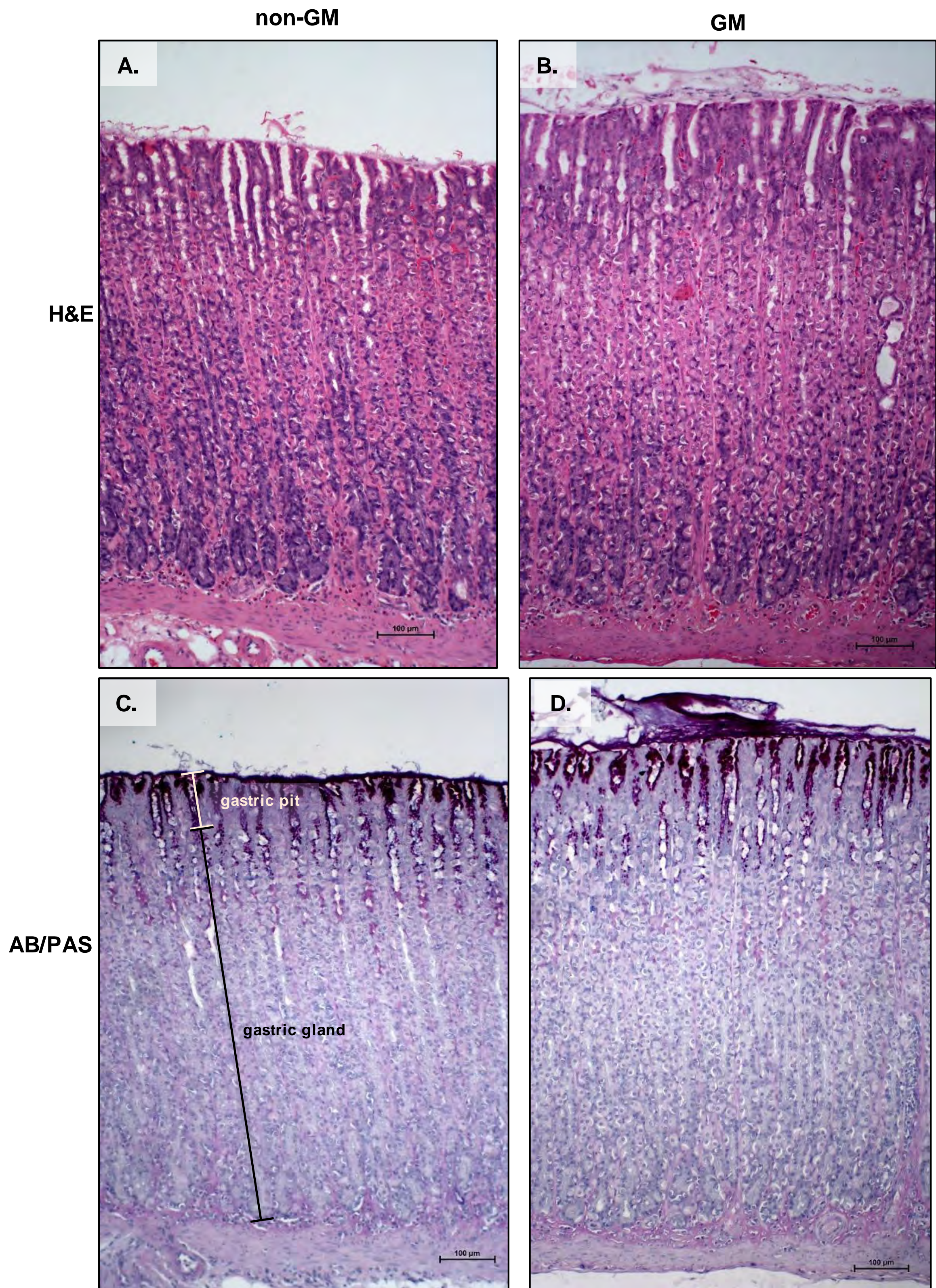



**Figure 19. Sections of the stomach (fundus) from rats fed a 60% GM or non-GM corn diet.** A.) Non-GM-fed rat, and B.) GM-fed rat stained with H&E; and C.) non-GM-fed rat and D.) GM-fed rat stained with AB/PAS. Scale bar = 100μm.

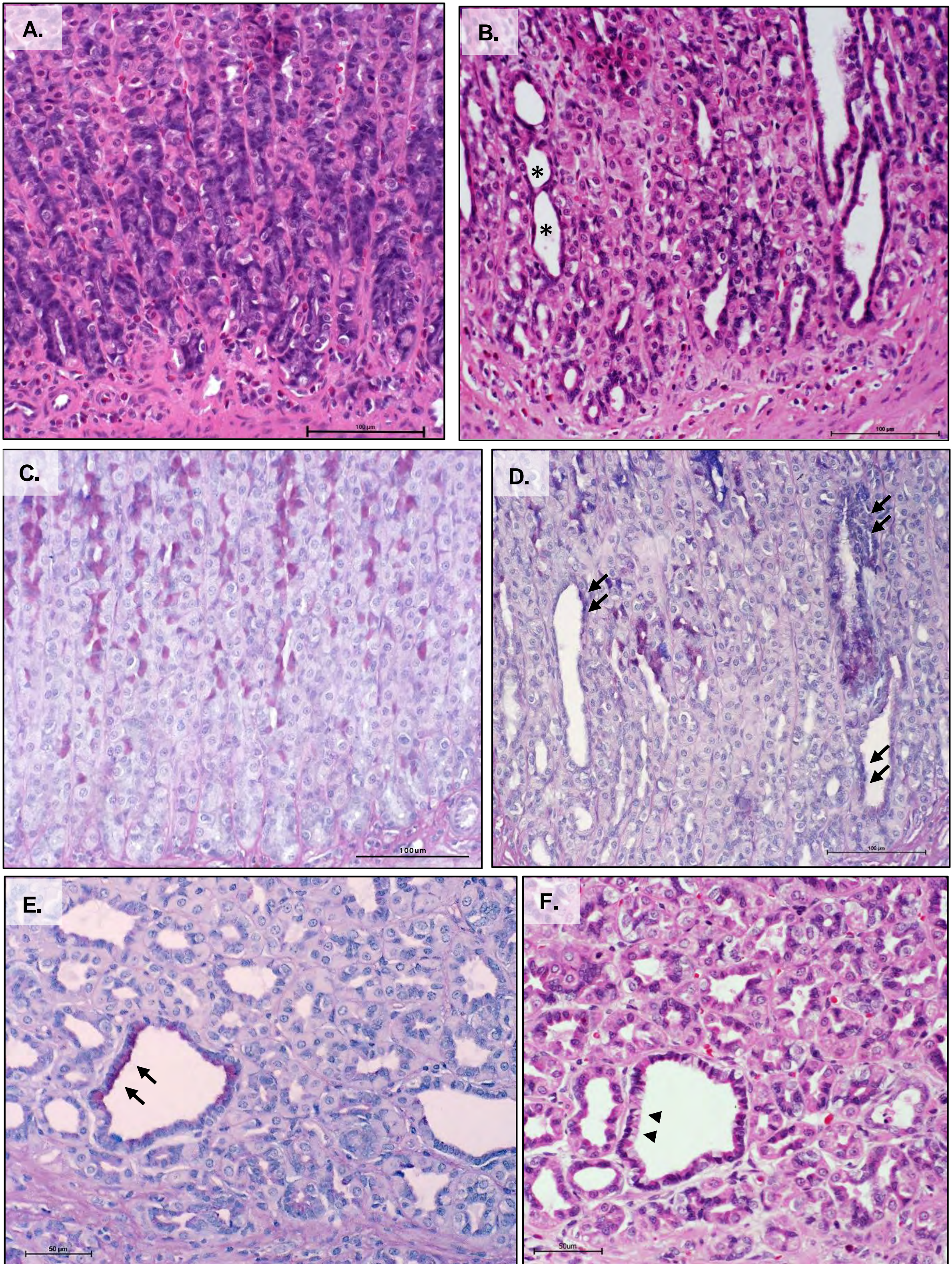



**Figure 20. Gastric glands of the rat stomach (fundus) from rats fed a 60% GM or non-GM corn diet.**

A. and B.) Sections stained with H&E from non-GM-fed rat (A.) showing normal gland appearance, and from GM-fed rat (B.) showing glandular dilatations with epithelial cell elongation (*). Scale bar = 100µm;

C. and D.) Sections stained with AB/PAS from non-GM-fed rat (C.) showing normal gland appearance, and from GM-fed rat (D.) showing intense staining in glandular dilatations (arrows). Scale bar = 100µm;

E. and F.) Glandular dilatation in GM-fed rat stained with AB/PAS (E.) showing epithelial dysplasia stained with PAS (arrows), and corresponding H&E-stained section (F.) of gland dilatations showing cuboidal cells (arrowheads). Scale bar = 50µm.

## Immunohistochemistry results

In the fundus of the stomach, caspase 3 positive cells were scant (i.e. 0-3 cells in the whole fundic region of the stomach of each rat) and in the positive cells, labelling was specifically localised in the nucleus (Figure 21). Negative controls did not show any specific localisation of the antibody (Figure 21C and E). The breast tumour positive control expressed caspase 3 in the centre of the lesion (Figure 21D).

Positive labelling with Ki67 antibody (localised in the nucleus), indicated a significant decrease (p = 0.028) in the number of proliferating cells in the GM-fed group (Table 12). In addition, a significant decrease (p = 0.033) was seen in the number of proliferating cells per pit/gland depth in the GM-fed group (Table 12). The anti-Ki67 labelling was seen primarily at the base of the gastric pits of the non-GM-fed group, while in the GM-fed group these cells were widely distributed in the distal pit and proximal glandular regions (Figure 22). Two rats (one GM and one non-GM-fed rat) had additional labelling of cells along the full length of an occasional gastric gland. Negative controls did not show any specific localisation of the antibody (Figure 22E). The breast tumour positive control expressed Ki67 at the periphery (Figure 22F).

**Table 12. Dividing cells in the glandular stomach mucosa of the rat fed a 60% GM or non-GM corn diet.** Mean and standard deviation (SD) shown.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050)[a] | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | n | Mean | SD | n | | |
| Proliferative index: % dividing cells / total count | **12.75** | **3.09** | **10** | **10.07** | **1.43** | **10** | **P = 0.028*** | **-21.02** |
| dividing cells/ pit + gland depth | **0.26** | **0.09** | **10** | **0.18** | **0.05** | **10** | **P = 0.033*** | **-30.77** |

[a] Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

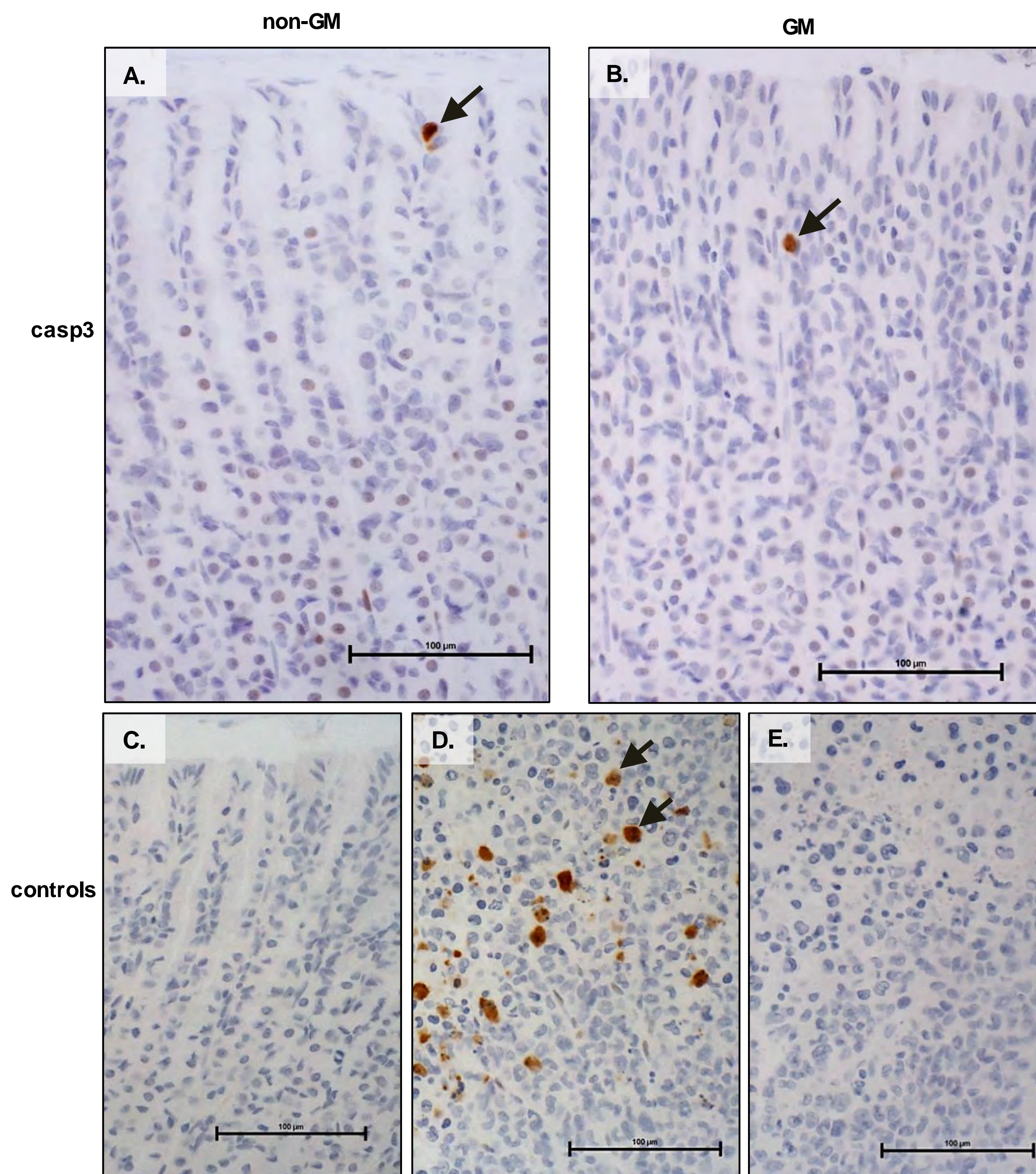



**Figure 21. Sections of the gastric pits of stomachs (fundus) labelled with anti-caspase 3 from rats fed a 60% GM or non-GM corn diet.**

A. and B.) Labelling indicated by arrows in non-GM-fed rat (A.), and GM-fed rat (B.);

C-E.) Controls: C.) Rat stomach negative control showing no specific labelling; D.) Rat tumour positive control showing specific labelling (arrows); E.) Rat tumour, negative control showing no specific labelling.

Scale bar = 100μm

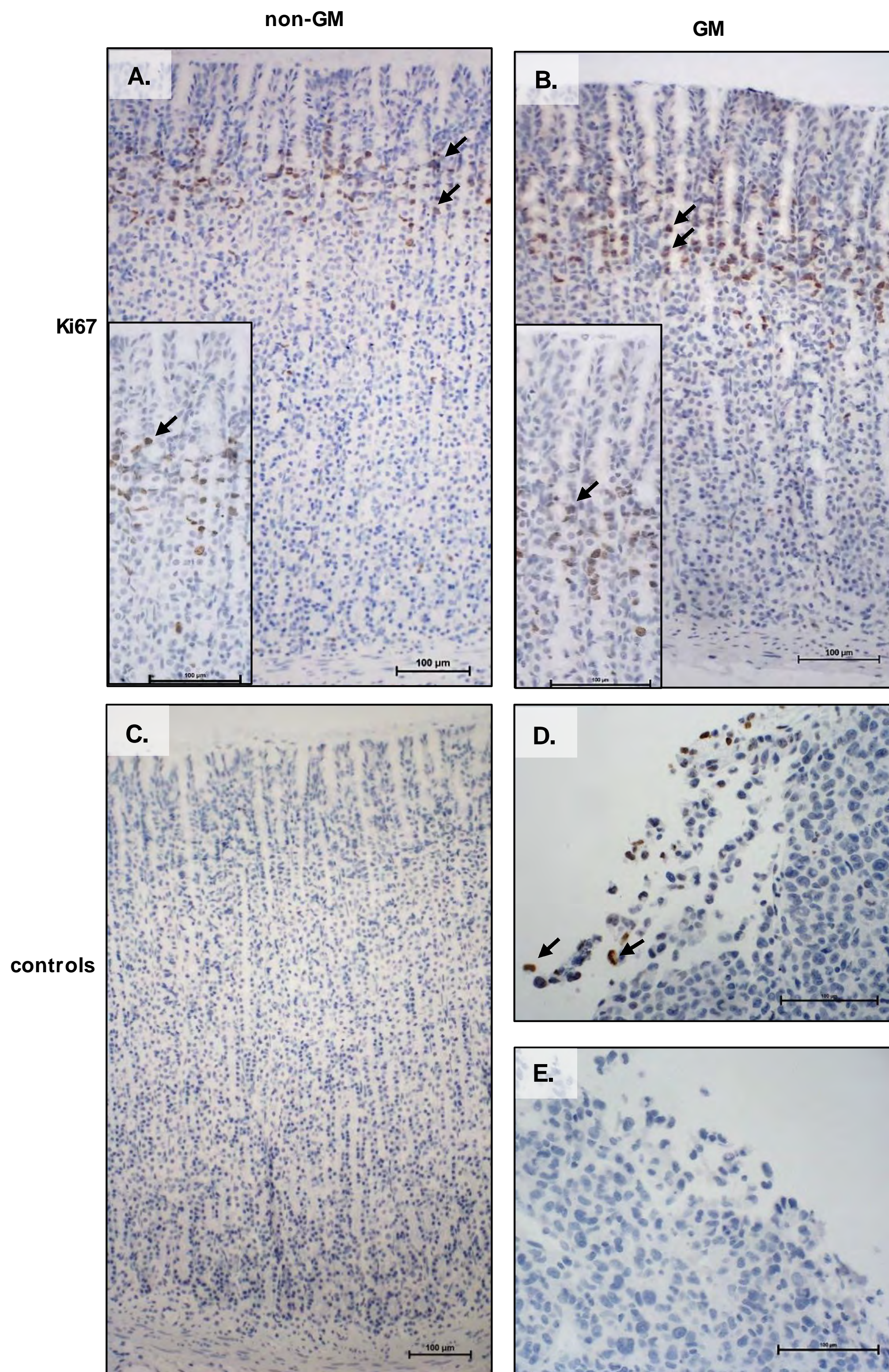



**Figure 22. Sections of the stomach (fundus) labelled with anti-Ki67 from rats fed a 60% GM or non-GM corn diet.**

A. and B.) Labelling indicated with arrows in non-GM-fed rat (A.) and GM-fed rat (B.). Insert: pits at higher magnification.

C-E.) Controls: C.) Rat stomach negative control showing no specific labelling; D.) Rat tumour positive control showing specific labelling (arrows); E.) Rat tumour, negative control showing no specific labelling.

Scale bar = 100µm

## *Small intestine (ileum)*

### Light microscopy

The morphometric analysis indicated a decrease in mucosal thickness, villi height (p = 0.049) and crypt depth in GM-fed rats compared with the non-GM fed rats (Table 13; Figure 23 and 25). There was a slight increase in number of enterocytes per villi height (p = 0.010). Additionally, IELs were slightly elevated in the non-GM-fed group, but were within the normal range in the GM-fed group; however, this finding was not statistically significant. Goblet cell counts were equally slightly elevated in both groups; however, this was not statistically significant (Tables 13 and 14; Figures 23 and 24).

**Table 13. Mucosal measurements and cell counts of the ileum of rats fed a 60% GM or non-GM corn diet.** Values show mean or median and standard deviation (SD) or interquartile range (IQR) depending on whether the data are normally distributed. Statistically significant values are given in bold.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050)[b] | Change (%)[c] |
|---|---|---|---|---|---|---|---|---|
| | Mean or median[a] | SD or IQR[a] | n | Mean or median[a] | SD or IQR[a] | n | | |
| average mucosa thickness (μm) | 534.87[a] | 465.24-623.09[a] | 8 | 417.88[a] | 416.57-442.98[a] | 10 | NS | -21.87 |
| **average villi height (μm)** | **358.03** | **79.62** | 8 | **291.93** | **51.93** | **10** | **P = 0.049*** | **-18.46** |
| average crypt depth (μm) | 167.60 | 37.16 | 8 | 139.17 | 29.84 | 10 | NS | -16.96 |
| average crypt depth (μm) (complete sample size) | 156.89 | 40.43 | 10 | 139.17 | 29.84 | 10 | NS | -11.29 |
| Average lamina propria thickness below crypt (μm) | 7.68[a] | 6.62-9.55[a] | 8 | 7.48[a] | 4.62-12.58[a] | 10 | NS | -2.60 |
| Average lamina propria thickness below crypt (μm) (complete sample size) | 7.03[a] | 3.94-8.53[a] | 10 | 7.48[a] | 4.62-12.58[a] | 10 | NS | 6.40 |
| % villi height / mucosa thickness | 66.69 | 4.72 | 8 | 66.36 | 5.85 | 10 | NS | -0.49 |
| % crypt / mucosa thickness | 31.51 | 4.69 | 8 | 31.64 | 5.03 | 10 | NS | 0.41 |
| % lamina thickness below crypt / mucosa thickness | 1.81 | 1.11 | 8 | 2.00 | 1.02 | 10 | NS | 10.50 |
| **average no. of enterocytes / villi height** | **1.24** | **0.30** | 8 | **1.68** | **0.32** | **10** | **P = 0.010*** | **35.48** |
| average no. of goblet cells / villi height | 0.32 | 0.09 | 8 | 0.43 | 0.12 | 10 | NS | 34.38 |
| average no. of IELs / villi height | 0.30 | 0.46 | 8 | 0.36 | 0.77 | 10 | NS | 20.00 |
| % goblet cells / enterocytes | 25.68 | 3.14 | 8 | 25.28 | 4.35 | 10 | NS | -1.56 |
| % IEL / enterocytes | 26.06[a] | 15.89-50.31[a] | 8 | 19.18[a] | 12.20-47-02[a] | 10 | NS | -26.40 |

[a] Not normally distributed data, the median and interquartile range (IQR) are given.

[b] Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001

[c] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

**Table 14. Population of cells per enterocyte in the ileum of rats fed a 60% GM or non-GM corn diet.** Normal population of goblet cells per enterocyte is 20%. Normal population of IELs per enterocyte is 0-20%.

| | GM | | non-GM | | Relative risk | 95% confidence interval | Statistical significance ($P \leq 0.050$)[a] |
|---|---|---|---|---|---|---|---|
| | Outcome of interest | | Outcome of interest | | | | |
| | present | absent | present | absent | | | |
| Percentage of goblet cells per enterocyte above 20% | 8 | 2 | 8 | 0 | 0.80 | 0.59 – 1.09 | NS |
| Percentage of goblet cells per enterocyte below 20% | 1 | 9 | 0 | 8 | 0.90[b] | 0.07-12.38[b] | NS |
| Percentage of IELs per enterocyte above 20% | 4 | 6 | 5 | 3 | 0.64 | 0.25 – 1.62 | NS |

[a] Statistical analysis performed was the Fisher's exact test.

[b] Relative risk and confidence interval was calculated by putting a "1" into the cell with zero. Statistical significance, p < 0.050.

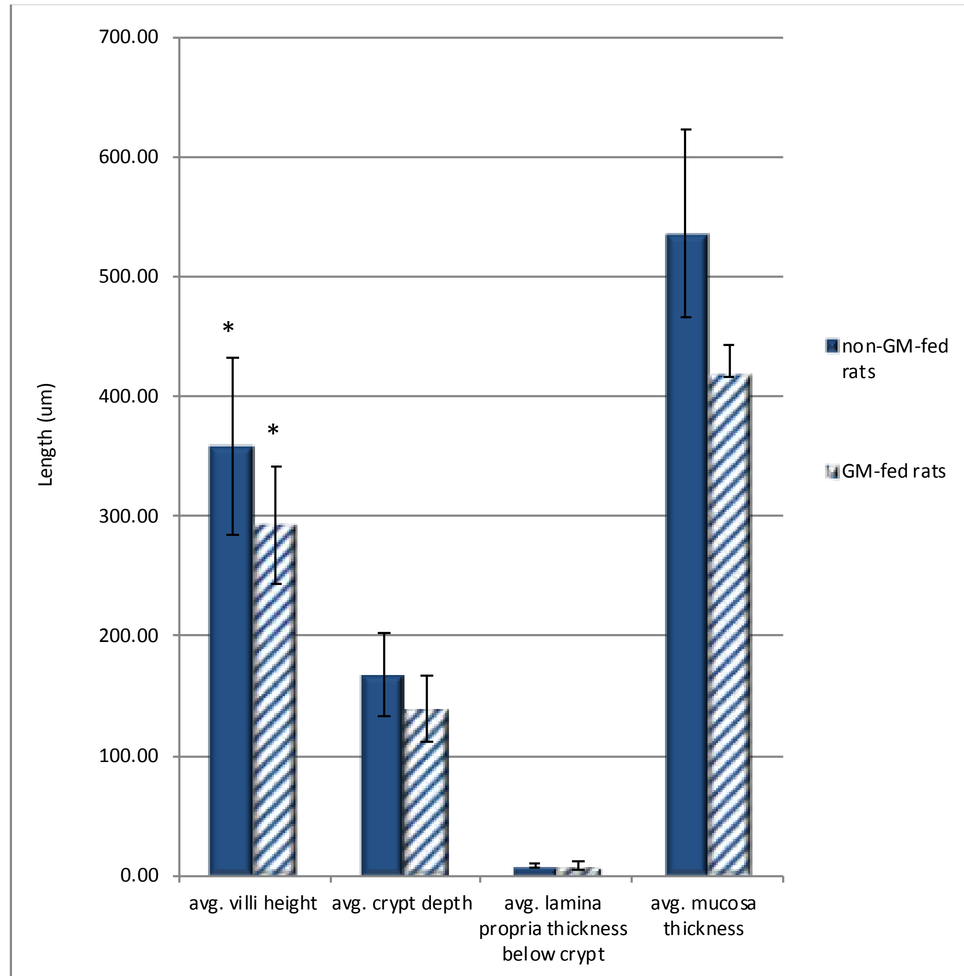



**Figure 23. Average mucosal measurements of the ileum of rats fed 60% GM or non-GM corn diet.** Graph shows mean or median and bars indicate standard deviation or interquartile range depending on whether the data are normally distributed. Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001

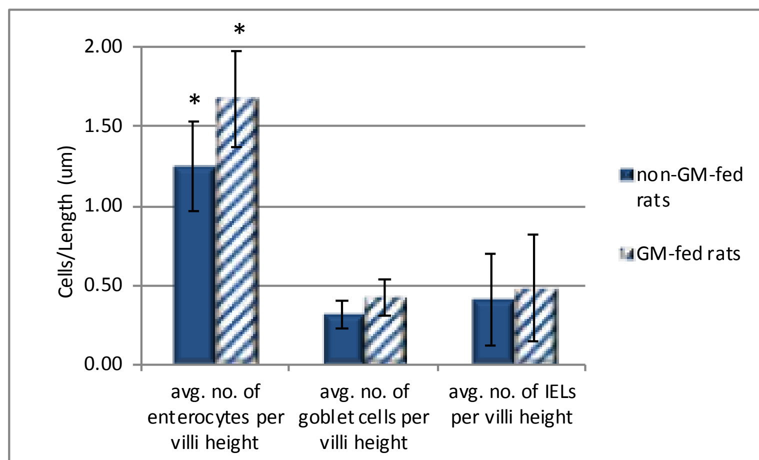



**Figure 24. Average number of enterocytes, goblet cells or IELs per villi height in the ileum of rats fed 60% GM or non-GM corn diet.** Bars indicate standard deviation. Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001

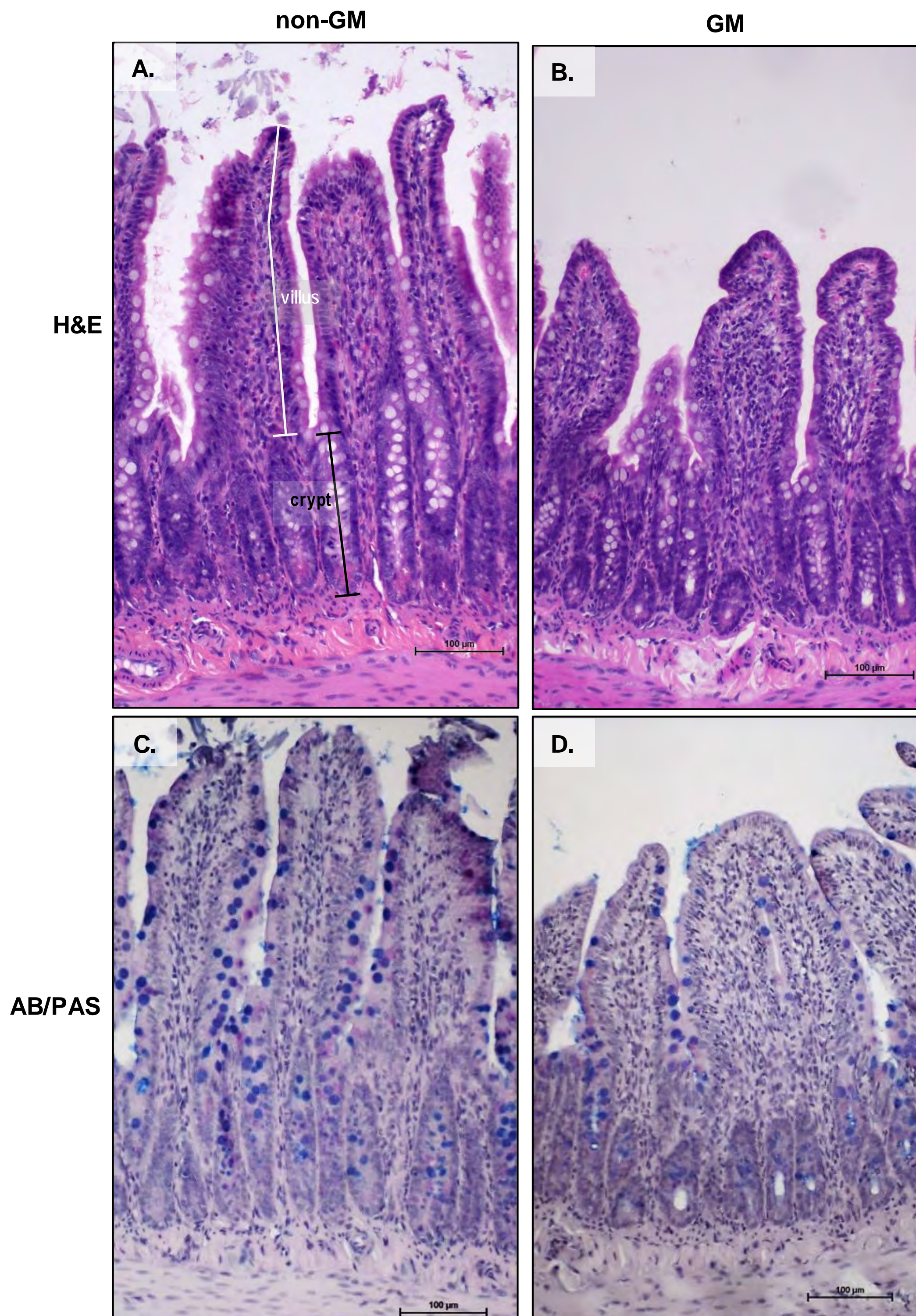



**Figure 25. Sections of the ileum from rats fed a 60% GM or non-GM corn diet.** A.) Non-GM-fed rat and B.) GM-fed rat stained with H&E; and C.) non-GM-fed rat and D.) GM-fed rat stained with AB/PAS. Scale bar = 100μm

### Immunohistochemistry

In the crypts of the ileum, caspase 3 positive cells were scant (i.e. 0-3 cells in the crypts of each animal) in both feeding groups. In the positive cells, labelling was specifically localised to the nucleus. Positive labelling was seen in the tips of the villi, where cells are being sloughed off (Figure 26A and B). Negative controls did not show any specific localisation of the antibody (Figure26C and E). The breast tumour positive control expressed caspase 3 in the centre of the lesion (Figure 26D).

Positive labelling for Ki67 was localised to the nuclei of the cells. Such cells were seen along the epithelium of the ileal crypts (Figure 27A-D). A count of the Ki67 positive cells in the crypts showed no statistically significant difference between the two groups (Table 15). In the base of the crypts (proliferative region), there were 2-4 unlabelled cells in most regions of the ileum (Figure 27C and D). Negative controls did not show any specific localisation of the antibody (Figure 27E). The breast tumour positive control expressed Ki67 at the periphery (Figure 27F).

**Table 15. Dividing cells in the ileum crypt of rats fed a 60% GM or non-GM corn diet.** Values show mean and standard deviation (SD).

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[a] |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | n | Mean | SD | n | | |
| Proliferative index: % dividing cells / total no. cells in crypt | 57.54 | 18.62 | 10 | 62.86 | 10.52 | 10 | NS | 9.25 |
| no. dividing cells / average crypt depth | 0.20 | 0.10 | 10 | 0.21 | 0.05 | 10 | NS | 5.00 |
| no. dividing cells per 10 crypts (Sukhotnik *et al.* method) | 219.44 | 99.01 | 9 | 167.11 | 46.70 | 10 | NS | -23.85 |
| % dividing cells in 10 crypts | 57.60 | 17.75 | 9 | 63.83 | 9.46 | 10 | NS | 10.82 |

[a] The effect of eating the GM diet compared with the non-GM diet as a percentage change. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

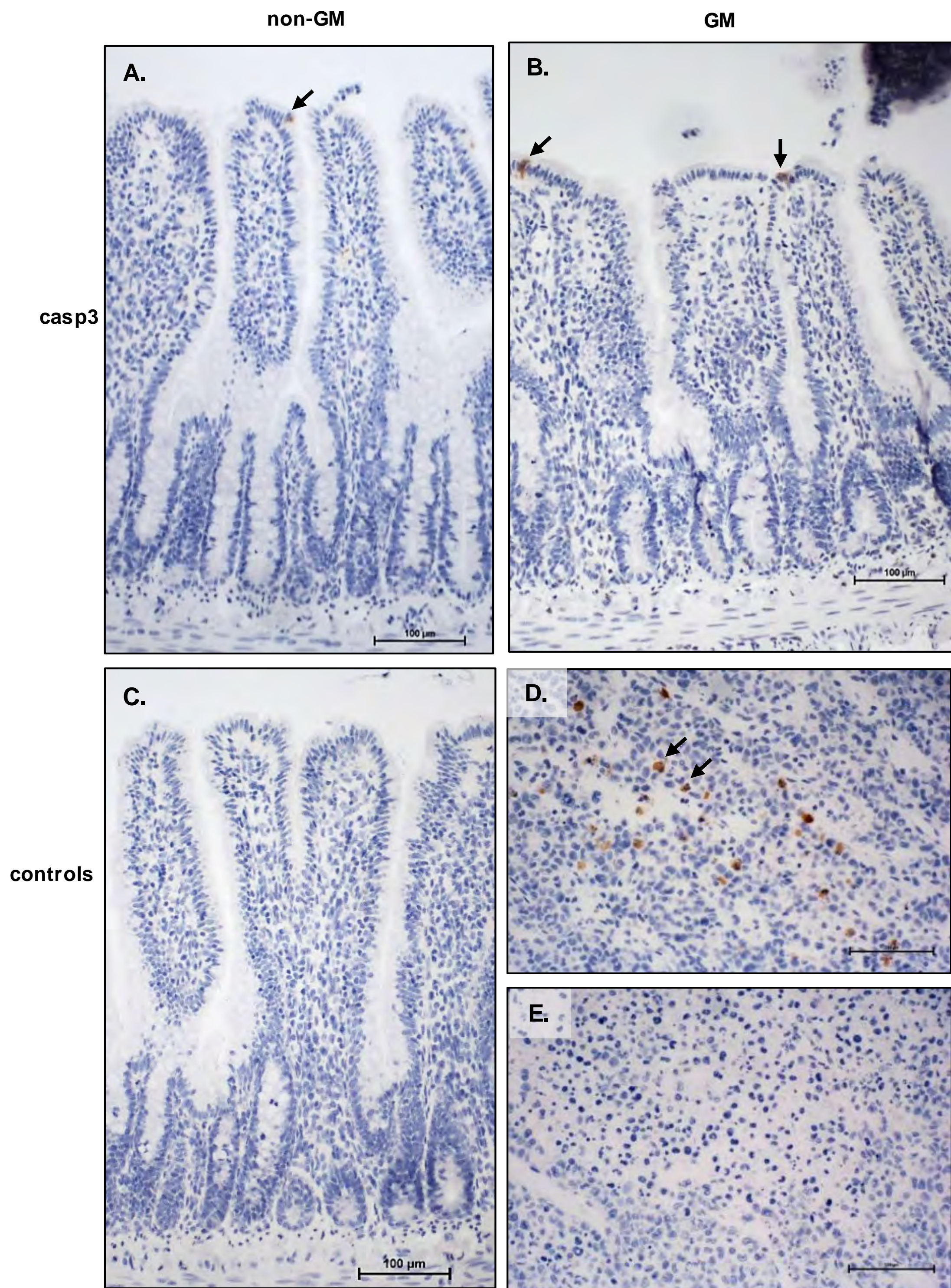



**Figure 26. Sections of the ileum labelled with anti-caspase 3 from rats fed a 60% GM or non-GM corn diet.**

A. and B.) Anti-caspase 3 labelled cells (arrows) at tips of villi in non-GM-fed (A.) and GM-fed (B.) rats.

C.-E.) Controls: C.) Rat ileum negative control showing no specific labelling; D.) Rat tumour positive control showing specific labelling (arrows); E.) Rat tumour negative control showing no specific labelling.

Scale bar = 100µm

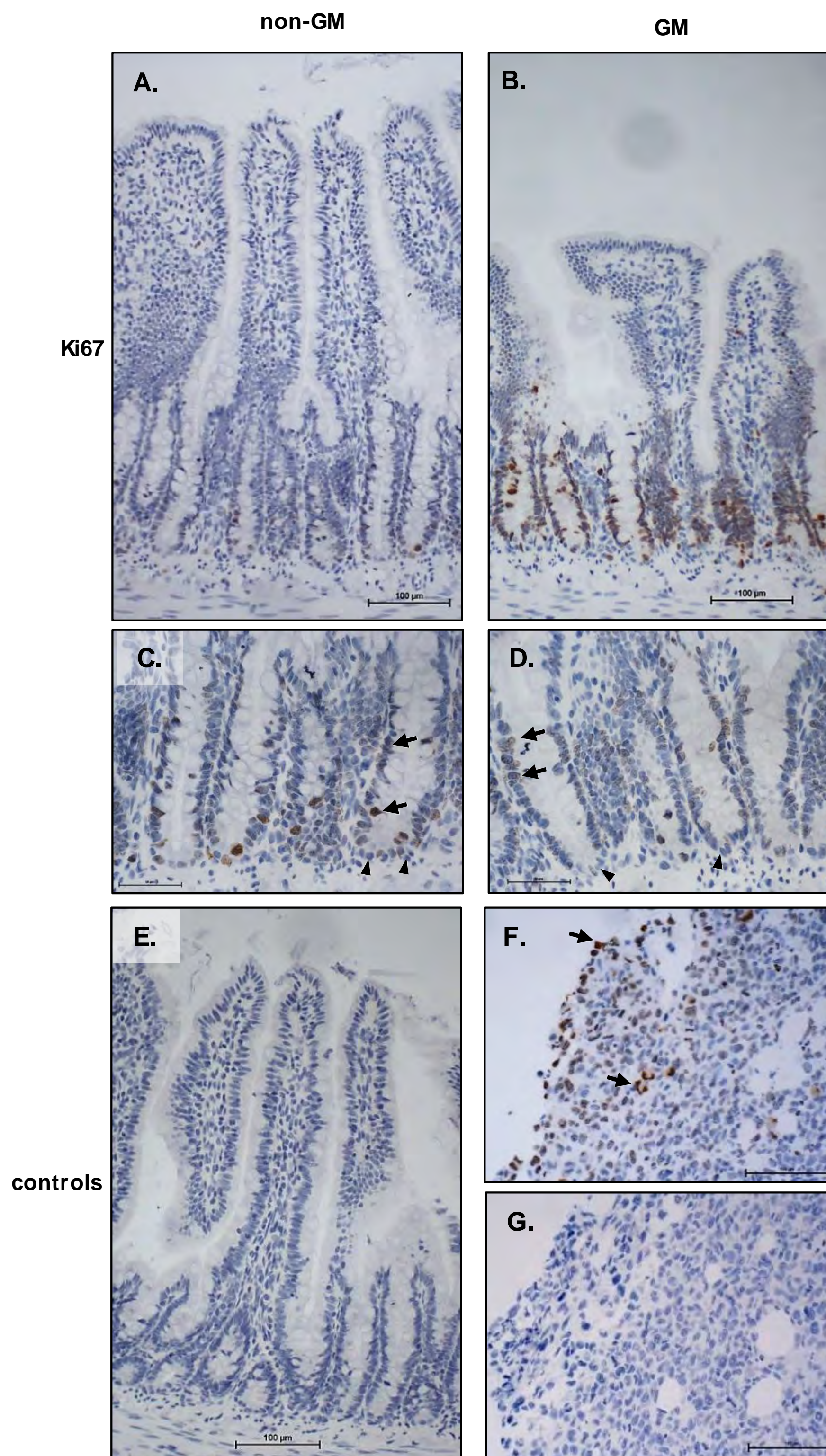



**Figure 27. Sections of the ileum labelled with anti-Ki67 from rats fed a 60% GM or non-GM corn diet.**
A-D.) Anti-Ki67 labelled cells (arrows) in non-GM (A.) and GM-fed (B.) rats. Labelling in crypts in non-GM (C.) and GM-fed (D.) rat. A few unlabelled cells can be seen at the base of the crypts (arrowheads).
E.-G.) Controls: E.) Rat stomach negative control showing no specific labelling; F.) Rat tumour positive control with ant-Ki67 labelled cells (arrows); G.) Rat tumour negative control showing no specific labelling.
Scale bar = 100μm

## *Electron Microscopy*

### Stomach

Transmission electron microscopic investigations of the fundic region of the stomach, demonstrated a significant loss (p = 0.006) of tight junction apposition between the mucus-producing cells of the surface and gastric pits of the GM-fed animals (Tables 16 and 17; Figures 28 and 29). The apposition loss was seen as either partial or complete loss, or as blebbing (Figures 29C-E).

**Table 16. Transmission electron microscopic investigation of tight junction apposition between mucus-producing cells in glandular stomachs (fundus) of rats fed a 60% GM or non-GM corn diet.** Values show median and interquartile range (IQR).

| | non-GM | | | GM | | | Statistical significance (P ≤0.050)[a] | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Median | IQR | n | Median | IQR | n | | |
| no. of poorly apposed TJ | 1.00 | 0.00-2.00 | 9 | 3.50 | 3.00-4.00 | 10 | P=0.006** | 250.00 |
| % poorly apposed TJ[c] | 7.14 | 0.00-26.09 | 9 | 35.42 | 24.17-44.44 | 10 | P=0.004** | 396.08 |

[a] Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change.

[c] Number of poorly apposed tight junctions as a percentage of total number of tight junctions counted.

**Table 17. Number of rats with poorly apposed tight junctions between the mucus-producing cells of the pit and luminal surface of the stomach (fundus) in rats fed a 60% GM or non-GM corn diet.**

| | GM | | non-GM | | Relative risk | 95% confidence interval | Statistical significance (P ≤0.050)[a] |
|---|---|---|---|---|---|---|---|
| | Outcome of interest | | Outcome of interest | | | | |
| | present | absent | present | absent | | | |
| poorly apposed tight junctions | 10 | 0 | 5 | 4 | 1.80 | 1.00 – 3.23 | P = 0.033* |

[a] Statistical analysis performed was the Fisher's Exact test. Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001

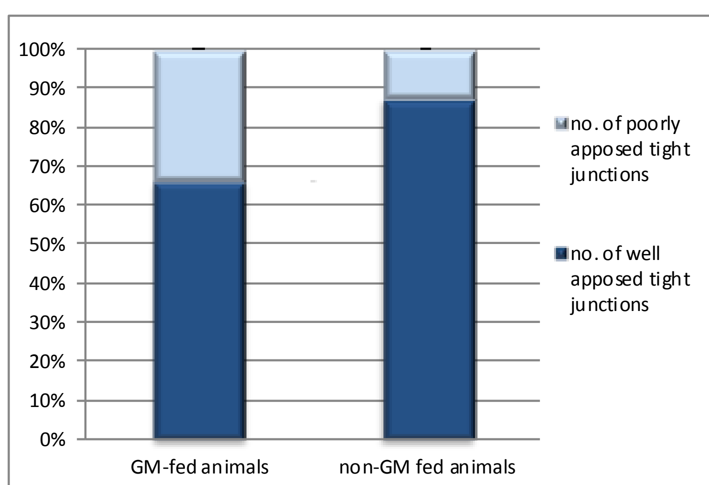



**Figure 28. Difference in frequency of tight junction appositions between the mucus-producing cells of the stomach mucosa (fundus) of rats fed a 60% GM or non-GM corn diet.**

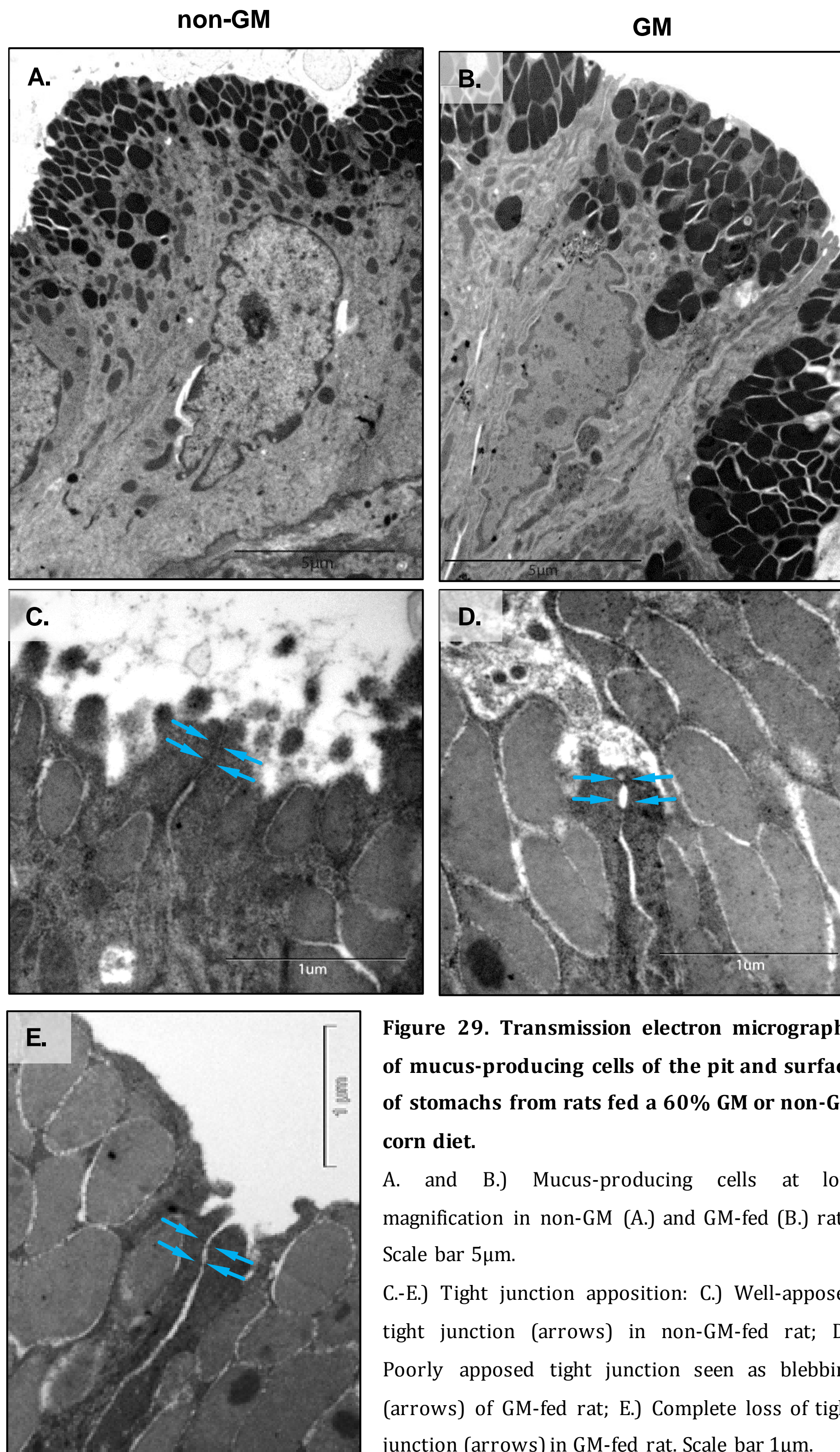



**Figure 29. Transmission electron micrographs of mucus-producing cells of the pit and surface of stomachs from rats fed a 60% GM or non-GM corn diet.**

A. and B.) Mucus-producing cells at low magnification in non-GM (A.) and GM-fed (B.) rats. Scale bar 5µm.

C.-E.) Tight junction apposition: C.) Well-apposed tight junction (arrows) in non-GM-fed rat; D.) Poorly apposed tight junction seen as blebbing (arrows) of GM-fed rat; E.) Complete loss of tight junction (arrows) in GM-fed rat. Scale bar 1µm.

## Small intestine (ileum)

Transmission electron microscopic investigations of the ileum demonstrated no loss in tight junction apposition (Table 18; Figure 30C and D). Microvilli density was higher in GM-fed animals compared with non-GM-fed animals. In addition, the length of the microvilli was increased in the GM-fed rats. However these results were not statistically significant (Table 18).

Changes in microvilli structure, such as fusion and blebbing were also observed on the apical surface of the enterocytes (Figures 30E-H). In some regions, the base of the microvilli (apical surface of the enterocytes), were at a uniform level, while in other regions it appeared irregular and jagged (Figure 30H). These features were seen in both groups (GM and non-GM-fed groups) (Table 19).

**Table 18. Transmission electron microscopic investigation of microvilli and tight junction apposition of enterocytes lining the villi in the ileum of rats fed a 60% GM or non-GM corn diet.** Values show mean or median and standard deviation (SD) or interquartile range (IQR) depending on whether the data are normally distributed.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Mean or median[a] | SD or IQR[a] | n | Mean or median[a] | SD or IQR[a] | n | | |
| average microvilli length (μm) | 1.16 | 0.13 | 5 | 1.31 | 0.13 | 5 | NS | 12.93 |
| % of area taken up by microvilli | 24.27 | 3.60 | 5 | 24.85 | 4.78 | 5 | NS | 2.39 |
| microvilli density per cellular width | 2.98[a] | 2.74-3.13[a] | 5 | 3.47[a] | 3.45-3.49[a] | 5 | NS | 16.44 |
| no. of poorly apposed TJ | 1.00[a] | 0.00-1.00[a] | 5 | 0.00[a] | 0.00-1.00[a] | 5 | NS | -100.00 |
| % of poorly apposed TJ[c] | 1.52[a] | 0.00-1.75[a] | 5 | 0.00[a] | 0.00-1.43[a] | 5 | NS | -100.00 |

[a] Not normally distributed data, the median and interquartile range (IQR) are given.

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

[c] Number of poorly apposed tight junctions as a percentage of total number of tight junctions counted

**Table 19. Abnormal observations seen in the TEM investigation of the ileum of 60% GM and non-GM corn fed rats.**

| | GM | | non-GM | | Relative risk | 95% confidence interval | Statistical significance (P ≤0.050)[a] |
|---|---|---|---|---|---|---|---|
| | Outcome of interest | | Outcome of interest | | | | |
| | present | absent | present | absent | | | |
| abnormal microvilli structure | 4 | 1 | 5 | 0 | 0.80 | 0.52 – 1.24 | NS |
| irregular surface below microvilli in enterocytes | 3 | 2 | 4 | 1 | 0.75 | 0.32 – 1.74 | NS |
| blebbing of enterocyte surface | 2 | 3 | 3 | 2 | 0.67 | 0.18 – 2.44 | NS |
| visual assessment: visible decrease in microvilli density | 1 | 4 | 2 | 3 | 0.50 | 0.06 – 3.91 | NS |
| necrotic cell(s) present | 1 | 4 | 2 | 3 | 0.50 | 0.06 – 3.91 | NS |
| poorly apposed tight junctions | 2 | 3 | 3 | 2 | 0.67 | 0.18 – 2.42 | NS |

[a] Statistical analysis performed was the Fisher's exact test

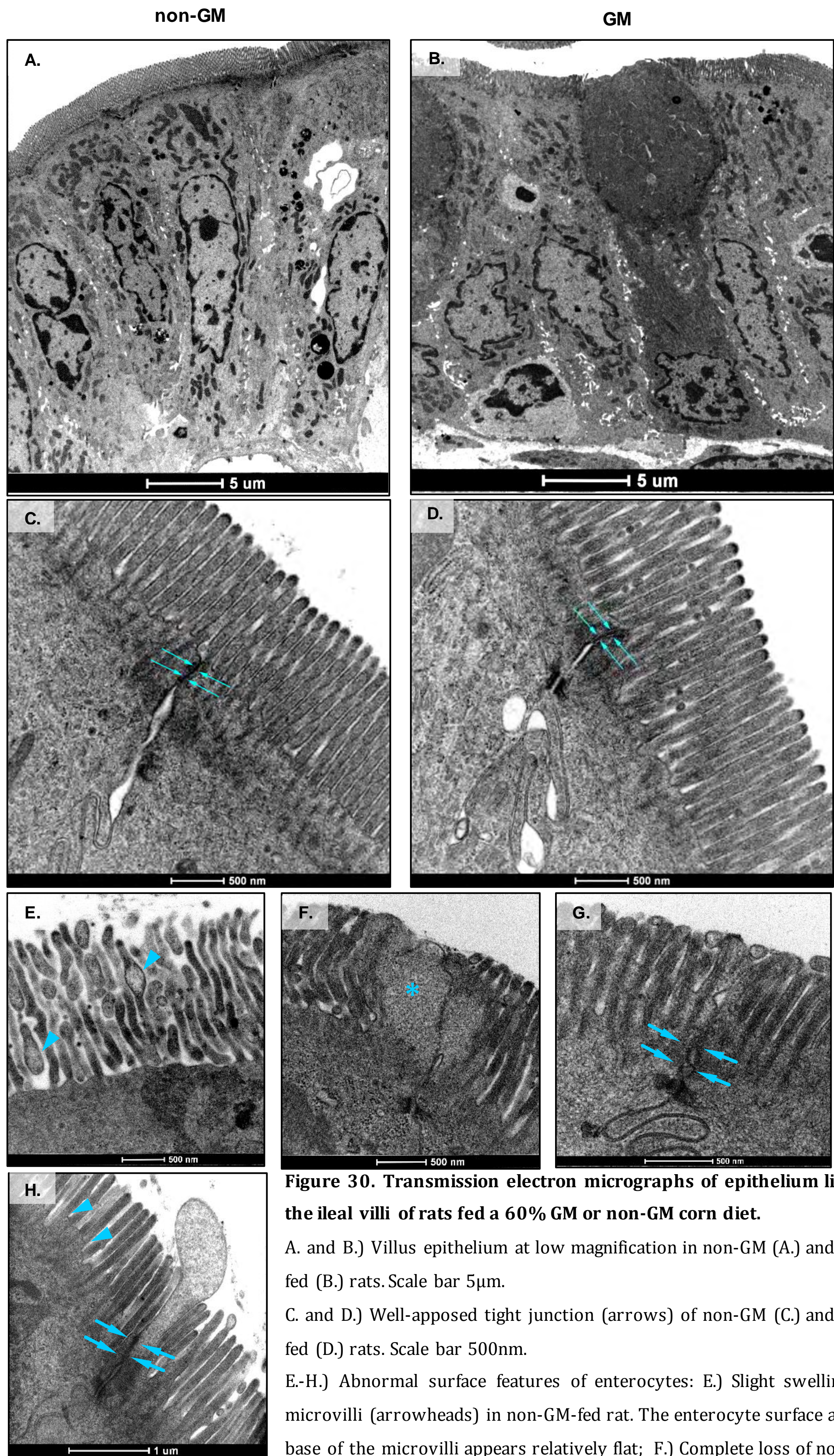



**Figure 30. Transmission electron micrographs of epithelium lining the ileal villi of rats fed a 60% GM or non-GM corn diet.**

A. and B.) Villus epithelium at low magnification in non-GM (A.) and GM-fed (B.) rats. Scale bar 5µm.

C. and D.) Well-apposed tight junction (arrows) of non-GM (C.) and GM-fed (D.) rats. Scale bar 500nm.

E.-H.) Abnormal surface features of enterocytes: E.) Slight swelling of microvilli (arrowheads) in non-GM-fed rat. The enterocyte surface at the base of the microvilli appears relatively flat; F.) Complete loss of normal microvilli structure (fused; *) superior to the tight junction in GM-fed rat; G.) Abnormal microvilli structure at surface superior to tight junction (arrows) in GM-fed animal. Tight junction appears to be poorly apposed. Scale bar 500nm; H.) Blebbing of apical surface near well-apposed tight junction (arrows) of non-GM-fed rat. The enterocyte surface at the base of the microvilli appears jagged (arrowheads). Scale bar 1µm.

## 3.4 Discussion

### 3.4.1 Animal feeding

During the animal feeding trial, the increase in body weight showed a similar pattern in both GM and non-GM groups (data not shown at collaborators' request). The sharp increase in body weight in the non-GM-fed group started later than in the GM-fed animals. This may not be an effect of the diet, because prior to this increase there was a reduced moisture level in both feeds. During that time, the feed intake was reduced, but the animals did not show signs of starvation or discomfort. In addition, the investigation of the non-glandular stomach did not reveal an increase in the thickness of the keratinized layer, which is a pathognomic sign of starvation (Kokue *et al.*, 1977) (Table 9 and Figure 16). This suggests that the animals in both groups had not suffered from long-term starvation.

The higher body weights of rats in the GM-fed group compared with those in the non-GM-fed group could have resulted from the reduced feed intake in the non-GM group due to the lower moisture content in the feed. Similarly, the higher stomach weight in the GM-fed group may also have resulted from the same reason, since the stomach to body weight ratios were not significantly different between the two groups (Table 8).

### 3.4.2 Chemically-induced damage

Several histopathological observations in the study support the notion that GM feed causes chemically-induced damage in the stomach and ileum. The most striking change was observed in the stomach, which was the loss in tight junction apposition between mucus-producing cells of the fundus (396.08% more than non-GM group; p = 0.004). This structural change is commonly associated with chemical gastropathy or bacterial infection (Eastwood and Erdmann, 1978; Meyer *et al.*, 1986; Amieva *et al.*, 2003); however, bacterial infections are typically accompanied by inflammation, while chemical gastropathy lacks such a severe response (Owen, 2003). The lack of a severe inflammatory response in the current study, therefore, suggests that the observed changes in the tight junctions in the GM-fed group may be a sign of chemical gastropathy. Chemical gastropathy in severe cases is characterised by an increase in glandular depth and pit coiling (Dixon *et al.*, 1996; Owen, 2003). Although not statistically significant, the GM-fed group demonstrated an increase in mucosal

thickness, particularly in the glandular depth (4.85% more than non-GM group). Pit depth was also greater in the GM-fed group, but pit coiling was not observed. The absence of pit coiling in GM-fed rats may indicate that the effect is mild rather than severe. Alternatively, pit coiling may be a feature seen only in human stomachs.

The most obvious diagnostic feature of chemical gastropathy is the absence of severe inflammation (Dixon *et al.*, 1996). In the present study, on average, rats of both groups showed a mild infiltrate of granular leukocytes (as assessed using the Updated Sydney System, Figure 10). This therefore, would not take away from the diagnosis of chemical gastropathy because the mild inflammation was seen in both the GM and non-GM-fed groups. Some other component, which was common to both feeds, would have caused the mild infiltration of granular leukocytes.

In chemical gastropathy, an increase in mitotic cells in the glands has been identified as a diagnostic feature, with most stem cells differentiating into parietal or enterochromaffin-like cells (Fenoglio-Preiser, 1998). In the current study, an overall decrease in mitotic cells was seen in the stomach mucosa of GM–fed animals (20.02% less than non-GM; p=0.028). The mitotic cells were identified using an immunohistochemical method for the Ki67 protein. In these sections, it was difficult to identify the demarcation between the pit and glands thus the exact location of the mitotic cells is not certain. Therefore, the results could only be presented as the number of mitotic cells per total count of the pits and glands. In addition, in the present study, an increase in the number of mitotic cells in the glands could have occurred in the initial phases of GM feeding, and at the time of death (26 weeks) the growth would have been completed, thus an increase in mitotic cells may not be present, but an increase in parietal or enterochromaffin-like cells may be evident.

The absence of an inflammatory response together with tight junction apposition loss has been observed in chemical gastropathy (Eastwood and Erdmann, 1978). This suggests that the loss of tight junction apposition is not enough to cause an increase in mucosal barrier permeability that would lead to serious damage of underlying tissue, and trigger an inflammatory response (Martin and Wallace, 2006). The presence of the selective barrier, the basement membrane or the mucous layer protecting the mucosal surface of the stomach, may be preventing this (Paimela *et al.* 1995). In addition, changes in the tight junction protein properties may alter the function of the junction other than its permeability (Huber et al., 2000). For example, in an *in vitro* study, an induced mutation of one of the tight junction proteins caused inhibition of neutrophil

migration, but no change in the selective paracellular permeability or transepithelial electrical resistance of the epithelium (Huber et al., 2000). In other words, the effect/consequence of tight junction apposition loss in gut tissue is dependent on which protein is affected.

In the small intestine, a common indicator of chemically-induced damage is a decrease in villi height and sometimes an increase in crypt depth (Isaacs *et al.*, 1987). The present study quantitatively observed a decrease in mucosa thickness, with both a reduction in villi height (18.46%) and crypt depth (11.29%) in the GM-fed compared with the non-GM-fed group; however, only the difference in villi height was statistically significant ($p = 0.049$). The few GM feeding studies that have performed morphometric analyses, have observed a decrease in ileum crypts of rats fed a GM potato diet (Ewen and Pusztai, 1999), a decrease in the mucosal thickness of the distal intestine of fish fed a GM-soy diet (Sissener *et al.*, 2009), and a slight decrease in mucosa thickness, villi height and crypt depth in rats (Tutel'ian *et al.*, 2010). (The latter study, however, failed to indicate, which section of the small intestine was investigated.) Furthermore, ileac perforation has been observed by pig farmers as a cause of death in pigs fed a GM diet. When the farmers switched to feeding their pigs a non-GM diet, the incidence of death due to ileac perforation was significantly reduced (Personal Communication, Carman, 2012). Other published studies, investigating the effects of GM-crops on health, have rarely indicated that a morphometric analysis of the small intestine mucosa has been performed (Zdziarski *et al.*, 2014). They appear to have performed a non-quantitative assessment of the tissue using light microscopy. Consequently, similar changes may have occurred, but without quantitative analyses, these may have been easily overlooked and thus have remained unreported.

The villous atrophy is a feature of chemically-induced enterocolitis in the small intestine (Isaacs *et al.*, 1987), therefore, the villous atrophy reported in this study and in the above mentioned studies, may be an indication that GM feed has toxic properties. This change in the small intestine was observed in four different feeding studies investigating a variety of diets. These diets contained: a) a GM corn that produces two *Bt* toxins and has glyphosate-tolerant properties (the present study), b) a GM potato containing the snowdrop lectin, *Galanthus nivalis* agglutinin (GNA) that is toxic to aphids (Ewen and Pusztai, 1999), c) a GM soy variety that has glyphosate-tolerant properties (Sissener *et al.*, 2009), or d) another GM soy variety that also has glyphosate-tolerant properties (Tutel'ian *et al.*, 2010). It is possible that the villous atrophy was caused by a

common factor in all four diets or by the individual traits of the GM crop. Also, the other components of the feed may have had a synergistic or potentiating effect in causing the features of chemically-induced enterocolitis. Further investigations using detailed quantitative methods are necessary to isolate the cause.

### 3.4.3 The *Bt* toxin as a source of toxicity

The loss of tight junction apposition could be the result of the presence of the insecticidal, *Cry* proteins that the GM crop has been designed to produce. The action of the *Cry* proteins is believed to be species specific, thus safe for mammalian consumption. However, histopathological or immunotoxicological examinations of the effects of the proteins on animal physiology are scarce (Vazquez-Padron *et al.*, 2000). In addition, the *Cry* proteins that are inserted into the GM crop have been changed, that is, they are truncated and/or have had alterations made to the genome sequence (Vaeck *et al.*, 1987; ISAAA, 2013). Therefore, these changes may impact the species-specificity and thus make them capable of interacting with non-target epithelial cells of the mammalian gut.

In an *in vitro* study on bovine intestinal cells, Shimada *et al.* (2006) found that the *Cry1Ab* protein was binding to the apical surface of the enterocytes. Specifically, the toxin was binding to actin, a major protein of the cytoskeleton. Actin's interaction with tight junction proteins may have an effect on tight junction integrity (Desai *et al.*, 2002; Capaldo and Nusrat, 2009). Remodelling of actin in epithelial cells has been reported alongside changes in tight junction structure and epithelial barrier dysfunction in several tissue types (Capaldo and Nusrat, 2009). In such cases, it is believed that the remodelling of actin and the reorganisation of the tight junction proteins are triggered by cytokines or apoptosis (Soler *et al.*, 1996; Desai *et al.*, 2002). In the current study, the trigger could be the binding of one or both of the *Cry* proteins to actin. Shimada *et al.* (2006) proposed that the interaction of *Cry1Ab* with actin would only be present in the *in vitro* model. In an *in vivo* study, they suggested that the actin would not be exposed; therefore, the *Cry* protein would not be able to bind to it. However, other studies investigating *Cry* proteins, have demonstrated that the proteins bind to mammalian intestinal epithelium in *in vivo* and *in situ* models (Vazquez-Padron *et al.*, 2000; de Souza Freire *et al.*, 2014).

Unlike in the stomach, tight junction apposition loss was not observed between the villous enterocytes in the ileum of the present study. If the *Cry* proteins are responsible for the tight junction apposition loss in the stomach, by the time they reach the ileum,

they may have been digested down and may no longer be in their active form. Thus, they may no longer have the ability to bind to the epithelium and cause tight junctional changes in the ileum. However, tight junctions may be affected at the proximal end of the small intestine, such as in the duodenum. The duodenum and the jejunum were not investigated in this study.

Bt toxins have been known to survive digestion in several animal species. For example, Chowdhury et al. (2003a), Chowdhury et al. (2003b) and Walsh et al. (2012) found Bt proteins in the stomachs, duodenum, ileum, caecum and rectum of pigs and calves fed a GM corn diet containing the Bt protein, *Cry1Ab*. This provides evidence that at least one of the Bt toxins present in the GM corn variety that was fed in this study is resistant to digestion through the entire GI tract of at least two mammalian species. There does not appear to be any similar studies on rats. Furthermore, the activation and binding properties of a Bt protein appear to be the most important factor for mammalian GI tract health rather than the digestion of the Cry proteins per se (Hofte and Whiteley, 1989; Schnepf et al., 1998; Pigott and Ellar, 2007; Soberón et al., 2010). In addition, alterations made to the structure of Bt proteins, during the process of GM crop development (e.g. truncation), may affect the species-specific mode of action of the Bt toxins, which may change their toxicity to mammalian gut cells.

An observable change in the ileum of the present study, was a significant increase (35.48% more than non-GM group; $p = 0.010$) in enterocyte number per villous height. Such an increase has also been reported in mice fed a diet containing either a transgenic potatoes that produced *Cry1* protein or a diet containing potatoes treated with a wild-type δ-endotoxin (i.e. all the types of *Bt* toxins produced by *B. thuringiensis* var. *kurstaki*) (Fares and El-Sayed, 1998). The greatest significant increase was seen in the rats fed the wild-type δ-endotoxin diet (29.78% more than non-GM; $p < 0.0001$). Fares and El-Sayed (1998) also found that 50% of the enterocytes in the affected villi were multinucleated and several enterocytes had loss of microvilli and associated cytoplasmic fragments of variable sizes. Similar observations, but to a lesser degree, were recorded in the ileum of mice fed the transgenic potato feed, and the authors concluded that the δ-endotoxin may have caused the change. The wild-type δ-endotoxin diet contains all types of *Bt* toxins produced by *B. thuringiensis* var. *kurstaki*, but the transgenic potato contains only one *Bt* toxin, the *Cry1* protein. Therefore, if the described changes are caused by the *Bt* toxins, a change of a lesser degree would be expected in this latter group containing the one *Cry1* protein. The present study investigated a GM corn that produces two *Bt* toxins, *Cry1Ab*

and *Cry3Bb1*. Hence, changes of a lesser degree would be expected. The results of the present study found changes to enterocyte numbers and ultrastructural changes to enterocyte apical surface including loss of microvilli. However, ultrastructural changes were prevalent in both the test and control groups and were not statistically significant (Table 19 and Figure 30). These results suggest that all *Bt* toxins have an effect on the enterocyte population, and only the *Cry1* and the wild-type δ-endotoxin have an effect on the microvilli of the ileum enterocytes.

### 3.4.4 Other observed changes

In some of the GM-fed animals, glandular dilatations with epithelial dysplasia and elongation were observed in the glandular stomach near its junction with the non-glandular part (p = 0.043 with 1-tailed t-test) (Table 11). Glandular dilatations are a natural occurrence, particularly in aged rats (Frantz *et al.*, 1991). However, they have also been observed in association with certain disease or pathological conditions (Frantz *et al.*, 1991; Tarnawski *et al.*, 1991; Şener *et al.*, 2004; Kikuchi *et al.*, 2010). In the current study, both the GM-fed and the non-GM-fed animals had glandular dilatations. However, some of the gland dilatations of the GM-fed rats contained epithelial dysplasia and cellular elongation (Table 11 and Figure 20). These changes could be an effect of the GM-feed. They were most often seen at the junctional region between the non-glandular and glandular stomach areas. The non-glandular stomach is a compartment were ingested products reside for a longer period (Gärtner, 2002). If the GM-feed has a toxic component, the effects are more likely to be observed in the junctional region of the glandular stomach, because of the prolonged holding of the feed in that compartment. In the non-glandular stomach adverse changes are not likely to be seen due to the protective function of the keratinized layer.

Epithelial dysplasia seen in the glandular dilatations could indicate the presence of immature cells that had migrated from the proliferative zone. Injury can alter the differentiation of cells from the proliferative zone, that is, it can change stomach cell lineages (Kikuchi *et al.*, 2010) and thus alter the staining-properties of the gastric gland cells. Non-steroidal anti-inflammatory drugs, ethanol and *Helicobacter pylori* infection have been known to cause damage/changes to the stomach proliferative zone and consequently initiate the migration of immature cells (Kikuchi *et al.*, 2010). The method of regeneration following such damage is still poorly understood; however, immunohistochemistry for certain progenitor cell markers and metaplasia can better

reveal the nature of the change (Figure 31). In particular the markers for proliferating cell nuclear antigen (PCNA), doublecortin and calcium/calmodulin-dependent protein kinase-like-1 (DCAMKL1), spasmolytic polypeptide-expressing metaplasia (SPEM), and trefoil family factor 2 (TFF2) (Kikuchi *et al.*, 2010).

The presence of immature cells, with altered staining properties, along with cystic glandular dilatations may be a sign of ulcer healing, or gastric epithelia dysplasia (Figure 31) (Kikuchi *et al.*, 2010). The ulcer healing is characterised by the predominant presence of mucous-like cells in the gastric glands and neck regions (Kikuchi *et al.,* 2010). According to the stage of healing, it may also be accompanied by an increase in mucosal connective tissue, and a decrease in mucosal thickness (Tarnawski *et al.,* 1990). The present study observed cells with altered staining properties in the glands, but did not observe an increase in mucosal connective tissue, or a decrease in mucosal thickness. Immunohistochemistry for markers of progenitor cells can better determine the nature of the changes, in particular the distribution of TFF2, DCAMK1 and PCNA-labelled cells (Figure 31) (Kikuchi et al., 2010).

Gastric epithelial dysplasia is a pre-neoplastic condition that generally leads to cancer (Cui et al., 2001; Kikuchi et al., 2010). It is associated with an increase in proliferation of epithelial cells and an abnormal distribution of such cells in the gastric mucosa. In addition, there is an impairment of apoptosis (Cui et al., 2001). In the present study, an increase in proliferative cells was not observed. In fact, a decrease in proliferation was seen (20.02% less than non-GM; $p = 0.028$). In addition, only two animals, one from each group, had abnormally distributed proliferative cells. There was also low numbers of apoptotic cells in both groups. Therefore, gastric epithelial dysplasia is the unlikely cause of the presence of immature cells and gland dilatations. However, immunohistochemistry for markers of progenitor cells can better evaluate this; in particular the markers, TFF2, DCAMK1, PCNA, and SPEM (Figure 31) (Kikuchi et al., 2010).

Elongation of epithelial cells seen in the glandular dilatations could indicate the glandular epithelium is undergoing repair. Elongation of epithelial cells in gut mucosa is a rapid repair mechanism (restitution) that occurs following a superficial insult to the mucosal epithelium (Hirst, 2011). The mechanism involves the elongation of the remaining viable cells to cover the basal lamina to maintain mucosal barrier integrity and continuity until other mechanisms, such as, cellular proliferation and/or an extensive inflammatory response ensue (Paimela *et al.*, 1995; Hirst, 2011). Restitution is

much faster than cell division and occurs within minutes to an hour after insult (Paimela *et al.*, 1995). Restitution is most often seen at the liminal surface and the pits (Ito *et al.*, 1984), however, if the epithelium of the gastric glands were damaged, this same mechanism of repair may occur to ensure that the basal lamina is not breached.

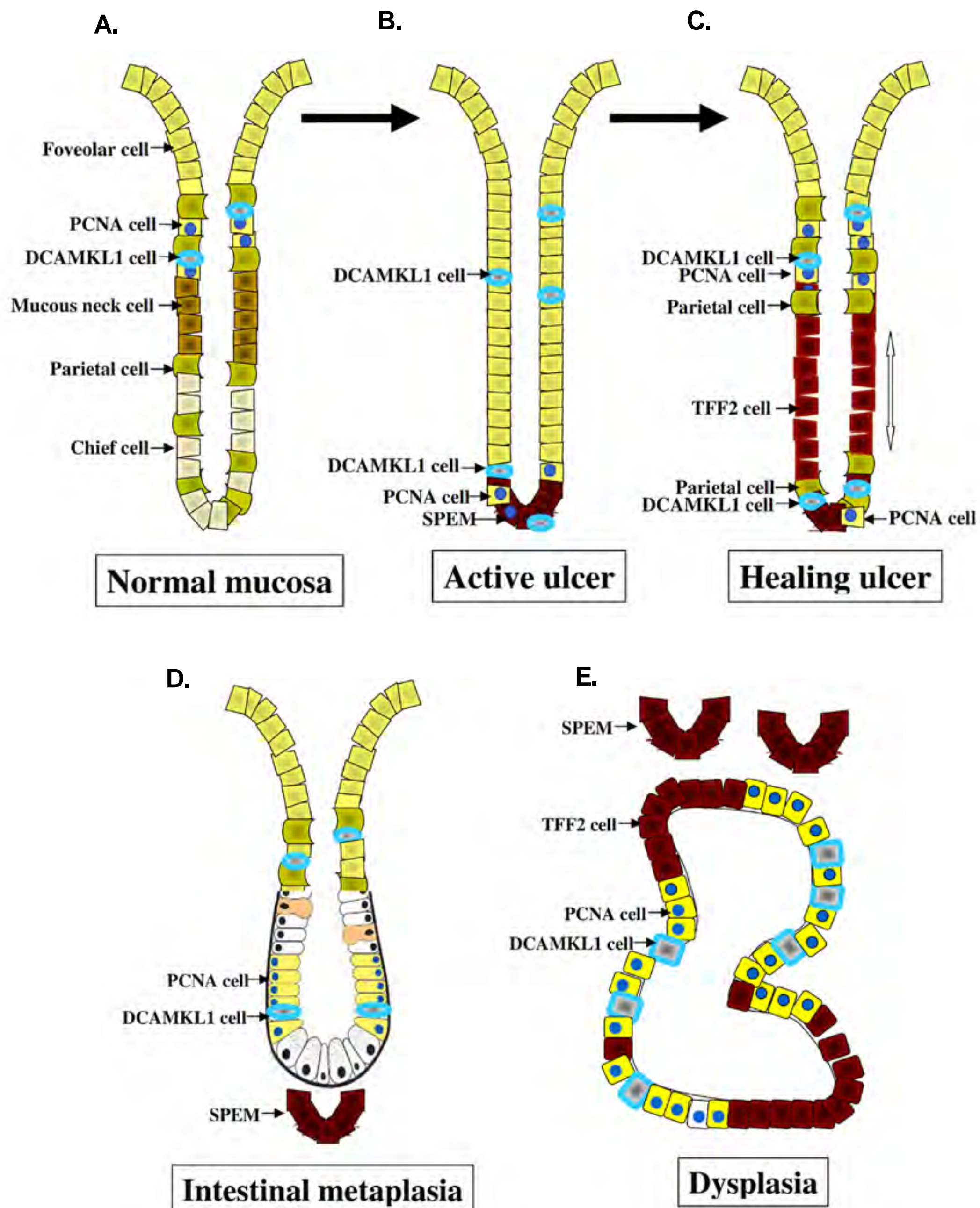



**Figure 31. Gastric mucosa showing various changes in cell population that may arise following gastric mucosal injury**

**A.) Structure of the normal/healthy mucosa showing the normal distribution of epithelial cells**.

**B.) Changes in epithelial cell lineages and their distribution in the margin of an active ulcer**. In the gastric mucosa neighbouring an active ulcer, parietal and chief cells are absent, there are a few DCAMKL1 cells in the pits, neck and deep gland regions, PCNA cells are few and the deep gland is primarily lined my SPEM cells.

**Figure 31. (cont.)**

**C.) Changes in epithelial cell lineages and their distribution in the margin of a healing ulcer**. In the healing ulcer, parietal cells reappear in the neck and gastric glands. Mucous-like cells predominate in the neck and glandular regions, with most expressing TTF2. Some PCNA and DCAMKL1 cells appear in the neck region and deep in the glands below the TTF2-expressing cells.

**D.) Changes in epithelial cell lineages in intestinal metaplasia.** In affected areas, intestinal-like cells predominate, many of which express PCNA. DCAMKL1 cells are also present in the deep pit region and in the gland distal to the PCNA cells. TTF2-expresing cells, consistent with SPEM appearance, are found in the deep mucosal region below the gastric glands that have intestinal metaplasia.

**E.) Changes in gland structure (cystic dilatation) and gastric gland cell lineages in gastric epithelial dysplasia**. Cells lining the cystic glands are cuboidal and predominantly express TTF2 or PCNA. There are a few DCAMKL1 cells also in the dilated glands. In the glands neighbouring the cystic glands, SPEM cells are often observed.

Abbreviations: PCNA = proliferating cell nuclear antigen; DCAMKL1 = doublecortin and calcium/calmodulin-dependent protein kinase-like-1; SPEM = spasmolytic polypeptide-expressing metaplasia; TFF2 = trefoil family factor 2.

(Source: Kikuchi et al. 2010).

## 3.5 Conclusion

Results demonstrate marked changes to gut morphology following long-term ingestion of feed containing GM corn. Several of the changes suggest a chemically-induced model of toxicity, in particular, the changes to tight junction apposition in the mucus-producing cells of the stomach, as well as villous atrophy in the ileum. A trend of pit and gland hyperplasia also suggests this model of damage. Several of these changes may implicate the *Bt* proteins as the source of toxicity. However, the lack of morphometric analyses in the few published GM-crop feeding studies, makes it difficult to associate or determine the exact cause of the change.

## Summary table of Study 1 results

| | Non-GM-fed | GM-fed | Significant difference |
|---|---|---|---|
| Initial body weight of animals (g) – *mean (SD)* | 49.40 (8.36) | 48.40 (7.72) | NS |
| **Final body weights of animals (g) - *mean (SD)*** | **355.30 (34.90)** | **443.40 (28.98)** | **P = 0.000*** ** |
| Stomach | | | |
| **Average weight of stomach (g) - *mean (SD)*** | **1.69 (0.21)** | **2.08 (0.48)** | **P = 0.038*** |
| Stomach weight : final body weight (%) - *median (IQR)* | 0.46 (0.44-0.50) | 0.43 (0.40-0.49) | NS |
| **Morphometric analyses:** | | | |
| *Non-glandular stomach* | | | |
| Mucosa thickness (µm) - *mean (SD)* | 59.80 (8.90) | 57.41 (6.15) | NS |
| Keratinized layer thickness (µm) - *median (IQR)* | 8.53 (7.65-8.75) | 8.13 (7.40-8.23) | NS |
| Non-keratinized layer thickness (µm) - *mean (SD)* | 51.57 (7.90) | 48.41 (3.62) | NS |
| Keratinized layer : mucosa thickness (%) - *median (IQR)* | 13.79 (12.23-15.12) | 13.83 (13.47-14.85) | NS |
| Non-keratinized layer : mucosa thickness (%) - *median (IQR)* | 86.71 (84.88-87.77) | 86.17 (85.15-86.53) | NS |
| *Glandular stomach – fundus* | | | |
| Mucosa thickness (µm) – *mean (SD)* | 738.51 (100.31) | 774.34 (89.53) | NS |
| Pit depth (µm) – *mean (SD)* | 94.21 (15.58) | 101.94 (11.25) | NS |
| Gland depth (µm) – *mean (SD)* | 606.28 (95.62) | 627.18 (87.52) | NS |
| Pit + gland depth (mean(µm) – *mean (SD)* | 700.49 (95.57) | 729.11 (85.32) | NS |
| Lamina propria thickness below gland (µm) – *mean (SD)* | 38.02 (9.66) | 45.22 (20.20) | NS |
| Pit depth : mucosa thickness (%) – *mean (SD)* | 12.96 (2.79) | 13.34 (2.23) | NS |
| Gland depth : mucosa thickness (%) – *mean (SD)* | 81.88 (2.92) | 80.86 (3.45) | NS |
| Pit + gland depth : mucosa thickness (%) - *median (IQR)* | 94.71 (94.24-95.36) | 95.25 (93.93-95.81) | NS |
| Lamina propria thickness below gland : mucosa thickness (%) – *median (IQR)* | 5.29 (4.64-5.76) | 4.75 (4.21-6.07) | NS |
| Granular leukocytes : area of lamina propria below gland (%) – *mean (SD)* | 0.16 (0.10) | 0.11 (0.05) | NS |
| **Proliferative index (dividing cells per total count) – *mean (SD)*** | **12.75 (3.09)** | **10.07 (1.43)** | **P = 0.028*** |
| **No. of proliferating cells per pit + gland depth – *mean (SD)*** | **0.26 (0.09)** | **0.18 (0.05)** | **P = 0.033*** |
| Apoptotic index | 0.00 | 0.00 | NS |
| Histological observations in fundus: | | | |
| Rats with gland dilatations (no. of affected animals) | 4/10 | 6/10 | NS |
| Rats with gland dilatations exhibiting AB/PAS or PAS positive cells (no. of affected animals) | 4/10 | 6/10 | NS |
| Rats with gland dilatations exhibiting elongated epithelium (no. of affected animals) | 4/10 | 6/10 | NS |
| Gland dilatations with AB/PAS or PAS positive cells | 0/4 | 4/6 | NS |
| Gland dilatations with elongated epithelium | 0/4 | 4/6 | NS |
| **Tight junction study in fundus:** | | | |
| **No. of poorly apposed tight junctions - *median (IQR)*** | **1.00 (0.00-2.00)** | **3.50 (3.00-4.00)** | **P = 0.006**** |
| **Poorly apposed tight junctions per total count (%) – *median (IQR)*** | **7.14 (0.00-26.09)** | **35.42 (24.17-44.44)** | **P = 0.004**** |
| **No. of rats with poorly apposed tight junctions** | **5/9** | **10/10** | **P = 0.033*** |

Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001
Abbreviations: SD = standard deviation; IQR = interquartile range

| | Non-GM-fed | GM-fed | Significant difference |
|---|---|---|---|
| Ileum | | | |
| **Morphometric analyses:** | | | |
| Mucosa thickness (μm) - *median (IQR)* | 534.87 (465.24-623.09) | 417.88 (416.57-442.98) | NS |
| **Villi height (μm) – *mean (SD)*** | **358.03 (79.62)** | **291.93 (51.93)** | **P = 0.049*** |
| Crypt depth (μm) – *mean (SD)* | 167.60 (37.16) | 139.17 (29.84) | NS |
| Lamina propria thickness below crypt (μm) - *median (IQR)* | 7.68 (6.62-9.55) | 7.48 (4.62-12.58) | NS |
| Villi height : mucosa thickness (%) – *mean (SD)* | 66.69 (4.72) | 66.36 (5.85) | NS |
| Crypt depth : mucosa thickness (%) – *mean (SD)* | 31.51 (4.69) | 31.64 (5.03) | NS |
| Lamina propria thickness below crypt : mucosa thickness (%) – *mean (SD)* | 1.81 (1.11) | 2.00 (1.02) | NS |
| **No. of enterocytes per villi height – *mean (SD)*** | **1.24 (0.30)** | **1.68 (0.32)** | **P = 0.010*** |
| No. of goblet cells per villi height – *mean (SD)* | 0.32 (0.09) | 0.43 (0.12) | NS |
| No. of IELs per villi height – *mean (SD)* | 0.30 (0.46) | 0.36 (0.77) | NS |
| Goblet cells : enterocytes (%) – *mean (SD)* | 25.68 (3.14) | 25.28 (4.35) | NS |
| IEL : enterocytes (%) - *median (IQR)* | 26.06 (15.89-50.31) | 19.18 (12.20-47.02) | NS |
| Proliferative index – *mean (SD)* | 57.54 (18.62) | 62.86 (10.52) | NS |
| Proliferating cells per crypt depth – *mean (SD)* | 0.20 (0.10) | 0.21 (0.05) | NS |
| Apoptotic index | 0.00 | 0.00 | NS |
| **Microvilli TEM study:** | | | |
| Microvilli length (μm) – *mean (SD)* | 1.16 (0.13) | 1.31 (0.13) | NS |
| Microvilli density : cytoplasm (%) – *mean (SD)* | 24.27 (3.60) | 24.85 (4.78) | NS |
| Microvilli density (no. of microvilli per surface length) – *median (IQR)* | 2.98 (2.74-3.13) | 3.47 (3.45-3.49) | NS |
| **Tight junction TEM study:** | | | |
| No. of poorly apposed tight junctions - *median (IQR)* | 1.00 (0.00-1.00) | 0.00 (0.00-1.00) | NS |
| Poorly apposed tight junctions per total count (%) - *median (IQR)* | 1.52 (0.00-1.75) | 0.00 (0.00-1.43) | NS |
| **TEM observations** (no. of affected animals): | | | |
| Abnormal microvilli structure | 5/5 | 4/5 | NS |
| Irregular surface bellow microvilli in enterocytes | 4/5 | 3/5 | NS |
| Blebbing of enterocyte surface | 3/5 | 2/5 | NS |
| Visual assessment: visible decrease in microvilli density | 2/5 | 1/5 | NS |
| Necrotic cell(s) present | 2/5 | 1/5 | NS |
| Poorly apposed tight junctions | 3/5 | 2/5 | NS |

Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001
Abbreviations: SD = standard deviation; IQR = interquartile range; IEL = intraepithelial lymphocyte

# 4. Study 2: Long-term feeding study of rats fed 30% corn

## 4.1 Introduction

The role of the second study was to investigate the effects of feeding a triple-stack GM corn diet at a lower dose (30%). The effects were investigated in the same organs as previously, with the addition of the jejunum.

## 4.2 Materials and Methods

All procedures were performed under two ethics approvals:

1) Animal Ethics Committee project no. M-2012-103A, University of Adelaide, South Australia.
2) Animal Welfare Committee project no. 646/07, Flinders University, South Australia.

All animal work was performed in accordance with the South Australian Prevention of Cruelty to Animals Act (1985) and with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).

All procedures were performed under blinded conditions.

### 4.2.1 Diet

The feed was prepared as described in Section 3.2.2. The experimental diets were semi-purified diets, formulated by Specialty Feeds (Glen Forrest, Australia) to meet the nutritional requirements for growth and well-being of rats (i.e. comparable to the standard rat diet, AIN-93G Growth Purified Diet; Appendix A1.2). The corn content in both GM and non-GM diets was 30%.

### 4.2.2 Animal Feeding

Thirty male Sprague Dawley rats weighing 78g ± 12g were obtained after weaning at 3 weeks of age. They were randomly placed into two groups, GM (n=15) and non-GM-fed (n=15) and fed a diet containing either 30% GM corn or 30% non-GM corn for 26 weeks. Rats were housed in pairs with *ad libitum* access to water and feed. Animal rooms were maintained at 21$^{o}$C ± 2$^{o}$C temperature, 40-60% humidity and a 12h light/dark cycle. The rats were monitored daily and weighed weekly. After 26 weeks, the rats were weighed, anaesthetised with isoflurane and euthanized via exsanguination. The stomach, jejunum and ileum were removed immediately post-mortem by a certified veterinarian.

### Stomach

At post mortem, stomachs were not weighed, so as to minimise the degeneration of the tissue by placing them into fixative as soon as possible. The stomachs were processed for LM and TEM as described in Section 3.2.3. Curling of the stomach during fixation was prevented by mounting the tissue onto a small piece of cardboard.

### Small intestine

Based on the experience of the first study, a method of sectioning the gut was modified to obtain a larger number of well-orientated, measurable villi. Instead of preparing histological slides by cross-sectioning the gut, longitudinal sections were obtained. On the cross sections of the small intestine, only 5 villi were well-orientated on average. Longitudinal sections allowed observation of on average 10 well-orientated villi in one slide. This reduced the number of sections needed for morphometric measurements or counts.

Sections of the jejunum and ileum were collected, cut along the mesentery, flushed with a PBS, mounted on a small piece of cardboard and fixed in 10% formalin for light microscopy. In addition, 1mm long rings of the jejunum and ileum were fixed in a solution containing 2% glutaraldehyde, 3% paraformaldehyde and 0.1M phosphate buffer (pH 7.4), then cut into 1mm cubes and processed for electron microscopy.

## 4.2.3 Histopathology

### *Light microscopy*

Fixed samples were processed and embedded in paraffin wax as described in Section 3.2.4. Small intestine (jejunum and ileum) sections were cut longitudinally into two halves and embedded as longitudinal bands.

### Stomach

Morphometric analysis and cell counts were performed as described in Section 3.2.4. In addition, the frequency of gland dilatations was investigated in three areas of the glandular stomach: 1) 3mm from non-glandular/glandular junction; 2) next 3mm; 3) anywhere else; 4) 1mm from pylorus. A dilated gland was defined as having a lumen-area of a minimum of 400μm$^2$. Dilated glands that appeared to be in the same column-axis, were counted as one.

### Small intestine (jejunum and ileum)

Morphometric analysis and cell counts were performed as described in Section 3.2.4. In sections where a Peyer's patch was present, measurements and counts were obtained 1mm away from the Peyer's patch.

### *Immunohistochemistry*

Immunohistochemistry for proliferation (Ki67) and apoptosis (caspase 3) were performed in the stomach and small intestine as described in Section 3.2.4.

### *Electron microscopy*

Transmission electron microscopic processing and investigation of stomach and small intestine (jejunum and ileum) were carried out using a FEI Tecnai G2 Spirit Microscope, as described in Section 3.2.4. In the jejunum and ileum sections, the point-counting method used Image J software and a 199cm$^2$ point graticule overlaying transmission electron micrographs (5-18 micrographs per rat per GI tract region) of the enterocyte apical surface taken at 20,500 magnification.

### 4.2.4 Statistical analysis

Statistical analyses were performed as described in Section 3.2.5. Based on the first study, where a potential directional change was likely, a one-tailed t-test was used. That is, where an increase in a parameter was predicated, the one-tailed test would only reveal differences in that direction. A large reduction in that parameter would not be significant. The converse would be true, where a decrease was predicated. A $p \leq 0.050$ was considered significant.

## 4.3 Results

### 4.3.1 Animal feeding

The average initial weights of the GM-fed and the non-GM-fed rats were similar (Table 20). During the feeding trial, rats of both groups gained weight at a normal rate (data not shown at the collaborators' request). Final body weights were comparable between the two groups (Table 20).

**Table 20. Initial and final body weights of rats fed 30% corn diet for 26 weeks.** Values show mean and standard deviation (SD).

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[a] |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | n | Mean | SD | n | | |
| average initial weight of rats (g) | 78.87 | 12.02 | 15 | 77.93 | 12.49 | 15 | NS | -1.19 |
| average final weight of rats (g) | 545.00 | 56.34 | 15 | 550.60 | 50.69 | 15 | NS | 1.03 |

[a] The effect of eating the GM diet compared with the non-GM diet as a percentage change. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

## 4.3.2 Histopathology

### *Non-glandular stomach*

Mucosal thickness measurements revealed no differences between the groups (Table 21; Figure 32). In either group, no histopathological changes were observed (Figure 33).

**Table 21. Non-glandular stomach morphometric analyses from rats fed a 30% GM or non-GM corn diet.** Values show mean and standard deviation (SD).

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[a] |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | n | Mean | SD | n | | |
| average mucosa thickness (μm) | 38.27 | 3.72 | 15 | 38.69 | 4.68 | 15 | NS | 1.10 |
| average thickness of keratinized layer (μm) | 45.15 | 4.11 | 15 | 45.47 | 4.86 | 15 | NS | 0.71 |
| average thickness of non-keratinized layer (μm) | 6.87 | 0.95 | 15 | 6.78 | 0.94 | 15 | NS | -1.31 |
| % keratinized/mucosa thickness | 15.25 | 1.84 | 15 | 14.76 | 2.21 | 15 | NS | -3.21 |
| % non-keratinized/mucosa thickness | 84.75 | 1.84 | 15 | 84.98 | 2.25 | 15 | NS | 0.27 |

[a] The effect of eating the GM diet compared with the non-GM diet as a percentage change. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

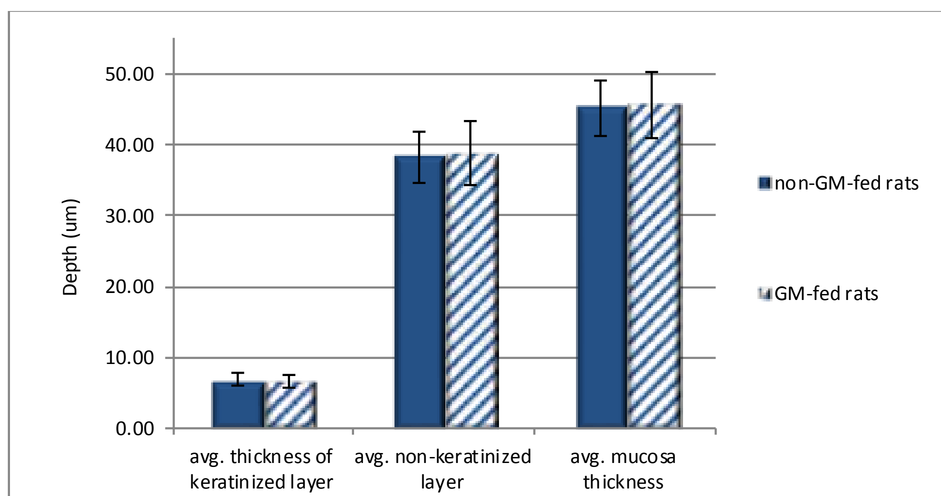



**Figure 32. Average mucosal measurements of the non-glandular stomach of rats fed a 30% GM or non-GM corn diet.** Bars indicate standard deviation.

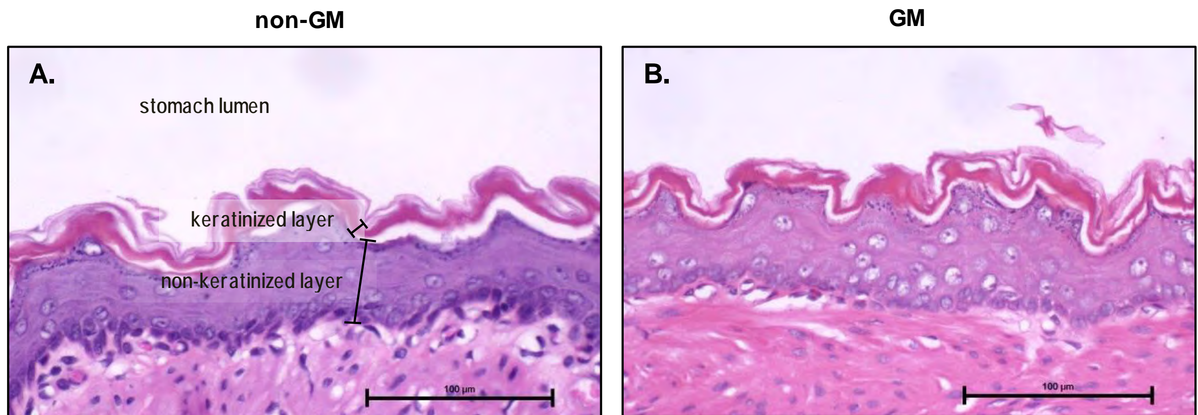



**Figure 33. Sections of the non-glandular stomach, stained with H&E, from rats fed a 30% GM or non-GM corn diet.** A.) Non-GM-fed rat and B.) GM-fed rat. Scale bar = 100µm

## *Glandular stomach*

### Light microscopy

In several rats of both groups, there was poor preservation of gastric pits; however, glands were well preserved. In regions were both the pits and glands were well-preserved (i.e. nuclei appeared normal) and well-orientated, mucosal measurements were performed. One to six mucosal measurements were performed in five rats of the GM-fed and four of the non-GM-fed group. These results showed an increase in gland depth ($p = 0.039$), as well as an increase in the combined pit and gland depth in the GM-fed group ($p = 0.046$) (Table 23; Figures 28 and 29). Mucosa thickness and pit depth were higher in the GM-fed group compared with the non-GM-fed group; however these results were not statistically significant.

There was a mild increase of granular leukocytes in the lamina propria of the glandular region of the mucosa of both feeding groups (as assessed using the Updated Sydney System, Figure 10). Lymphocytes were rarely seen in the lamina propria of the pit and glandular region of the mucosa.

**Table 22. Glandular stomach (fundus) morphometric analyses and cell counts from rats fed a 30% GM or non-GM corn diet.** Values show mean and standard deviation (SD). Statistically significant values are given in bold.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050)[a] | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | n | Mean | SD | n | | |
| average mucosa thickness (μm) | 714.56 | 79.27 | 4 | 812.38 | 77.12 | 5 | NS | 13.69 |
| average pit depth (μm) | 92.16 | 23.90 | 4 | 100.39 | 18.93 | 5 | NS | 8.93 |
| **average gland depth (μm)** | **588.53** | **64.78** | **4** | **682.56** | **70.06** | **5** | **P = 0.039*** | **15.98** |
| **average pit + gland depth (μm)** | **680.70** | **77.65** | **4** | **782.95** | **78.26** | **5** | **P = 0.046*** | **15.02** |
| average thickness of lamina propria below glands (μm) | 33.87 | 10.62 | 4 | 29.43 | 3.09 | 5 | NS | -13.11 |
| % pit/ mucosa thickness | 12.88 | 2.69 | 4 | 12.35 | 1.96 | 5 | NS | -4.11 |
| % gland/ mucosa thickness | 82.37 | 1.35 | 4 | 83.99 | 2.14 | 5 | NS | 1.97 |
| % pit + gland/ mucosa thickness | 95.25 | 1.37 | 4 | 96.34 | 0.57 | 5 | NS | 1.14 |
| % lamina propria thickness below gland/ mucosa thickness | 4.75 | 1.37 | 4 | 3.66 | 0.57 | 5 | NS | -22.95 |

[a] Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

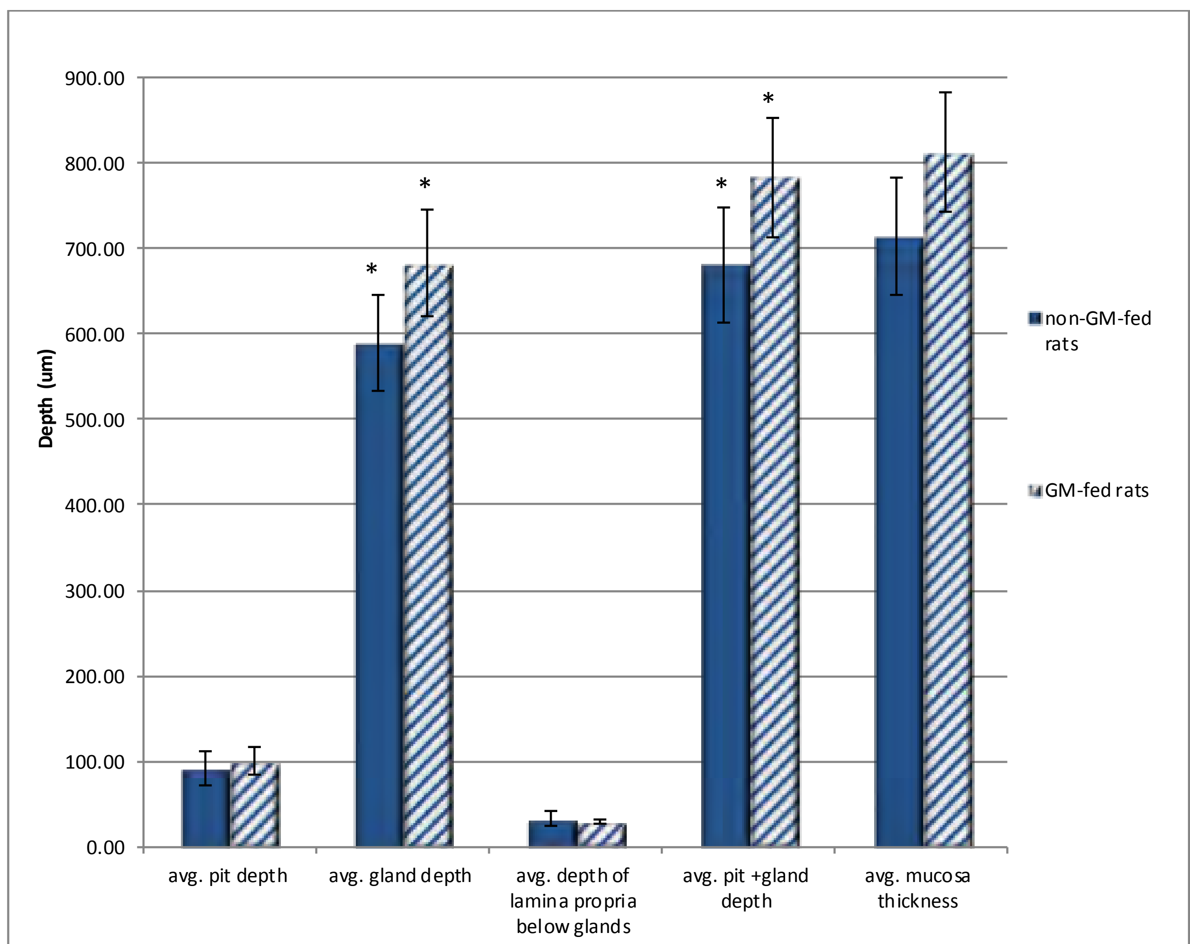



**Figure 34. Average mucosal measurements for glandular stomach (fundus) of rats fed a 30% GM or non-GM corn diet.** Bars indicate standard deviation. Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001

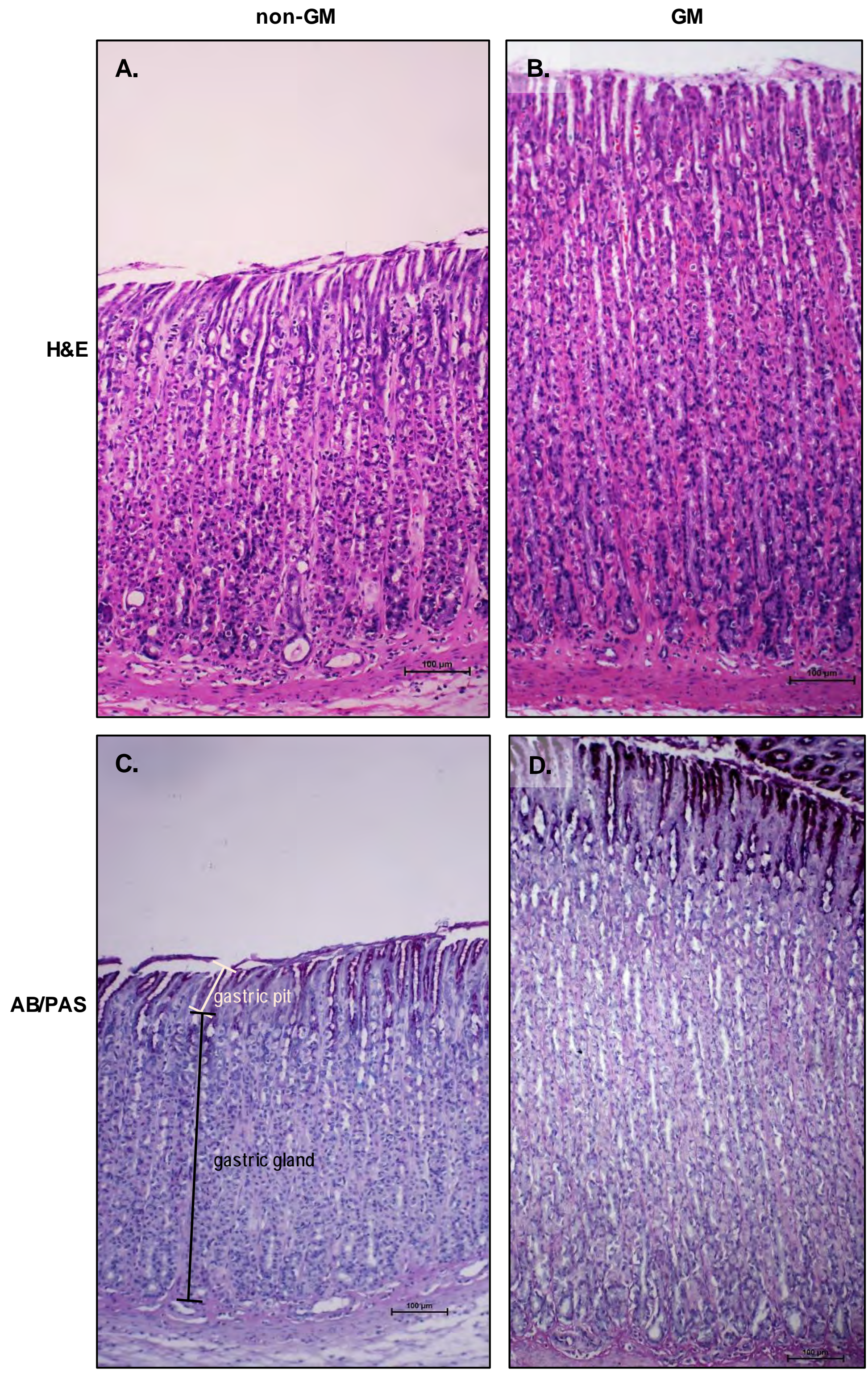



**Figure 35. Sections of the stomach (fundus) from rats fed a 30% GM or non-GM corn diet.** A.) Non-GM-fed rat, and B.) GM-fed rat stained with H&E; and C.) non-GM-fed rat and D.) GM-fed rat stained with AB/PAS. Scale bar = 100µm

The frequency of glandular dilatations in all 4 areas of the stomach in both groups revealed no statistically significant differences (Table 23). All animals of the GM-fed group (15/15) and 10 out of 14 in the non-GM-fed group had glandular dilatations (p = 0.042; Table 24). Glandular dilatations were most common in area 3 of both the GM and non-GM-fed groups. Both groups of rats had gland dilatations with AB/PAS or PAS positive cells and/or with elongated epithelial cells (Table 24).

**Table 23. Number of glandular dilatations observed in the glandular stomach (fundus) of rats fed a 30% GM or non-GM corn diet.** The stomach was divided into three areas: 1) 3mm from glandular/non-glandular junction; 2) next 3mm; 3) anywhere else; 4) 1mm from pylorus. Values show median and interquartile range (IQR).

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%) |
|---|---|---|---|---|---|---|---|---|
| | Median | IQR | n | Median | IQR | n | | |
| no. of gland dilatations in area 1 | 0.00 | 0.00-075 | 14 | 1.00 | 0.00-0.45 | 15 | NS | NA |
| no. of gland dilatations in area 2 | 1.00 | 0.00-2.74 | 14 | 0.00 | 0.00-2.50 | 15 | NS | -100.00 |
| no. of gland dilatations in area 3 | 2.50 | 0.00-3.75 | 14 | 3.00 | 1.00-6.25 | 14 | NS | 20.00 |
| no. of gland dilatations in area 4 | 0.00 | 0.00-1.00 | 5 | 0.00 | 0.00-0.00 | 6 | NS | -100.00 |
| total no. of gland dilatations (areas 1-4) | 3.50 | 0.50-13.50 | 14 | 8.00 | 2.50-14.50 | 15 | NS | 128.57 |
| total no. of gland dilatations (areas 1-3) | 3.50 | 0.50-12.75 | 14 | 8.00 | 2.50-14.50 | 15 | NS | 128.57 |

[a] The effect of eating the GM diet compared with the non-GM diet as a percentage change.

**Table 24. Number of animals with glandular dilatations in the gastric glands of the fundus of rats fed a 30% GM or non-GM corn diet.** The stomach was divided into three areas: 1) 3mm from glandular/non-glandular junction; 2) next 3mm; 3) anywhere else; 4) 1mm from pylorus. Statistically significant results are given in bold.

| | GM | | non-GM | | Relative risk | 95% confidence interval | Statistical significance (P ≤0.050)[a] |
|---|---|---|---|---|---|---|---|
| | Outcome of interest | | Outcome of interest | | | | |
| | present | absent | present | absent | | | |
| **Gland dilatations observed in all 4 areas** | **15** | **0** | **10** | **4** | **1.40** | **1.01 – 0.95** | **P = 0.042*** |
| **Gland dilatations observed in areas 1-3** | **15** | **0** | **10** | **4** | **1.40** | **1.01 – 0.95** | **P = 0.042*** |
| Gland dilatations with AB/PAS and/or PAS positive cells | 8 | 5 | 4 | 9 | 2.00 | 0.80 – 5.03 | NS |
| Gland dilatations with elongated cells | 7 | 6 | 4 | 9 | 1.75 | 0.67 – 4.56 | NS |

[a] Statistical analysis performed was the Fisher's exact test. Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001

## *Small intestine (jejunum and ileum)*

### Jejunum

Mucosal measurements (Table 25) were higher in GM-fed than in non-GM-fed group; however, these were not statistically significant (Figures 36 and 38). Intraepithelial

lymphocyte counts were in the normal range in the GM-fed group, but were slightly elevated in the non-GM-fed group (p = 0.032). The ratio of goblet cells to enterocyte was slightly less than normal in both groups; however this was not statistically significant (Tables 25 and 26; Figure 37).

**Table 25. Mucosal measurements and cell counts of the jejunum of rats fed a 30% GM or non-GM corn diet.** Values show mean and standard deviation (SD). Statistically significant values are given in bold.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050)[a] | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | n | Mean | SD | n | | |
| average mucosa thickness (μm) | 735.12 | 49.57 | 14 | 746.08 | 67.66 | 14 | NS | 1.49 |
| average villi height (μm) | 597.09 | 49.22 | 14 | 602.97 | 52.16 | 14 | NS | 0.98 |
| average crypt depth (μm) | 131.96 | 16.71 | 14 | 141.08 | 19.16 | 14 | NS | 6.91 |
| average crypt depth (μm) (complete sample size) | 129.71 | 18.31 | 15 | 139.35 | 19.63 | 15 | NS | 7.43 |
| average thickness of lamina propria below crypt (μm) | 6.48 | 1.77 | 14 | 6.79 | 1.92 | 14 | NS | 4.78 |
| % villi height / mucosa thickness | 81.18 | 2.44 | 14 | 80.87 | 1.99 | 14 | NS | -0.38 |
| % crypt depth / mucosa thickness | 17.98 | 2.21 | 14 | 18.91 | 1.88 | 14 | NS | 5.17 |
| % lamina propria thickness below crypt / mucosa thickness | 0.89 | 0.27 | 14 | 0.91 | 0.22 | 14 | NS | 2.25 |
| average no. of enterocytes / villi height | 0.90 | 0.08 | 14 | 0.09 | 0.10 | 14 | NS | -90.00 |
| average no. of goblet cells / villi height | 0.16 | 0.03 | 14 | 0.16 | 0.02 | 14 | NS | 0.00 |
| **average no. of IELs / villi height** | **0.24** | **0.08** | **14** | **0.18** | **0.07** | **14** | **P = 0.038*** | **-25.00** |
| % goblet cells / enterocytes | 18.17 | 2.68 | 14 | 17.60 | 2.90 | 14 | NS | -3.14 |
| **% IEL / enterocytes** | **26.55** | **7.91** | **14** | **19.84** | **7.71** | **14** | **P = 0.032*** | **-25.27** |

[a] Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

**Table 26. Population of cells per enterocyte in the jejunum of rats fed a 30% GM or non-GM corn diet.** Normal population of goblet cells per enterocyte is 20%. Normal population of IELs per enterocyte is 0-20%.

| | GM | | non-GM | | Relative risk | 95% confidence interval | Statistical significance (P ≤0.050)[a] |
|---|---|---|---|---|---|---|---|
| | Outcome of interest | | Outcome of interest | | | | |
| | present | absent | present | absent | | | |
| Percentage of goblet cells per enterocyte above 20% | 2 | 12 | 3 | 11 | 0.67 | 0.13 – 3.40 | NS |
| Percentage of goblet cells per enterocyte below 20% | 9 | 5 | 9 | 5 | 1.00 | 0.58 – 1.74 | NS |
| Percentage of IELs per enterocyte above 20% | 7 | 7 | 10 | 4 | 0.70 | 0.38 – 1.30 | NS |

[a] Statistical analysis performed was the Fisher's exact test.

800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Length (um)
avg. villi height
avg. crypt depth
avg. thickness of lamina propria below crypts
avg. mucosa thickness
non-GM-fed rats
GM-fed rats

**Figure 36. Average mucosal measurements of the jejunum of rats fed 30% GM or non-GM corn diet.** Bars indicate standard deviation.

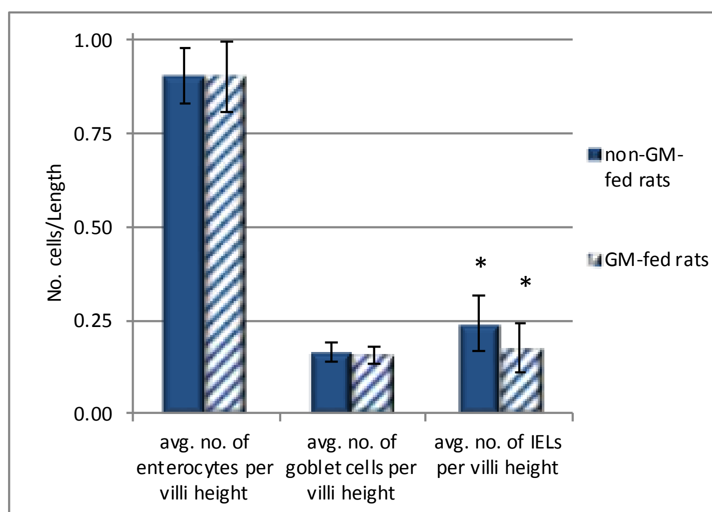



**Figure 37. Average number of enterocytes, goblet cells and IELs of jejunal villi of rats fed 30% GM or non-GM corn diet.** Bars indicate standard deviation. Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001

### *Jejunum*

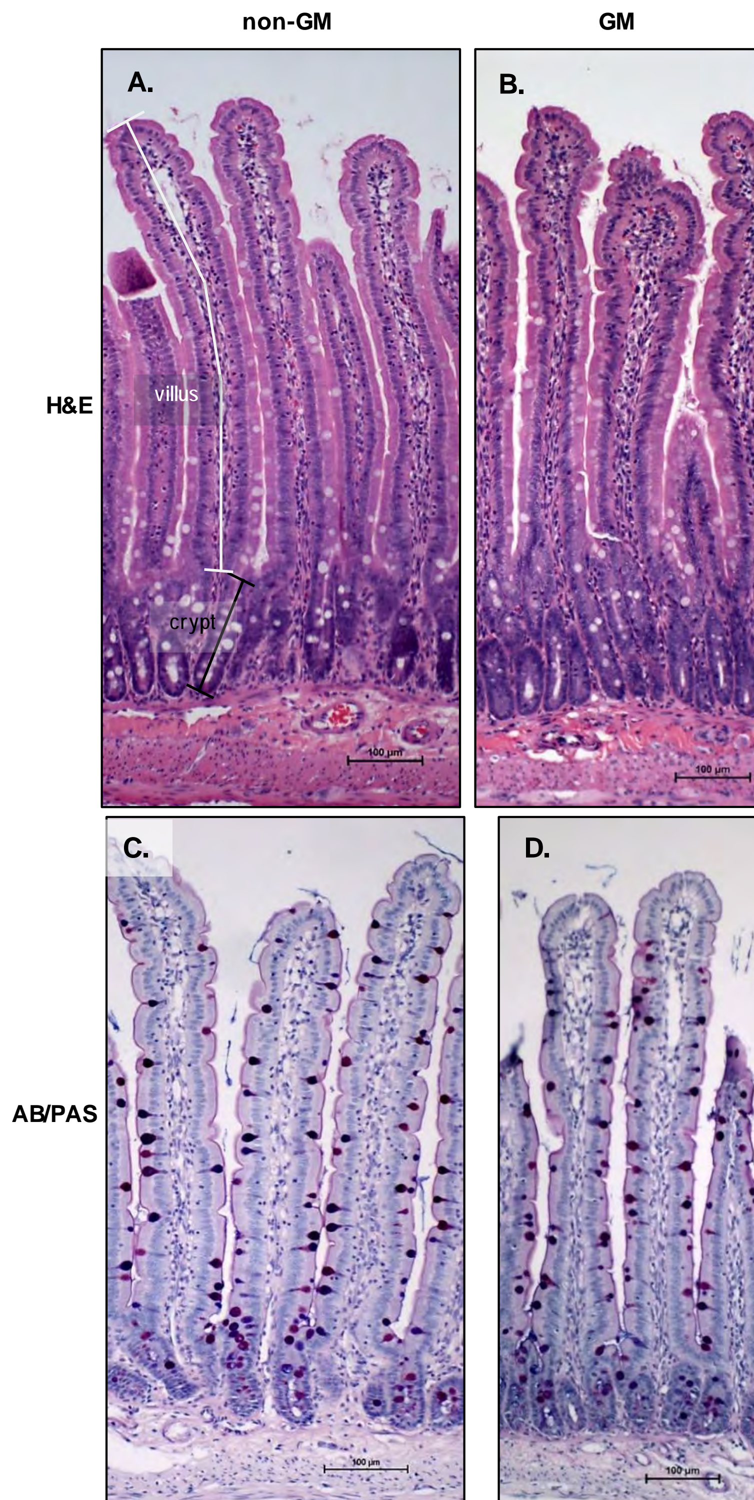



**Figure 38. Sections of the jejunum of rats fed a 30% GM or non-GM corn diet.** A.) Non-GM-fed rat, and B.) GM-fed rat stained with H&E; and C.) non-GM-fed rat and D.) GM-fed rat stained with AB/PAS. Scale bar = 100μm

## Ileum

Mucosal measurements revealed an increase in mucosa thickness and villi height, and a decrease in crypt depth in GM-fed versus non-GM-fed animals. However, these results were not statistically significant (Table 27; Figures 39 and 41). The number of intraepithelial lymphocytes were slightly elevated in the non-GM-fed group, but were in the normal range in the GM-fed group; however this was not statistically significant. The population of goblet cells per enterocyte was slightly higher than normal in both groups; however this was not statistically significant (Tables 27 and 28; Figure 40).

**Table 27. Mucosal measurments and cell counts of the ileum of rats fed a 30% GM or non-GM corn diet.** Values show mean or median and standard deviation (SD) or interquartile range (IQR) depending on whether the data are normally distributed.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Mean or median[a] | SD or IQR[a] | n | Mean or median[a] | SD or IQR[a] | n | | |
| average mucosa thickness (μm) | 403.38 | 51.94 | 13 | 415.19 | 46.77 | 13 | NS | 2.93 |
| average villi height (μm) | 277.11 | 38.54 | 13 | 290.99 | 36.27 | 13 | NS | 5.01 |
| average crypt depth (μm) | 121.13 | 22.25 | 13 | 119.19 | 13.14 | 13 | NS | -1.60 |
| average thickness of lamina propria below crypt (μm) | 4.99 | 1.46 | 13 | 4.31 | 0.90 | 13 | NS | -13.63 |
| % villi height / mucosa thickness | 68.72 | 3.64 | 13 | 70.01 | 1.83 | 13 | NS | 1.88 |
| % crypt depth / mucosa thickness | 29.97 | 3.57 | 13 | 28.76 | 1.66 | 13 | NS | -4.04 |
| % lamina thickness below crypt / mucosa thickness | 1.27 | 0.47 | 13 | 1.05 | 0.27 | 13 | NS | -17.32 |
| average no. of enterocytes / villi height | 0.13 | 0.00 | 12 | 0.13 | 0.01 | 13 | NS | 0.00 |
| average no. of goblet cells / villi height | 0.03 | 0.00 | 12 | 0.03 | 0.00 | 13 | NS | 0.00 |
| average no. of IELs / villi height | 0.03[a] | 0.03-0.05[a] | 12 | 0.03[a] | 0.02-0.03[a] | 13 | NS | 0.00 |
| % goblet cells / enterocytes | 25.02 | 2.73 | 12 | 25.35 | 3.28 | 13 | NS | 1.32 |
| % IEL / enterocytes | 25.01[a] | 21.66-41.28[a] | 12 | 21.63[a] | 20.47-23.26[a] | 13 | NS | -13.51 |

[a] Not normally distributed data, the median and interquartile range is given.

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

**Table 28. Population of cells per enterocyte in the ileum of rats fed a 30% GM or non-GM corn diet.** Normal population of goblet cells per enterocyte is 20%. Normal population of IELs per enterocyte is 0-20%.

| | GM | | non-GM | | Relative risk | 95% confidence interval | Statistical significance (P ≤0.050)[a] |
|---|---|---|---|---|---|---|---|
| | Outcome of interest | | Outcome of interest | | | | |
| | present | absent | present | absent | | | |
| Percentage of goblet cells per enterocyte above 20% | 11 | 2 | 11 | 1 | 0.92 | 0.69 – 1.23 | NS |
| Percentage of goblet cells per enterocyte below 20% | 1 | 12 | 1 | 11 | 0.92 | 0.07 – 13.18 | NS |
| Percentage of IELs per enterocyte above 20% | 9 | 4 | 11 | 1 | 0.76 | 0.51 – 1.13 | NS |

[a] Statistical analysis performed was the Fisher's exact test

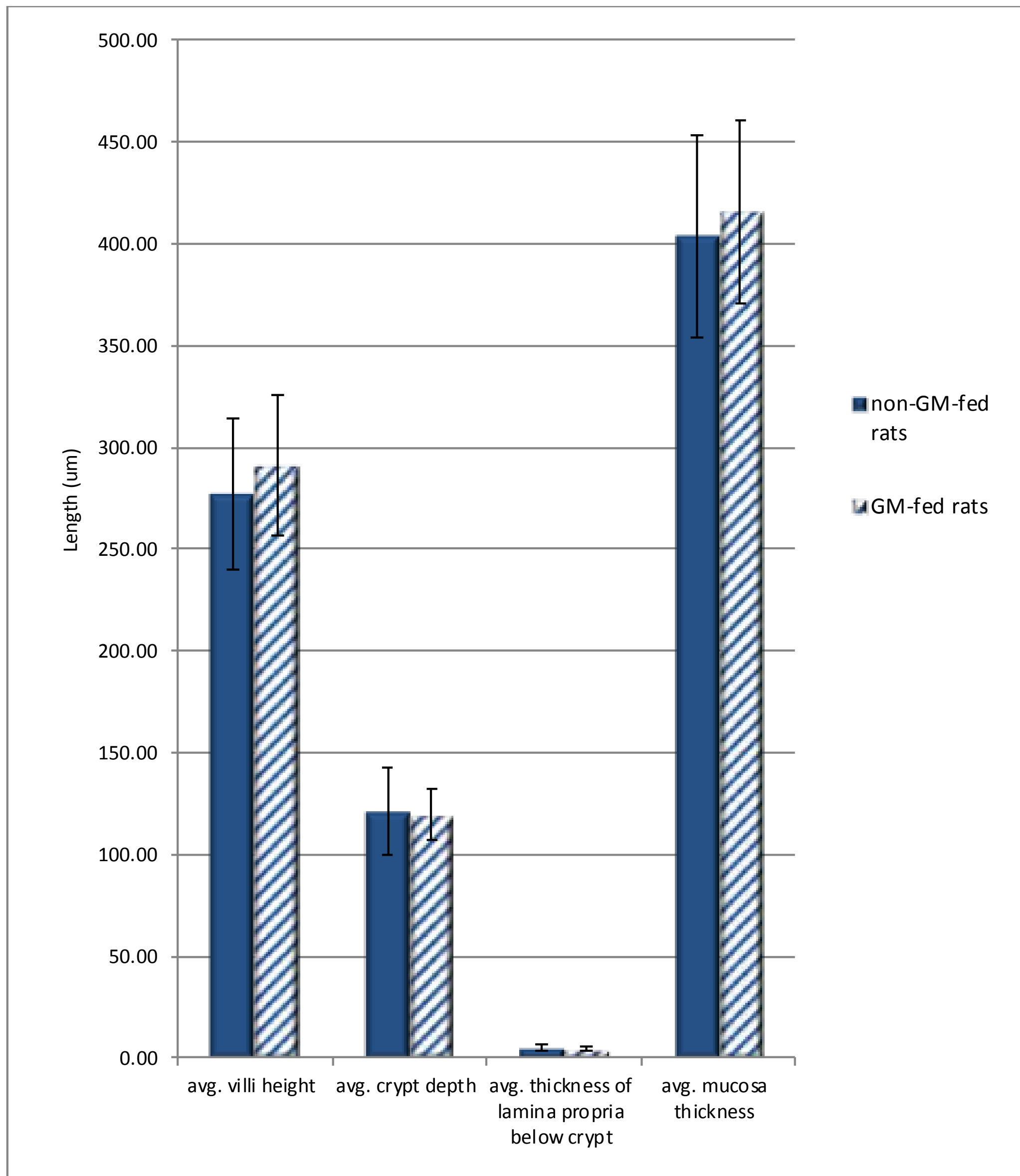



**Figure 39. Average mucosal measurements of the ileum of rats fed 30% GM or non-GM corn diet.** Bars indicate standard deviation.

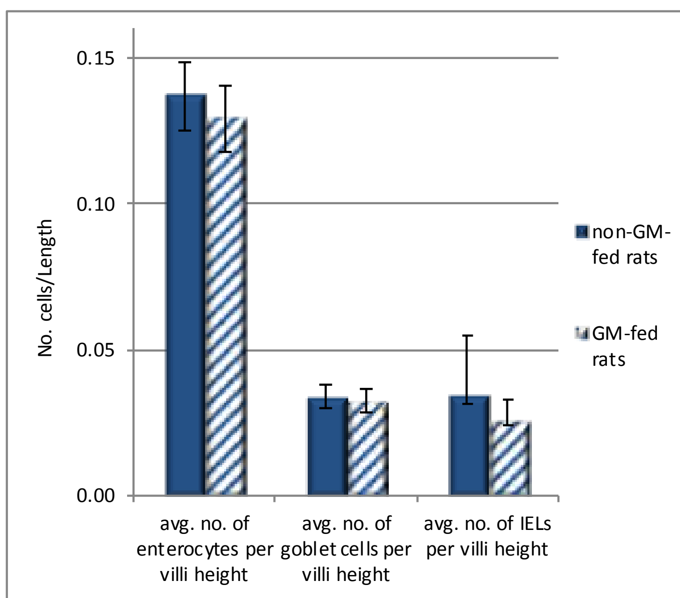



**Figure 40. Average number of enterocytes, goblet cells and IELs of ileal villi of rats fed 30% GM or non-GM corn diet.** Bars indicate standard deviation.

## *Ileum*

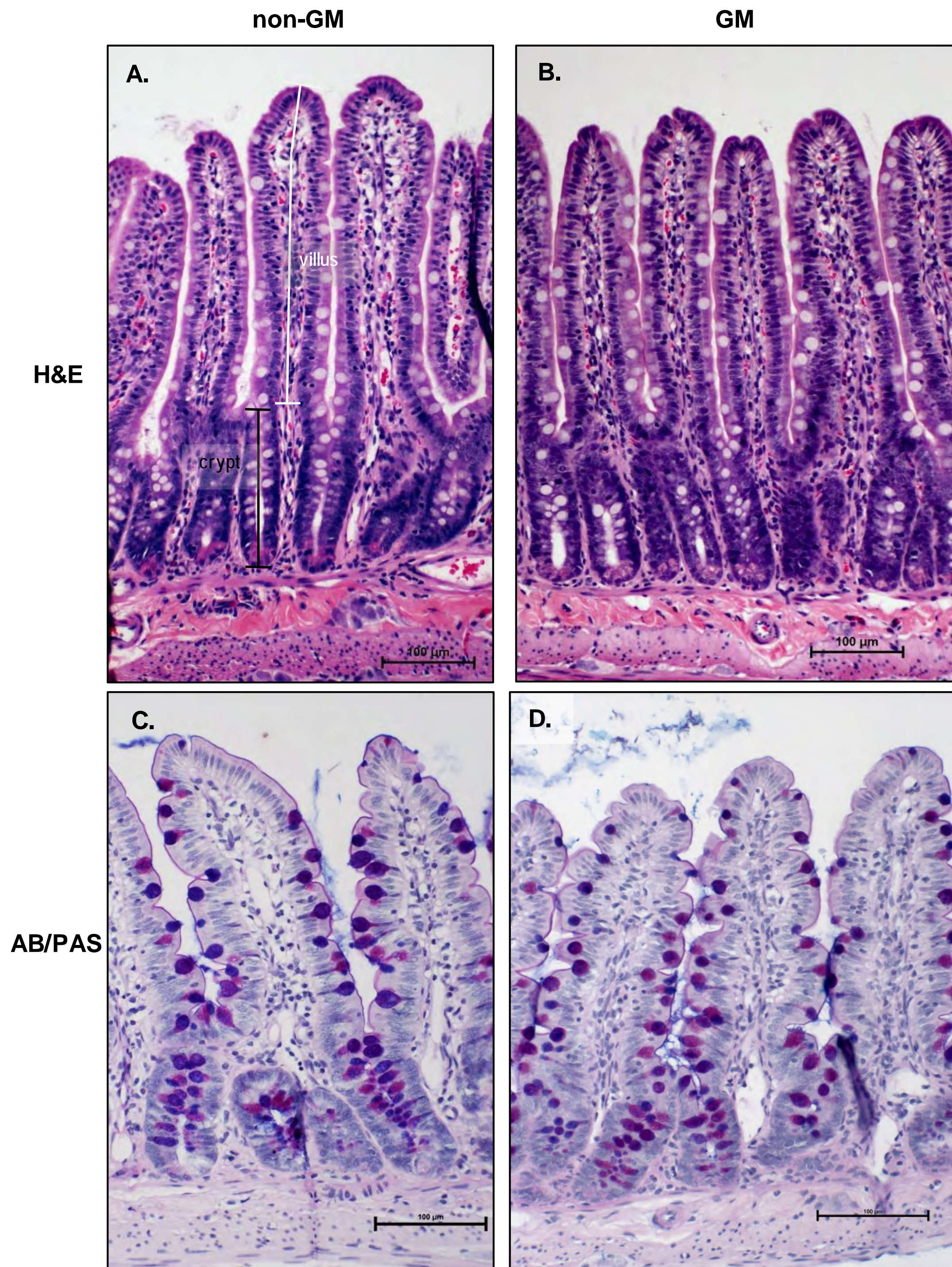



**Figure 41. Sections of the ileum of rats fed a 30% GM or non-GM corn diet.** A.) non-GM-fed rat, and B.) GM-fed rat stained with H&E; and C.) non-GM-fed rat, and D.) GM-fed rat stained with AB/PAS. Scale bar = 100µm

## Branching villi

In the jejunum and ileum, branching villi were observed in several animals of both groups (14/14 in GM and 14/15 non-GM; Figure 42). Their frequency was not statistically significant (Table 29 and 30). In some animals they appeared at regular intervals (every 5-6 villi) (Figure 42B). Sometimes the branches were thin with minimal amount of lamina propria, other times they had a crypt-like, glandular appearance (Figure 42C). Occasionally, branching villi appeared fused to form large structures that occupied a broader area with multiple branches (Figure 42D). In sections treated with anti-Ki67, some of the nuclei of the branching-villus epithelium were labelled (Figure 42F).

**Table 29. Number of branching villi seen in the small intestines of rats fed a 30% GM or non-GM corn diet.** Values show median and interquartile range (IQR).

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%) |
|---|---|---|---|---|---|---|---|---|
| | Median | IQR | n | Median | IQR | n | | |
| *Jejunum* | | | | | | | | |
| 1-2 villi affected per area | 1.00 | 1.00-2.00 | 13 | 2.00 | 0.00-2.25 | 12 | NS | 100.00 |
| larger area affected | 0.00 | 0.00-1.00 | 13 | 0.00 | 0.00-1.00 | 12 | NS | NA |
| total no. of branching villi | 1.00 | 1.00-3.00 | 13 | 2.50 | 0.75-3.00 | 12 | NS | 150.00 |
| *Ileum* | | | | | | | | |
| 1-2 villi affected per area | 2.00 | 0.00-4.00 | 13 | 3.00 | 2.00-4.00 | 13 | NS | 50.00 |
| larger area affected | 1.00 | 0.00-1.00 | 13 | 1.00 | 0.00-2.00 | 13 | NS | 0.00 |
| total no. of branching villi | 2.00 | 1.00-4.00 | 13 | 5.00 | 2.00-7.00 | 13 | NS | 150.00 |

[a] The effect of eating the GM diet compared with the non-GM diet as a percentage change.

**Table 30. The number of rats with branching villi in the jejunum and ileum of rats fed a 30% GM or non-GM corn diet.**

| | GM | | non-GM | | Relative risk | 95% confidence interval | Statistical significance (P ≤0.050)[a] |
|---|---|---|---|---|---|---|---|
| | Outcome of interest | | Outcome of interest | | | | |
| | present | absent | present | absent | | | |
| *Jejunum* | | | | | | | |
| 1-2 villi affected per area | 8 | 4 | 10 | 3 | 0.87 | 0.53 – 1.43 | NS |
| larger area affected | 5 | 7 | 4 | 9 | 1.35 | 0.47 – 3.89 | NS |
| total no. of branching villi | 9 | 3 | 11 | 2 | 0.89 | 0.59 – 1.32 | NS |
| *Ileum* | | | | | | | |
| 1-2 villi affected per area | 12 | 1 | 9 | 4 | 1.33 | 0.90 – 1.98 | NS |
| larger area affected | 8 | 5 | 7 | 6 | 1.14 | 0.59 – 2.22 | NS |
| total no. of branching villi | 13 | 0 | 11 | 2 | 1.18 | 0.94 – 1.49 | NS |

[a] Statistical analysis performed was the Fisher's exact test

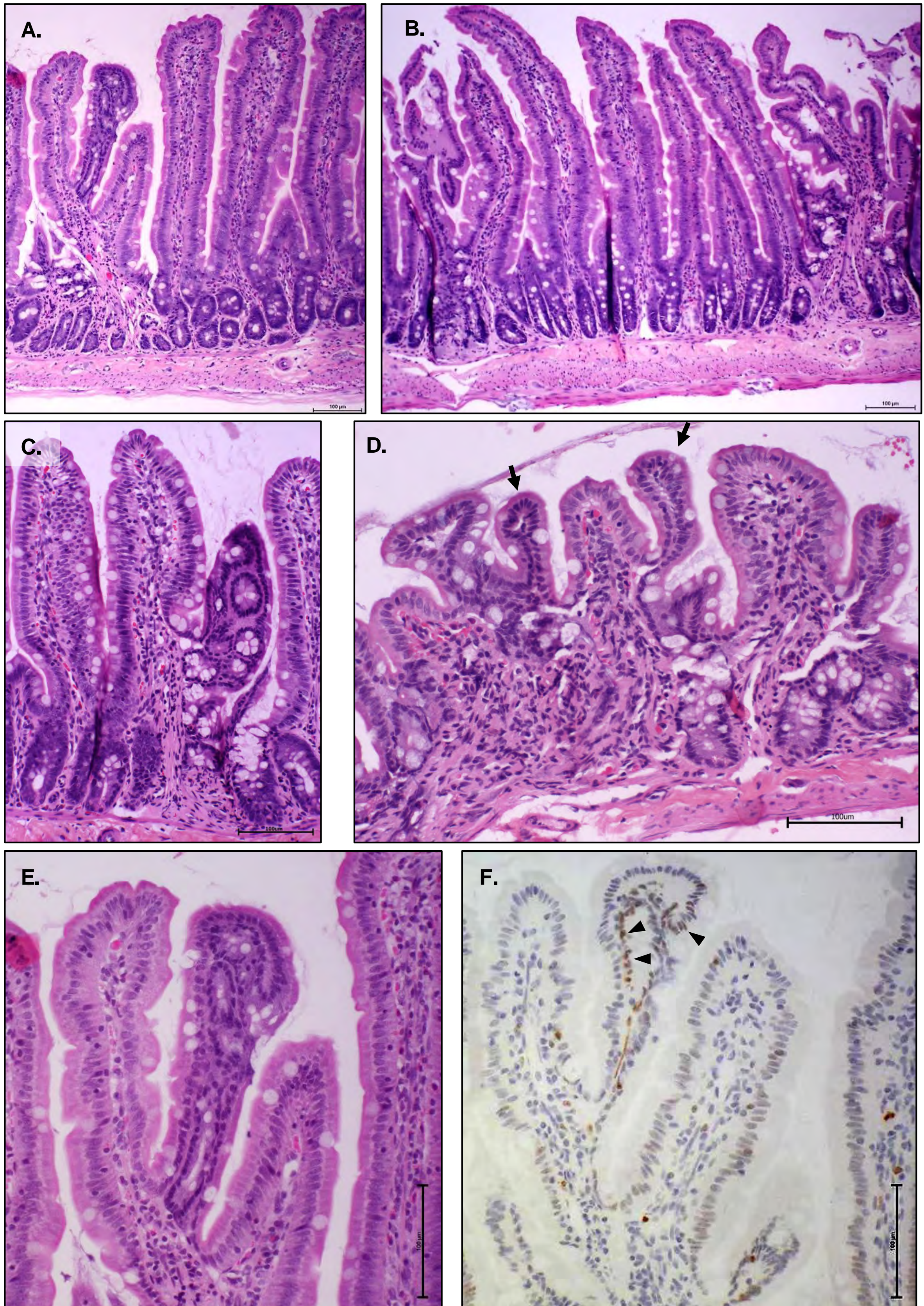



**Figure 42. Branching villi in rats fed a 30% GM or non-GM corn diet.** A.) Jejunal branching villus next to well-orientated villi (H&E); B.) jejunal branching villi at regular intervals among well-orientated villi, along the length of the intestine (H&E); C.) ileal branching villus with glandular appearance (H&E); D.) in ileum, larger area affected showing thin branch-like structures (arrows) extending from a mass that has lost normal villous structure (H&E); E.) jejunal branching villus at higher magnification (H&E), and F.) the corresponding branching villus at higher magnification showing cells labelled with anti-Ki67 (arrowheads). Scale bar = 100μm.

## Immunohistochemistry results

### *Stomach*

In several rats of both groups, there was poor preservation of gastric pits; however, the glands were well preserved. Not enough regions were observed that had well-orientated and well-preserved pit/gland units to obtain apoptotic and proliferative indexes.

### *Small intestine*

In the crypts of the jejunum and ileum, caspase 3 positive cells were scant (i.e. 0-3 cells in the crypts of each animal). In the positive cells, labelling was specifically localised to the nucleus. Positive labelling for caspase 3 was seen in the tips of the villi, where cells are being sloughed off (Figure 43 and 45).

Positive labelling for Ki67 was localised to the nuclei of the cells. Such cells were seen along the epithelium of the jejunal and ileal villi and crypts (Figures 44A and B, and 46A and B). In the base of the crypts (proliferative region), there were 2-3 unlabelled cells in most regions of the ileum.

In the jejunum, the proliferative index was slightly higher (p = 0.003) in the GM than in the non-GM-fed group (Table 31). In the ileum, it was also higher, but was not statistically significant (Table 31).

**Table 31. Dividing cells in the ileum crypt of rats fed a 30% GM or non-GM corn diet.** Values show mean or median and standard deviation (SD) or interquartile range (IQR) depending on whether the data are normally distributed. Statistically significant values are given in bold.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[c] |
|---|---|---|---|---|---|---|---|---|
| | Mean or median[a] | SD or IQR[a] | n | Mean or median[a] | SD or IQR[a] | n | | |
| *Jejunum* | | | | | | | | |
| Proliferative index: **% dividing cells / total no. cells in crypt** | **86.16** | **3.04** | **15** | **89.30** | **2.10** | **15** | **P = 0.003**** | **3.64** |
| no. dividing cells / average crypt depth | 0.29 | 0.03 | 15 | 0.29 | 0.04 | 15 | NS | 0.00 |
| *Ileum* | | | | | | | | |
| Proliferative index: % dividing cells / total no. cells in crypt | 82.41[a] | 79.87-86.77[a] | 13 | 86.63[a] | 79.61-88.35[a] | 13 | NS | 5.12 |
| no. dividing cells / average crypt depth | 0.34 | 0.07 | 13 | 0.31 | 0.04 | 13 | NS | -8.82 |

[a] Not normally distributed data, the median and interquartile range (IQR) are given.

[b] Statistical significance: p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001

[c] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

## *Jejunum*

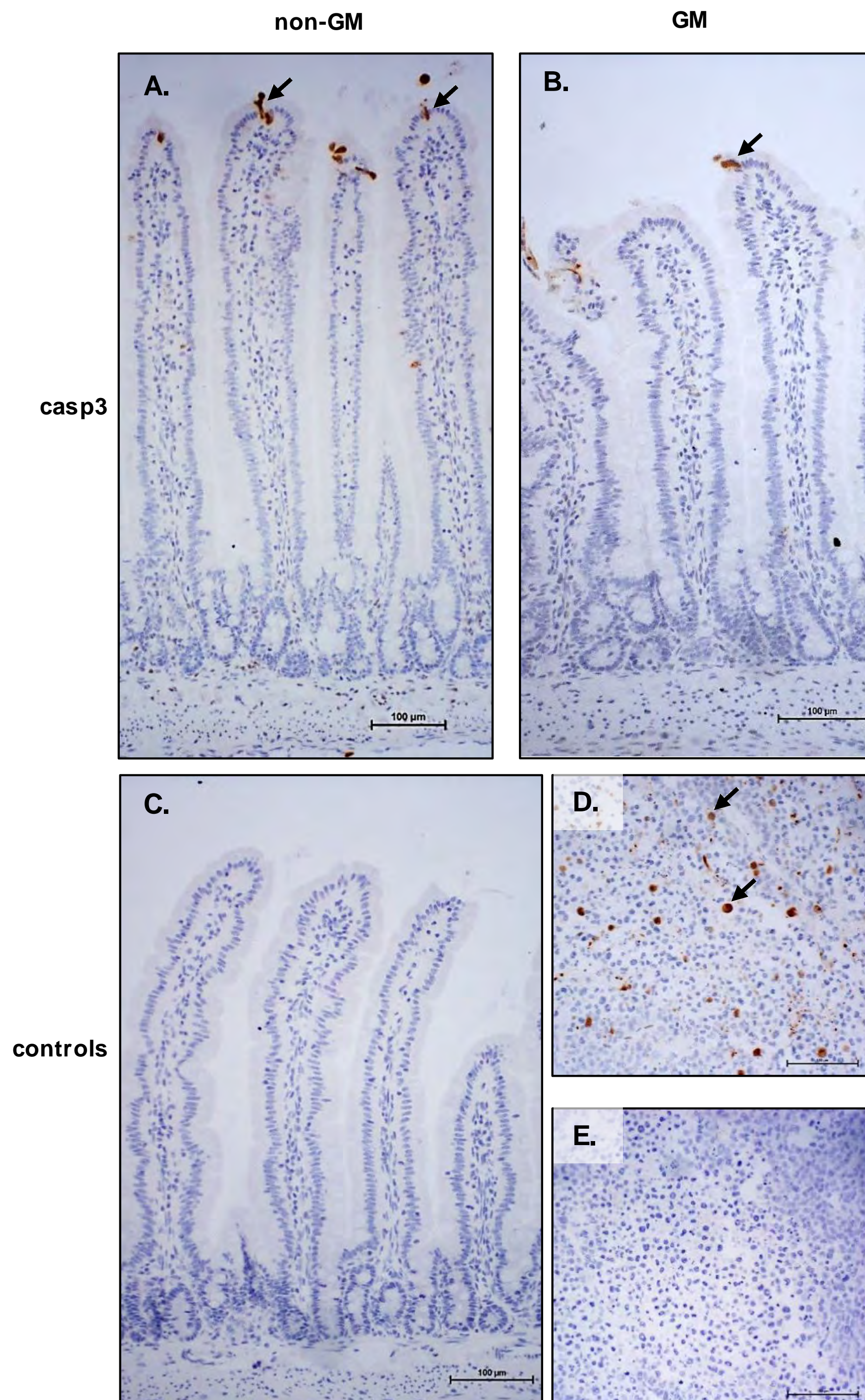



**Figure 43. Sections of the jejunum labelled with anti-caspase 3 (arrows) from rats fed a 30% GM or non-GM corn diet.**

A. and B.) Anti-caspase-3 labelled cells (arrows) in non-GM (A.) and GM-fed (B.) rat.

C.-E.) Controls: C). Jejunum, negative control showing no specific labelling; D.) rat tumour, positive control showing specific labelling of nuclei (arrows); E.) rat tumour, negative control showing no specific labelling. Scale bar = 100µm

## *Jejunum*

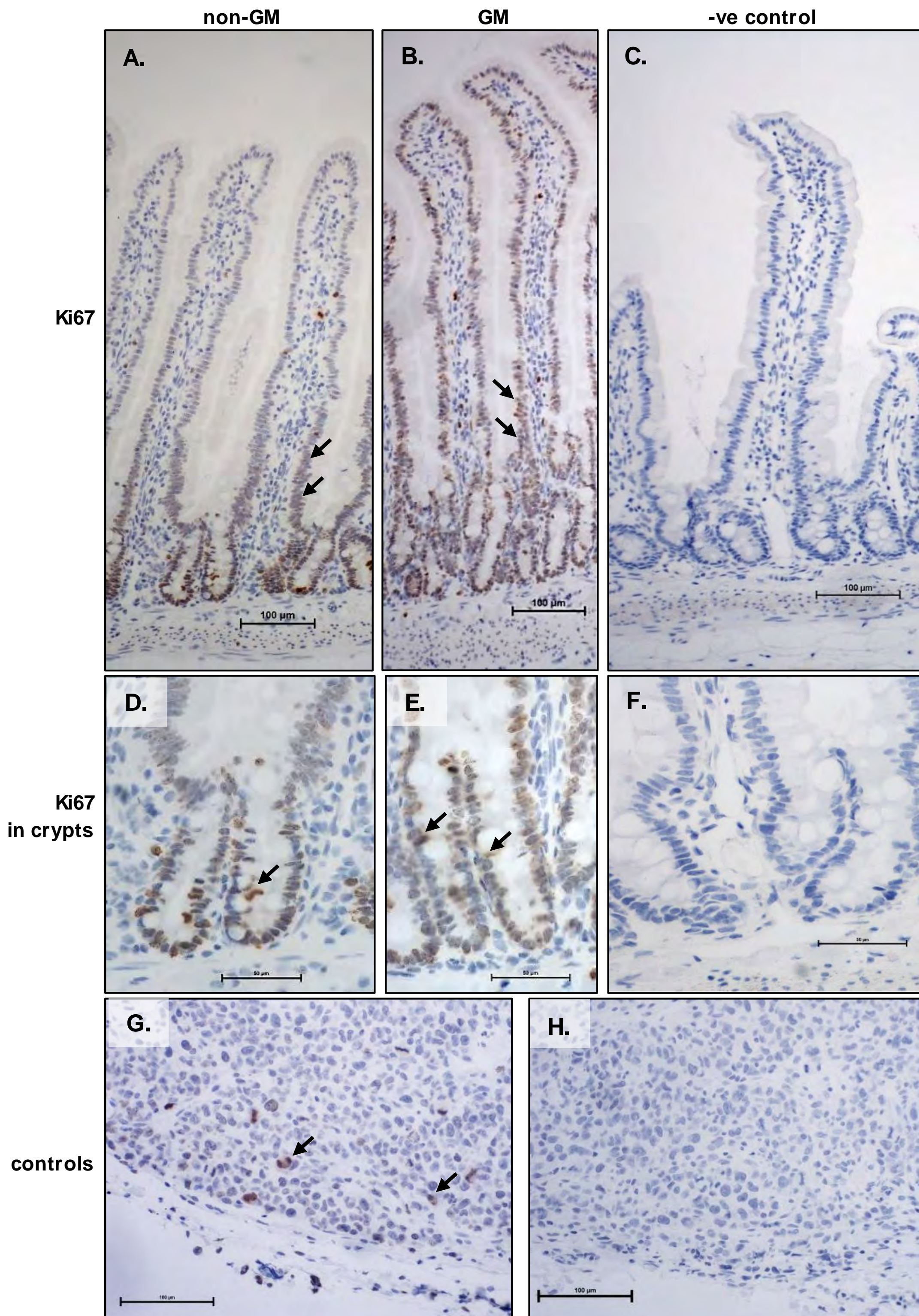



**Figure 44. Sections of the jejunum labelled with anti-Ki67 (arrows) from rats fed a 30% GM or non-GM corn diet.**

A.-C.) Anti-Ki67 labelled cells in the crypts and along the villi (arrows) of non-GM (A.) and GM-fed (B.) rat; No labelling in negative jejunum control rat tissue (C.). Scale bar = 100µm.

D.-F.) Labelled crypt cells (arrows) of non-GM (D.) and GM-fed rat (E.). No labelling in the crypts of the negative jejunum control rat tissue (F.). Scale bar = 50µm;

G.-E.) Controls: G.) rat tumour, positive control showing specific labelling of nuclei (arrows); E.) rat tumour, negative control showing no specific labelling. Scale bar = 100µm

## *Ileum*

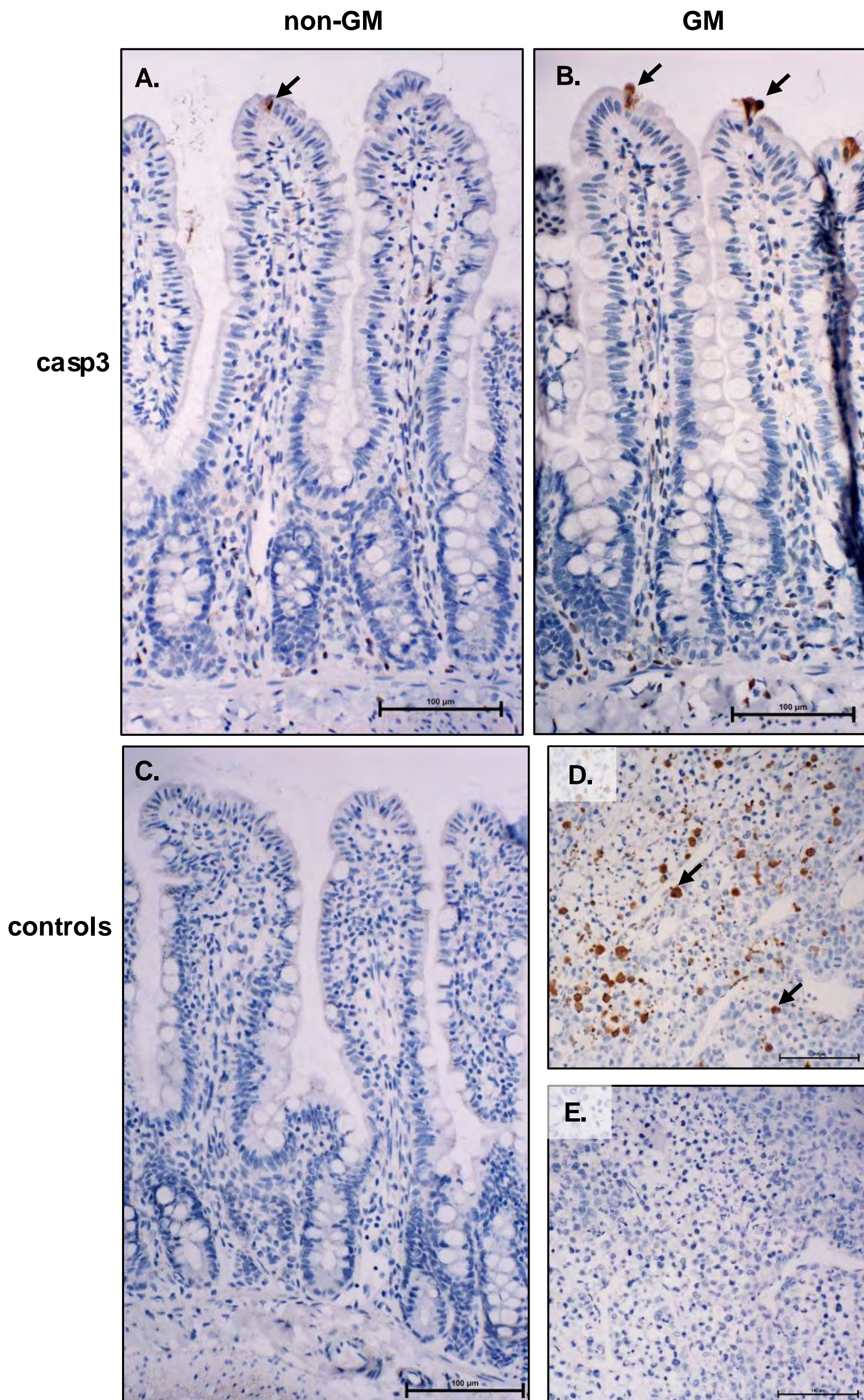



**Figure 45. Sections of the ileum labelled with anti-caspase 3 (arrows) from rats fed a 30% GM or non-GM corn diet.**

A. and B.) Anti-caspase-3 labelled cells (arrows) in non-GM (A.) and GM-fed (B.) rat.

C.-E.) Controls: C). Ileum, negative control showing no specific labelling; D.) rat tumour, positive control showing specific labelling of nuclei (arrows); E.) rat tumour, negative control showing no specific labelling. Scale bar = 100μm

## *Ileum*

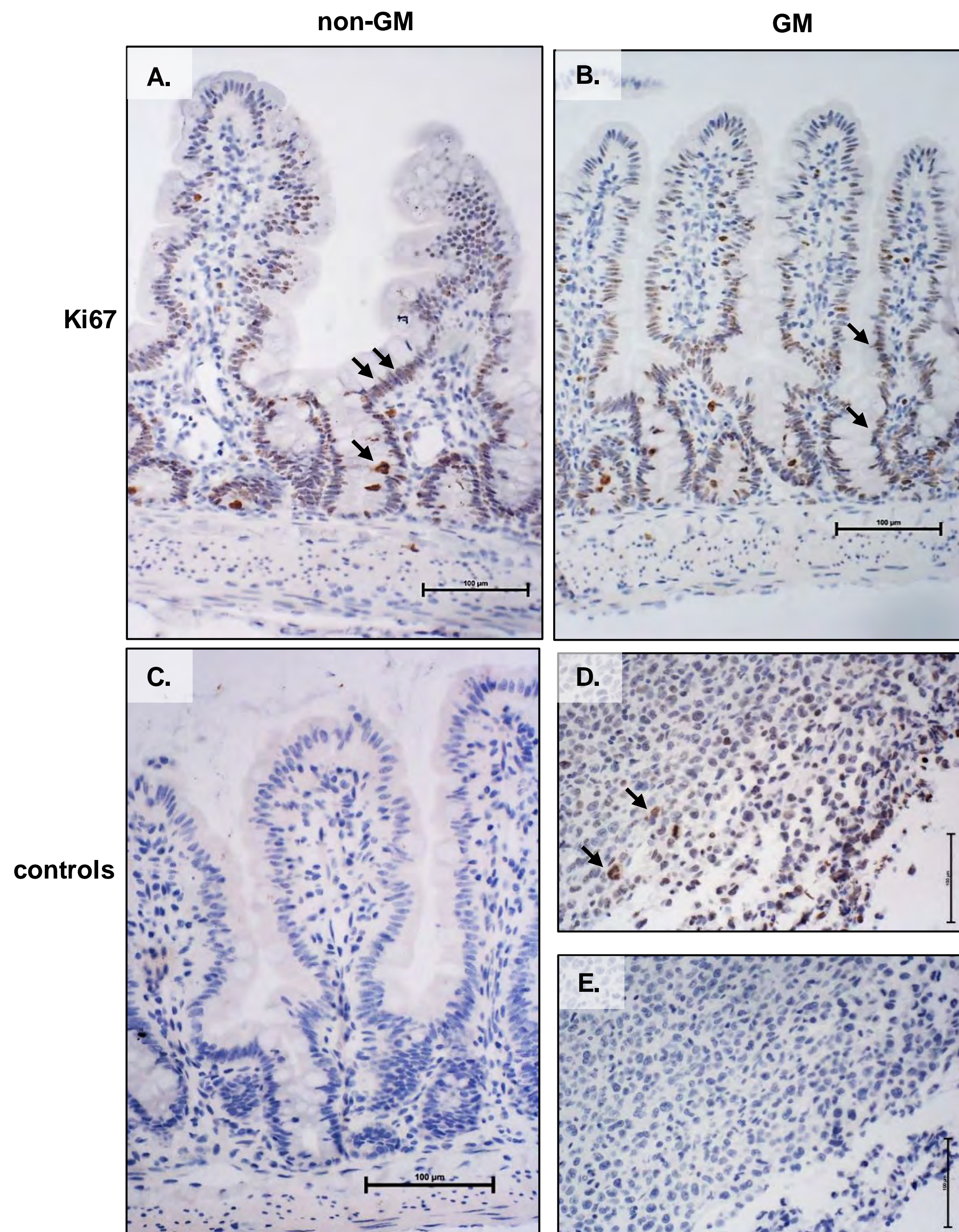



**Figure 46. Sections of the ileum labelled with anti-Ki67 from rats fed a 30% GM or non-GM corn diet.**

A. and B.) Anti-Ki67 labelled cells in crypts and along the villi (arrows) in non-GM (A.) and GM-fed (B.) rat.

C.-E.) Controls: C). Ileum, negative control showing no specific labelling; D.) rat tumour, positive control showing specific labelling of nuclei (arrows); E.) rat tumour, negative control showing no specific labelling. Scale bar = 100µm

## Stomach

The number of poorly apposed tight junctions between the mucus-producing cells was not significantly different between the two groups (Table 32; Figures 47 and 48). The incidence of tight junction apposition loss was seen in all animals of both groups (5/5 for GM; 9/9 for non-GM). However, in the non-GM-fed group, only four out of the nine had more than two poorly apposed tight junctions per rat compared with four out of five in the GM-fed group (Table 33). These yield a relative risk of 1.80, which was not statistically significant (Table 33).

In one non-GM-fed rat, there was a section of the stomach that contained glandular dilatations. Some of the cells lining the glandular dilatation were elongated or cuboidal (Figure 38).

**Table 32. Transmission electron microscopic investigation of tight junction apposition between mucus-producing cells in glandular stomachs (fundus) of rats fed a 30% GM or non-GM corn diet.** Values show median and interquartile range (IQR).

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[a] |
|---|---|---|---|---|---|---|---|---|
| | Median | IQR | n | Median | IQR | n | | |
| no. of poorly apposed TJ | 2.00 | 2.00-10.00 | 9 | 9.00 | 4.00-11.00 | 5 | NS | 350.00 |
| % poorly apposed TJ[b] | 12.50 | 10.00-26.32 | 9 | 31.71 | 16.00-33.33 | 5 | NS | 153.68 |

[a] The effect of eating the GM diet compared with the non-GM diet as a percentage change. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

[b] Number of poorly apposed tight junctions as a percentage of total number of tight junctions counted

**Table 33. Number of rats with more than three poorly apposed tight junctions between the mucus-producing cells of the pit and luminal surface of the stomach (fundus) in rats fed a 30% GM or non-GM corn diet.**

| | GM | | non-GM | | Relative risk | 95% confidence interval | Statistical significance (P ≤0.050)[a] |
|---|---|---|---|---|---|---|---|
| | Outcome of interest | | Outcome of interest | | | | |
| | present | absent | present | absent | | | |
| Poorly apposed tight junctions | 4 | 1 | 4 | 5 | 1.80 | 0.77 – 4.22 | NS |

[a] Statistical analysis performed was the Fisher's Exact test

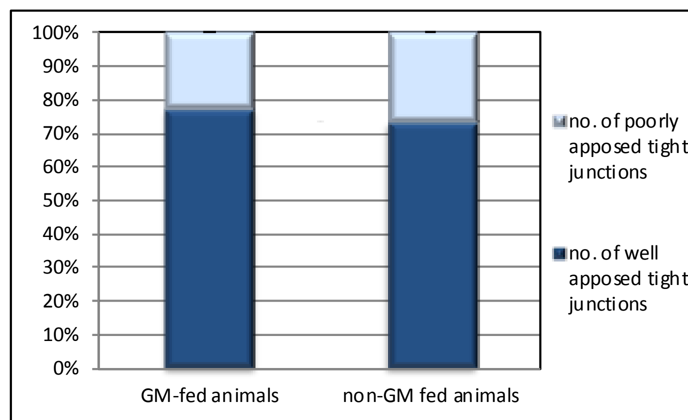



**Figure 47. Difference in frequency of tight junction appositions between the mucus-producing cells of the stomach (fundus) mucosa of rats fed a 30% GM or non-GM corn diet.**

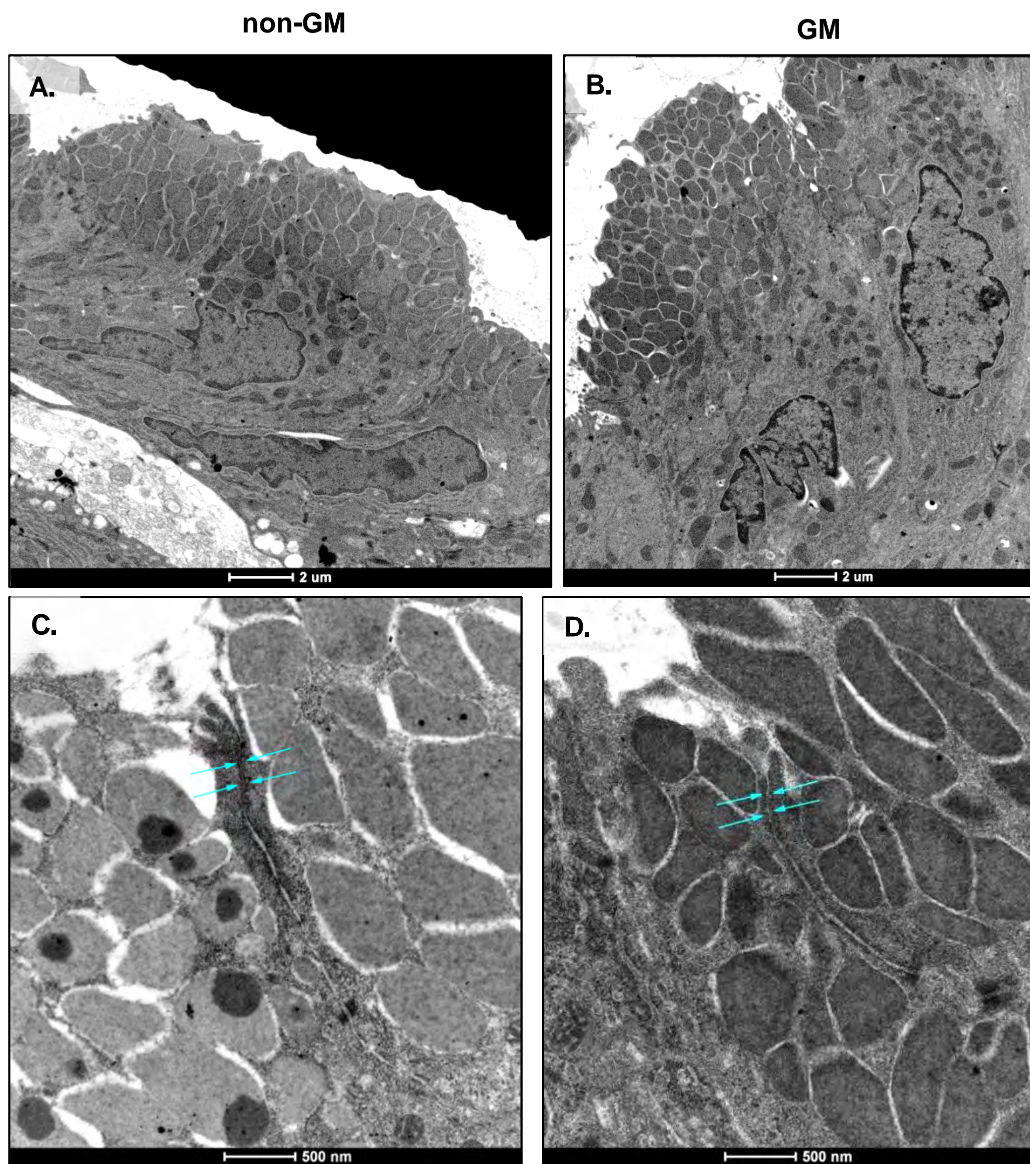



**Figure 48. Transmission electron micrographs of mucus-producing cells of the pit and surface of stomachs from rats fed a 60% GM or non-GM corn diet.**

A. and B.) Mucus-producing cells at low magnification in non-GM (A.) and GM-fed (B.) rats. Scale bar 2μm.

C. and D.) Well-apposed tight junctions (arrows) in non-GM (C.) and GM (D.) rats. Scale bar 500nm

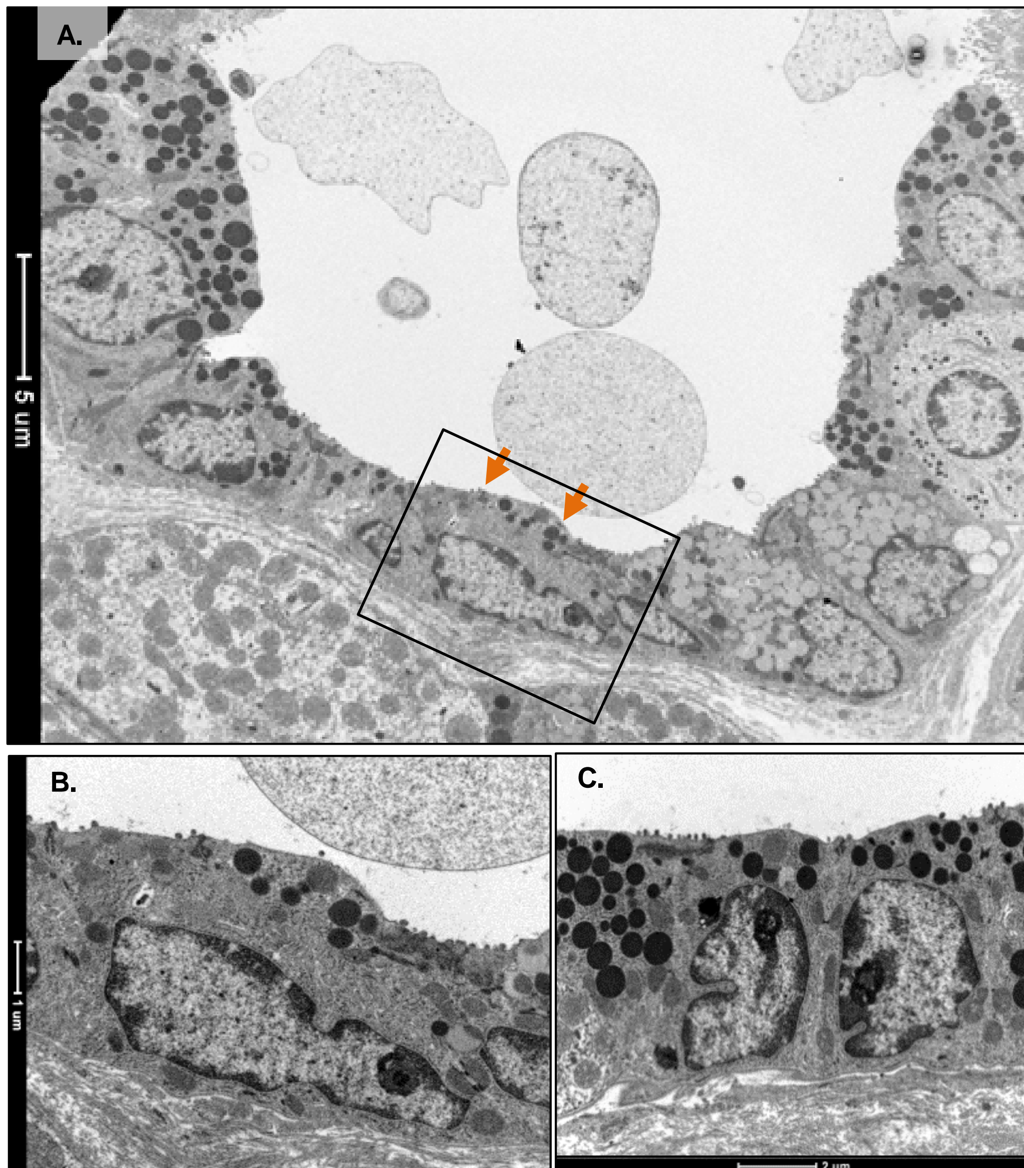



**Figure 49. Transmission electron micrographs of glandular dilatations of stomachs of rats fed a 60% non-GM corn diet.**

A.) Glandular dilatation lined with an elongated epithelial cells (arrows). Scale bar 5μm.

B.) High magnification of the area within the rectangle in Figure A, showing an elongated epithelial cell with an elongated nucleus and very few cytoplasmic granules. Scale bar 1μm.

C.) Cuboidal epithelium lining a glandular dilatation in another area of the section. Scale bar 2μm

## Small intestine (jejunum and ileum)

Transmission electron microscopic investigations of the jejunum and ileum demonstrated no loss in tight junction apposition. There was no difference in microvilli density or length between groups in either region of the small intestine (Table 34-37).

Changes in microvilli structure, such as fusion and blebbing were observed on the apical surface of the enterocytes in both the jejunum and ileum of GM and non-GM-fed rats (Figures 50 and 51). In some regions, the base of the microvilli (apical surface of

the enterocytes), were at a uniform level, while in other regions it appeared irregular and jagged (Figure 51F).

One animal in the jejunum had microvilli that appeared to be breaking up (Figure 50E). One section of the jejunum contained a branching villus. The cells lining this villus often had abnormal or a loss of microvilli (Figure 50F).

### *Jejunum*

**Table 34. Transmission electron microscopic investigation of microvilli and tight junction apposition of enterocytes lining the villi in the jejunum of rats fed a 30% GM or non-GM corn diet.** Values show mean or median and standard deviation (SD) or interquartile range (IQR) depending on whether the data are normally distributed

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Mean or median[a] | SD or IQR[a] | n | Mean or median[a] | SD or IQR[a] | n | | |
| average microvilli length (μm) | 1.72 | 0.33 | 4 | 1.68 | 0.18 | 6 | NS | -2.33 |
| % of area taken up by microvilli | 40.80[a] | 30.07-42.76[a] | 4 | 42.80[a] | 36.96-43.15[a] | 6 | NS | 4.90 |
| microvilli density per cellular width | 2.59 | 0.35 | 4 | 2.75 | 0.38 | 6 | NS | 6.18 |
| no. of poorly apposed TJ | 1.25 | 1.89 | 4 | 2.83 | 2.04 | 6 | NS | 126.40 |
| % of poorly apposed TJ[c] | 2.60 | 3.77 | 4 | 5.47 | 3.75 | 6 | NS | 110.38 |

[a] Not normally distributed data, the median and interquartile range is given.

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

[c] Number of poorly apposed tight junctions as a percentage of total number of tight junctions counted

**Table 35. Abnormal features seen in the TEM investigation of the jejunum of 30% GM and non-GM corn fed rats.**

| | GM | | non-GM | | Relative risk | 95% confidence interval | Statistical significance (P ≤0.050)[a] |
|---|---|---|---|---|---|---|---|
| | Outcome of interest | | Outcome of interest | | | | |
| | present | absent | present | absent | | | |
| Abnormal microvilli structure | 3 | 3 | 2 | 2 | 1.00 | 0.28 – 3.54 | NS |
| Blebbing of enterocyte surface | 2 | 4 | 1 | 3 | 1.33 | 0.17 – 10.25 | NS |
| Cells with complete loss of microvilli | 3 | 3 | 1 | 4 | 2.50 | 0.36 – 17.17 | NS |
| Visual assessment: visible decrease in microvilli density | 2 | 4 | 1 | 3 | 1.33 | 0.17 – 10.25 | NS |
| Necrotic cell(s) present | 0 | 6 | 0 | 4 | NA | NA | NA |
| Poorly apposed tight junctions | 5 | 1 | 2 | 2 | 1.67 | 0.59 – 4.73 | NS |

[a] Statistical analysis performed was the Fisher's exact test.

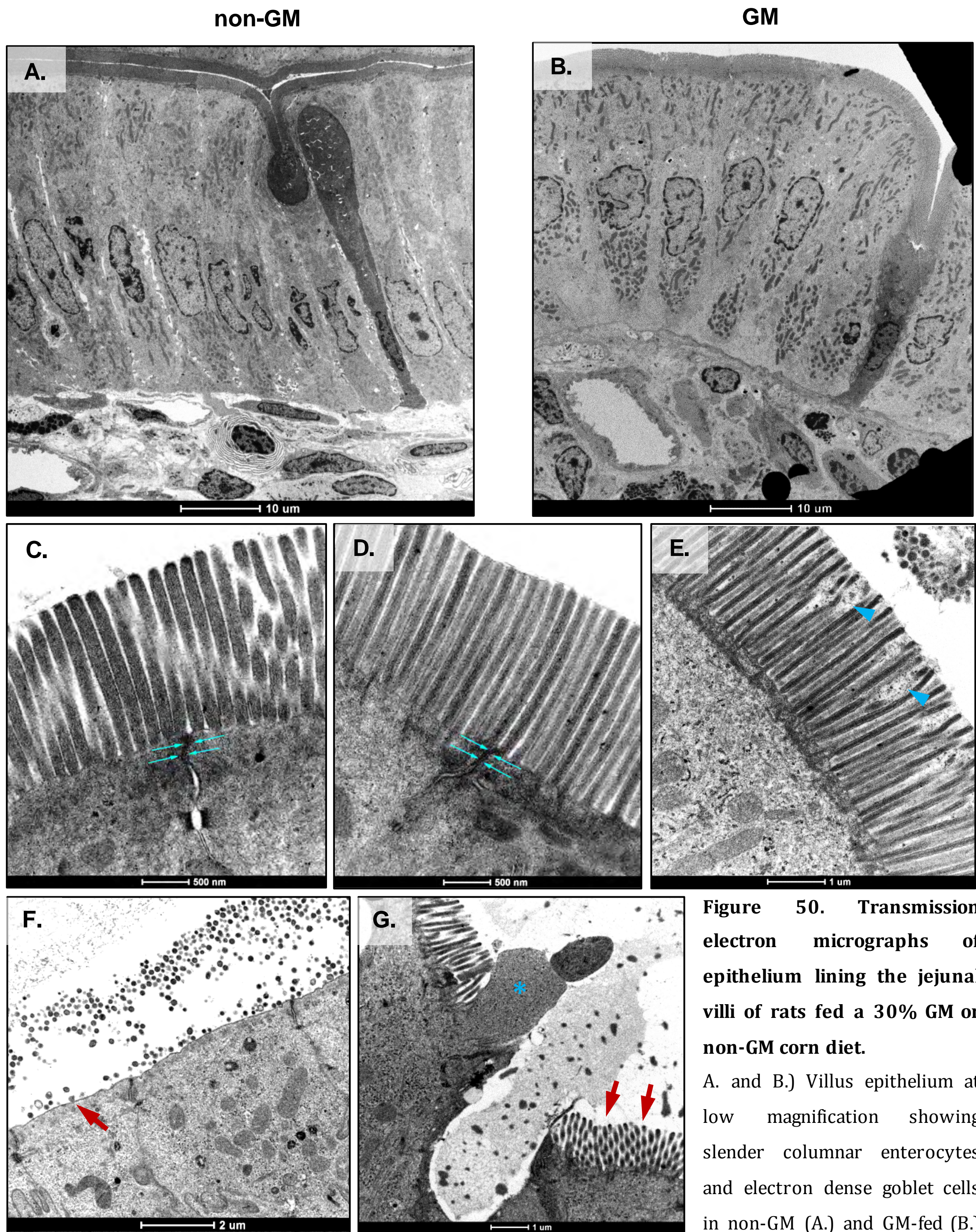



**Figure 50. Transmission electron micrographs of epithelium lining the jejunal villi of rats fed a 30% GM or non-GM corn diet.**

A. and B.) Villus epithelium at low magnification showing slender columnar enterocytes and electron dense goblet cells in non-GM (A.) and GM-fed (B.) rats. Scale bar 10µm.

C. and D.) Apical surface of enterocyte at higher magnification showing well-apposed tight junctions (thin arrows) and long, slender microvilli in non-GM (C.) and GM-fed (D.) rats. Scale bar 500nm.

E.-G.) Abnormal surface features of enterocytes: E.) Microvilli appear to be “breaking up” (arrowheads) in non-GM-fed animal; F.) loss of microvilli on surface of enterocytes of a branching villus in non-GM-fed rat. At the luminal surface, small vesicle-like structures appear. Only one appears to be continuous with the surface membrane (thick red arrow); G.) Blebbing (asterisk) of enterocyte surface in GM-fed rat. Also note the normal appearance of microvilli in cross-section (thick red arrows).

## *Ileum*

**Table 36. Transmission electron microscopic investigation of microvilli and tight junction apposition of enterocytes lining the villi in the ileum of rats fed a 30% GM or non-GM corn diet.** Values show mean and standard deviation (SD). Values show mean and standard deviation (SD).

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Mean | SD | n | Mean | SD | n | | |
| average microvilli length (µm) | 1.38 | 0.24 | 4 | 1.31 | 0.15 | 6 | NS | -5.07 |
| % of area taken up by microvilli | 34.31 | 6.44 | 4 | 36.94 | 9.86 | 6 | NS | 7.67 |
| microvilli density per cellular width | 2.35 | 0.61 | 4 | 2.32 | 0.66 | 6 | NS | -1.28 |
| no. of poorly apposed TJ | 2.00 | 1.83 | 4 | 1.83 | 1.33 | 6 | NS | -8.50 |
| % of poorly apposed TJ[b] | 4.60 | 3.51 | 4 | 4.48 | 3.24 | 6 | NS | -2.61 |

[a] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

[b] Number of poorly apposed tight junctions as a percentage of total number of tight junctions counted

**Table 37. Abnormal features seen in the TEM investigation of the ileum of 30% GM and non-GM corn fed rats.**

| | GM | | non-GM | | Relative risk | 95% confidence interval | Statistical significance (P ≤0.050)[a] |
|---|---|---|---|---|---|---|---|
| | Outcome of interest | | Outcome of interest | | | | |
| | present | absent | present | absent | | | |
| Abnormal microvilli structure | 1 | 5 | 1 | 3 | 0.67 | 0.06 – 7.85 | NS |
| Irregular surface below microvilli in enterocytes | 1 | 5 | 2 | 2 | 0.33 | 0.04 – 2.56 | NS |
| Blebbing of enterocyte surface | 1 | 5 | 1 | 3 | 0.67 | 0.06 – 7.85 | NS |
| Cells with complete loss of microvilli | 3 | 3 | 3 | 1 | 0.67 | 0.25 – 1.78 | NS |
| Necrotic cell(s) present | 0 | 6 | 1 | 3 | 0.57[b] | 0.05-6.86[b] | NS |
| Poorly apposed tight junctions | 2 | 3 | 3 | 2 | 0.67 | 0.18 – 2.42 | NS |

[a] Statistical analysis performed was the Fisher's exact test

[b] Relative risk and confidence interval was calculated by putting a "1" into the cell with zero

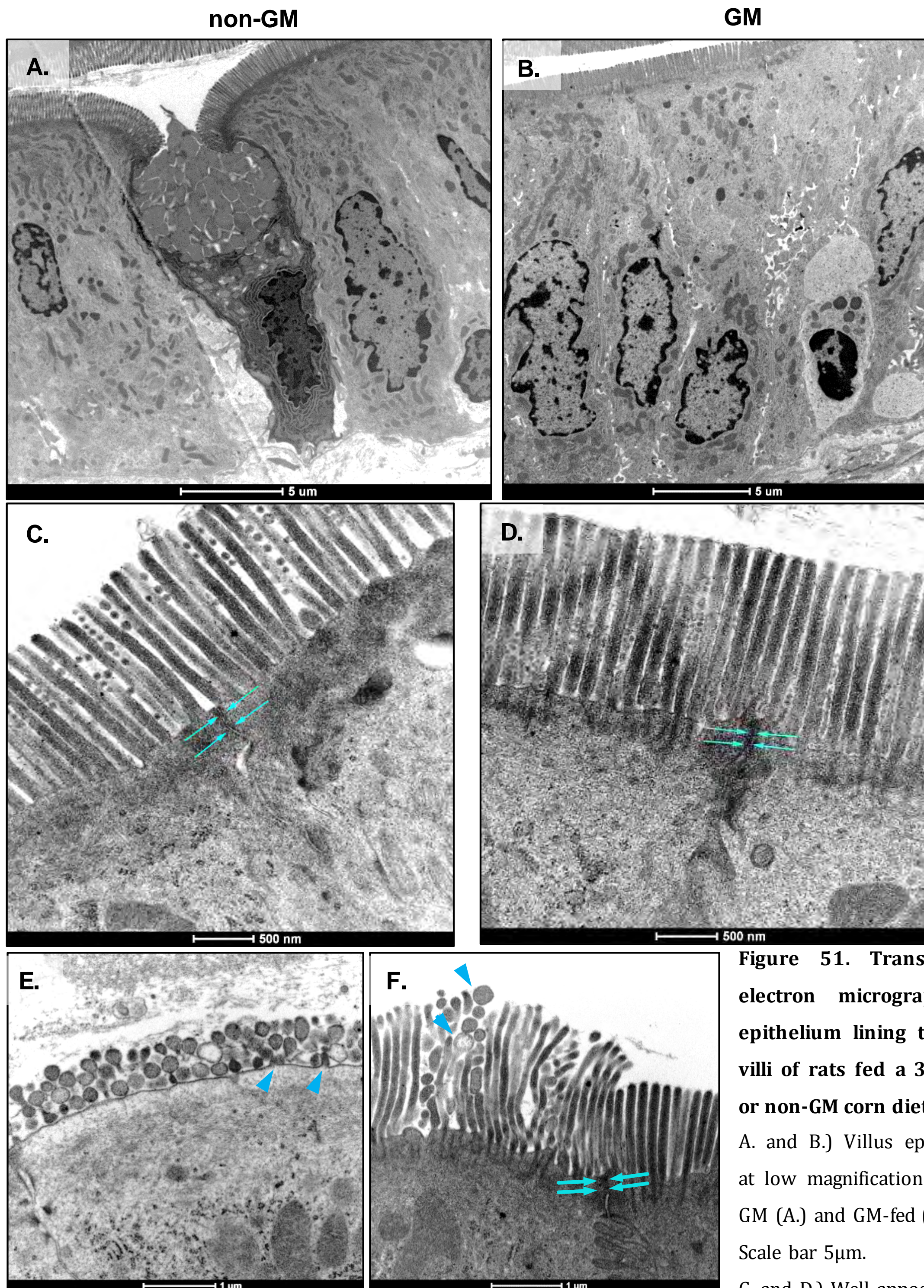



**Figure 51. Transmission electron micrographs of epithelium lining the ileal villi of rats fed a 30% GM or non-GM corn diet.**

A. and B.) Villus epithelium at low magnification in non-GM (A.) and GM-fed (B.) rats. Scale bar 5μm.

C. and D.) Well-apposed tight junctions (arrows) of non-GM (C.) and GM-fed (D.) rats. In both animals the microvilli are long and slender. Scale bar 500nm.

E. and F.) Abnormal surface features of enterocytes: E.) Loss of microvilli on surface of enterocyte of non-GM-fed rat. The microvilli have the appearance of vesicles and only two appear to be continuous with the surface membrane (arrowhead); F.) Microvilli of two neighbouring enterocytes of GM-fed rat. Tight junction indicated by arrows. One of the cells appears to have longer microvilli with some being swollen (arrowheads), while the other cell's microvilli have a regular appearance. Both cells appear to have an irregular surface bellow the microvilli, but more so the cell with the swollen microvilli. Scale bar 1μm.

## 4.4 Discussion

### 4.4.1 Chemically-induced damage

The results of the present study suggest chemically-induced damage of a milder form than in the 60% corn-fed study or alternatively it suggests a different model of damage. The most obvious change, observed in this study, was the increase in gastric gland depth (15.98% more; p = 0.039) in the GM-fed group (Table 22; Figures 34 and 35). A similar change was seen in the 60% corn study (3.45%, although not statistically significant; Table 10), and, in a short-term feeding study investigating GM potatoes transformed to produce the snowdrop lectin, GNA protein (15.31% when fed boiled, p = 0.02; 23.75% when fed raw, not statistically significant). In the latter study, Ewen and Pusztai (1999), found that the increase in gland depth was also seen in the rats fed non-GM potatoes supplemented with GNA. Therefore, the authors suggested that the cause was the presence of GNA protein in the GM feed. However, it is possible that another mechanism is responsible for the change, particularly since an increase in gland depth was seen in the present two studies with a GM corn content of 30% and 60%. Studies investigating GM crops rarely report changes to stomach tissue (Zdziarski *et al.,* 2014). It is possible that a mechanism, common to different types of GM crops, is causing an increase in gland depth, but since there is no requirement for morphometric analyses to be part of histopathological investigations, similar changes may have occurred, but have remained unreported.

The tight junction apposition loss, which is sometimes observed in chemical gastropathy (Eastwood and Kirchner, 1974; Eastwood and Erdmann, 1978; Meyer *et al.*, 1986), was seen in both the GM and non-GM-fed groups although it did not reach statistical significance. In contrast, rats fed 60% corn-feed revealed significant tight junction apposition loss in the stomachs of rats fed GM-corn diet (Tables 16 and 17). This could suggest a dose-dependent response or imply a different mechanism of damage.

In the small intestine, villous atrophy is a common feature of chemically-induced damage (Isaacs *et al.*, 1987). Unlike in the 60% corn study, the present 30% corn study, showed no decrease in villi height in the ileum. In fact, there was an increase in villi height (5.01% more than non-GM fed group), although this was not statistically

significant. These results may suggest a dose-dependent response or a different model of damage.

In the jejunum, crypt hyperplasia was seen in the GM-fed rats compared with the non-GM-fed group. The proliferative index showed a 3.64% increase ($p = 0.003$), and crypt depth was 7.43% higher than non-GM-fed group, although the latter result was not statistically significant. These results may suggest that the observed hyperplasia may be a response to GM toxicity. In chemically-induced damage, crypt hyperplasia is a compensatory/repair mechanism to villous atrophy (Isaacs *et al.,* 1987; Thompson *et al.,* 2013). It is not, however, clear whether, crypt hyperplasia and villous atrophy occur simultaneously. At some stage of this repair mechanism, villi height may return to normal or increase, as a result of the proliferation of the crypt epithelium. These features were observed in the present study with both jejunal and ileal villi either being of normal length or at an increase (Tables 25 and 27). These results also suggest a dose-dependent response to the GM corn diet, since the higher dose (60%) diet, showed villous atrophy (Table 13) (indicating on-going damage to ileum mucosa), and the lower does (30%) diet, showed no change or an increase in villi height, with accompanying increase in crypt depth in the jejunum (Table 25), or no change in crypt depth in the ileum (Table 27). These changes may demonstrate the end stage of the repair mechanism. In the higher-dose (60%) study, the jejunum was not investigated; therefore, it is possible that the 60% corn diet caused jejunal villi atrophy. This warrants further investigation.

Crypt hyperplasia is seen in several other pathologies, such as, bacterial infections (Percy and Barthold, 2008), and pre-neoplastic lesions (Thompson et al., 2013), including, reactive hyperplasia (Mohr, 1997). The mechanism of development and repair in such pathologies, are similar to those demonstrated above, however, bacterial infection and reactive hyperplasia also feature leukocytic infiltration and sometimes focal ulceration (Mohr, 1997; Percy and Barthold, 2008). In reactive hyperplasia, there is also a decrease or loss of goblet cell development (Mohr, 1997). In the present study, neither epithelial leukocytic infiltration nor a significant decrease in goblet cells was observed. In addition, the lamina propria was similar in rats of both studies and did not show evidence of inflammation. Therefore, neither reactive hyperplasia nor bacterial infection were evident.

In the development of neoplastic lesions, from the pre-neoplastic stage is associated with ongoing crypt hyperplasia. This hyperplasia, leads to mutagenesis and tumour formation (Thompson et al., 2013). To identify the development or presence of such lesions, a feeding study of 12 to 24 months has been recommended (Pozharisski, 1975; OECD, 2008a). The present study was of six months duration and therefore, only pre-neoplastic changes, such as, crypt hyperplasia will be evident.

An increase in crypt depth in the jejunum and ileum has also been observed in rats fed a GM potato diet containing the snowdrop lectin, GNA (Ewen and Pusztai, 1999). The gene cassette used in the transformation of the GM potato, also contained the CaMV promoter genes. It has been suggested that if the CaMV promoter genes were to be incorporated into the gut epithelium, they may cause uncontrolled proliferation of the epithelial cells and thus result in the observed crypt hyperplasia (Ewen and Pusztai, 1999). The present study, investigated a triple-stacked GM corn that contains at least four such promoter genes, which were in synthetic, enhanced/modified, or unaltered forms (Table 1). If the CaMV promoter genes were responsible for crypt hyperplasia, such a result would be expected in the present study, particularly in the higher-dose (60%) investigation. However, crypt hyperplasia was only observed in the jejunums of the lower-dose GM corn-fed rats. The jejunum was not investigated in the higher-dose (60%) study, therefore, it may have remained undetected. Also, the ileum of both present studies (60% and 30% GM corn diet studies) did not show crypt hyperplasia. It is possible that in the GM corn feed, the CaMV promoter genes were digested before reaching the ileum, thus they would no longer be in a form that could cause epithelial hyperplasia.

### 4.4.2 The *Bt* toxin as a source of toxicity

In the jejunum sections of the present study, there was a significant increase in IEL population in non-GM-fed and a decrease in the GM-fed rats (25.00% less than non-GM group; p = 0.038; Table 25). Although not statistically significant, such a trend was also seen in the ileum of both the 30% and 60% studies. Intraepithelial lymphocytes play an important role in the first sign of defence in the small intestine, with each type of lymphocyte playing a specific role. In a study investigating gut lymphocyte populations associated with a GM-corn diet (MON810), Finamore *et al.* (2008) demonstrated that the number of each type or subtype of lymphocyte could significantly vary. For example, the

number of $CD3^+$ T cells was significantly higher, while the number of $CD19^+$ B cells was significantly lower in young rats fed the GM diet for 30d. In the T cell subpopulations, $CD4^+$ and $\alpha\beta^+$ T cells were found to be significantly lower, and $\gamma\delta^+$ T cells were found to be significantly higher. In their 90d studies, there were also significant differences. Velimirov *et al.* (2008) also found a similar relationship between the population of $CD3^+$ T cells (higher) and $CD19^+$ B cells (lower) in the jejunums of mice fed a GM corn diet (MON810xNK603 corn). In both these studies, the diet contained the gene cassette for MON810, which encodes for the *Cry1Ab* protein. The present study also contained corn with the gene cassette MON810. Therefore, the similar types and subtype of lymphocytes may also be present in the current study. This warrants further investigation.

## 4.5 Conclusions

The study suggests dose-dependent responses to feeding rats a GM-corn diet. The changes in the stomach and small intestine of rats fed the lower-dose GM-diet imply that a toxic effect is still present, but not to the degree seen in the maximum-dose study. Some of these changes suggest chemically-induced gastropathy and enterocolitis.

## Summary table of Study 2 results

| | Non-GM-fed | GM-fed | Significant difference |
|---|---|---|---|
| Initial body weight of animals (g) – *mean (SD)* | 78.87 (12.02) | 77.93 (12.49) | NS |
| Final body weights of animals (g) - *mean (SD)* | 545.00 (56.34) | 550.60 (50.69) | NS |
| Stomach | | | |
| Morphometric analyses: | | | |
| *Non-glandular stomach* | | | |
| Mucosa thickness (µm) - *mean (SD)* | 38.27 (3.72) | 38.69 (4.68) | NS |
| Keratinized layer thickness (µm) - *mean (SD)* | 45.15 (4.11) | 45.47 (4.86) | NS |
| Non-keratinized layer thickness (µm) - *mean (SD)* | 6.87 (0.95) | 6.78 (0.94) | NS |
| Keratinized layer : mucosa thickness (%) - *mean (SD)* | 15.25 (1.84) | 14.76 (2.21) | NS |
| Non-keratinized layer : mucosa thickness (%) - *mean (SD)* | 84.75 (1.84) | 84.98 (2.25) | NS |
| *Glandular stomach – fundus* | | | |
| Mucosa thickness (µm) - *mean (SD)* | 714.56 (79.27) | 812.38 (77.12) | NS |
| Pit depth (µm) - *mean (SD)* | 92.16 (23.90) | 100.39 (18.93) | NS |
| **Gland depth (µm) - *mean (SD)*** | **588.53 (64.78)** | **682.56 (70.06)** | **P = 0.039*** |
| **Pit + gland depth (µm) - *mean (SD)*** | **680.70 (77.65)** | **782.95 (78.26)** | **P = 0.046*** |
| Lamina propria thickness below gland (µm) - *mean (SD)* | 33.87 (10.62) | 29.43 (3.09) | NS |
| Pit depth : mucosa thickness (%) - *mean (SD)* | 12.88 (2.69) | 12.35 (1.96) | NS |
| Gland depth : mucosa thickness (%) - *mean (SD)* | 82.37 (1.35) | 83.99 (2.14) | NS |
| Pit + gland depth : mucosa thickness (%) - *mean (SD)* | 95.25 (1.37) | 96.34 (0.57) | NS |
| Lamina propria thickness below gland : mucosa thickness (%) - *mean (SD)* | 4.75 (1.37) | 3.66 (0.57) | NS |
| Histological observations in fundus: | | | |
| **Rats with gland dilatations (no. of affected animals)** | **10/14** | **15/15** | **P = 0.042*** |
| Rats with gland dilatation exhibiting AB/PAS or PAS positive cells (no. of affected animals) | 4/13 | 8/13 | NS |
| Rats with gland dilatation exhibiting elongated cells (no. of affected animals) | 4/13 | 6/13 | NS |
| Frequency of gland dilatations per animal - *median (IQR)* | 3.50 (0.50-13.50) | 8.00 (2.50-14.50) | NS |
| Tight junction study in fundus: | | | |
| No. of poorly apposed tight junctions - *median (IQR)* | 2.00 (2.00-10.00) | 9.00 (4.00-11.00) | NS |
| Poorly apposed tight junctions per total count (%) - *median (IQR)* | 12.50 (10.00-26.32) | 31.71 (16.00-33.33) | NS |
| No. of rats with poorly apposed tight junctions | 4/9 | 4/5 | NS |

Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001
Abbreviations: SD = standard deviation; IQR = interquartile range

| | Non-GM-fed | GM-fed | Significant difference |
|---|---|---|---|
| Jejunum | | | |
| Morphometric analyses: | | | |
| Mucosa thickness (µm) - *mean (SD)* | 735.12 (49.57) | 746.08 (67.66) | NS |
| Villi height (µm) - *mean (SD)* | 597.09 (49.22) | 602.97 (52.16) | NS |
| Crypt depth (µm) - *mean (SD)* | 131.96 (16.71) | 141.08 (19.16) | NS |
| Lamina propria thickness below crypt (µm) - *mean (SD)* | 6.48 (1.77) | 6.79 (1.92) | NS |
| Villi height : mucosa thickness (%) - *mean (SD)* | 81.18 (2.44) | 80.87(1.99) | NS |
| Crypt depth : mucosa thickness (%) - *mean (SD)* | 17.98 (2.21) | 18.91 (1.88) | NS |
| Lamina propria thickness below crypt : mucosa thickness (%) - *mean (SD)* | 0.89 (0.27) | 0.91 (0.22) | NS |
| No. of enterocytes per villi height - *mean (SD)* | 0.90 (0.08) | 0.09 (0.10) | NS |
| No. of goblet cells per villi height - *mean (SD)* | 0.16 (0.03) | 0.16 (0.02) | NS |
| No. of IELs per villi height - *mean (SD)* | 0.24 (0.08) | 0.18 (0.07) | NS |
| Goblet cells : enterocytes (%) - *mean (SD)* | 18.17 (2.68) | 17.60 (2.90) | NS |
| **IEL : enterocytes (%)** - ***mean (SD)*** | **26.55 (7.91)** | **19.84 (7.71)** | **P = 0.032*** |
| **Proliferative index** - ***mean (SD)*** | **86.16 (3.04)** | **89.30 (2.10)** | **P = 0.003**** |
| Proliferating cells per crypt depth - *mean (SD)* | 0.29 (0.03) | 0.29 (0.04) | NS |
| Apoptotic index | 0.00 | 0.00 | NS |
| Histological observations: | | | |
| Total number of branching villi - *median (IQR)* | 1.00 (1.00-3.00) | 2.50 (0.75-3.00) | NS |
| No. of animals with branching villi | 11/13 | 9/12 | NS |
| **Microvilli TEM study:** | | | |
| Microvilli length (µm) - *mean (SD)* | 1.72 (0.33) | 1.68 (0.18) | NS |
| Microvilli density : cytoplasm (%) - *median (IQR)* | 40.80 (30.07-42.76) | 42.80 (36.96-43.15) | NS |
| Microvilli density (no. of microvilli per surface length) - *mean (SD)* | 2.59 (0.35) | 2.75 (0.38) | NS |
| **Tight junction TEM study:** | | | |
| No. of poorly apposed tight junctions - *mean (SD)* | 1.25 (1.89) | 2.83 (2.04) | NS |
| Poorly apposed tight junctions per total count (%) - *mean (SD)* | 2.60 (3.77) | 5.47 (3.75) | NS |
| **TEM observations** (no. of affected animals): | | | |
| Abnormal microvilli structure | 2/4 | 3/6 | NS |
| Blebbing of enterocyte surface | 1/4 | 2/6 | NS |
| Cells with complete loss of microvilli | 1/5 | 3/6 | NS |
| Visual assessment: visible decrease in microvilli density | 1/4 | 2/6 | NS |
| Necrotic cell(s) present | 0/4 | 0/6 | NS |
| Poorly apposed tight junctions | 2/4 | 5/6 | NS |

Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001
Abbreviations: SD = standard deviation; IQR = interquartile range; IEL = intraepithelial lymphocyte

| | Non-GM-fed | GM-fed | Significant difference |
|---|---|---|---|
| **Ileum** | | | |
| Morphometric analyses: | | | |
| Mucosa thickness - *mean (SD)* | 403.38 (51.94) | 415.19 (46.77) | NS |
| Villi height - *mean (SD)* | 277.11 (38.54) | 290.99 (36.27) | NS |
| Crypt depth - *mean (SD)* | 121.13 (22.25) | 119.19 (13.14) | NS |
| Lamina propria thickness below crypt - *mean (SD)* | 4.99 (1.46) | 4.31 (0.90) | NS |
| Villi height : mucosa thickness (%) - *mean (SD)* | 68.72 (3.64) | 70.01 (1.83) | NS |
| Crypt depth : mucosa thickness (%) - *mean (SD)* | 29.97 (3.57) | 28.76 (1.66) | NS |
| Lamina propria thickness below crypt : mucosa thickness (%) - *mean (SD)* | 1.27 (0.47) | 1.05 (0.27) | NS |
| No. of enterocytes per villi height - *mean (SD)* | 0.13 (0.00) | 0.13 (0.01) | NS |
| No. of goblet cells per villi height - *mean (SD)* | 0.03 (0.00) | 0.03 (0.00) | NS |
| No. of IELs per villi height - *median (IQR)* | 0.03 (0.03-0.05) | 0.03 (0.02-0.03) | NS |
| Goblet cells : enterocytes (%) - *mean (SD)* | 25.02 (2.73) | 25.35 (3.28) | NS |
| IEL : enterocytes (%) - *median (IQR)* | 25.01 (21.66-41-28) | 21.63 (20.47-23.26) | NS |
| Proliferative index - *median (IQR)* | 82.41 (79.87-86.77) | 86.63 (79.61-88.35) | NS |
| Proliferating cells per crypt depth - *mean (SD)* | 0.34 (0.07) | 0.31 (0.04) | NS |
| Apoptotic index | 0.00 | 0.00 | NS |
| Histological observations: | | | |
| Total number of branching villi - *median (IQR)* | 2.00 (1.00-4.00) | 5.00 (2.00-7.00) | NS |
| No. of animals with branching villi | 11/13 | 13/13 | NS |
| **Microvilli TEM** study: | | | |
| Microvilli length - *mean (SD)* | 1.38 (0.24) | 1.31 (0.15) | NS |
| Microvilli density : cytoplasm (%) - *mean (SD)* | 34.31 (6.44) | 36.94 (9.86) | NS |
| Microvilli density (no. of microvilli per surface length) - *mean (SD)* | 2.35 (0.61) | 2.32 (0.66) | NS |
| **Tight junction TEM study:** | | | |
| No. of poorly apposed tight junctions - *mean (SD)* | 2.00 (1.83) | 1.83 (1.33) | NS |
| Poorly apposed tight junctions per total count (%) - *mean (SD)* | 4.60 (3.51) | 4.48 (3.24) | NS |
| **TEM observations** (no. of affected animals): | | | |
| Abnormal microvilli structure | 1/4 | 1/6 | NS |
| Irregular surface bellow microvilli in enterocytes | 2/4 | 1/6 | NS |
| Blebbing of enterocyte surface | 1/4 | 1/6 | NS |
| Cells with complete loss of microvilli | 3/4 | 3/6 | NS |
| Necrotic cell(s) present | 1/4 | 0/6 | NS |
| Poorly apposed tight junctions | 3/5 | 2/5 | NS |

Statistical significance: *p<0.050 to 0.010, ** p<0.010 to 0.001, ***p<0.001
Abbreviations: SD = standard deviation; IQR = interquartile range; IEL = intraepithelial lymphocyte

# 5. Study 3: Expression of tight junction proteins in the stomachs of 60% corn-fed rats

## 5.1 Introduction

Study 1, which investigated the long-term effects of feeding rats a 60% GM-corn diet, observed a loss of tight junction apposition in the stomachs of the GM-fed compared with the non-GM-fed rats ($p = 0.004$). Tight junctions are intercellular-binding structures that are made up of several different transmembrane and intracellular proteins. The most common of these are the occludin, claudin, JAM and ZO proteins. Studies into the expression and physiological properties of tight junction proteins indicate a correlation between the type of tight junction protein and the function of the specific segment of the digestive tract (Fujita *et al.*, 2008; Markov *et al.*, 2010). Markov *et al.* (2010) found that the claudins responsible for permeability and "seal" properties of the junction were highest in the duodenum, which correlates with its need to resist the action of low gastric pH and emulsifying bile salts. Based on these observations it could be assumed that these permeability and "seal"-responsible proteins may also have high expression in the stomach. Occludin, claudin-1 and ZO-1 are known to be responsible or add to the "seal" function of the tight junction (Van Itallie and Anderson, 1997; Markov *et al.*, 2010; Oshima and Miwa, 2016). Therefore, the aim of this study was to investigate if occludin, claudin-1 and ZO-1 were involved in the loss of tight junction apposition seen in the TEM study, using both immunohistochemistry LM and immunofluorescence-confocal microscopy.

## 5.2 Materials and Methods

Immunohistochemical light and immunofluorescence-confocal microscopic investigations were performed on stomach tissue from the 60% corn study (Study 1). Stomach tissue was taken from 20 rats (10 rats from GM and 10 rats from non-GM-fed group).

### 5.2.1 Immunohistochemical light microscopic investigation

#### Occludin and Claudin-1

Sections of the stomach were cut at 4μm, mounted on DAKO slides, and incubated at 30°C overnight. Slides were deparaffinised in histolene, rehydrated in graded ethanol solutions to distilled water. Antigen retrieval was performed using high pH antigen retrieval solution (DAKO) and set on 20 min cycle at 100°C (occludin) or 97°C (claudin-

1) (DAKO PT Link). Using an automated cycle (DAKO Autostainer Plus), sections were treated with 3% hydrogen peroxide (FLEX peroxidase blocker, DAKO) for 5min to quench the endogenous peroxidases and then immersed in Protein Block (DAKO) for 30 min, followed by a 60 min incubation in primary antibody (1:200 dilution with actual concentration of occludin at 0.125mg/mL, Invitrogen 71-1500; 1:300 dilution with actual concentration of claudin-1 at 0.083mg/mL, Invitrogen 51-9000). Sections were labelled with anti-rabbit HRP polymer (DAKO) and developed with DAB (DAB FLEX, DAKO) for 10min. Sections were rinsed with water and counterstained with Harris haematoxylin for 10 sec, blued in ammonia solution for 1min, dehydrated in a graded series of ethanol, then placed into histolene and coverslipped using DPEX mounting medium.

Rat colon and stomach from a rat known to have been fed a non-GM diet were used as the controls in the following way:

1) Positive control: colon treated with primary antibody (occludin or claudin-1)
2) Negative control: stomach and colon treated with buffer and no primary antibody

#### Semi-quantitative evaluation of tissue

Using an Olympus BH2 light microscope and a x40 objective lens, the intensity of labelling in the gastric pits and glands of four well-orientated areas was scored using a 0-4 scale (0 = no expression; 1 = weak expression; 2 = moderate expression; 3 = strong expression; 4 = extremely strong expression) (Gross *et al.*, 2003). The tight junctions of the gastric pits were the focus of this study; therefore, observations of non-glandular areas were only made cursorily. Labelling in two areas of the non-glandular stomach was scored used the same scale. The first area was of the limiting ridge (the area at the non-glandular/glandular junction). The second area was at least 50µm from the junction.

### 5.2.2 Immunofluorescence confocal microscopic investigation

Sections were cut at 4µm, mounted on silane-coated slides and incubated at 30$^{o}$C overnight. There was one section per slide per rat. Slides were dried for 1hr at 64$^{o}$C, dewaxed in xylene and hydrated in graded ethanol to water. Antigen retrieval was performed according to antibody manufacturer specifications: Sections to be labelled with anti-occludin and anti-ZO-1 were treated with peroxidase (*Stroptomyces grisens*, SIGMA P-5147 for occludin; SIGMA P-6911 for ZO-1) at 37$^{o}$C for 10 min. Slides to be

labelled with anti-claudin-1, were placed in EDTA solution (pH7.4) and heated in a water bath to 80-100$^{o}$C for 20 min, followed by rapid cooling in ice-cold water bath for 15 min. After antigen retrieval, slides were washed in PBS and then blocked in 10% goat or rabbit serum for 30 min. Sections were then labelled with primary antibodies, rabbit anti-occludin (1:200 dilution with actual antibody concentration at 0.125mg/mL, Invitrogen 71-15000), rabbit anti-claudin-1 (1:200 dilution with actual antibody concentration at 0.125mg/mL, Invitrogen 51-9000), or rabbit anti-ZO-1 (1:200 dilution with actual antibody concentration at 0.125mg/mL, Invitrogen 61-7300) and incubated overnight at 4$^{o}$C. Negative control received PBS only. Non-specific IgG control was treated with normal rabbit anti-IgG (1:800 dilution with actual antibody concentration at 0.125mg/mL, R&D Systems AB-105-C). After incubation sections were rinsed twice in PBS and labelled with secondary antibody, Alexa Fluor 488 goat anti-rabbit (1:500 dilution with actual antibody concentration at 0.004mg/mL, Life Technologies A-11008) for 1hr in darkness at room temperature. Following two washes in PBS, sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI), washed three times in PBS and cover-slipped using Fluoromount Aqueous Mounting Medium (SIGMA F4680). Slides were examined using a scanning confocal microscope (occludin and claudin-1: Leica SP5 Spectral Scanning Confocal Microscope; ZO-1: Olympus FV-1000 Confocal Microscope) at x40 objective lens with oil immersion (zoom 4x).

Control sections (negative stomach, and negative and non-specific IgG colon tissue) were examined at the same gain as the test sections (Appendix A12). Confocal examinations were carried out using Leica LAS AF Lite software (Version 2.6.3 build 8173; anti-occludin and anti-claudin-1 labelled sections) or Olympus FV-1000 Confocal Microscope software (anti-ZO-1 labelled sections).

### Semi-quantitative evaluation of tissue

In sections labelled with anti-occludin and anti-claudin-1, a minimum of four well-orientated areas of the gastric pit were chosen and a "z-stack" was obtained (0.8 μm increments). The "Max image" function of the Leica LAS AF Lite software was used to assess the frequency of labelling of the z-stack images. In sections labelled with anti-ZO-1, a minimum of four well-orientated areas of the gastric pits were recorded at a depth where the labelling was most numerous. Frequency of labelling was scored using a 0-4 scale adapted from Armstrong *et al.* (2001) (0 = no immunopositive signals, 1 = very occasional scattered immunopositive signals in the pit region, 2 = several immuno

positive signals, 3 = numerous immunopositive signals seen throughout the gastric pit region, 4 = florid immunopositive signals seen throughout the whole pit region of the section).

The tight junctions of the gastric pits were the focus of this study; therefore, observations of gastric glands and non-glandular areas were only made cursorily. A minimum of two well-orientated areas of gastric gland and of non-glandular stomach mucosa were recorded at two depths where the labelling was most numerous. The frequency of labelling was scored using the same 0-4 scale as for the gastric pits. In the non-glandular stomach, the first area to be scored was at the limiting ridge. The second area was at least 50μm from the ridge.

### 5.2.3 Statistical analysis

Statistical analyses were performed as described in Section 3.2.5. Based on the first study, where a potential directional change was likely, a one-tailed t-test was used. That is, where an increase in a parameter was predicated, the one-tailed test would only reveal differences in that direction. A large reduction in that parameter would not be significant. The converse would be true, where a decrease was predicated. A $p \leq 0.050$ was considered significant.

## 5.3 Results

### 5.3.1 Immunohistochemical light microscopic investigation

#### Occludin

There was no difference in the intensity of labelling between the groups of any of the stomach regions (Table 38; Figure 52). In the gastric pits, labelling was seen in the cytoplasm and in some areas between mucus-producing cells, labelling was seen as dots connected by thin bands (Figure 53A and B). In the gastric glands, labelling was seen in the cytoplasm and outlining the cells (Figure 53D and E). In the non-glandular area, labelling was primarily in the cytoplasm and was of greater intensity at the limiting ridge than in the area further away from the non-glandular/glandular junction (Figure 53G-H and J-K).

**Table 38. Intensity of occludin labelling in the stomachs of rats fed a 60% GM or non-GM corn diet.** (0-4 scale: 0 = no labelling; 1 = faint labelling; 2 = faint-medium labelling; 3 = medium-intense labelling; 4 = intense labelling) Values show mean or median and standard deviation (SD) or interquartile range (IQR) depending on whether the data are normally distributed.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Mean or median[a] | SD or IQR[a] | n | Mean or median[a] | SD or IQR[a] | n | | |
| gastric pits of glandular stomach | 2.38 | 0.44 | 10 | 2.67 | 0.78 | 9 | NS | 12.18 |
| gastric glands of glandular stomach | 1.80 | 0.80 | 10 | 1.78 | 0.63 | 9 | NS | -1.11 |
| non-glandular stomach mucosa | 2.25[a] | 1.38-3.13[a] | 8 | 2.50[a] | 1.50-2.75[a] | 7 | NS | 11.11 |

[a] Not normally distributed data, the median and interquartile range (IQR) are given.

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

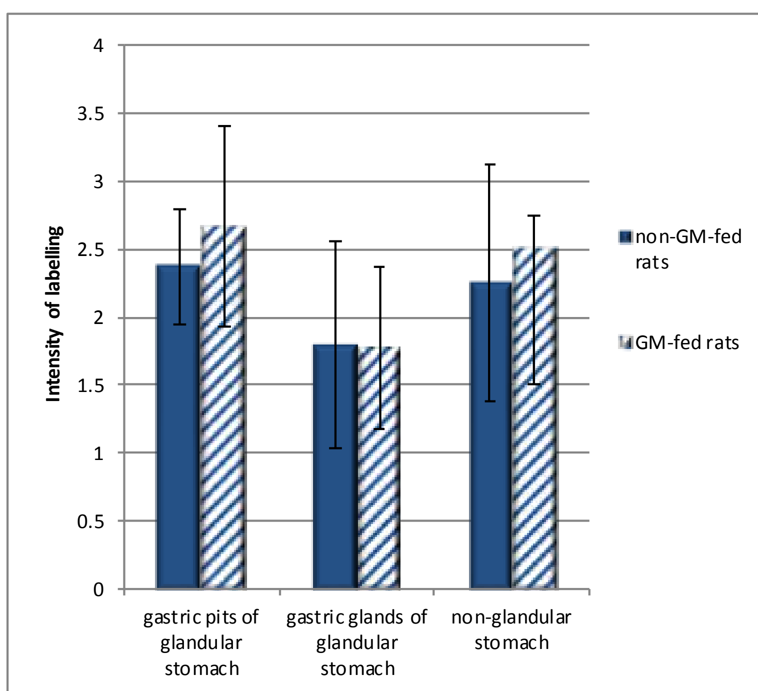



**Figure 52. Intensity of occludin labelling in the stomachs of rats fed a 60% GM or non-GM corn diet.** Graph shows mean or median and bars indicate standard deviation or interquartile range depending on whether the data are normally distributed.

## *Occludin*

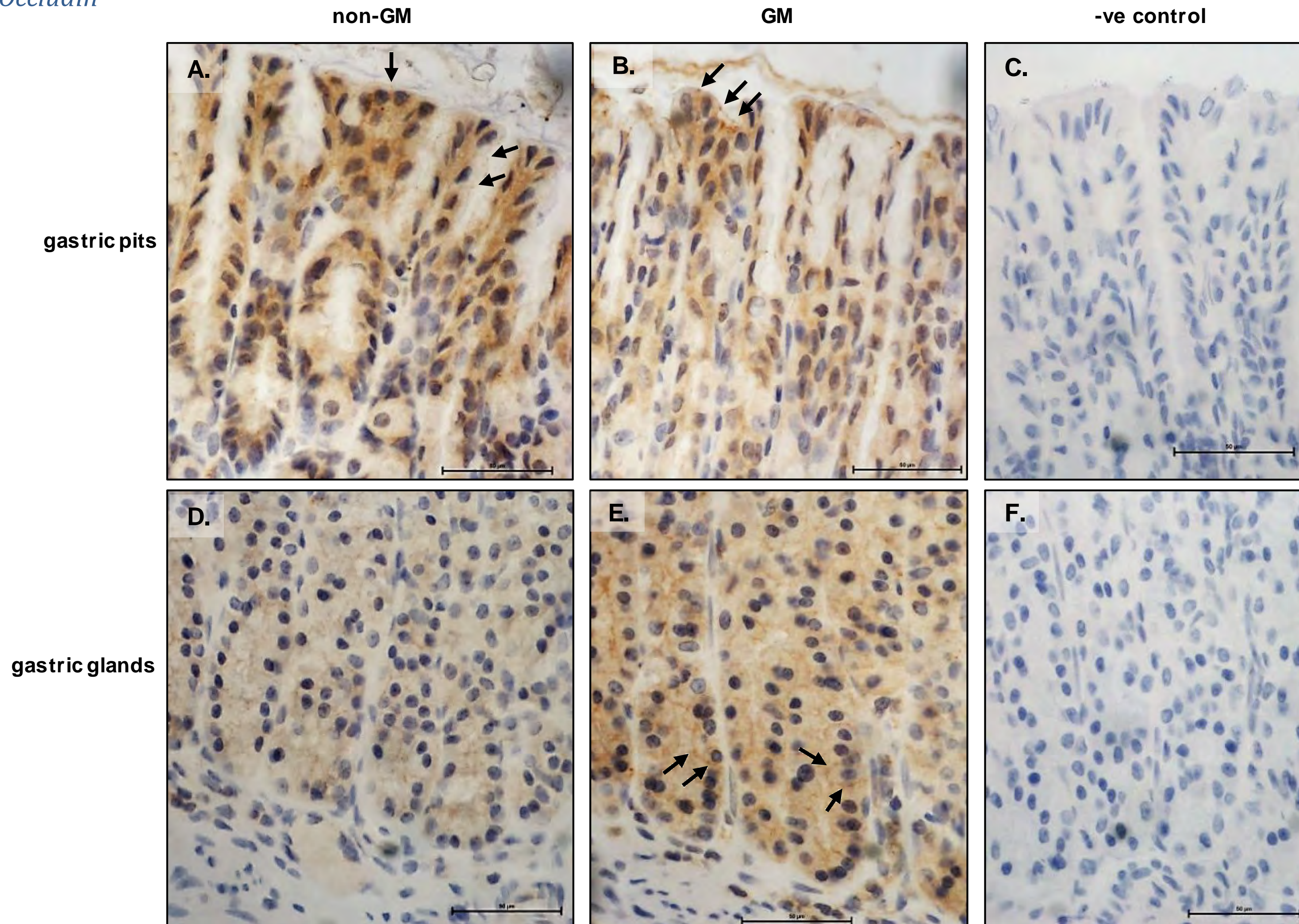



**Figure 53. Occludin expression in stomachs of rats fed a 60% GM or non-GM corn diet.**

A.-C.) *Gastric pits:* Medium-intense labelling in gastric pits of non-GM (A.) and GM-fed (B.) rats. Specific labelling (arrows) connected by thin bands seen at the apical surface of the gastric pit cells.

C.) Gastric pits of negative control showing no specific labelling.

Scale bar 50μm.

D.-F.) *Gastric glands:* D.) Faint-medium labelling in non-GM-fed rat; and E.) medium-intense labelling in the cytoplasm with specific labelling outlining the gastric gland cells (arrows) in GM-fed rat.

F.) Gastric glands of negative control showing no specific labelling.

Scale bar 50μm.

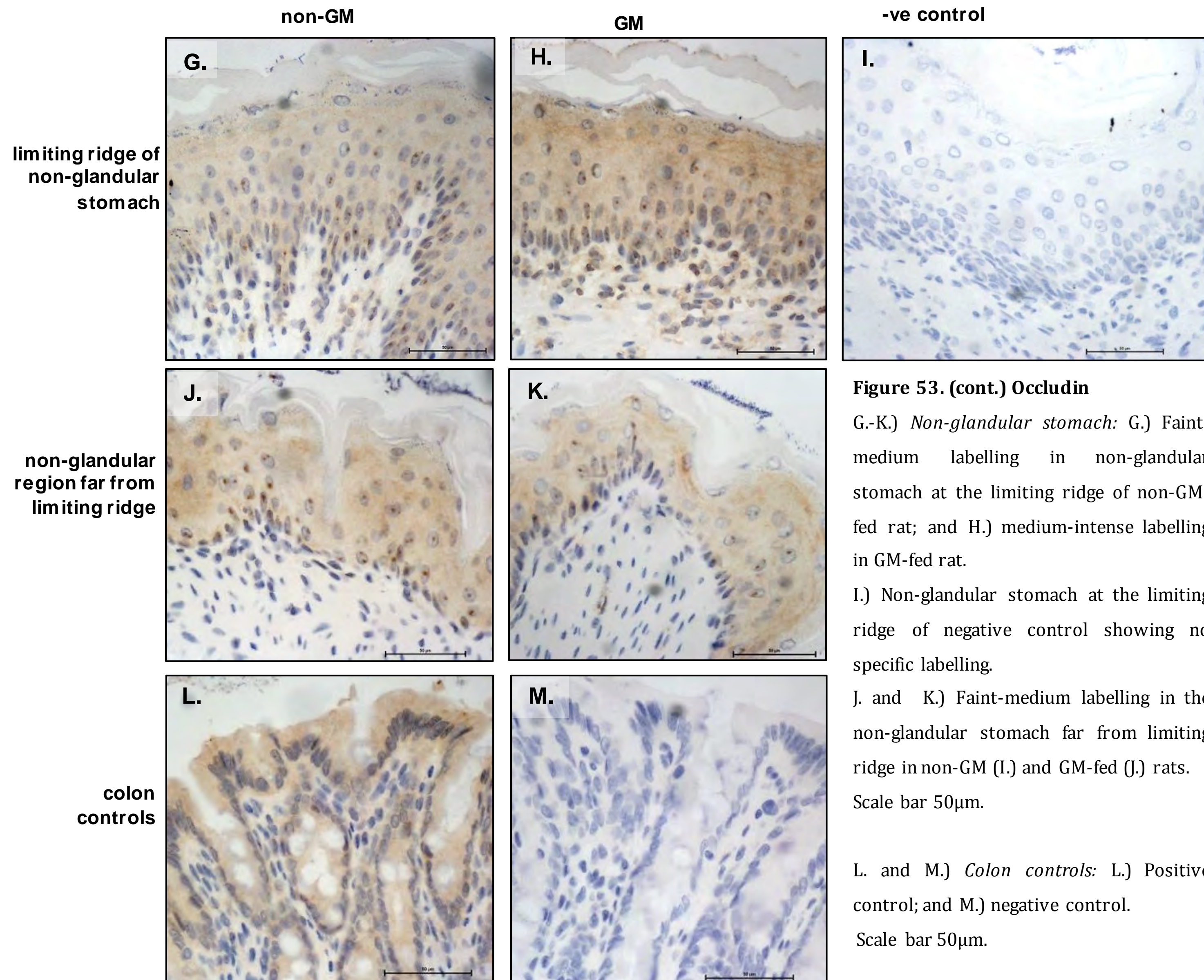



**Figure 53. (cont.) Occludin**

G.-K.) *Non-glandular stomach:* G.) Faint-medium labelling in non-glandular stomach at the limiting ridge of non-GM-fed rat; and H.) medium-intense labelling in GM-fed rat.

I.) Non-glandular stomach at the limiting ridge of negative control showing no specific labelling.

J. and K.) Faint-medium labelling in the non-glandular stomach far from limiting ridge in non-GM (I.) and GM-fed (J.) rats. Scale bar 50μm.

L. and M.) *Colon controls:* L.) Positive control; and M.) negative control.
Scale bar 50μm.

## Claudin-1

There was no difference in the intensity of labelling between the groups of any of the stomach regions (Table 39; Figure 54). In the pits, claudin-1 labelling was in the cytoplasm and at the apical membrane of the epithelial cells (Figure 55A and B). In the glands, labelling was seen outlining the cells (Figure 55D and E). In the non-glandular stomach, labelling was seen outlining the cells, with intensity being greater at the limiting ridge than in the area further away from the non-glandular/glandular junction (Figure 55G-H and J-K).

**Table 39. Intensity of claudin-1 labelling in the stomachs of rats fed a 60% GM or non-GM corn diet.** (0-4 scale: 0 = no labelling; 1 = faint labelling; 2 = faint-medium labelling; 3 = medium-intense labelling; 4 = intense labelling) Values show mean or median and standard deviation (SD) or interquartile range (IQR) depending on whether the data are normally distributed.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Mean or median[a] | SD or IQR[a] | n | Mean or median[a] | SD or IQR[a] | n | | |
| gastric pits of glandular stomach | 2.40 | 0.57 | 10 | 2.52 | 1.13 | 9 | NS | 5.00 |
| gastric glands of glandular stomach | 2.70 | 0.54 | 10 | 2.58 | 0.88 | 9 | NS | -4.44 |
| non-glandular stomach mucosa | 3.00[a] | 3.00-3.13[a] | 8 | 3.25[a] | 3.00-3.50[a] | 8 | NS | 10.21 |

[a] Not normally distributed data, the median and interquartile range is given.

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

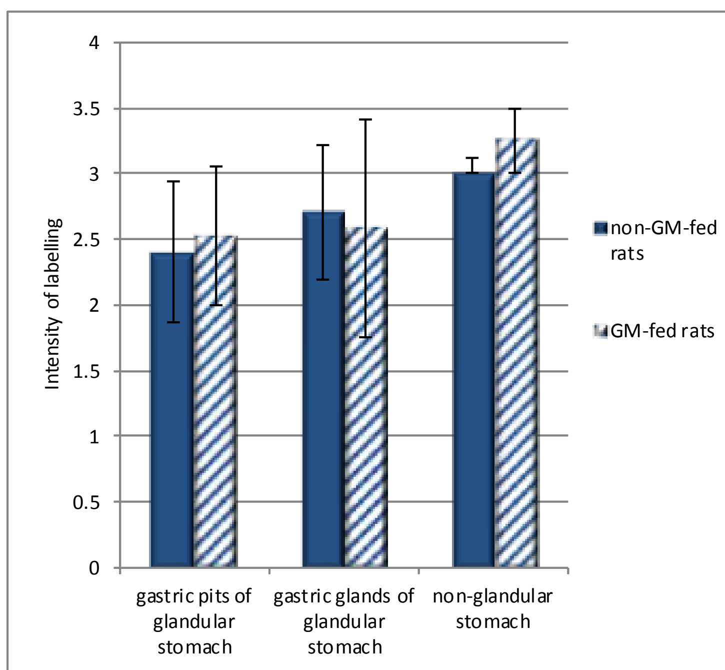



**Figure 54. Intensity of claudin-1 labelling in the stomachs of rats fed a 60% GM or non-GM corn diet.** Graph shows mean or median and bars indicate standard deviation or interquartile range depending on whether the data are normally distributed.

*Claudin-1*

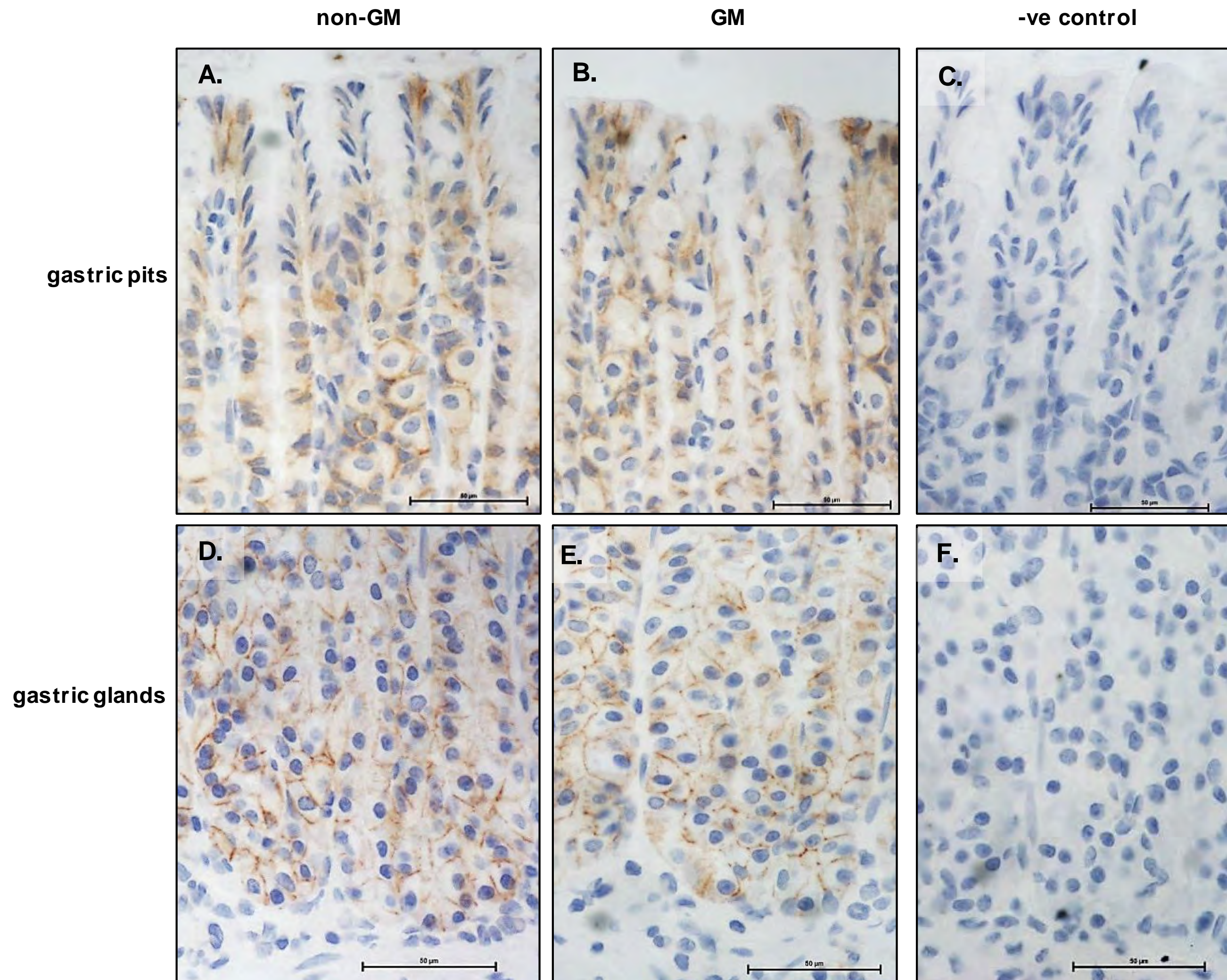



**Figure 55. Claudin-1 expression in stomachs of rats fed a 60% GM or non-GM corn diet.**

A-C) *Gastric pits:* Medium-intense labelling in gastric pits of non-GM (A) and GM-fed (B) rats.

C) Gastric pits of negative control showing no specific labelling.

Scale bar 50µm.

D-F) *Gastric glands:* Medium-intense labelling in non-GM (D) and GM-fed (E) rat. Labelling

F) Gastric glands of negative control showing no specific labelling.

Scale bar 50µm.

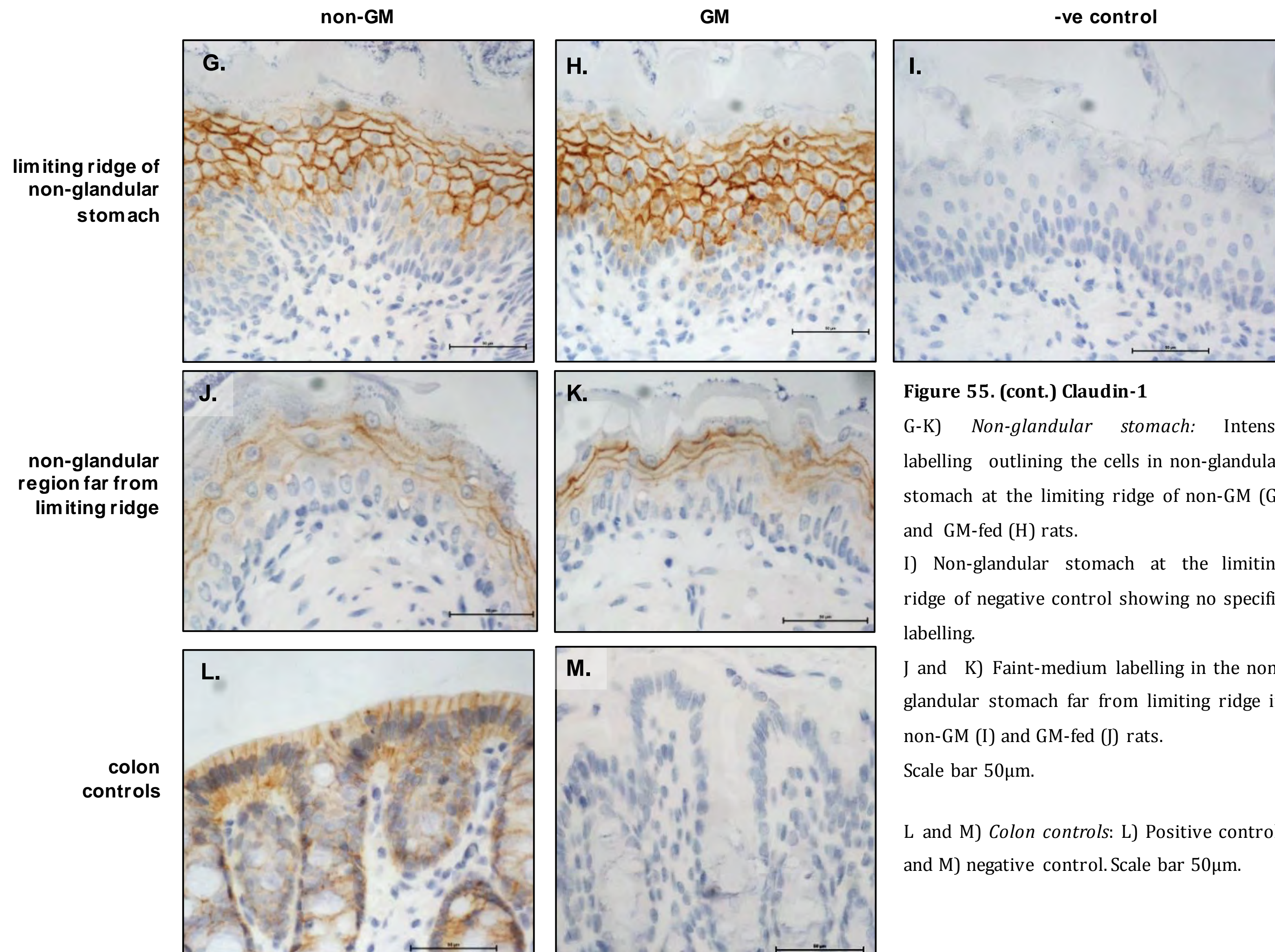



**Figure 55. (cont.) Claudin-1**

G-K) *Non-glandular stomach:* Intense labelling outlining the cells in non-glandular stomach at the limiting ridge of non-GM (G) and GM-fed (H) rats.

I) Non-glandular stomach at the limiting ridge of negative control showing no specific labelling.

J and K) Faint-medium labelling in the non-glandular stomach far from limiting ridge in non-GM (I) and GM-fed (J) rats.

Scale bar 50µm.

L and M) *Colon controls*: L) Positive control; and M) negative control. Scale bar 50µm.

## 5.3.2 Immunofluorescence confocal microscopic investigation

### Occludin

There was no decrease in occludin expression in any area of the stomach of either GM or non-GM fed groups (Table 40; Figure 56). Occludin expression in the gastric pits was seen as single dots connected by thin bands (Figure 56). In the gastric glands, autofluorescence of the granules in the epithelial cells made it impossible to see the labelling in several rats (3/10 GM and 1/9 non-GM). In non-glandular stomach labelling was seen as single dots surrounding the epithelial cells in the non-keratinized layer of the mucosa (Figure 56Q-T).

**Table 40. Occludin frequency of labelling in the stomachs of rats fed a 60% GM or non-GM corn diet** (0-4 scale: 0 = zero signals, 1 = a few signals, 2 = few-medium number of signals, 3 = medium-many signals, 4 = many signals). Values show mean or median and standard deviation (SD) or interquartile range (IQR) depending on whether the data are normally distributed.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Mean or median[a] | SD or IQR[a] | n | Mean or median[a] | SD or IQR[a] | n | | |
| gastric pits of glandular stomach | 3.75[a] | 3.25-4.00[a] | 9 | 3.50[a] | 3.06-3.69[a] | 10 | NS | -6.67 |
| gastric glands of glandular stomach | 2.94 | 0.68 | 8 | 2.86 | 0.99 | 7 | NS | -2.72 |
| non-glandular stomach mucosa | 2.50 | 0.50 | 5 | 2.42 | 0.78 | 8 | NS | -3.2 |

[a] Not normally distributed data, the median and interquartile range is given.

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

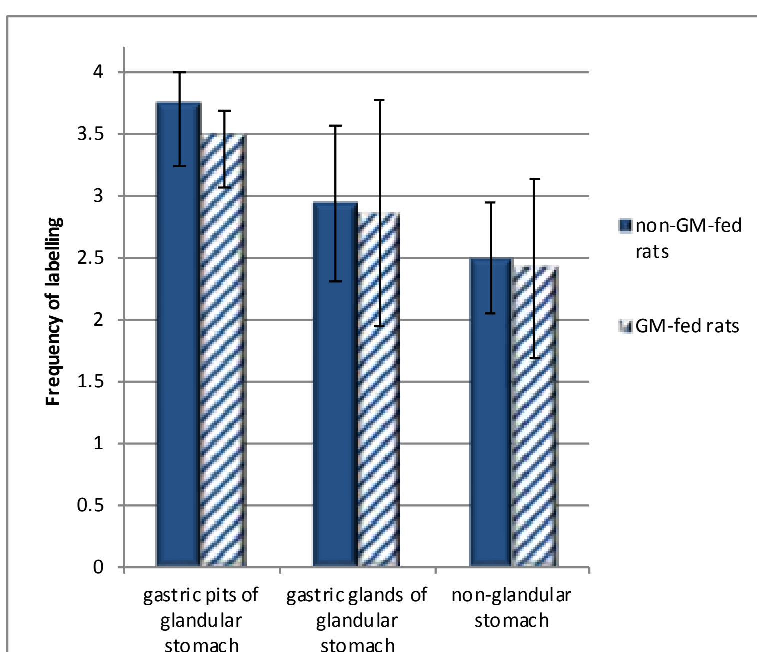



**Figure 56. Occludin frequency of labelling in the stomachs of rats fed a 60% GM or non-GM corn diet.** Graph shows mean or median and bars indicate standard deviation or interquartile range depending on whether the data are normally distributed.

### *Occludin – gastric pits*

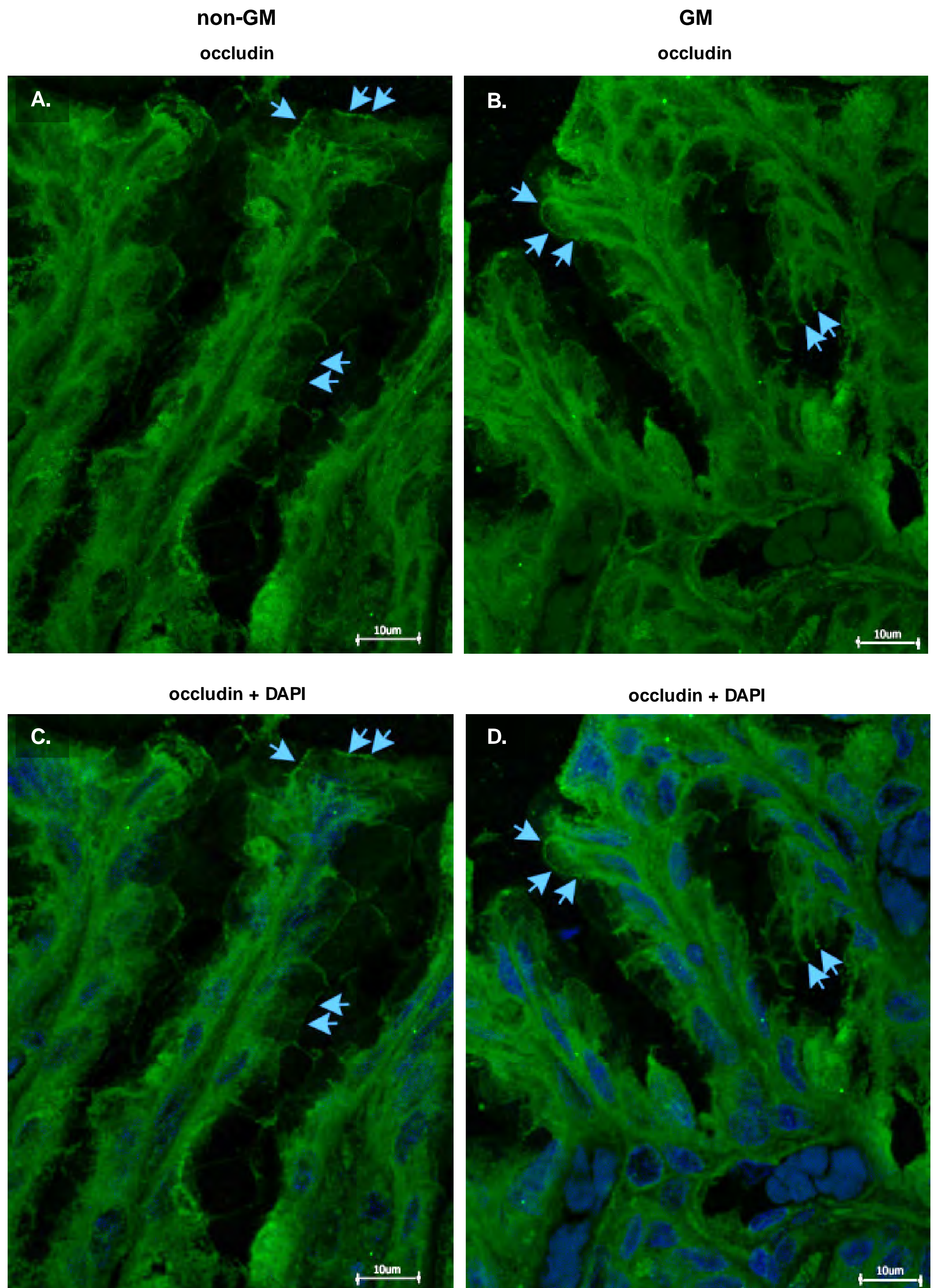



**Figure 57. Occludin expression in the stomachs of rats fed a 60% GM or non-GM corn diet.**

A.-D.) *Gastric pits:* Gastric pits labelled with anti-occludin, or with additional labelling of cell nuclei with DAPI in non-GM (A. and C.), and GM-fed (B. and D.) rats. Occludin labelling seen as specific dots (arrows) connected by a thin band.

*Occludin – gastric pits controls*

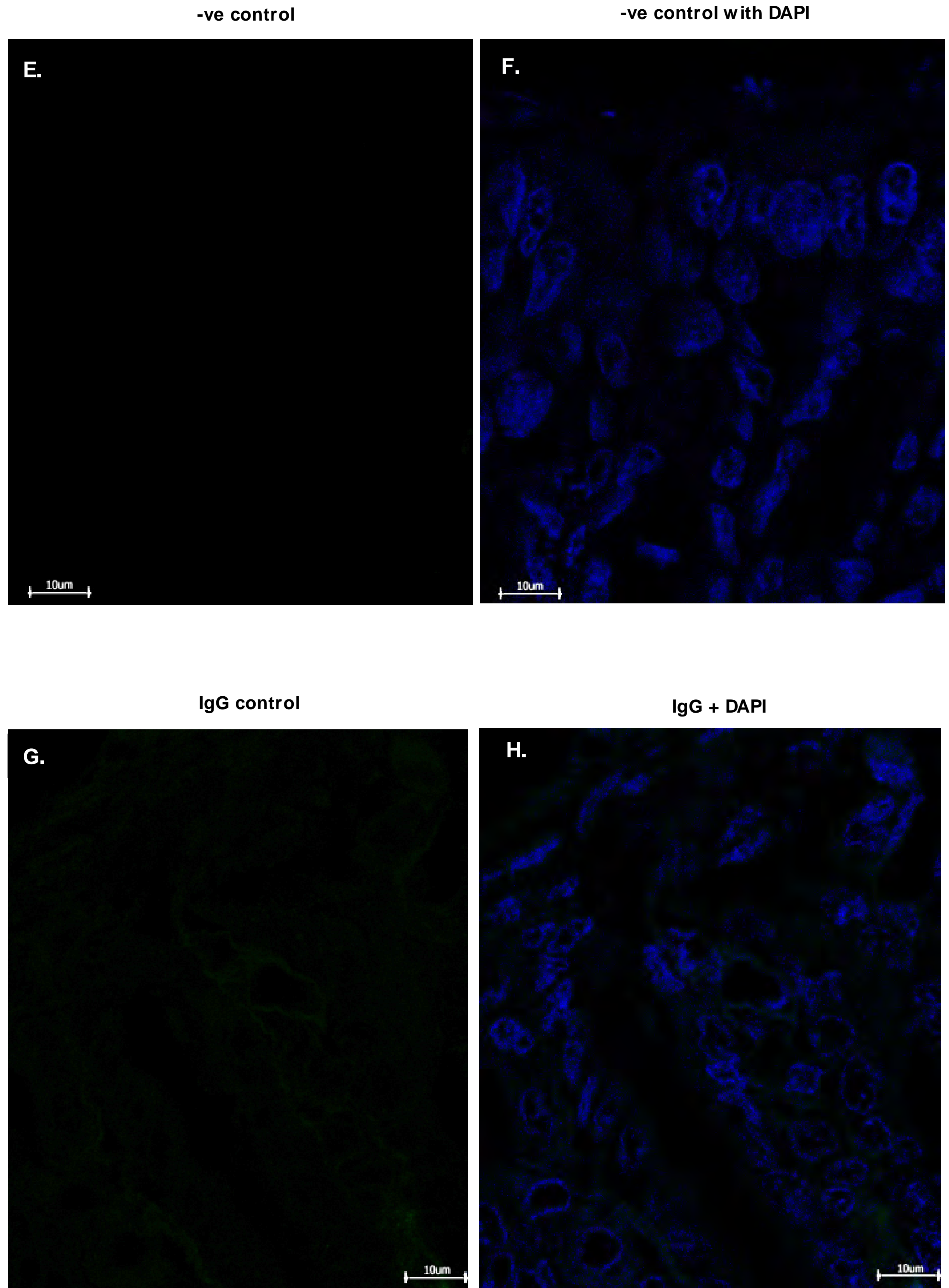



**Figure 57. (cont.) Occludin**

E.-H.) *Gastric pit controls:* Gastric pits of negative control (E. and F.) showing no specific labelling. Gastric pits treated with non-specific IgG (G. and H.) showing no specific labelling.

*Occludin – gastric glands*

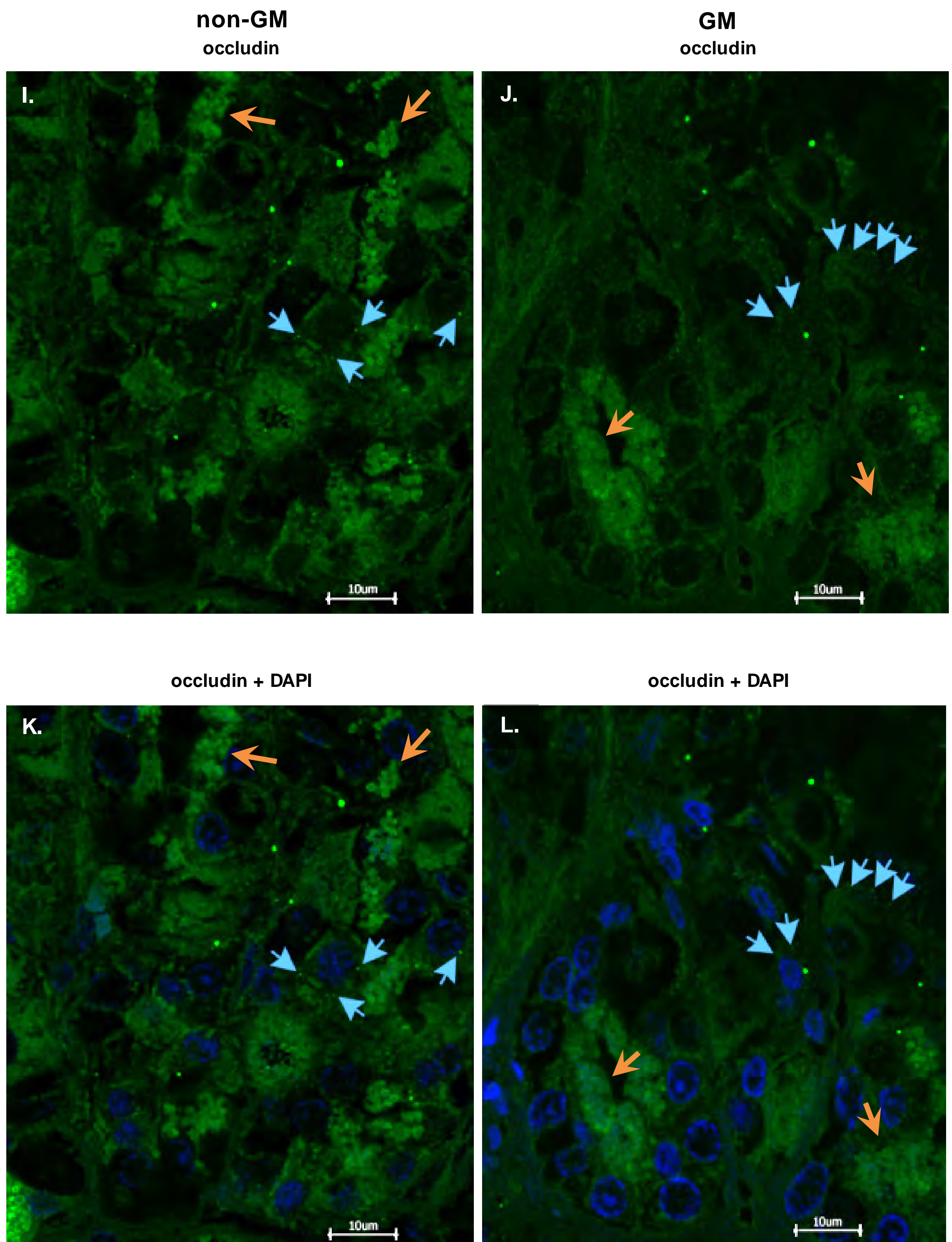



**Figure 57. (cont.) Occludin**

I.-P.) Gastric glands: Gastric glands labelled with anti-occludin, or with additional labelling of cell nuclei with DAPI in non-GM (I. and K.), and GM-fed (J. and L.) rats. Occludin labelling seen as specific small dots (blue arrows). In some areas the dots appear to encircle the epithelial cell. Some autofluorescence in seen in the cytoplasm of the gland epithelial cells (orange arrows).

## *Occludin – gastric gland controls*

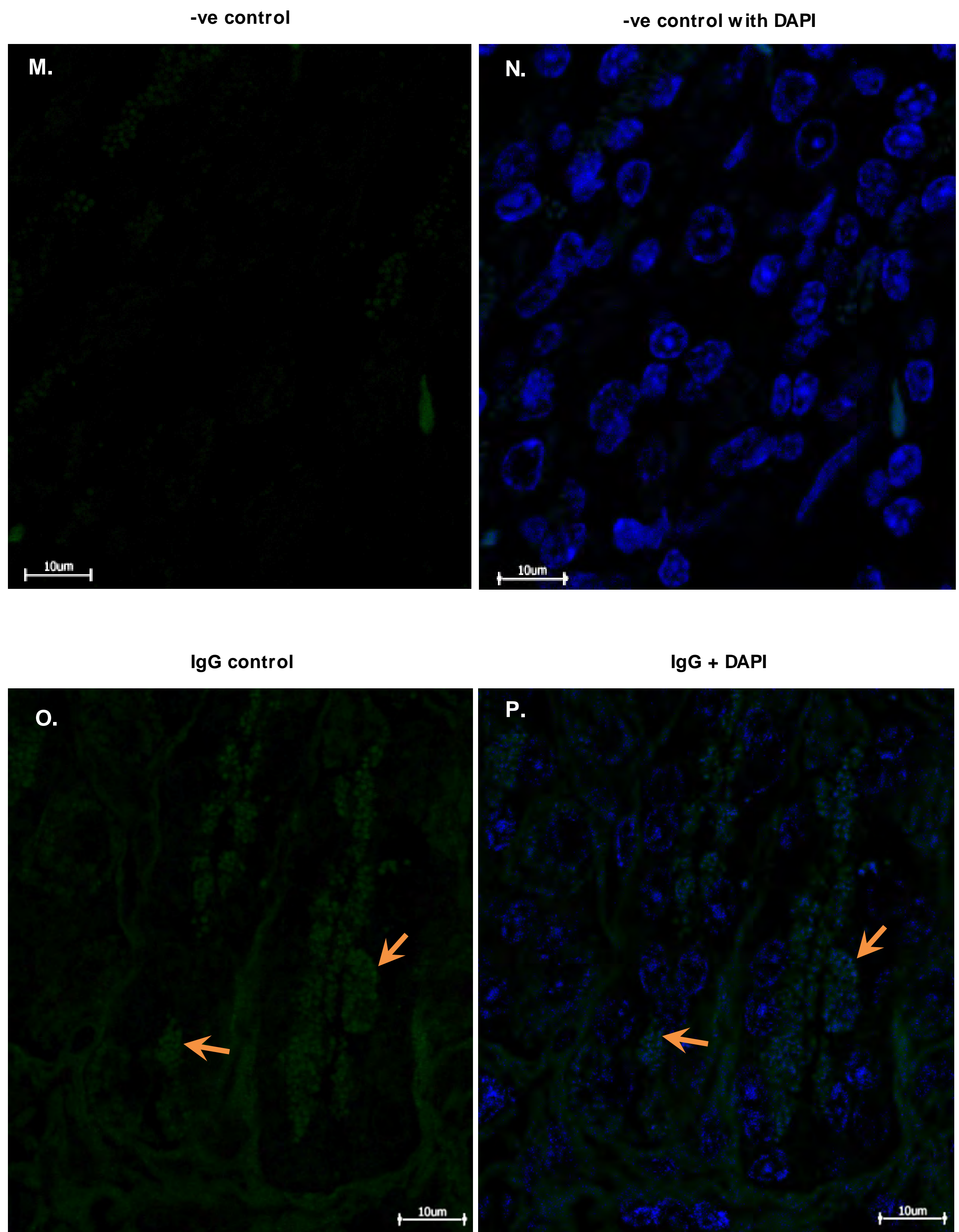



**Figure 57. (cont.) Occludin**

M.-P.) *Gastric gland controls:* Gastric glands of negative control (M. and N.) showing no specific labelling. Gastric glands treated with non-specific IgG (O. and P.) showing no specific labelling. Some autofluorescence seen in the cytoplasm of the gland epithelial cells (orange arrows).

## *Occludin – non-glandular stomach*

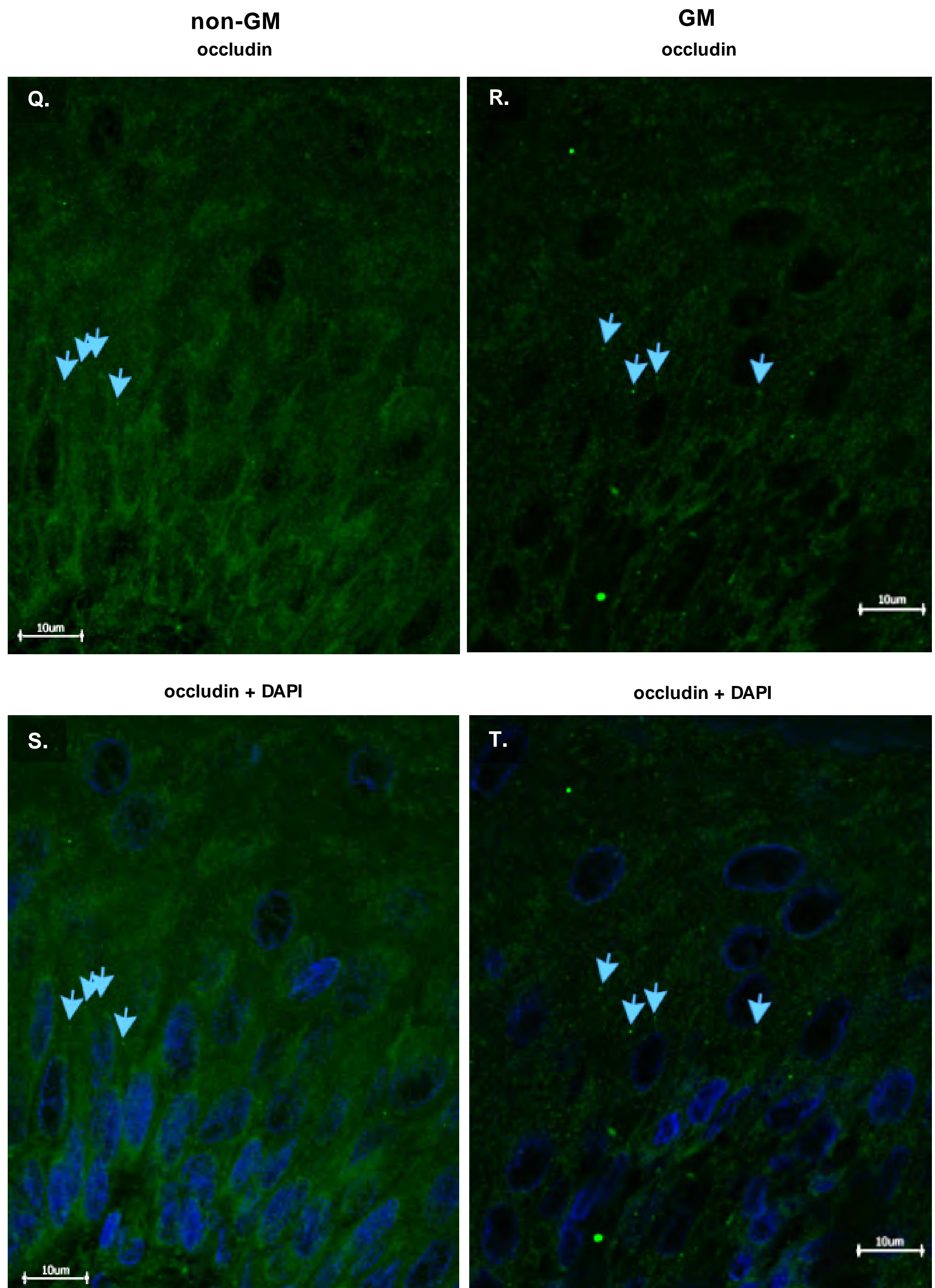



**Figure 57. (cont.) Occludin**

Q.-T.) Non-glandular stomach: Non-glandular stomach labelled with anti-occludin (arrows), or with additional labelling of cell nuclei with DAPI, in non-GM (Q. and S.) and GM-fed (R. and T.) rats. Occludin labelling seen as specific dots (arrows).

## *Occludin – colon controls*

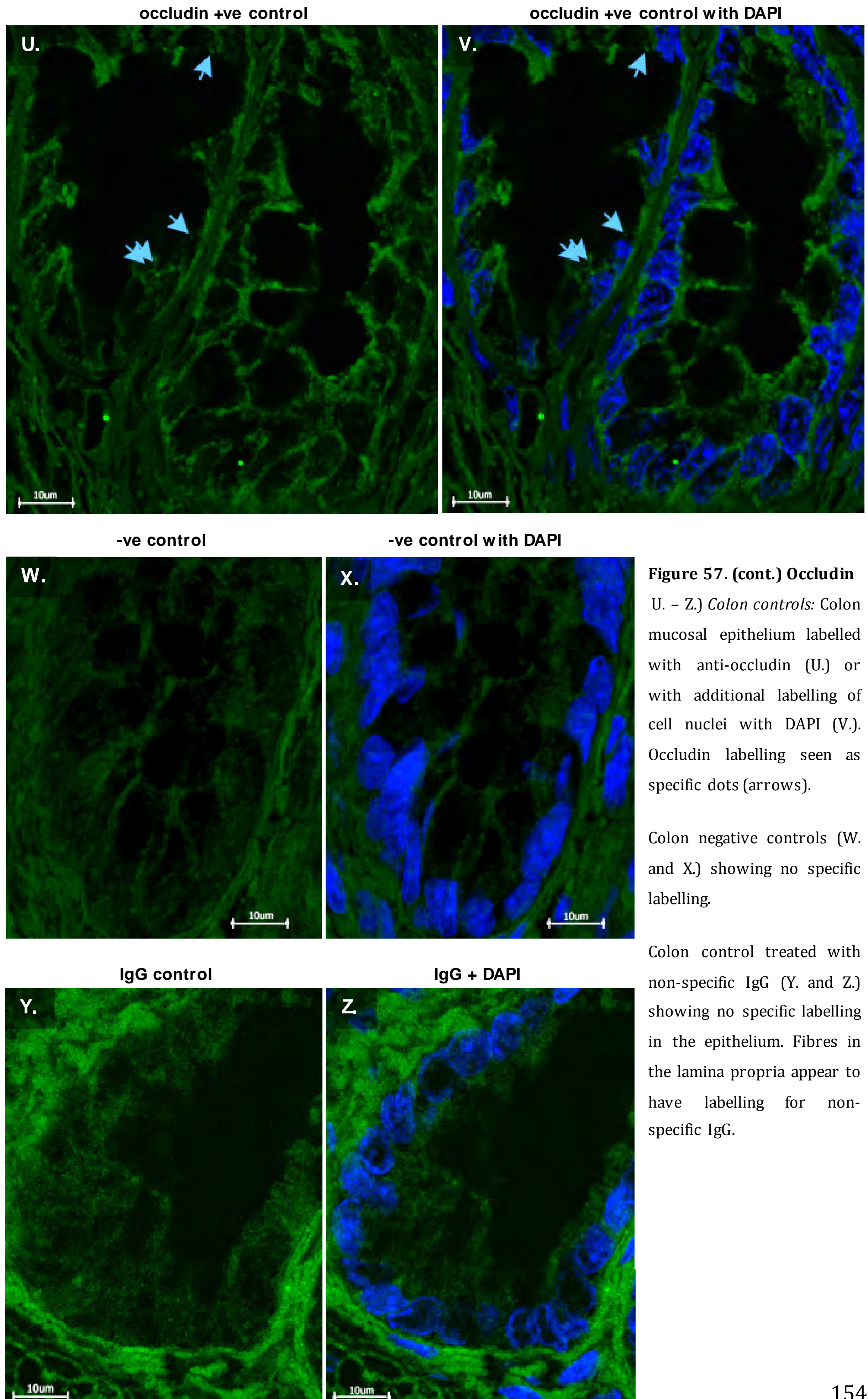



**Figure 57. (cont.) Occludin** U. – Z.) *Colon controls:* Colon mucosal epithelium labelled with anti-occludin (U.) or with additional labelling of cell nuclei with DAPI (V.). Occludin labelling seen as specific dots (arrows).

Colon negative controls (W. and X.) showing no specific labelling.

Colon control treated with non-specific IgG (Y. and Z.) showing no specific labelling in the epithelium. Fibres in the lamina propria appear to have labelling for non-specific IgG.

### Claudin-1

There was no difference in claudin-1 expression in the gastric pits or glands between GM or non-GM fed animals (Table 41; Figure 58). Expression was slightly higher in GM-fed animals in the non-glandular stomach, but this was not statistically significant. Claudin-1 expression in the gastric pits and glands was seen as single dots (Figure 59). In non-glandular stomach labelling was seen as single dots surrounding the epithelial cells in the non-keratinized layer of the mucosa (Figure 59Q-T).

**Table 41. Claudin-1 frequency of labelling in the stomachs of rats fed a 60% GM or non-GM corn diet** (0-4 scale: 0 = no signals, 1 = a few signals, 2 = few-medium number of signals, 3 = medium-many signals, 4 = many signals). Values show mean or median and standard deviation (SD) or interquartile range (IQR) depending on whether the data are normally distributed.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%) |
|---|---|---|---|---|---|---|---|---|
| | Mean or median[a] | SD or IQR[a] | n | Mean or median[a] | SD or IQR[a] | n | | |
| gastric pits of glandular stomach | 2.67 | 0.84 | 10 | 2.32 | 0.63 | 9 | NS | -13.11 |
| gastric glands of glandular stomach | 4.00[a] | 3.13-4.00[a] | 10 | 3.50[a] | 3.00-4.00[a] | 9 | NS | -12.50 |
| non-glandular stomach mucosa | 3.33[a] | 2.54-4.00[a] | 6 | 3.67[a] | 3.50-4.00[a] | 5 | NS | 10.21 |

[a] Not normally distributed data, the median and interquartile range is given.

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

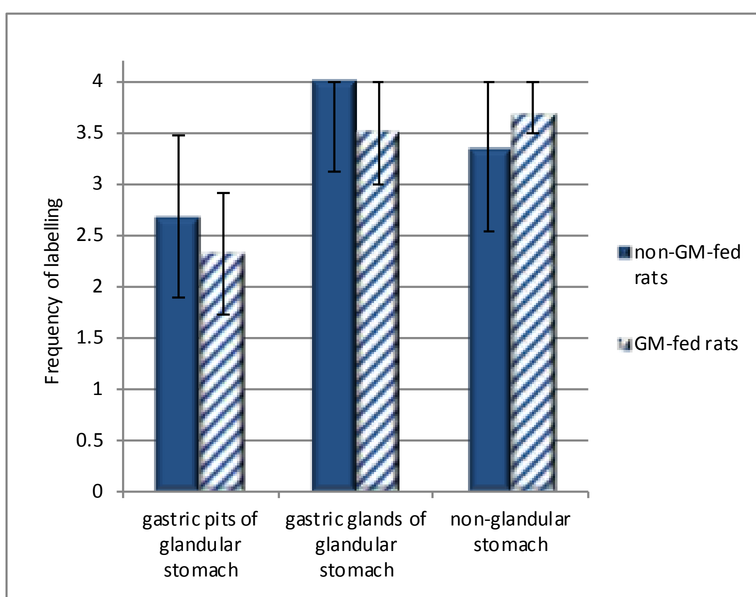



**Figure 58. Claudin-1 frequency of labelling in the stomachs of rats fed a 60% GM or non-GM corn diet.** Graph shows mean or median and bars indicate standard deviation or interquartile range depending on whether the data are normally distributed.

*Claudin-1 – gastric pits*

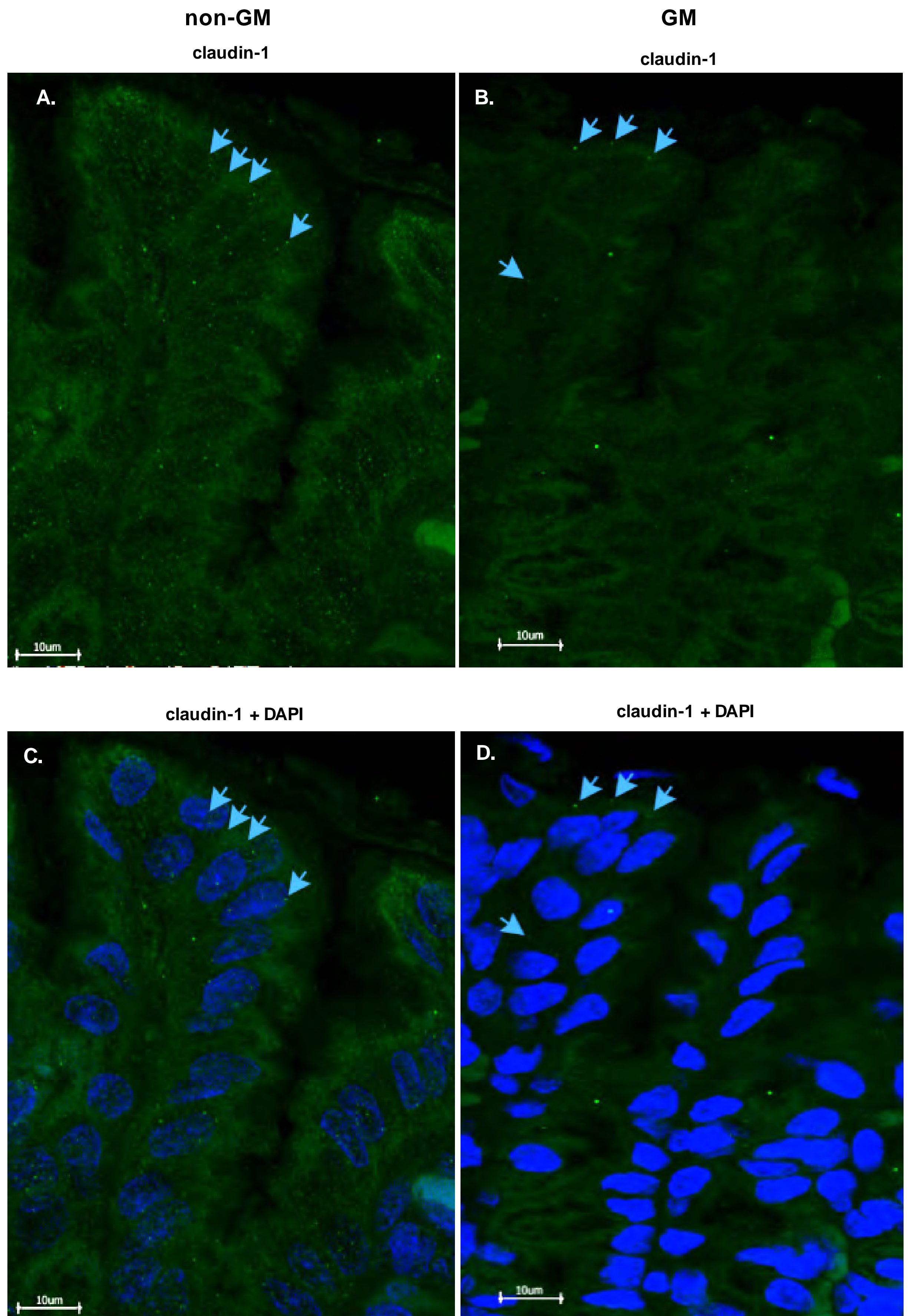



**Figure 59. Claudin-1 expression in the stomachs of rats fed a 60% GM or non-GM corn diet.**

A.-D.) *Gastric pits:* Gastric pits labelled with anti-claudin-1, or with additional labelling of cell nuclei with DAPI in non-GM (A. and C.), and GM-fed (B. and D.) rats. Claudin-1 labelling seen as specific dots (arrows).

## *Claudin-1 – gastric pit controls*

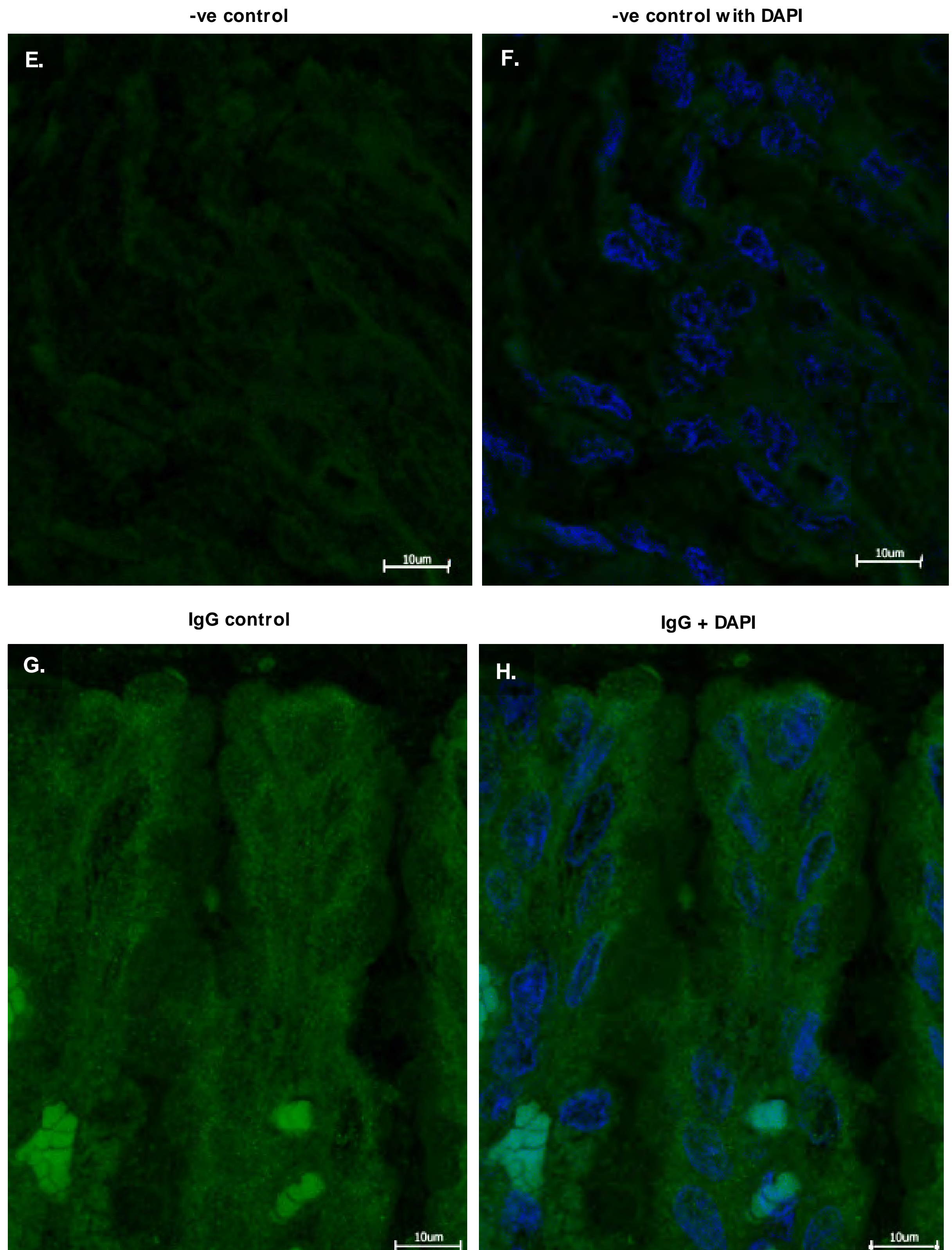



**Figure 59. (cont.) Claudin-1**

E.-H.) *Gastric gland controls:* Gastric pits of negative control (C. and F.) showing no specific labelling. Gastric pits treated with non-specific IgG (G. and H.) showing no specific labelling.

*Claudin-1 – gastric glands*

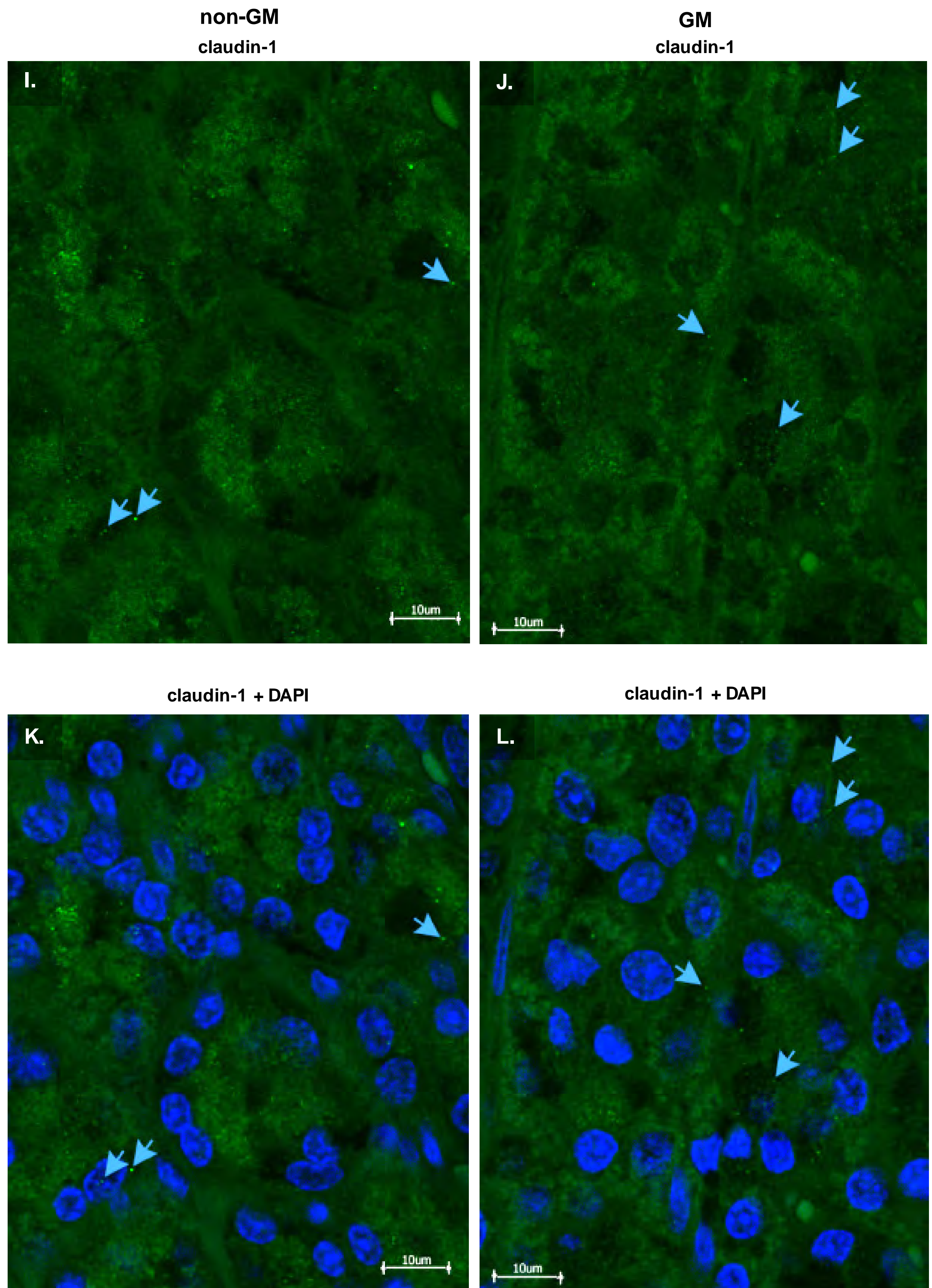



**Figure 59. (cont.) Claudin-1**

I.-L.) *Gastric glands*: Gastric glands labelled with anti-claudin-1, or with additional labelling of cell nuclei with DAPI in non-GM (I. and M.), and GM-fed (J. and M.) rats. Claudin-1 labelling seen as specific dots (arrows).

### *Claudin-1 – gastric gland controls*

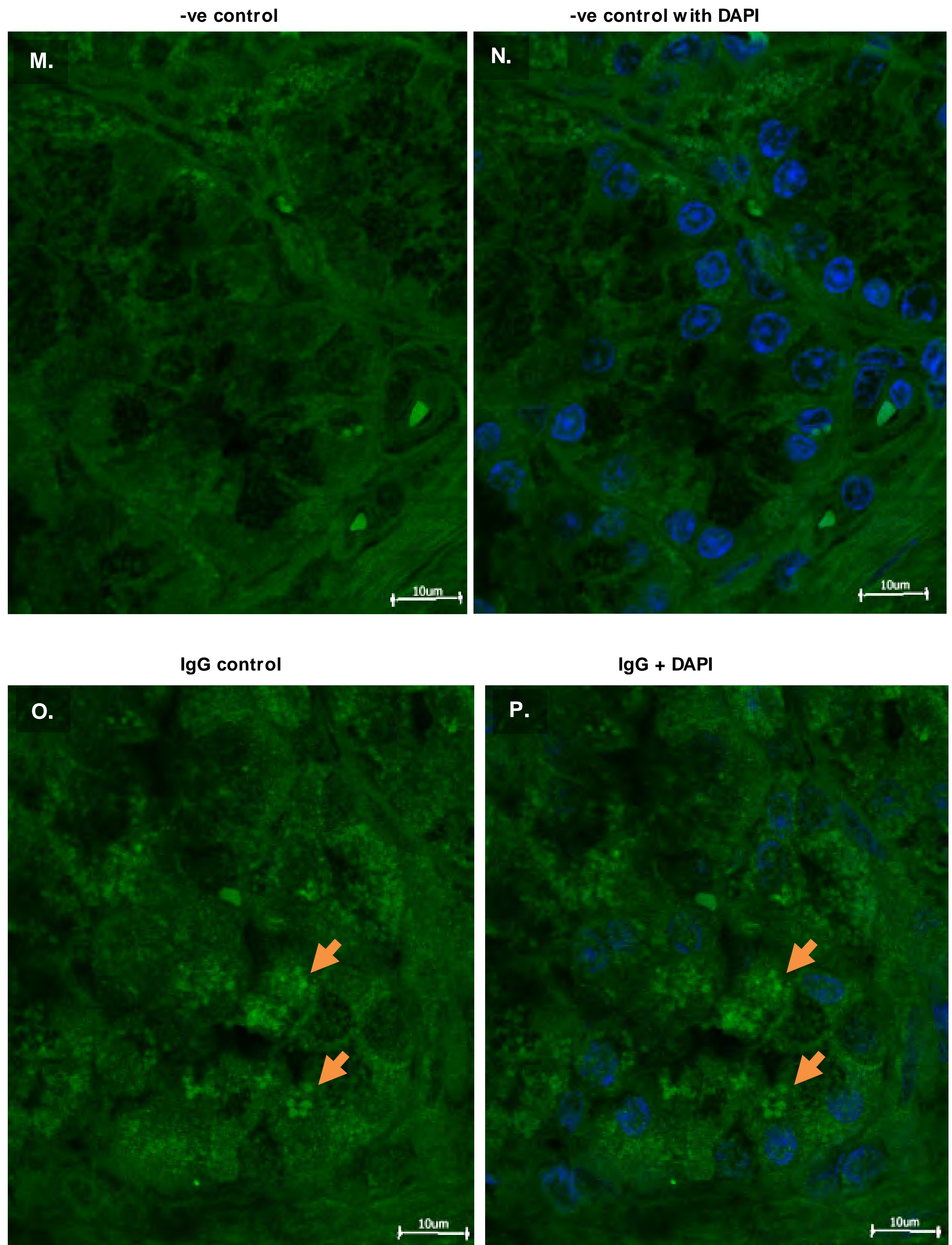



**Figure 59. (cont.) Claudin-1**

M.-P.) *Gastric gland controls*: Gastric glands of negative control (M. and N.) showing no specific labelling of epithelium. Gastric glands treated with non-specific IgG (O. and P.) showing no specific labelling of epithelium. Some autofluorescence in the cytoplasm of the gland cells (orange arrows).

### *Claudin-1 – non-glandular stomach*

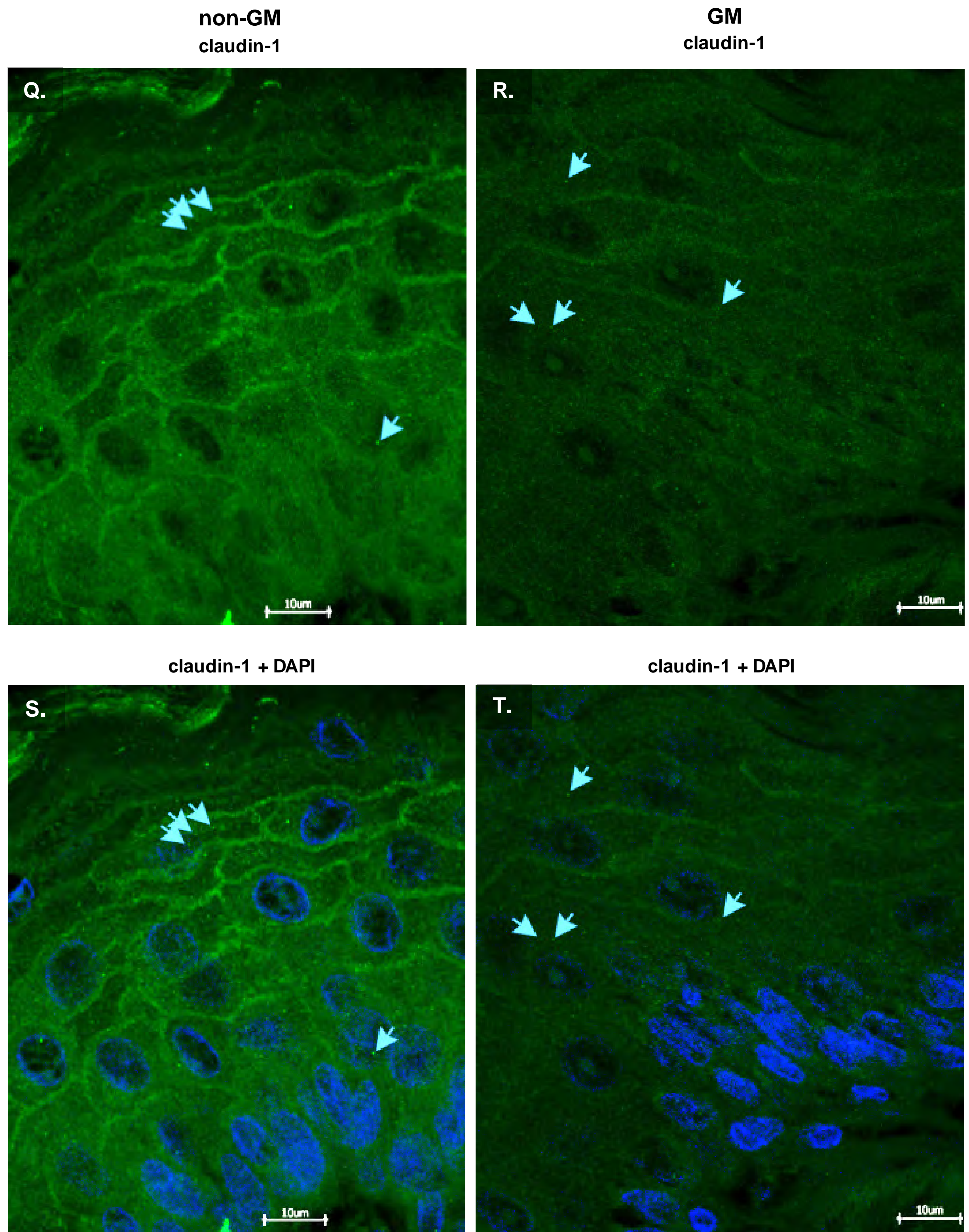



**Figure 59. (cont.) Claudin-1**

Q.-T.) *Non-glandular stomach:* Non-glandular stomach labelled with anti-claudin-1, or with additional labelling of cell nuclei with DAPI, in non-GM (Q. and S.) and GM-fed (R. and T.) rats. Claudin-1 labelling seen as specific dots (arrows) that appear to encircle the cells.

## *Claudin-1 – colon controls*

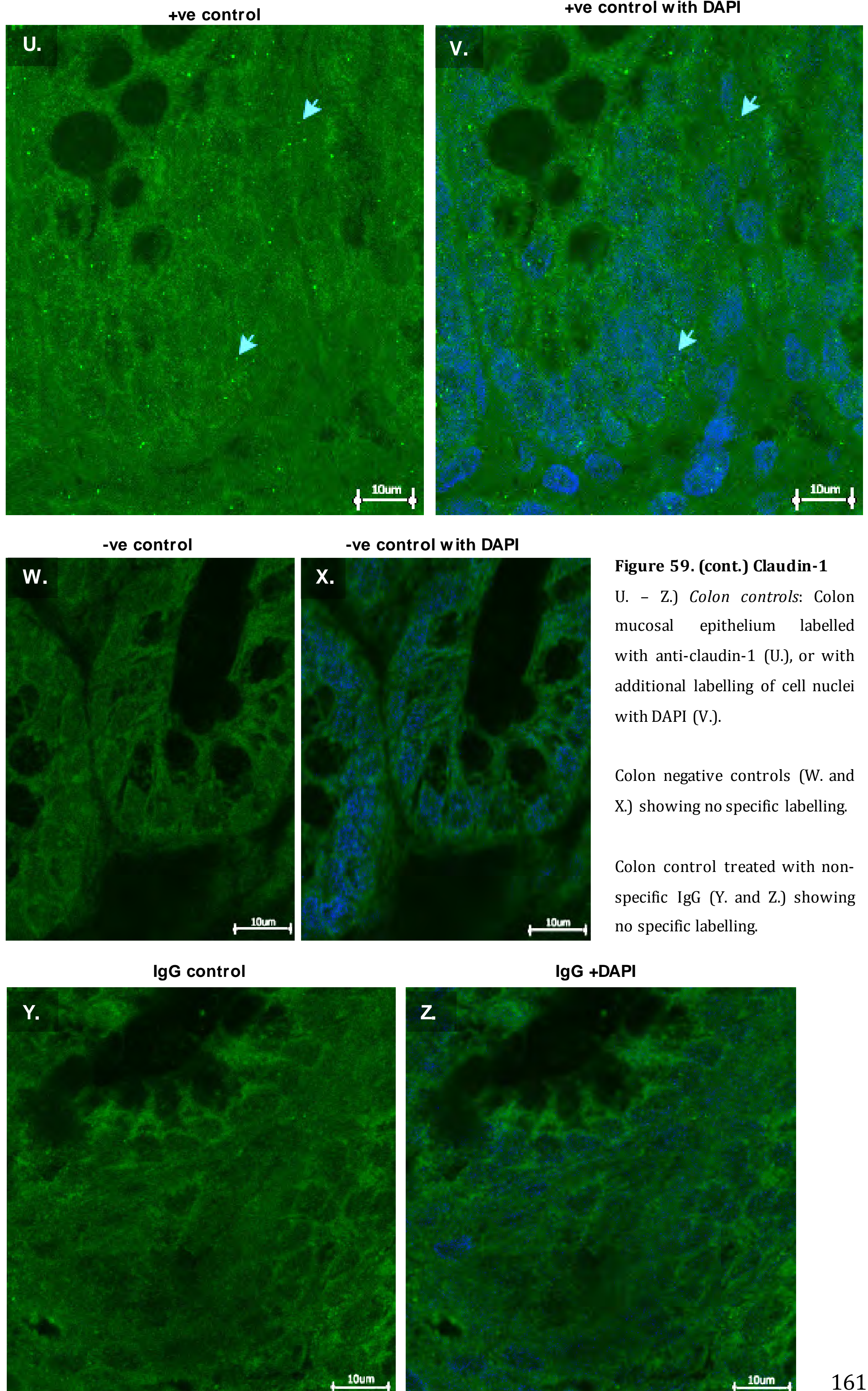



**Figure 59. (cont.) Claudin-1**

U. – Z.) *Colon controls*: Colon mucosal epithelium labelled with anti-claudin-1 (U.), or with additional labelling of cell nuclei with DAPI (V.).

Colon negative controls (W. and X.) showing no specific labelling.

Colon control treated with non-specific IgG (Y. and Z.) showing no specific labelling.

### ZO-1

In the gastric pits of negative and IgG controls, autofluorescence was observed at the stomach luminal surface. Therefore, this area was avoided in the assessment of the stomach tissues treated with the anti-ZO-1 in both the GM and non-GM-fed groups. There was no difference in ZO-1 expression in gastric pit or gland of either GM or non-GM fed groups (Table 42; Figure 60). Expression was slightly higher in GM-fed animals in the non-glandular stomach, but this was not statistically significant. ZO-1 expression in the gastric pits and glands was seen as single dots (Figure 61). In non-glandular stomach labelling was seen as single dots surrounding the epithelial cells in the non-keratinized layer of the mucosa (Figure 61M-N and P-Q).

**Table 42. ZO-1 frequency of labelling in the stomachs of rats fed a 60% GM or non-GM corn diet** (0-4 scale: 0 = zero signals, 1 = a few signals, 2 = few-medium number of signals, 3 = medium-many signals, 4 = many signals). Values show mean or median and standard deviation (SD) or interquartile range (IQR) depending on whether the data are normally distributed.

| | non-GM | | | GM | | | Statistical significance (P ≤0.050) | Change (%)[b] |
|---|---|---|---|---|---|---|---|---|
| | Mean or median[a] | SD or IQR[a] | n | Mean or median[a] | SD or IQR[a] | n | | |
| gastric pits of glandular stomach | 2.03 | 0.82 | 10 | 2.02 | 0.81 | 9 | NS | -0.49 |
| gastric glands of glandular stomach | 1.75[a] | 1.50-3.44[a] | 10 | 2.00[a] | 1.50-2.50[a] | 9 | NS | 14.29 |
| non-glandular stomach mucosa | 2.77 | 0.78 | 8 | 3.58 | 0.75 | 7 | NS | 29.24 |

[a] Not normally distributed data, the median and interquartile range is given.

[b] The effect of eating the GM diet compared with the non-GM diet as a percentage change. The means were compared, unless one or both variables were not normally distributed, then the medians were compared. A negative sign before the number indicates that the GM diet decreased the variable. No sign indicates that the GM diet increased the value of the variable.

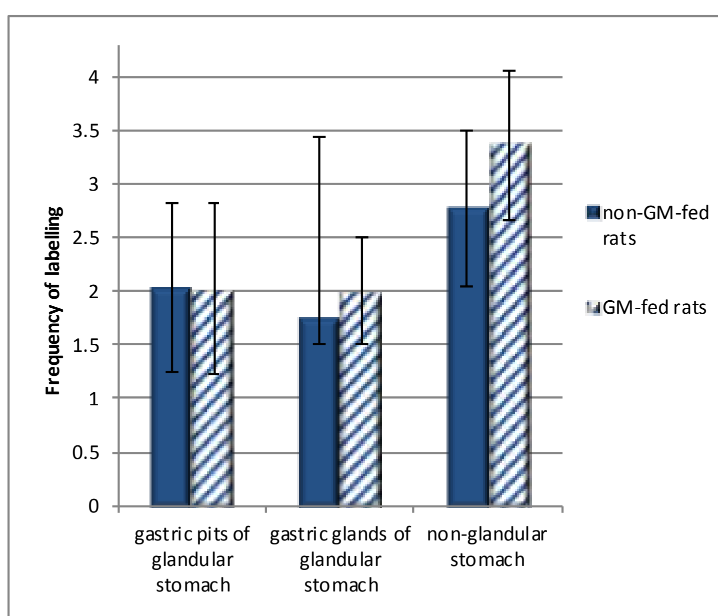



**Figure 60. ZO-1 frequency of labelling in the stomachs of rats fed a 60% GM or non-GM corn diet.** Graph shows mean or median and bars indicate standard deviation or interquartile range depending on whether the data are normally distributed.

### *ZO-1 – gastric pits*

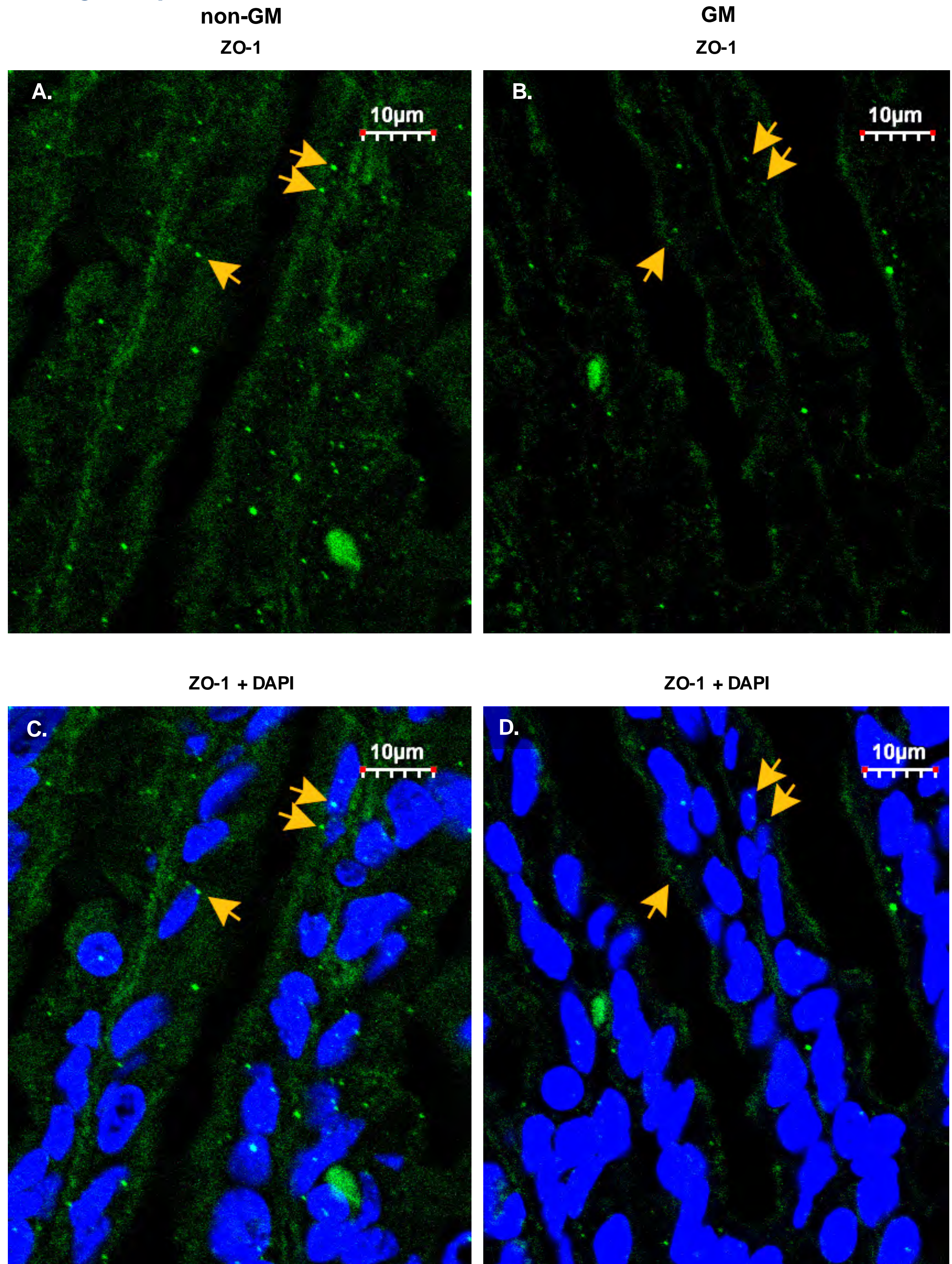



**Figure 61. ZO-1 expression in the stomachs of rats fed a 60% GM or non-GM corn diet.**

A.-D.) *Gastric pits:* Gastric pits labelled with anti-ZO-1, or with additional labelling of cell nuclei with DAPI in non-GM (A. and C.), and GM-fed (B. and D.) rats. ZO-1 labelling seen as specific dots (orange arrows). The intensity of DAPI-labelling of the nuclei is high. The gain could not be sufficiently adjusted to reduce the intensity of the DAPI labelling.

### *ZO-1 – gastric pit controls*

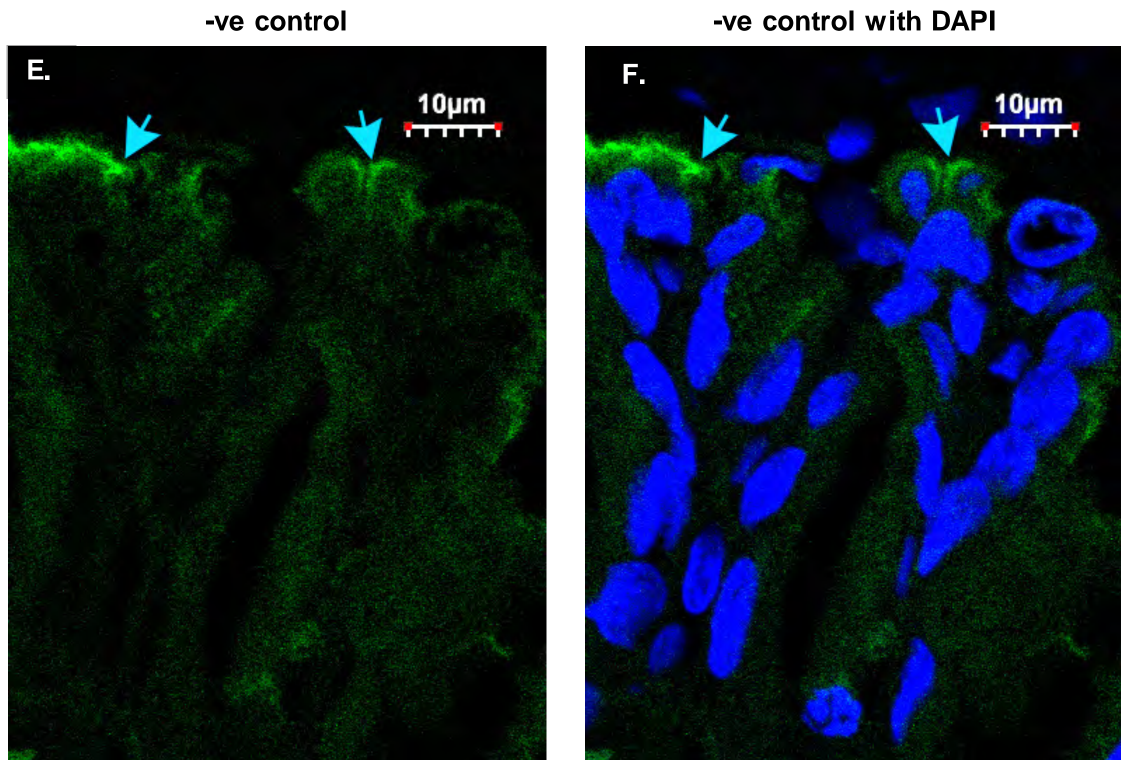



**Figure 61. ZO-1**

E. and F.) *Gastric pit controls:* Gastric pits of negative control (C. and F.) showing no specific labelling. Some autofluorescence is seen at the top edge of the section (blue arrows). The intensity of DAPI-labelling of the nuclei is high. The gain could not be sufficiently adjusted to reduce the intensity of the DAPI labelling.

### *ZO-1 – gastric glands*

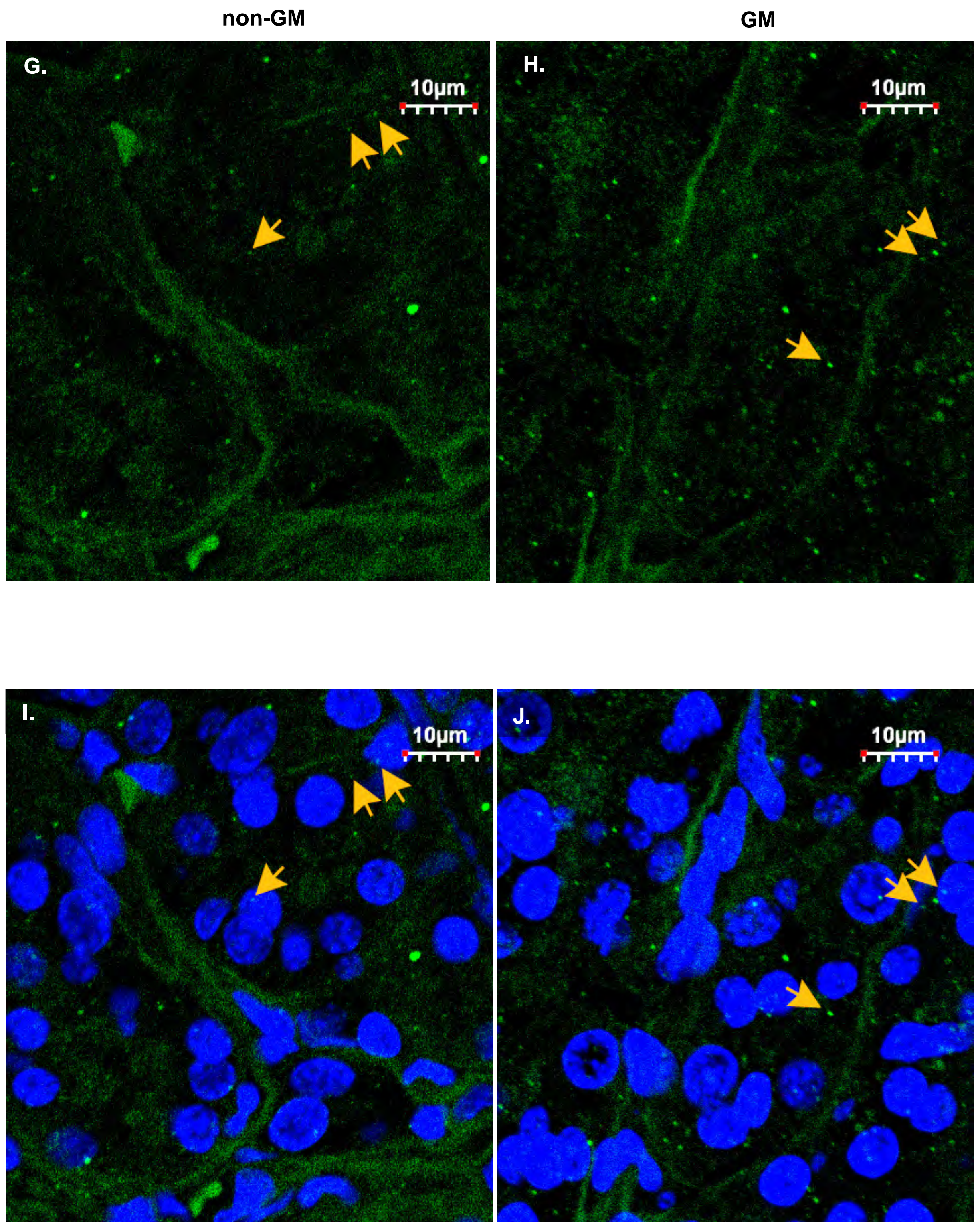



**Figure 61. (cont.) ZO-1**

G.-J.) *Gastric glands*: Gastric glands labelled with anti-ZO-1, or with additional labelling of cell nuclei with DAPI in non-GM (G. and I.), and GM-fed (H. and J.) rats. . ZO-1 labelling seen as specific dots (orange arrows).The intensity of DAPI-labelling of the nuclei is high. The gain could not be sufficiently adjusted to reduce the intensity of the DAPI labelling.

*ZO-1 – gastric gland controls*

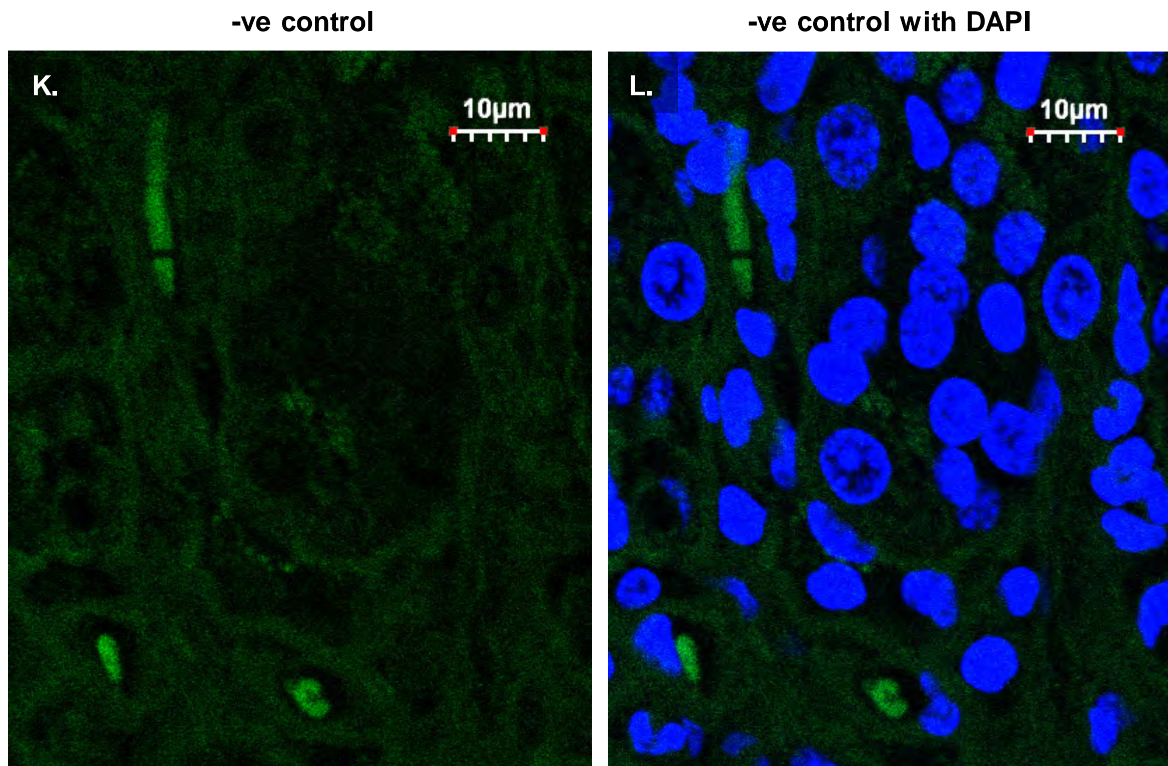



**Figure 61. (cont.) ZO-1**

G.-L.) *Gastric gland controls:* Gastric glands of negative control (K. and L.) showing no specific labelling. The intensity of DAPI-labelling of the nuclei is high. The gain could not be sufficiently adjusted to reduce the intensity of the DAPI labelling.

## *ZO-1 – non-glandular stomach*

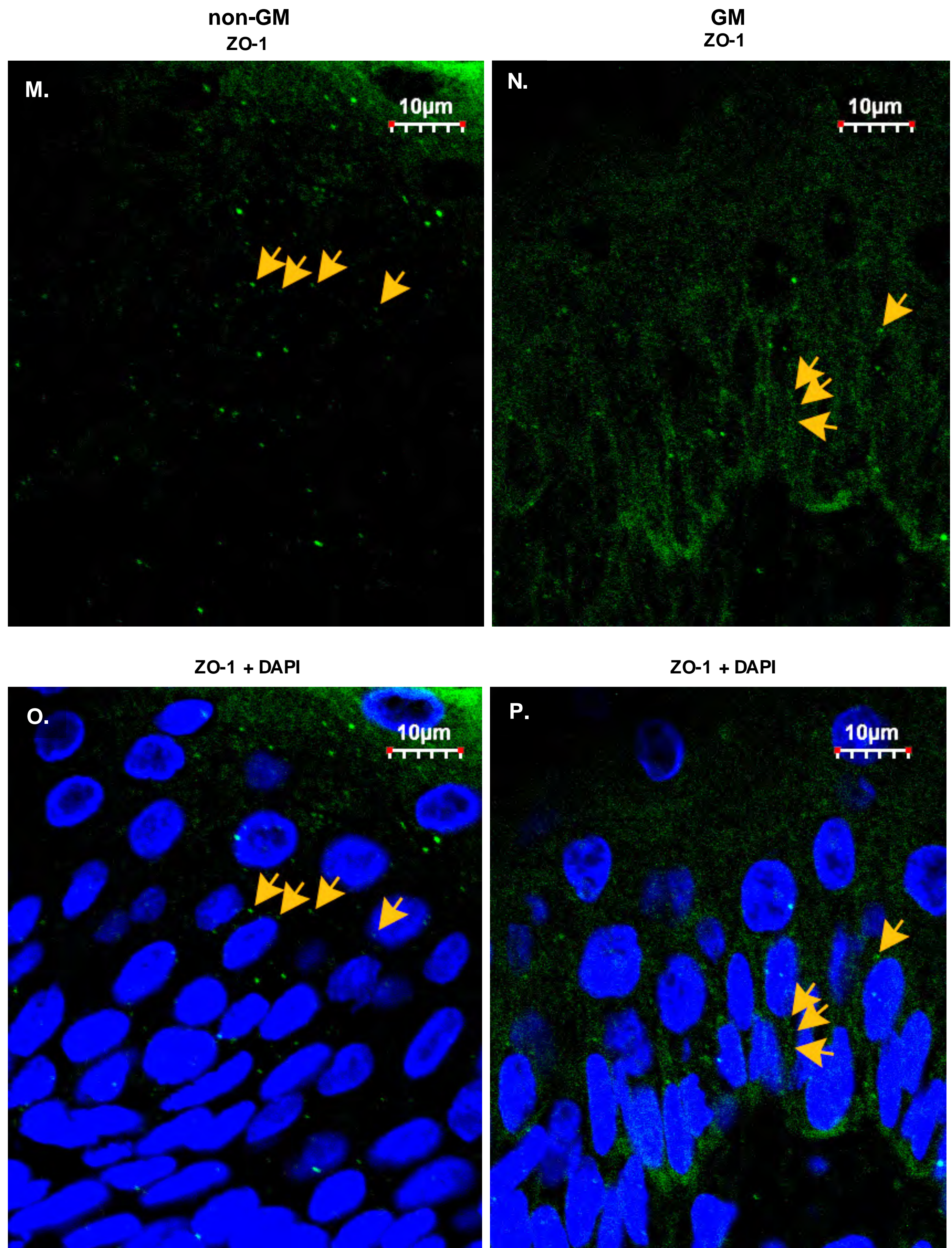



**Figure 61. (cont.) ZO-1**

M.-P.) *Non-glandular stomach*: Non-glandular stomach labelled with anti-ZO-1 (arrows), or with additional labelling of cell nuclei with DAPI, in non-GM (M. and N.) and GM-fed (O. and P.) rats. ZO-1 labelling seen as specific dots (orange arrows).The intensity of DAPI-labelling of the nuclei is high. The gain could not be sufficiently adjusted to reduce the intensity of the DAPI labelling.

### *ZO-1 – non-glandular stomach controls*

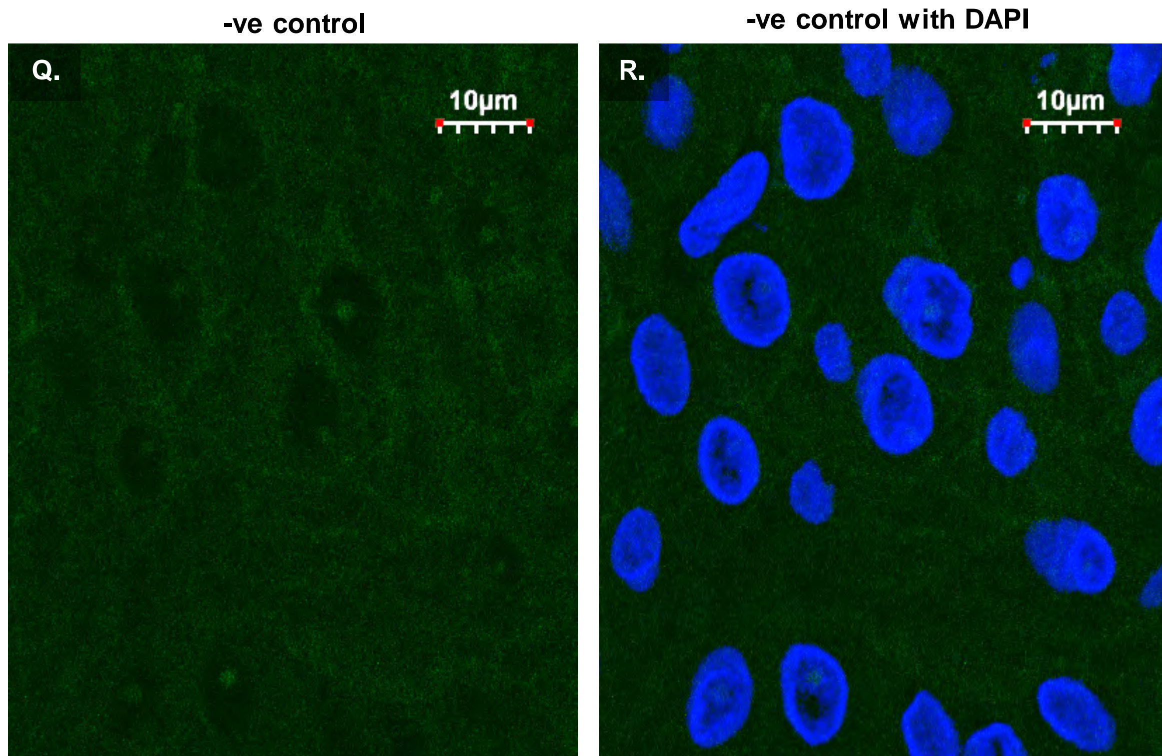



**Figure 61. (cont.) ZO-1**

Q.-R.) *Non-glandular stomach controls: N*on-glandular stomach of negative control (O. and R.) showing no specific labelling. The intensity of DAPI-labelling of the nuclei is high. The gain could not be sufficiently adjusted to reduce the intensity of the DAPI labelling.

### *ZO-1 – colon controls*

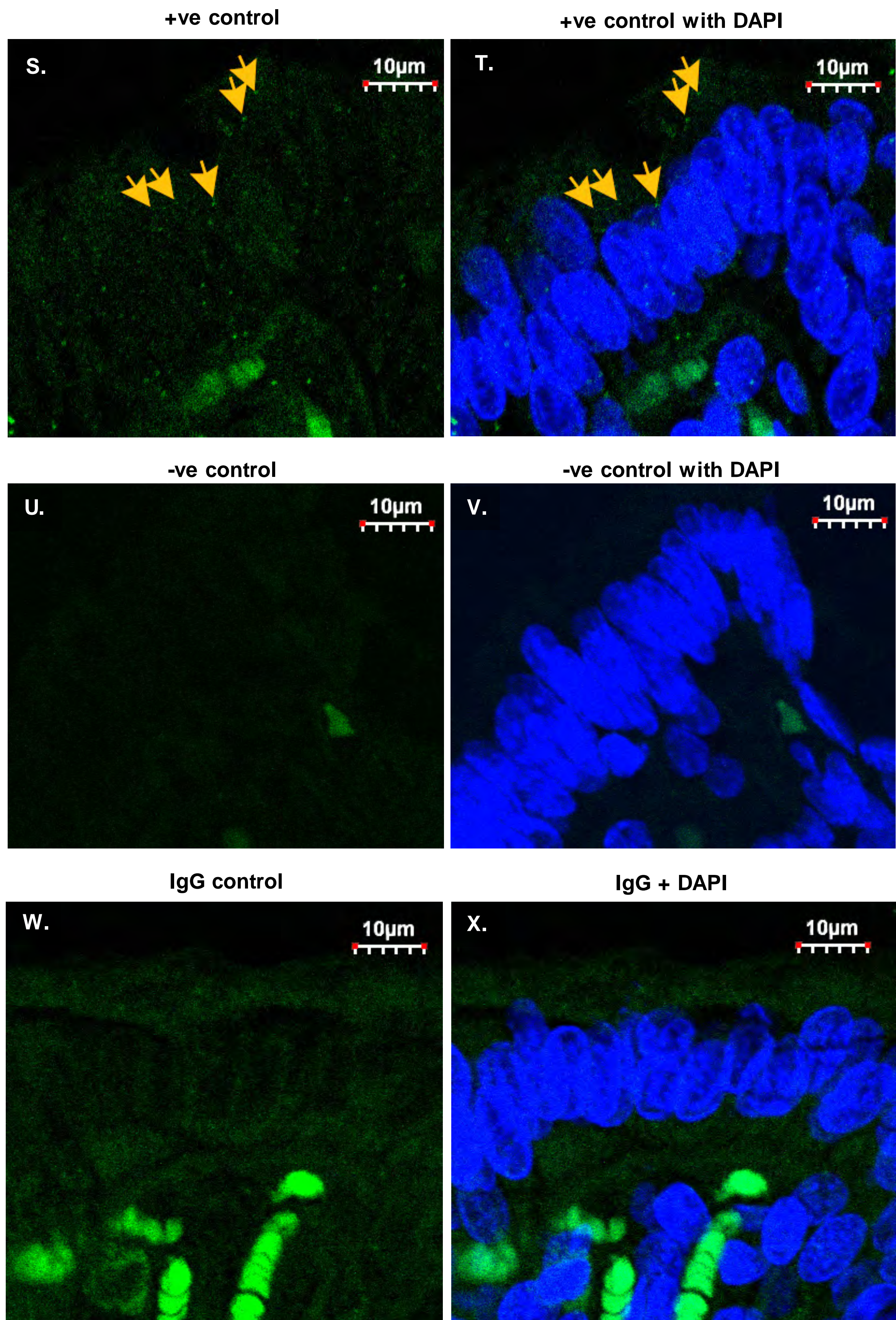



**Figure 61. (cont.) ZO-1**

S. – X.) *Colon controls:* Colon mucosal epithelium labelled with anti-ZO-1 (S.), or with additional labelling of cell nuclei with DAPI (T.).

Colon negative controls (U. and V.) showing no specific labelling of the epithelium.

Colon control treated with non-specific IgG (W. and X.) showing no specific labelling of the epithelium.

## 5.4 Discussion

There were no differences in the frequency or the intensity of expression of occludin, claudin-1 or ZO-1 in any of the regions of the stomach between the GM and non-GM-fed groups. Occludin, claudin-1 and ZO-1 are the most common of the tight junction proteins. There are, however, many other types of proteins, such as the JAMs or other isotypes of the ZO or claudin families (Wardill *et al.*, 2012).

Studies seem to suggest that certain tight junctional proteins (e.g. of the claudin family) are specific to certain areas of the digestive tract and their localisation correlates with the function of that region (Markov *et al.*, 2010). The tight junctions in the stomach have to withstand the acidic pH and at the same time, maintain the mucosal barrier. Therefore, it is more likely that the tight junction proteins expressed in this tissue will be occludin, claudins-1, -3, -4, -5, and -8, as well as ZO-1 and JAM-1, which have all been found to be associated with the "seal" function of the tight junction (Van Itallie and Anderson, 1997; Martìn-Padura *et al.*, 1998; Markov *et al.*, 2010). The present study, found no change in the expression of occludin, claudin-1 or ZO-1 between the treatment and non-treatment groups. Therefore, the appositional change seen in the 60% corn-fed TEM study (Figure 29) may be due to the expression of one or more of the other tight junction proteins. To elucidate whether tight junction apposition loss, was a result of the loss of "tightness"-conferring tight junction proteins, mucosal barrier permeability tests (e.g. resistance test using Ussing chambers) could be performed.

Loss of tight junction apposition may be a consequence of actin remodelling (Capaldo and Nusrat, 2009). This mechanism of change may be relevant to the present study, since *Cry* proteins have been found to bind to actin (Shimada *et al.,* 2006) and to the apical surface (Vazquez-Padron *et al.*, 2000; de Souza Freire *et al.*, 2014) of mice and bovine enterocytes. In addition, the effect of the *Cry* protein binding to the apical surface of mouse enterocytes had an adverse effect on the mucosal polarization (Vazquez-Padron *et al.*, 2000). This suggests that the enterocyte tight junctions may be affected. Therefore, in the present study, the *Cry* proteins, that the investigated GM crop was designed to produce, may be responsible for the tight junction apposition loss through the *Cry* proteins' interaction with the actin.

Actin is bound to tight junctions via ZO-1, -2 and -3 proteins (Fanning *et al.*, 1998; Wittchen *et al.,* 1999; Wittchen *et al.*, 2000; Hartsock and Nelson, 2008). The present study investigated the expression of ZO-1 and found no difference between the GM and

non-GM-fed groups. Therefore, it is possible that the change may be in the expression of ZO-2 and/or -3 proteins.

## 5.5 Conclusion

The tight junction abnormalities observed in the TEM investigation of the 60% corn study were not associated with the tight junction proteins, occludin, claudin-1, and ZO-1. Therefore, it must have resulted from abnormalities of other tight-junction-associated proteins. Literature suggests that the *Bt* toxin (the *Cry* proteins), introduced into the GM corn, may be affecting the actin in the epithelial cells, which in turn may be causing tight junction protein reorganisation.

## Summary table of Study 3 results

| | Intensity[a] (IHC-LM) | | Significant difference | Frequency[b] (IF-C) | | Significant difference |
|---|---|---|---|---|---|---|
| | Non-GM-fed | GM-fed | | Non-GM-fed | GM-fed | |
| ***Gastric pits – glandular stomach*** – mean (SD) or *median (IQR)* | | | | | | |
| Occludin | 2.38 (0.44) | 2.67 (0.78) | NS | *3.75 (3.25-4.00)* | *3.50 (3.06-3.69)* | NS |
| Claudin-1 | 2.40 (0.57) | 2.52 (1.13) | NS | 2.67 (0.84) | 2.32 (0.63) | NS |
| ZO-1 | NA | NA | NS | 2.03 (0.82) | 2.02 (0.81) | NS |
| ***Gastric glands – glandular stomach*** – mean (SD) or *median (IQR)* | | | | | | |
| Occludin | 2.38 (0.44) | 2.67 (0.78) | NS | 2.94 (0.68) | 2.86 (0.99) | NS |
| Claudin-1 | 2.70 (0.54) | 2.58 (0.88) | NS | *4.00 (3.13-4.00)* | *3.50 (3.00-4.00)* | NS |
| ZO-1 | NA | NA | NS | *1.75 (1.50-3.44)* | *2.00 (1.50-2.50)* | NS |
| ***Non-glandular stomach*** – mean (SD) or *median (IQR)* | | | | | | |
| Occludin | *2.25 (1.38-3.13)* | *2.50 (1.50-2.75)* | NS | 2.50 (0.50) | 2.42 (0.78) | NS |
| Claudin-1 | *3.00 (3.00-3.13)* | *3.25 (3.00-3.50)* | NS | *3.33 (2.54-4.00)* | *3.67 (3.50-4.00)* | NS |
| ZO-1 | NA | NA | NS | 2.77 (0.78) | 3.58 (0.75) | NS |

[a] Intensity 0-4 scale: 0 = no labelling; 1 = faint labelling; 2 = faint-medium labelling; 3 = medium-intense labelling; 4 = intense labelling

[b] Frequency 0-4 scale: 0 = zero signals, 1 = a few signals, 2 = few-medium number of signals, 3 = medium-many signals, 4 = many signals

Abbreviations: IHC-LM = Immunohistochemical light microscopy; IF-C = Immunofluorescence confocal microscopy; SD = standard deviation; IQR = interquartile range

# 6. Overall discussion

The observations documented in this thesis suggest that the GM-corn diet may have some effect on the gastrointestinal health of rats. The changes observed appear to be dose-dependent with some resembling chemically-induced damage, and others indicating that the *Cry* proteins may be harmful to the mammalian gut. Several published and unpublished reports and studies support these results, suggesting that a common model of toxicity may be involved (Fares and El-Sayed, 1998; Ewen and Pusztai, 1999; Personal communication, Carman, 2012). However, the number of published studies that perform morphometric analyses, cell counts and use specialised techniques, is limited (Zdziarski *et al.*, 2014) and thus make it difficult to determine whether this relationship is common to all GM crops or to only certain traits. The present study clearly portrays the need for studies to be undertaken with morphometric analyses, cell counts and specialised techniques, such as immunohistochemistry for proliferation and specific lymphocyte populations, as well as, TEM. It is only through these techniques that subtle changes may be detected. The majority of long-term rat-feeding studies performed have a duration of 90 days (Domingo and Bordonaba, 2011; Zdziarski *et al.*, 2014), which may not be long enough for obvious changes to arise. Hence, the need for sensitive morphometric investigations and the use of specialised techniques that will detect subtle and pre-neoplastic changes.

The present feeding studies were of 6 month duration. The changes observed were mild, but significant. A study of even longer duration may depict some of the more severe changes associated with, for example, chemically-induced gastropathy or enterocolitis, such as, loss of normal mucosal architecture with deposition of fibrous tissue and intestine stricture formation. It may also determine whether some of the changes observed were pre-neoplastic.

The present study clearly portrays the limit of the concept of *substantial equivalence*, even if the concept is used as the starting point of the safety evaluations. The concept of substantial equivalence works on the premise that the safety of GM foods can be determined through the assessment of its individual characteristics/components with compounds or organisms of known safety (FSANZ, 2007). The test does not treat the GM crop as a novel entity, the safety of which needs to be evaluated as a whole. The present study investigated the long-term feeding of a triple-stack corn variety deemed safe on

the premise that the single-stacked varieties, from which it was bred, were found to be substantially equivalent to all non-GM corn varieties. This study reported changes that were observed only in the GM-fed group, indicating that some factor in the feed affected the health of the digestive tract of rats. The concept of substantial equivalence appears to have failed in predicting these changes. Therefore, the test for equivalence has limits, for example, it does not take into account the changes that may arise during or after the transformation process. In addition, it cannot test for any synergistic or pleiotropic effects that may arise from the insertion of several different protein-encoding genes into one crop, nor can it predict the effect of consuming several different types of GM crops in one meal. Therefore, long-term feeding trials should be made compulsory and studies investigating single-stacked crops should be followed up with investigations of multiple-stacked crops. In addition, to further evaluate the potential synergistic or pleiotropic effects of GM feed, long-term feeding studies should be performed that investigate a diet containing several GM crop varieties. The latter is of great importance, since animals and humans most probably consume GM material and GM products of various traits in a single meal due to the high prevalence of GM crops on the market (Clive, 2014).

In addition, a GM crop or crops may also have effects on existing conditions or may have an effect on populations of humans and animals that are sensitive to the development of certain diseases or medical conditions, such as diabetes (Dyck *et al.* 2002), or gastritis (Mason *et al.*, 2013). The presence of the GM crop in the diet may exacerbate the problem, and as such, safety studies have to take this into account and report not only the presence of a change, but also the degree. In other words, both treatment and non-treatment groups may observe a change, but in the treatment group the change may be severe, particularly, if another component of the feed already causes damage/change. Such an observation was made in a study investigating the effect of a diet containing GM soy and GM corn on pigs (Carman *et al.*, 2013). The study found that the diet caused inflammation in stomachs, however, only the GM-fed animals showed signs of severe gastritis. Pigs are known to have delicate gut tissue, thus are more prone to inflammation, in particular with the consumption of ground corn (Mason *et al.*, 2013) or soy (Personal communication, Godlewski, 2013). It should be noted, that the Carman *et al.* (2013) study did not contain any histopathological investigations, but based their findings on the degree of redness of the mucosal surface. Although, histopathological investigations are ideal, the degree of redness is an acceptable and established grading

system of gastritis (Esaki *et al.,* 2002; Kubo *et al.*, 2014). It is primarily used in endoscopic examinations, however, it can be used post-mortem, but care must be taken that all stomachs are treated in the same manner and confirmed histopathologically.

The Carman *et al.* (2013) study, along with the other GM-feeding studies discussed in this thesis, portrays the inadequacies in the investigation of the effects of GM-feed on the health of humans and animals, and subsequent approvals for commercialisation. The inadequacies include:

1) no published long-term feeding trials for the majority of approved GM crops, particularly of multi-stacked crops (Section 1.4.3; Zdziarski *et al.*, 2014);

2) the few published studies lack quantitative evaluations of the effects of GM feed on tissue, particularly of GI tract (Zdziarski *et al.*, 2014);

3) the studies that have morphometric analyses, do not have a systematic approach to their investigations, i.e. lack of uniformity in protocols for the conduction of investigations and presentation of the findings (Zdziarski *et al.*, 2014);

4) no clear definition of what constitutes pathological changes or toxic effects (Zdziarski *et al.*, 2014);

5) no evaluation of the combined effect of GM products e.g. of several traits having a potentiating effect;

6) limited data exists for the safety of long-term consumption of each individual trait or component (e.g. truncated or fused *Cry* proteins or CaMV promoter genes)(Ho *et al.*, 1999; Hammond and Koch, 2012);

7) duration of the feeding trials are not long enough to evaluate the carcinogenicity of GM feed (OECD, 2008a);

8) no uniformity in animals or species used to investigate the effects of GM feed consumption (Snell *et al.,* 2012);

9) some of the earlier GM crops were approved for human and animal consumption based on produce quality and not on animal histopathological investigations. To date,

no histopathological data has been submitted to update those approvals (FSANZ, 2015);

In other evaluations of the safety of a novel compound, such as, pharmaceutical drugs, there are many detailed and systematic investigations prior to approval. In particular, the investigations evaluate the safety of each component of the drug and the combined effect of these components (Personal communication, Edwards, 2014). This same approach should be applied to the study and approval of GM crops for human and animal consumption.

## 6.1 The use of a non-isogenic variety of corn

Due to the commercial unavailability of the isogenic or near-isogenic line of corn, a different corn variety had to be used in the control diet. It is possible that the changes observed are associated with that particular variety of corn and not the GM diet. However, corn has been consumed for thousands of years and, as such, is deemed safe. Therefore, any deleterious changes (such as tight junction apposition loss) are unlikely to occur, thus the observed changes are more likely to be the result of the GM corn. Comparison of these findings with a group of rats fed an isogenic line is ideal and these findings could be verified.

# 7. Further research

The present study investigated a diet containing a triple-stacked corn. To further elucidate the source of damage, three separate long-term feeding studies should be performed each investigating one of the single-stacked corn varieties. Also, to further determine whether the results of the present study were due to cross-breeding GM varieties, a long-term feeding study should be performed investigating a diet that contained all three single-stacked GM corn varieties (i.e. consumed in one meal).

The GM crop investigated contains modified genes for the expression of two insecticidal, *Bt* proteins that bind to insect gut epithelium and cause cell death (Galitsky *et al.*, 2001; Bravo *et al.*, 2004). As was discussed in Section 3.4.3, evidence exists that the insecticidal, *Bt* proteins may bind to rodent intestinal cells. In particular the *Bt* toxin, *Cry1Ab* may be binding to actin, subsequently causing tight junction structure disorganisation or remodelling (Shimada *et al.*, 2006; Capaldo and Nusrat, 2009). Further studies into the expression of *Cry1Ab*, *Cry3Bb*, actin, and tight junction proteins in the stomach of rats fed a 60% GM corn diet, may reveal specific targets for the *Bt* toxin or other components of the GM feed.

Loss of tight junction apposition in the stomach may not correspond with an increase in mucosal barrier permeability. Tight junctions are an integral part of this barrier, however, changes to the tight junction protein properties may alter the functions other than permeability (Huber *et al.*, 2000). Different tight junction proteins have different functions. Occludin, and claudins-1, -3, -4, -5 and 8 appear to provide the "seal" function to the tight junction, while claudins-2, -7, and 12 mediate paracellular permeability (Van Itallie and Anderson, 1997; Markov *et al.*, 2010). To further elucidate whether tight junction apposition loss seen in the first study (in rats fed 60% GM corn diet) was a result of the loss of permeability- or "tightness"-conferring tight junction proteins, mucosal barrier permeability tests (e.g. using Ussing chambers) should be performed on freshly-collected stomach tissue from rats fed a 60% GM corn diet. In addition, the loss of tight junction apposition did not induce an inflammatory response. Whether, this was associated with a loss of continuity of the basement membrane warrants further investigation at an ultrastructural level.

Studies seem to suggest that *Bt* toxins may induce changes at the ultrastructural level (Fares and El-Sayed, 1998). Results of the present study appear to support this.

Investigations of the safety of *Bt* toxins are not adequately substantiated using ultrastructural and systematic chemical investigations. Therefore, studies should be performed to investigate the safety of long-term consumption of the *Cry* protein, not only in the form produced by the GM crop (i.e. in GM feed or extracted from the GM crop), but also in its wild-type form and in the form that it appears in the gene cassette (prior to insertion). This will help determine whether *Cry* proteins have an effect on mammalian tissue and whether that effect is confined to only certain forms of the protein, such as, the wild-type or the GM modified form. In addition, the Fares and El-Sayed (1998) study, suggests that *Bt* toxins may have a synergistic or potentiating effect, that is, the presence of more than one *Cry* protein may exacerbate the effect (discussed in Section 3.4.3). As such, these aspects need to be investigated before allowing human or animal consumption. Systematic studies into the effect of *Cry* proteins on mammalian tissue, may also help establish a dose-response relationship as well as provide a no-effect concentration to recommend to industries.

The present study was a general histopathological investigation, primarily at the light microscopy level. Further research could investigate other changes at the ultrustructural level, for example, gastric pit cell and parietal cell population and ultrastructure (Helander *et al.,* 1986; Karam and Leblond, 1993; Naoki *et al,.* 1998; Ogata and Yamasaki, 2000). Such investigations may reveal subtle changes to stomach morphology.

## 8. Final Conclusion

Long-term feeding of a diet that contained a triple-stacked GM corn caused changes that resemble chemically-induced injury. In the stomach, there was an increase in pit and gland depths, and tight junction apposition loss between the mucus-producing cells of the pit and luminal surfaces. In the small intestine, there were differences in villi heights, crypt depth, proliferation of crypt cells and IELs counts. These changes were dose-dependent and may be linked to the presence of the insecticidal, *Cry* proteins.

Findings of these studies, indicate the need for long-term feeding trials that include standardised/reproducible morphometric analyses and specialised microscopic techniques. These in turn will substantially help in determining whether the consumption of GM food is safe for animals and humans.

# Appendix A: Feed specifications

## A1.1 Composition of 60% corn diet

**Specialty Feeds**
www.specialtyfeeds.com

3150 Great Eastern Highway,
GLEN FORREST
Western Australia, 6071
Phone 61 8 9298 8111
Fax 61 8 9298 8700
E-mail info@specialtyfeeds.com

| DIET | |
|---|---|
| **SF07-011** | **Maize Diet** |

A semi-pure diet formulation for laboratory rats and mice based on AIN-93G. This formulation satisfies the nutritional requirements for growth of rats and mice. Some modifications have been made to the original formulation to suit locally available raw materials.

- Feed is manufactured as a 12 mm diameter cube, vacuum packed.

| Calculated Nutritional parameters | |
|---|---|
| Protein | 18 % |
| Total Fat | 6.0 % |
| Crude Fibre | 6.1 % |
| Acid Detergent Fibre | 6.1 % |
| Digestible Energy | 15.0 MJ / Kg |

**DIET FORM**

12 mm diameter pellets. Vacuum packed in 5 Kg oxygen impermeable bags under Nitrogen, bags are then packed in cartons to protect them during transit.

| Ingredients | |
|---|---|
| Casein (Acid) | 132 g/Kg |
| Ground Maize | 600 g/Kg |
| Canola oil | 40 g/Kg |
| DL Methionine | 3.0 g/Kg |
| Wheat Starch | 140 g/Kg |
| Cellulose | 50 g/Kg |
| Calcium Carbonate | 13 g/Kg |
| Sodium Chloride | 2.6 g/Kg |
| Potassium Citrate | 2.5 g/Kg |
| Potassium Dihydrogen Phosphate | 2.8 g/Kg |
| Potassium Sulphate | 0.4 g/Kg |
| AIN93G Trace minerals | 1.4 g/Kg |
| Choline Chloride (65%) | 2.5 g/Kg |
| AIN93G Vitamins | 10 g/Kg |

## Calculated Amino Acids

| Calculated Amino Acids | |
|---|---|
| Valine | 1.1 % |
| Leucine | 1.8 % |
| Isoleucine | 0.7 % |
| Threonine | 0.7 % |
| Methionine | 0.7 % |
| Cystine | 0.2 % |
| Lysine | 1.1 % |
| Phenylalanine | 0.9 % |
| Tyrosine | 0.9 % |
| Tryptophan | 0.2 % |

## Calculated Total Minerals

| Calculated Total Minerals | |
|---|---|
| Calcium | 0.46 % |
| Phosphorous | 0.33 % |
| Magnesium | 0.20 % |
| Sodium | 0.11 % |
| Chloride | 0.17 % |
| Potassium | 0.35 % |
| Sulphur | 0.2 % |
| Iron (note Fe concentration is always higher than calculated figure in pelletised diets) | 100 mg / Kg |
| Copper | 10 mg / Kg |
| Iodine | 0.22 mg / Kg |
| Manganese | 20 mg / Kg |
| Cobalt | no data |
| Zinc | 43 mg / Kg |
| Molybdenum | no data |
| Selenium | 0.25 mg / Kg |
| Cadmium | no data |
| Chromium | 2.0 mg / Kg |
| Lithium | 0.1 mg / Kg |
| Boron | 0.7 mg / Kg |
| Nickel | 0.55 mg / Kg |
| Vanadium | 0.10 mg / Kg |

## Calculated Total Vitamins

| Calculated Total Vitamins | |
|---|---|
| Vitamin A (Retinol) | 8,000 IU / Kg |
| Vitamin D3 (Cholecalciferol) | 1,000 IU / Kg |
| Vitamin E (• Tocopherol acetate) | 90 mg / Kg |
| Vitamin K (Menadione) | 1 mg / Kg |
| Vitamin C (Ascorbic acid) | none added |
| Vitamin B1 (Thiamine) | 8 mg / Kg |
| Vitamin B2 (Riboflavin) | 7 mg / Kg |
| Niacin (Nicotinic acid) | 43 mg / Kg |
| Vitamin B6 (Pyridoxine) | 11 mg / Kg |
| Pantothenic acid | 20 mg / Kg |
| Biotin | 240 ug / Kg |
| Folic acid | 2 mg / Kg |
| Inositol | none added |
| Vitamin B12 (Cyanocobalamin) | 100 mg / Kg |
| Choline | 2000 mg / Kg |

## Calculated Fat Composition

| Calculated Fat Composition | |
|---|---|
| Myristic Acid 14:0 | trace |
| Palmitic Acid 16:0 | 0.4 % |
| Stearic Acid 18:0 | 0.1 % |
| Palmitoleic Acid 16:1 | trace |
| Oleic Acid 18:1 | 2.7 % |
| Gadoleic Acid 20:1 | trace |
| Linoleic Acid 18:2 n6 | 2.0 % |
| a Linolenic Acid 18:3 n3 | 0.6 % |
| Arachadonic Acid 20:4 n6 | no data |
| EPA 20:5 n3 | no data |
| DHA 22:6 n3 | no data |
| Total Carotenoid | no data |
| Total Phospholipid | no data |
| Cholesterol | no data |

Calculated data uses information from typical raw material composition. It could be expected that individual batches of diet will vary from this figure.

We are happy to provide full calculated nutritional information for all of our products, however we would like to emphasize that these diets have been specifically designed for manufacture by Specialty Feeds

## A1.2 Composition of 30% corn diet

| Diet SF12-079 | 30% Maize Rodent Diet |
|---|---|

A 30% Maize rodent diet, based on customer specifications.

| Calculated Nutritional Parameters | |
|---|---|
| Protein | 16.0% |
| Total Fat | 6.0% |
| Total Carbohydrate | 37.0% |
| Crude Fibre | 5.4% |
| AD Fibre | 5.4% |
| Digestible Energy | 15.4 MJ / Kg |
| % Total calculated digestible energy from lipids | 14.2% |
| % Total calculated digestible energy from protein | 17.2% |

**Diet Form and Features**

- Semi pure and maize diet. 12 mm diameter pellets.
- Pack size 5 Kg, vacuum packed in oxygen impermeable plastic bags, under nitrogen. Bags are packed into cardboard cartons to protect them during transit. Smaller pack quantity on request.
- Diet suitable for irradiation but not suitable for autoclave.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.

| Ingredients | |
|---|---|
| Casein (Acid) | 138 g/Kg |
| Maize | 300 g/Kg |
| Canola Oil | 50 g/Kg |
| Cellulose | 50 g/Kg |
| Wheat Starch | 420 g/Kg |
| DL Methionine | 3.0 g/Kg |
| Calcium Carbonate | 14.1 g/Kg |
| Sodium Chloride | 2.6 g/Kg |
| AIN93 Trace Minerals | 1.4 g/Kg |
| Potassium Citrate | 2.5 g/Kg |
| Potassium Dihydrogen Phosphate | 3.5 g/Kg |
| Potassium Sulphate | 1.4 g/Kg |
| Choline Chloride (75%) | 2.5 g/Kg |
| AIN93 Vitamins | 10 g/Kg |

| Calculated Amino Acids | |
|---|---|
| Valine | 0.99% |
| Leucine | 1.56% |
| Isoleucine | 0.69% |
| Threonine | 0.64% |
| Methionine | 0.72% |
| Cystine | 0.10% |
| Lysine | 1.10% |
| Phenylanine | 0.80% |
| Tyrosine | 0.82% |
| Tryptophan | 0.21% |

| Calculated Total Minerals | |
|---|---|
| Calcium | 0.50% |
| Phosphorous | 0.30% |
| Magnesium | 0.14% |
| Sodium | 0.12% |
| Chloride | 0.17% |
| Potassium | 0.35% |
| Sulphur | 0.21% |
| Iron | 78 mg/Kg |
| Copper | 8.9 mg/Kg |
| Iodine | 0.2 mg/Kg |
| Manganese | 21 mg/Kg |
| Cobalt | No data |
| Zinc | 51 mg/Kg |
| Molybdenum | 0.15 mg/Kg |
| Selenium | 0.3 mg/Kg |
| Cadmium | No data |
| Chromium | 1.0 mg/Kg |
| Fluoride | 1.0 mg/Kg |
| Lithium | 0.1 mg/Kg |
| Boron | 1.6 mg/Kg |
| Nickel | 0.5 mg/Kg |
| Vanadium | 0.1 mg/Kg |

| Calculated Total Vitamins | |
|---|---|
| Vitamin A (Retinol) | 6 040 IU/Kg |
| Vitamin D (Cholecalciferol) | 1 000 IU/Kg |
| Vitamin E (a Tocopherol acetate) | 84 mg/Kg |
| Vitamin K (Menadione) | 1 mg/Kg |
| Vitamin C (Ascorbic acid) | None added |
| Vitamin B1 (Thiamine) | 7.3 mg/Kg |
| Vitamin B2 (Riboflavin) | 6.5 mg/Kg |
| Niacin (Nicotinic acid) | 37 mg/Kg |
| Vitamin B6 (Pryridoxine) | 9 mg/Kg |
| Pantothenic Acid | 18 mg/Kg |
| Biotin | 218 ug/Kg |
| Folic Acid | 2 mg/Kg |
| Inositol | None added |
| Vitamin B12 (Cyancobalamin) | 102 ug/Kg |
| Choline | 1 610 mg/Kg |

| Calculated Fatty Acid Composition | |
|---|---|
| Saturated Fats C12:0 and less | 0.01% |
| Myristic Acid 14:0 | Trace |
| Palmitic Acid 16:0 | 0.32% |
| Stearic Acid 18:0 | 0.12% |
| Palmitoleic Acid 16:1 | 0.01% |
| Oleic Acid 18:1 | 3.02% |
| Gadoleic Acid 20:1 | 0.05% |
| Linoleic Acid 18:2 n6 | 1.69% |
| a Linolenic Acid 18:3 n3 | 0.71% |
| Arachadonic Acid 20:4 n6 | No data |
| EPA 20:5 n3 | No data |
| DHA 22:6 n3 | No data |
| Total n3 | 0.71% |
| Total n6 | 1.69% |
| Total Mono Unsaturated Fats | 3.09% |
| Total Polyunsaturated Fats | 2.38% |
| Total Saturated Fats | 0.49% |

Calculated data uses information from typical raw material composition. It could be expected that individual batches of diet will vary from this figure. **Diet post treatment by irradiation or auto clave could change these parameters**. We are happy to provide full calculated nutritional information for all of our products, however we would like to emphasise that these diets have been specifically designed for manufacture by Specialty Feeds.

# Appendix B: Protocols and microscope settings

## B1.1 Haematoxylin and eosin staining procedure

| | |
|---|---|
| Histolene | 2 x 15min (minimum) |
| Rehydration: | |
| 100% ethanol | 2 x 2min |
| 70% ethanol | 2min |
| 50% ethanol | 2min |
| Deionised water | 2min |
| Harris haematoxylin | 2-3min |
| Wash in running tap water | |
| Hydrochloric acid | 1-2 very quick immersions |
| Wash in running tap water | |
| Ammonia solution | 1min |
| Wash in running tap water | |
| Optimal staining: nuclei - dark purple; connective tissue - slightly grey | |
| Eosin | 2-3min |
| Wash in running tap water | |
| Dehydration: | |
| 100% ethanol | a few vigorous immersions |
| 100% ethanol | 2 x 3min |
| Histolene | 2 x 5min (minimum) |
| Coverslip using DPX mounting medium | |

| Harris haematoxylin | |
|---|---|
| Haematoxylin | 4g/L |
| Aluminium ammonium sulphate | 80g/L |
| 100% ethanol | 100mL/L |
| Sodium iodate | 02g/L |
| Distilled water | 800mL/L |

| Eosin | |
|---|---|
| Eosin Y (1% in water) | 100mL/L |
| Phloxine b (1% in water) | 10mL/L |
| 95% ethanol | 750mL/L |
| Acetic acid | 4mL/L |

## B1.2 Combined alcian blue and periodic acid Schiff staining procedure (pH2.5)

| Step | Time |
|---|---|
| Histolene | 2 x 15min (minimum) |
| Rehydration: | |
| 100% ethanol | 2 x 2min |
| 70% ethanol | 2min |
| 50% ethanol | 2min |
| Deionised water | 2min |
| Alcian blue (pH 2.5) | 5min |
| Wash in water | |
| SPIT test for PAS | 30min |
| 3% periodic acid | 5min |
| Schiffs' reagent | |
| Wash in running water | 10-15min |
| Counter stain in Harris haematoxylin | 10sec |
| Wash in running water | |
| Hydrochloric acid | 1-2 very quick immersion |
| Wash in running water | |
| Ammonia solution | 1min |
| Wash in running water | |
| CHECK that nuclei are stained enough | |
| Dehydration: | |
| 100% ethanol | a few vigorous immersion |
| 100% ethanol | 2 x 3min |
| Histolene | 2 x 5min (minimum) |
| Coverslip using DPX mounting medium | |

### Alcian blue (pH 2.5)

| | |
|---|---|
| 3% glacial acetic acid | 100.0 mL |
| Alcian blue | 1.0 gm |

Mix and adjust pH to 2.5, using acetic acid.

### 3% Periodic acid

| | |
|---|---|
| Acetic acid | 3.0mL |
| Distilled water | 100.0mL |

### Schiffs’ reagent

| | |
|---|---|
| Fuchsin (pararosaniline) | 1g |
| Conc. HCl | 2.55mL/200mL |
| Sodium or potassium metabisulfite | 1g |
| Activated charcoal | 2g |

*Preparation*

Bring 200mL of distilled water to the boil. Add fuchsin, slightly stir and filter. Allow solution to cool to 50°C. Add 200mL of 0.15N HCl. Stir and allow solution to cool to 25°C. Add sodium or potassium metabisulfite, stir solution and store in dark place at room temperature for 16-24h. Next day add activated charcoal to the solution, shake and filter into a dark bottle. Store at 4°C in darkness.

## B1.3 Immunohistochemistry – DAKO automated machine procedure

Fixation: 10% buffered formalin (pH 7.4)

Paraffin embedded sections (4µm thick) mounted on DAKO slides

**Staining procedure:**

| | |
|---|---|
| Histolene | 2 x 5min (minimum) |
| Rehydrate: | |
| 100% ethanol | 2 x 2min |
| 70% ethanol | 2min |
| 50% ethanol | 2min |
| Distilled water | 2min |
| Antigen retrieval (DAKO Pt Link) Buffer | 20min at 97$^{o}$C or 100$^{o}$C |

*Automated cycle (DAKO Autostainer Plus):*

| | |
|---|---|
| Buffer wash x2 | |
| 3% hydrogen peroxidase (FLEX Peroxidase blocker, DAKO) | 5min |
| Buffer wash x 2 | |
| Protein block | 30min |
| Buffer wash x2 | |
| 1$^{o}$ antibody | 60min |
| Buffer wash x2 | |
| Polymer (anti-rabbit HRP, DAKO) | 30min |
| Buffer wash x2 | |
| DAB (DAKO) | 10min |
| Buffer wash x2 | |
| Deionised water rinse | |

*Manual steps:*

| | |
|---|---|
| Counter stain in Harris haematoxylin | 10sec |
| Wash in running tap water | |
| Hydrochloric acid | 1-2 very quick immersions |
| Wash in running tap water | |
| Ammonia solution | 1min |

Wash in running tap water

Optimal staining: nuclei slightly blue

Dehydration

| | |
|---|---|
| 100% ethanol | a few vigorous immersions |
| 100% ethanol | 2 x 3min |
| Histolene | 2 x 5min (minimum) |

Coverslip using DPX mounting medium

## Buffer solution

### Stock solution (pH 7.4) 30x concentrate

| | |
|---|---|
| NaCl | 263.0g |
| $NaH_2PO_4$ anahydrous | 36.0g |
| (or $NaH_2PO_4.H_2O$ | 41.4g) |

Dissolve $NaH_2PO_4$ in 250mL of distilled water while heating and stirring. Add 700mL of distilled water and dissolve NaCl into the solution. Cool to room temperature. Adjust pH to 6.23-6.24 using 5N NaOH (approximately 47.5mL).

### Buffer solution

| | |
|---|---|
| Stock solution | 33.32mL |
| Deionised water | 966.68mL |
| Tween 20 | 10.00mL |

## B1.4 Immunofluorescence method for confocal microscopy

Fixation: 10% buffered formalin (pH 7.4)

Paraffin embedded section (4µm thick) mounted on silane coated slides

| Step | Time |
|---|---|
| Slide dryer (temp. 64°C) | 1hr |
| Dewaxing: | |
| Xylene | 10min |
| Xylene | 5-10min |
| 1:1, xylene : ethanol | 5min |
| Rehydration: | |
| 100% ethanol | 2 x 5min |
| 96% ethanol | 5min |
| 70% ethanol | 5min |
| 50% ethanol | 5min |
| Deionised water | 2 x 5min |
| Antigen retrieval | |
| Protease (occludin and ZO-1) | 10min at 37°C |
| EDTA (claudin-1) | 30min at 80-100°C and 15min cooling |
| Wash with PBS | 2 x 5min |
| Blocking solution (5-10%) | 30min |
| 1° antibody diluted in PBS | 24hr at 4°C |
| Wash with PBS | 2 x 5min |

Following procedures performed in darkness or in minimum light:

| Step | Time |
|---|---|
| 2° antibody (with fluorescent tag) diluted in PBS | 1hr at room temp. |
| Wash with PBS | 2 x 5min |
| DAPI | 5-8min |
| Return slides to previous PBS | |
| then wash in fresh PBS | 3 x 5min |
| Cover slip with fluoromont | allow to set 24hrs |
| Store slides in 4°C, covered in alfoil | |

## Antigen retrieval solutions

### EDTA (pH 9) to make 1.5L

| | |
|---|---|
| Tris | 1.815g |
| EDTA | 0.555g |
| Deionised water | 1500mL |
| Tween 20 | 0.5mL |

Adjust pH to 9 using 5N NaOH

### Protease

| | |
|---|---|
| Protease *(Streptomyces grisen)* | 1-2mg |
| Deionised water | 1mL |

## Confocal microscope settings

### Occludin and Claudin-1:

The occludin and claudin-1 part of the confocal microscopic investigation was carried out using the Leica SP5 Spectral Scanning Confocal Microscope at Adelaide Microscopy, University of Adelaide, South Australia. . Microscope was set at the following specification.

| Leica SP5 Spectral Scanning Confocal Microscope settings | |
|---|---|
| **Scanner settings** | |
| ScanMode | xyz |
| Pinhole [m] | 111.5 µm |
| Pinhole [airy] | 1.00 |
| Size-Width | 122.9 µm |
| Size-Height | 122.9 µm |
| Zoom | 4 |
| Resolution | 8 bits |
| Format-Width | 1024 pixels |
| Format-Height | 1024 pixels |
| **Hardware settings** | |
| UV Lens FW | Lens 40x/1.25 Oil |
| Laser (405 Diode, Chaser UV) | 800 nm |
| Laser (Argon, visible) | 458 nm |
| Laser (Argon, visible) (Power) | 30% |
| Laser (DPSS 561, visible) | 561 nm |
| Laser (HeNe 633, visible) | 633 nm |
| Scan Field Rotation | 0 |
| Scan Speed | 400 Hz |
| Objective | HCX PL APO 40.0x1.25 OIL |
| Numerical aperture (Obj.) | 1.2 |
| Refraction index | 1.33 |
| Emission bandwidth PMT1: begin – end | 412.0nm – 480.0nm |
| Emission bandwidth PMT3: begin – end | 496.0nm – 580.0nm |
| Occludin - Alexa Fluor 488 Gain | 681.8 |
| Claudin-1 - Alexa Fluor 488 Gain | 739.0 |

### ZO-1:

The ZO-1 part of the confocal microscopic investigation was carried out using Olympus FV-1000 Confocal Microscope at the Department of Pathology, Children's Memorial Health Institute, Warsaw, Poland, under the direction Dr Joanna Bierła. Microscope was set at the routine settings used in this laboratory.

## 1º Antibody specifications

| Immunohistochemistry light microscopy | | | | |
|---|---|---|---|---|
| Antibody | Catalogue no. | Manufacturers' antibody concentration | Dilution | Actual antibody concentration used |
| Caspase 3 | abcam, ab4051 | 0.3mg/mL | 1:1000 | 0.003mg/mL |
| Ki67 | abcam, ab16667 | Estimated concentration: 10-50µg/ml (Tissue culture supernatant, therefore not purified.) | 1:1000 | Estimated concentration: 0.01-0.05µg/mL |
| Occludin | Invitrogen, 71-1500 | 0.25mg/mL | 1:200 | 0.125mg/mL |
| Claudin-1 | Invitrogen, 51-9000 | 0.25mg/mL | 1:300 | 0.083mg/mL |
| Immunofluorescence – confocal microscopy | | | | |
| Occludin | Invitrogen 71-1500 | 0.25mg/mL | 1:200 | 0.125mg/mL |
| Claudin-1 | Invitrogen 51-9000 | 0.25mg/mL | 1:200 | 0.125mg/mL |
| ZO-1 | Invitrogen 61-7300 | 0.25mg/mL | 1:200 | 0.125mg/mL |
| Normal rabbit IgG | R&D Systems AB-105-C | 0.25mg/mL | 1:800 | 0.125mg/mL |

## 2º Antibody specifications

| Immunofluorescence – confocal microscopy | | | | |
|---|---|---|---|---|
| Antibody | Catalogue no. | Manufacturers' antibody concentration | Dilution | Actual antibody concentration used |
| Alexa Fluor 488 | Life Technologies A-11008 | 2mg/mL | 1:500 | 0.004mg/mL |

## B1.5 Processing samples for TEM

Small pieces of tissue 1x1x2mm

1) Fixation: 2% glutaraldehyde, 3% paraformaldehyde, 0.1M phosphate buffer (pH7.4)

2) Wash in phosphate buffer — 2 x 30min (minimum time)

3) Post-fix in 1% osmium tetroxide in phosphate buffer — 1hr (1.5hr maximum)

4) Wash in phosphate buffer — 1 x 30min

5) Dehydrate in ethanol graded series:

| | |
|---|---|
| 30% | 1 x 30min |
| 50% | 1 x 30min |
| 70% | 1 x 30min -> preferably left overnight |
| 80% | 1 x 30min |
| 90% | 1 x 30min |
| 100% | 1 x 45min |
| 100% with desiccating buds | 3 x 60min |

6) Intermediate: Propylene oxide — 2 x 60min

7) Infiltration:

| | |
|---|---|
| 1:2, epoxy resin : propylene oxide | overnight |
| 2:1, epoxy resin : propylene oxide | 9am – 4pm |
| Pure resin | 4pm – 2pm next day |

8) Embed in pure resin (Epoxy) and polymerised at 60°C for three days

## Toludine Blue stain for TEM thick sections

| | |
|---|---|
| 0.05% toludine blue | 1mL |
| 0.05M sodium borate | 1mL |

## Reference List


Aaziz, R. & Tepfer, M. 1999. Recombination in RNA viruses and in virus-resistant transgenic plants. *The Journal of General Virology,* 80 (Pt 6)**,** 1339-1346.

Abdo, E. M., Barbary, O. M. & Shaltout, O. E.-S. 2014. Feeding study with Bt corn (MON810: Ajeeb YG) on Rats: Biochemical analysis and liver histopathology. *Food and Nutrition Sciences,* 5(No. 2)**,** 11.

Aktories, K. & Barbieri, J. T. 2005. Bacterial cytotoxins: Targeting eukaryotic switches. *Nature Reviews Microbiology,* 3**,** 397-410.

Allen, A. & Flemstrom, G. 2005. Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin. *American Journal of Physiology. Cell Physiology,* 288**,** C1-19.

Altpeter, F., Baisakh, N., Beachy, R., Bock, R., Capell, T., Christou, P., Daniell, H., Datta, K., Datta, S., Dix, P. J., Fauquet, C., Huang, N., Kohli, A., Mooibroek, H., Nicholson, L., Nguyen, T. T., Nuget, G., Raemakers, K., Romano, A., Somers, D. A., Stoger, E., Taylor, N. & Visser, R. 2005. Particle bombardment and the genetic enhancement of crops: Myths and realities. *Molecular Breeding,* 15**,** 305-327.

Alverdy, J., Chi, H.S., & Sheldon, G.F., 1985. The effect of parenteral nutrition on gastrointestinal immunity: The importance of enteral stimulation. *Annals of Surgery*, 202, 681-684.

Amieva, M. R., Vogelmann, R., Covacci, A., Tompkins, L. S., Nelson, W. J. & Falkow, S. 2003. Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. *Science,* 300**,** 1430-1434.

Anderson, J. M. & Van Itallie, C. M. 2009. Physiology and function of the tight junction. *Cold Spring Harbor Perspectives in Biology,* 1(2)**,** a002584.

Antushevich, H., Pawlina, B., Kapica, M., Krawczynska, A., Herman, A. P., Kuwahara, A., Kato, I. & Zabielski, R. 2013. Influence of fundectomy and intraperitoneal or intragastric administration of apelin on apoptosis, mitosis, and DNA repair enzyme OGG1,2 expression in adult rats gastrointestinal tract and pancreas. *Journal of Physiology and Pharmacology: An Official Journal of the Polish Physiological Society,* 64**,** 423-428.

Armstrong, R. J. E., Harrower, T. P., Hurelbrink, C. B., McLaughin, M., Ratcliffe, E. L., Tyers, P., Richards, A., Dunnett, S. B., Rosser, A. E. & Barker, R. A. 2001. Porcine neural xenografts in the immunocompetent rat: Immune response following grafting of expanded neural precursor cells. *Neuroscience,* 106**,** 201-216.

Atisook, K. & Madara, J. L. 1991. An oligopeptide permeates intestinal tight junctions at glucose-elicited dilatations. Implications for oligopeptide absorption. *Gastroenterology,* 100**,** 719-724.

*Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7th edition* 2004. Australian Government, National Health and Medical Research Council, Canberra. Available: http://www.nhmrc.gov.au/guidelines-publications/ea16

Avtandilov, G. G. 1982. *[Introduction to Quantifying Pathological Morphology]* Moscow, Medicine.

Avtandilov, G. G. 1990. *[Medical morphometry. Manual]*, Moscow: Medicine.

Azadi, H., Ghanian, M., Ghoochani, O. M., Rafiaani, P., Taning, C. N., Hajivand, R. Y. & Dogot, T. 2015. Genetically modified crops: Towards agricultural growth, agricultural development, or agricultural sustainability? *Food Reviews International*, 31, 195-221.

Barker, N., Ridgway, R. A., van Es, J. H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A. R., Sansom, O. J. & Clevers, H. 2009. Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature,* 457**,** 608-611.

Bazzoni, G., Martinez-Estrada, O. M., Orsenigo, F., Cordenonsi, M., Citi, S. & Dejana, E. 2000. Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. *The Journal of Biological Chemistry,* 275**,** 20520-20526.

BCH 2015. *Gene and DNA Sequence Registry* [Online]. Biosafety Clearing-House. Available: https://bch.cbd.int/database/gene-registry/ [Accessed 30 November 2015].

Benbrook, C. M. 2016. Trends in glyphosate herbicide use in the United States and globally. *Environmental Sciences Europe*, 28(3) 1-15.

Bertram, T. A., Markovits, J. E. & Juliana, M. M. 1996. Non-proliferative Lesions of the Alimentary Canal in Rats. *Guides for Toxicologic Pathology.* Washington: STP/ARP/AFIP.

Betton, G.R. 2013. A review of the toxicology and pathology of the gastrointestinal tract. *Cell Biology and Toxicology*, 29 (5), 321-338.

Bravo, A., Gomez, I., Conde, J., Munoz-Garay, C., Sanchez, J., Miranda, R., Zhuang, M., Gill, S. S. & Soberon, M. 2004. Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. *Biochimica et Biophysica Acta,* 1667**,** 38-46.

Bruewer, M., Utech, M., Ivanov, A. I., Hopkins, A. M., Parkos, C. A. & Nusrat, A. 2005. Interferon-gamma induces internalization of epithelial tight junction proteins via a macropinocytosis-like process. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology,* 19**,** 923-933.

Burnett, P. 2014. *Monsanto GM crops, Egypt*. [Online]. Available: https://ejatlas.org/conflict/monsanto-gm-crops-egypt [Accessed 30 November 2015].

Buzoianu, S. G., Walsh, M. C., Rea, M. C., Quigley, L., O'Sullivan, O., Cotter, P. D., Ross, R. P., Gardiner, G. E. & Lawlor, P. G. 2013. Sequence-based analysis of the intestinal microbiota of sows and their offspring fed genetically modified maize expressing a truncated form of *Bacillus thuringiensis* Cry1Ab protein (Bt Maize). *Applied and Environmental Microbiology,* 79**,** 7735-7744.

Campbell, I. 2012a. Digestion and absorption. *Anaesthesia and Intensive Care Medicine,* 13**,** 62-63.

Campbell, I. 2012b. The mouth, stomach and intestines. *Anaesthesia and Intensive Care Medicine,* 13**,** 56-58.

Capaldo, C. T. & Nusrat, A. 2009. Cytokine regulation of tight junctions. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* 1788**,** 864-871.

Carman, J. A. 2012. *Deaths of pigs in USA abatoirs.* Personal communication.

Carman, J. A. 2004. Is GM Food Safe to Eat? *In:* Hindmarsh, R. & Lawrence, G. (eds.) *Recoding Nature Critical Perspectives on Genetic Engineering.* Sydney: UNSW Press.

Carman, J. A., Vlieger, H. R., Ver Steeg, L. J., Sneller, V. E., Robinson, G. W., Clinch-Jones, C. A., Haynes, J. I. & Edwards, J. W. 2013. A long-term toxicology study on pigs fed a combined genetically modified (GM) soy and GM maize diet. *Journal of Organic Systems,* 8**,** 38-54.

Casella, G., Villanacci, V., Fisogni, S., Cambareri, A. R., Di Bella, C., Corazzi, N., Gorla, S., Baldini, V. & Bassotti, G. 2009. Colonic left-side increase of eosinophils: A clue to drug-related colitis in adults. *Alimentary Pharmacology and Therapeutics* 29**,** 535-541.

CERA 2012. *GM Crop Database.* [Online]. Washington D.C.: Center for Environmental Risk Assessment (CERA). ILSI Research Foundation. Available: http://cera-gmc.org/index.php?action=gm_crop_database [Accessed 26 June 2012].

Chandra, M., Riley, M. I. & Johnson, D. 1992. Spontaneous neoplasms in aged Sprague-Dawley rats. *Archives of Toxicology,* 66**,** 496-502.

Chen, M., Shelton, A. & Ye, G. Y. 2011. Insect-resistant genetically modified rice in China: From research to commercialization. *Annual Review of Entomology,* 56**,** 81-101.

Chowdhury, E. H., Kuribara, H., Hino, A., Sultana, P., Mikami, O., Shimada, N., Guruge, K. S., Saito, M. & Nakajima, Y. 2003a. Detection of corn intrinsic and recombinant DNA fragments and Cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11. *Journal of Animal Science*, 81, 2546-2551.

Chowdhury, E. H., Shimada, N., Murata, H., Mikami, O., Sultana, P., Miyazaki, S., Yoshioka, M., Yamanaka, N., Hirai, N. & Nakajima, Y. 2003b. Detection of Cry1Ab protein in gastrointestinal contents but not visceral organs of genetically modified Bt11-fed calves. *Veterinary and Human Toxicology*, 45, 72-75.

Christou, P. 1992. Genetic transformation of crop plants using microprojectile bombardment. *The Plant Journal,* 2**,** 275-281.

Christou, P., McCabe, D. E. & Swain, W. F. 1988. Stable transformation of soybean callus by DNA-coated gold particles. *Plant Physiology,* 87**,** 671-674.

Cleary, P., Sinnott, M., Hari, B., Bakalis, S. & Harrison, S. 2015. Modelling Food Digestion. *In:* S. Bakalis, Knoerzer, K. & Fryer, P. (eds.) *Modeling Food Processing Operations.* Woodhead Publishing.

Clive, J. 2014. *Global Status of Commercialized Biotech/GM Crops: 2014,* Ithaca, NY., ISAAA.

Clive, J. & Krattiger, A. F. 1996. Global Review of the Field Testing and Commercialization of Transgenic Plants, 1986 to 1995: The First Decade of Crop Biotechnology. *ISAAA Briefs No. 1.* Ithaca, NY ISAAA.

Corazza, G. R., Frazzoni, M., Dixon, M. F. & Gasbarrini, G. 1985. Quantitative assessment of the mucosal architecture of jejunal biopsy specimens: A comparison between linear measurement, stereology, and computer aided microscopy. *Journal of Clinical Pathology,* 38**,** 765-770.

Coruzzi, G. 2010. Overview of gastrointestinal toxicology. *Current Protocols in Toxicology,* Chapter 21**,** Unit 21.1.

Crickmore, N., Zeigler, D. R., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Bravo, A. & Dean, D. H. 2012. *Bacillus thuringiensis Toxin Nomenclature* [Online]. Available: http:// www.lifesci.sussex.ac.uk/ Home/Neil_Crickmore/Bt/ [Accessed 16 April 2012].

Cummins, J., Ho, M. W. & Ryan, A. 2000. Hazardous CaMV promoter? *Nature Biotechnology,* 18**,** 363.

Cui, R. T., Cai, G., Yin, Z. B., Cheng, Y., Yang, Q. H. & Tian, T. 2001. Transretinoic acid inhibits rats gastric epithelial dysplasia induced by N-methyl-N-nitro-N-nitrosoguanidine: Influences on cell apoptosis and expression of its regulatory genes. *World Journal of Gastroenterology,* 7(3), 394-398.

Cuvelier, C., Demetter, P., Mielants, H., Veys, E. M. & De Vos, M. 2001. Interpretation of ileal biopsies: Morphological features in normal and diseased mucosa. *Histopathology,* 38**,** 1-12.

Dai, S., Zheng, P., Marmey, P., Zhang, S., Tian, W., Chen, S., Beachy, R. N. & Fauquet, C. 2001. Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment. *Molecular Breeding,* 7**,** 25-33.

Daleprane, J. B., Chagas, M. A., Vellarde, G. C., Ramos, C. F. & Boaventura, G. T. 2010. The impact of non- and genetically modified soybean diets in aorta wall remodeling. *Journal of Food Science,* 75**,** T126-T131.

Daleprane, J. B., Feijó, T. S. & Boaventura, G. T. 2009. Organic and genetically modified soybean diets: Consequences in growth and in hematological indicators of aged rats. *Plant Foods for Human Nutrition,* 64**,** 1-5.

Davies, P. 2015. *Crop breeding and development of GM crops.* Personal communication.

De Souza Freire, I., Miranda-Vilela, A. L., Barbosa, L. C., Martins, E. S., Monnerat, R. G. & Grisolia, C. K. 2014. Evaluation of cytotoxicity, genotoxicity and hematotoxicity of the recombinant spore-crystal complexes Cry1Ia, Cry10Aa and Cry1Ba6 from *Bacillus thuringiensis* in Swiss mice. *Toxins,* 6**,** 2872-2885.

De Vendomois, J. S., Roullier, F., Cellier, D. & Seralini, G. E. 2009. A comparison of the effects of three GM corn varieties on mammalian health. *International Journal of Biological Sciences,* 5(7)**,** 706-726.

Delannay, X., Bauman, T. T., Beighley, D. H., Buettner, M. J., Coble, H. D., Defelice, M. S., Derting, C. W., Diedrick, T. J., Griffin, J. L., Hagood, E. S., Hancock, F. G., Hart, S. E., Lavallee, B. J., Loux, M. M., Lueschen, W. E., Matson, K. W., Moots, C. K., Murdock, E., Nickell, A. D., Owen, M. D. K., Paschal Ii, E. H., Prochaska, L. M., Raymond, P. J., Reynolds, D. B., Rhodes, W. K., Roeth, F. W., Sprankle, P. L., Tarochione, L. J., Tinius, C. N., Walker, R. H., Wax, L. M., Weigelt, H. D. & Padgette, S. R. 1995. Yield evaluation of a glyphosate-tolerant soybean line after treatment with glyphosate. *Crop Science,* 35**,** 1461-1467.

Denning, T. L., Wang, Y.-C., Patel, S. R., Williams, I. R. & Pulendran, B. 2007. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nature Immunology,* 8(10)**,** 1086-1094.

Desai, T. R., Leeper, N. J., Hynes, K. L. & Gewertz, B. L. 2002. Interleukin-6 causes endothelial barrier dysfunction via the protein kinase C pathway. *The Journal of Surgical Research,* 104(2)**,** 118-123.

DeSesso, J. M. & Jacobson, C. F. 2001. Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. *Food and Chemical Toxicology,* 39(3)**,** 209-228.

Dixon, M. F., Genta, R. M., Yardley, J. H. & Correa, P. 1996. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *The American Journal of Surgical Pathology,* 20(10)**,** 1161-1181.

Domingo, J. L. 2007. Toxicity studies of genetically modified plants: A review of the published literature. *Critical Reviews in Food Science and Nutrition,* 47(8)**,** 721-733.

Domingo, J. L. & Bordonaba, J. G. 2011. A literature review on the safety assessment of genetically modified plants. *Environment International,* 37(3)**,** 734-742.

Domingo, J. L. 2016. Safety assessment of GM plants: An updated review of the scientific literature. *Food and Chemical Toxicology*, 95, 12-18.

Dong, B., Xie, Y.-Q., Chen, K., Wang, T., Tang, W., You, W.-C. & Li, J.-Y. 2005. Differences in biological features of gastric dysplasia, indefinite dysplasia, reactive hyperplasia and discriminant analysis of these lesions. *World Journal of Gastroenterology: WJG,* 11(23)**,** 3595-3600.

Dong, W., Yang, L., Shen, K., Kim, B., Kleter, G. A., Marvin, H. J., Guo, R., Liang, W. & Zhang, D. 2008. GMDD: A database of GMO detection methods. *BMC Bioinformatics,* 9(1)**,** 260.

Dyck, R., Klomp, H., Tan, L. K., Turnell, R. W., & Boctor, M. A. 2002. A comparison of rates, risk factors, and outcomes of gestational diabetes between aboriginal and non-aboriginal women in the Saskatoon health district. *Diabetes Care*, 25, 487-493.

Eastwood, G. L. & Erdmann, K. R. 1978. Effect of ethanol on canine gastric epithelial ultrastructure and transmucosal potential difference. *The American Journal of Digestive Diseases,* 23**,** 429-435.

Eastwood, G. L. & Kirchner, J. P. 1974. Changes in the fine structure of mouse gastric epithelium produced by ethanol and urea. *Gastroenterology,* 67**,** 71-84.

Edelblum, K. L. & Turner, J. R. 2009. The tight junction in inflammatory disease: Communication breakdown. *Current Opinion in Pharmacology,* 9(6)**,** 715-720.

Edwards, J. W. 2014 *Safety evaluation of drugs and environmental toxins.* Personal communication.

EFSA 2008. *Application for authorization of MON 863 × MON 810 ×NK603 maize in the European Union, according to Regulation (EC) No 1829/2003 on genetically modified food and feed. Part II Summary*. [Online]. Available: http://www.gmo-compass.org/pdf/regulation/maize/MON863xMON810xNK603_maize_apllication_food_feed.pdf [Accessed 16 April 2012].

EFSA 2010. Scientific Opinion on application (EFSA-GMO-CZ-2008-62) for the placing on the market of insect resistant and herbicide tolerant genetically modified maize MON 89034 x 1507 x MON 88017 x 59122 and all sub-combinations of the individual events as present in its segregating progeny, for food and feed uses, import and processing under Regulation (EC) No 1829/2003 from Dow AgroSciences and Monsanto *EFSA Journal,* 8(9)**,** 1781 [37 pp.].

EFSA 2013. EFSA guidance on the submission of applications for authorisation of genetically modified plants under Regulation (EC) No 1829/2003. *EFSA Journal* 11(12), 3491 [21 pp.].

El-Shamei, Z., Gab-Alla, A., Shatta, A., Moussa, E. & Rayan, A. 2012. Histopathological changes in some organs of male rats fed on genetically modified corn (Ajeeb YG). *Journal of American Science,* 8(10)**,** 684-696.

Esaki, M., Matsumoto, T., Nakamura, S., Kawasaki, M., Iwai, K., Hirakawa, K., Tarumi, K.-I., Yao, T. & Iida, M. 2002. GI involvement in Henoch-Schönlein purpura. *Gastrointestinal Endoscopy*, 56, 920-923.

Ewen, S. W. B. & Pusztai, A. 1999. Effect of diets containing genetically modified potatoes expressing Galanthus nivalis lectin on rat small intestine. *The Lancet,* 354(9187), 1353-1354.

Fanning, A. S. & Anderson, J. M. 2009. Zonula occludens-1 and -2 are cytosolic scaffolds that regulate the assembly of cellular junctions. *Annals of the New York Academy of Sciences*, 1165, 113-120.

Fanning, A. S., Jameson, B. J., Jesaitis, L. A. & Anderson, J. M. 1998. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *The Journal of Biological Chemistry,* 273(45), 29745-29753.

FAO/WHO 2000. Safety Aspects of Genetically Modified Foods of Plant Origin. Report of a Joint FAO/WHO Expert Consultation on Foods derived from Biotechnology (29 May–2 June, 2000). Geneva.

Fares, N. H. & El-Sayed, A. K. 1998. Fine structural changes in the ileum of mice fed on delta-endotoxin-treated potatoes and transgenic potatoes. *Natural Toxins,* 6, 219-233.

Fasano, A. & Nataro, J. P. 2004. Intestinal epithelial tight junctions as targets for enteric bacteria-derived toxins. *Advanced Drug Delivery Reviews,* 56, 795-807.

Faure, M., Moënnoz, D., Montigon, F., Mettraux, C., Mercier, S., Schiffrin, E. J., Obled, C., Breuillé, D. & Boza, J. 2003. Mucin production and composition is altered in dextran sulfate sodium-induced colitis in rats. *Digestive Diseases and Sciences,* 48, 1366-1373.

Fenoglio-Preiser, C. 1998. Creating a framework for diagnosing the benign gastric biopsy. *Current Diagnostic Pathology,* 5, 2-6.

Finamore, A., Roselli, M., Britti, S., Monastra, G., Ambra, R., Turrini, A. & Mengheri, E. 2008. Intestinal and peripheral immune response to MON810 maize ingestion in weaning and old mice. *Journal of Agricultural and Food Chemistry*, 56(23), 11533-11539.

Fink, M. P. 2003. Intestinal epithelial hyperpermeability: Update on the pathogenesis of gut mucosal barrier dysfunction in critical illness. *Current Opinion in Critical Care,* 9, 143-151.

Fox, J. G. & Wang, T. C. 2007. Inflammation, atrophy, and gastric cancer. *The Journal of Clinical Investigation,* 117(1), 60-69.

Francescon, S. 2001. The new directive 2001/18/EC on the deliberate release of genetically modified organisms into the environment: Changes and perspectives. *Review of European Community and International Environmental Law,* 10, 309-320.

Frantz, J., Betton, G., Cartwright, M., Crissman, J., Macklin, A. & Maronpot, R. 1991. Proliferative Lesions of the Non-glandular and Glandular Stomach in Rats. *Guides for Toxicologic Pathology.* Washington: STP/ARP/AFIP.

FSANZ 2007. The role of animal feeding studies in the safety assessment of genetically modified foods. Report of a workshop hosted by Food Standards Australia New Zealand (Friday 15th June 2007). Canberra: FSANZ.

FSANZ. 2010. Food derived from GM plants containing stacked genes. [Online]. Available: http://www.foodstandards.gov.au/consumer/gmfood/stackedgene/Pages/default.aspx [Accessed 24/06/2013].

FSANZ. 2011. *GM Current Applications and Approvals* [Online]. Available: http://www.foodstandards.gov.au/consumerinformation/gmfoods/gmcurrentapplication1030.cfm [Accessed 1 November 2011].

FSANZ. 2015. *Current GM applications and approvals* [Online]. Available: http://www.foodstandards.gov.au/consumer/gmfood/applications/Pages/default.aspx [Accessed 24 December 2015].

Fujita, H., Sugimoto, K., Inatomi, S., Maeda, T., Osanai, M., Uchiyama, Y., Yamamoto, Y., Wada, T., Kojima, T., Yokozaki, H., Yamashita, T., Kato, S., Sawada, N. & Chiba, H. 2008. Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca2+ absorption between enterocytes. *Molecular Biology of the Cell,* 19, 1912-1921.

Furuse, M., Sasaki, H., Fujimoto, K. & Tsukita, S. 1998. A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *The Journal of Cell Biology,* 143, 391-401.

Gab-Alla, A. A., El-Shamei, Z., Shatta, A. A., Moussa, E. A. & Rayan, A. M. 2012. Morphological and biochemical changes in male rats fed on genetically modified corn (Ajeeb YG). *Journal of American Science,* 8(9), 1117-1123.

Galitsky, N., Cody, V., Wojtczak, A., Ghosh, D., Luft, J. R., Pangborn, W. & English, L. 2001. Structure of the insecticidal bacterial delta-endotoxin Cry3Bb1 of *Bacillus thuringiensis*. *Acta crystallographica. Section D, Biological crystallography,* 57(Pt 8), 1101-1109.

Gärtner, K. 2002. The forestomach of rats and mice, an effective device supporting digestive metabolism in muridae (review). *Journal of Experimental Animal Science,* 42, 1-20.

Gasson, M. 2003. The Safety Evaluation of Genetically Modified Foods. *In:* D'Mello, J. P. F. (ed.) *Food Safety: Contaminants and Toxins.* Wallingford: CABI Publishing.

Geboes, K., Hertogh, G. D. & Ectors, N. 2006. Drug-induced pathology in the large intestine. *Current Diagnostic Pathology,* 12(4), 239-247.

Gelvin, S. B. 2003. *Agrobacterium*-mediated plant transformation: The biology behind the "gene-jockeying" tool. *Microbiology and Molecular Biology Reviews,* 67, 16-37.

Gill, S. S., Cowles, E. A. & Pietrantonio, P. V. 1992. The mode of action of *Bacillus thuringiensis* endotoxins. *Annual Review of Entomology* 37, 615-636.

Glare, T. R. & O'Callaghan, M. 2000. *Bacillus thuringiensis: Biology, Ecology and Safety,* Chichester, John Wiley & Sons.

Godlewski, M. M. 2013 *Histopathoogy of the intestines and the impact of various diets on GIT of rats and pigs.* Personal communication.

Godlewski, M. M., Bierla, J. B., Strzalkowski, A., Martinez-Puig, D., Pajak, B., Kotunia, A., Chetrit, C. & Zabielski, R. 2009. A novel cytometric approach to study intestinal mucosa rebuilding in weaned pigs fed with dietary nucleotides. *Livestock Science,* 123, 215-220.

Godlewski, M. M., Slazak, P., Zabielski, R., Piastowska, A. & Garlak, M. A. 2006. Quantitative study of soybean-induced changes in proliferation and programmed cell death in the intersinal mucosa of young rats. *Journal of Physiology and Pharmacology,* 57, Supp 7, 125-133.

Groschwitz, K. R. & Hogan, S. P. 2009. Intestinal barrier function: Molecular regulation and disease pathogenesis. *The Journal of Allergy and Clinical Immunology,* 124(1), 3-20; quiz 21-22.

Gross, M. L., El-Shakmak, A., Szabo, A., Koch, A., Kuhlmann, A., Munter, K., Ritz, E. & Amann, K. 2003. ACE-inhibitors but not endothelin receptor blockers prevent podocyte loss in early diabetic nephropathy. *Diabetologia,* 46, 856-868.

Guarner, F. & Malagelada, J. R. 2003. Gut flora in health and disease. *Lancet,* 361, 512-519.

Gutiérrez-González, L. & Wright, N. A. 2008. Biology of intestinal metaplasia in 2008: More than a simple phenotypic alteration. *Digestive and Liver Disease,* 40, 510-522.

Halldorsdottir, A. M., Sigurdardottrir, M., Jonasson, J. G., Oddsdottir, M., Magnusson, J., Lee, J. R. & Goldenring, J. R. 2003. Spasmolytic polypeptide-expressing metaplasia (SPEM) associated with gastric cancer in Iceland. *Digestive Diseases and Sciences,* 48(3), 431-41.

Hammond, B., Dudek, R., Lemen, J. K. & Nemeth, M. A. 2006a. Results of a 90-day safety assurance study with rats fed grain from corn borer-protected corn. *Food and Chemical Toxicology,* 44, 1092-1099.

Hammond, B., Lemen, J., Dudek, R., Ward, D., Jiang, C., Nemeth, M. & Burns, J. 2006b. Results of a 90-day safety assurance study with rats fed grain from corn rootworm-protected corn. *Food and Chemical Toxicology,* 44, 147-160.

Hammond, B. G., Dudek, R., Lemen, J. K. & Nemeth, M. 2004. Results of a 13 week safety assurance study with rats fed grain from glyphosate tolerant corn. *Food and Chemical Toxicology,* 42, 1003-1014.

Hammond, B. G., Vicini, J. L., Hartnell, G. F., Naylor, M. W., Knight, C. D., Robinson, E. H., Fuchs, R. L., & Padgette, S. R. 1996. The feeding value of soybeans fed to rats, chickens, catfish and dairy cattle is not altered by genetic incorporation of glyphosate tolerance. *The Journal of Nutrition*, 126, 717-727.

Hammond, B.G. & Koch, M.S. 2012. A Review of the Food Safety of Bt Crops. *In:* E. Sansinenea (ed.) *Bacillus thuringiensis Biotechnology*. Dordrecht: Springer.

Hartsock, A. & Nelson, W. J. 2008. Adherens and tight junctions: Structure, function and connections to the actin cytoskeleton. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1778, 660-669.

Healy, C., Hammond, B. & Kirkpatrick, J. 2008. Results of a 13-week safety assurance study with rats fed grain from corn rootworm-protected, glyphosate-tolerant MON 88017 corn. *Food and Chemical Toxicology,* 46**,** 2517-2524.

Helander, H. F., Leth, R. & Olbe, L. 1986. Stereological investigations on human gastric mucosa: I. Normal oxyntic mucosa. *The Anatomical Record*, 216, 373-380.

Hirst, B. H. 2011. The gastric mucosal barrier. *Comprehensive Physiology.* Supplement 18: Handbook of Physiology, The Gastrointestinal System, Salivary, Gastric, Pancreatic, and Hepatobiliary Secretion, 279-308.

Ho, M.-W., Ryan, A. & Cummins, J. 1999. Cauliflower Mosaic Viral Promoter - A Recipe for Disaster? *Microbial Ecology in Health and Disease,* 11**,** 194-197.

Hoffmann, W. 2008. Regeneration of the gastric mucosa and its glands from stem cells. *Current Medicinal Chemistry,* 15**,** 3133-3144.

Hofte, H. & Whiteley, H. R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Reviews,* 53(2)**,** 242-255.

Hosoyamada, Y. & Sakai, T. 2005. Structural and mechanical architecture of the intestinal villi and crypts in the rat intestine: Integrative reevaluation from ultrastructural analysis. *Anatomy and Embryology,* 210**,** 1-12.

Howarth, G. S., Francis, G. L., Cool, J. C., Xu, X., Byard, R. W. & Read, L. C. 1996. Milk growth factors enriched from cheese whey ameliorate intestinal damage by methotrexate when administered orally to rats. *The Journal of Nutrition,* 126**,** 2519-2530.

Huber, D., Balda, M. S. & Matter, K. 2000. Occludin modulates transepithelial migration of neutrophils. *The Journal of Biological Chemistry,* 275**,** 5773-5778.

Ibrahim, M. a. A. & Okasha, E. F. 2016. Effect of genetically modified corn on the jejunal mucosa of adult male albino rat. *Experimental and Toxicologic Pathology*, 68, 579-588.

Implementing Regulation (Eu) 2013. COMMISSION IMPLEMENTING REGULATION (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006. *Official Journal of the European Union*.

ISAAA 2013. *ISAAA's GM Approval Database.* [Online]. Available: http://www.isaaa.org/gmapprovaldatabase/ [Accessed 14 March 2013].

Isaacs, P. E., Sladen, G. E. & Filipe, I. 1987. Mefenamic acid enteropathy. *Journal of Clinical Pathology* 40**,** 1221-1227.

Ishii, T. & Araki, M. 2016. Consumer acceptance of food crops developed by genome editing. *Plant Cell Reports*, 35, 1507–1518.

Ito, S., Lacy, E. R., Rutten, M. J., Critchlow, J., & Silen, W. 1984. Rapid repair of injured gastric mucosa. *Scandinavian Journal of Gastroenterology. Supplement*, 101, 87-95.

Kania, J., Konturek, S. J., Marlicz, K., Hahn, E. G. & Konturek, P. C. 2003. Expression of survivin and caspase-3 in gastric cancer. *Digestive Diseases and Sciences,* 48**,** 266-271.

Karam, S. M. & Leblond, C. P. 1993. Dynamics of epithelial cells in the corpus of the mouse stomach. II. Outward migration of pit cells. *The Anatomical Record*, 236, 280-296.

Kaspareit, J. & Rittinghausen, S. 1999. Spontaneous neoplastic lesions in Harlan Sprague-Dawley rats. *Experimental and Toxicologic Pathology,* 51**,** 105-107.

Kawabata, T. K. 1996. Immunotoxicology of the Gastrointestinal Tract. *In:* Smialowicz, R. J. & Holsapple, M. P. (eds.) *Experimental Immunotoxicology.* Boca Raton: CRC Press.

Keefe, D. M., Brealey, J., Goland, G. J. & Cummins, A. G. 2000. Chemotherapy for cancer causes apoptosis that precedes hypoplasia in crypts of the small intestine in humans. *Gut,* 47**,** 632-637.

Keefe, D. M., Cummins, A. G., Dale, B. M., Kotasek, D., Robb, T. A. & Sage, R. E. 1997. Effect of high-dose chemotherapy on intestinal permeability in humans. *Clinical Science,* 92**,** 385-389.

Kikuchi, M., Nagata, H., Watanabe, N., Watanabe, H., Tatemichi, M. & Hibi, T. 2010. Altered expression of a putative progenitor cell marker DCAMKL1 in the rat gastric mucosa in regeneration, metaplasia and dysplasia. *BMC Gastroenteroogyl,* 10**,** 65 [14 pp.].

Kimura, Y., Shiozaki, H., Hirao, M., Maeno, Y., Doki, Y., Inoue, M., Monden, T., Ando-Akatsuka, Y., Furuse, M., Tsukita, S. & Monden, M. 1997. Expression of occludin, tight-junction-associated protein, in human digestive tract. *The American Journal of Pathology,* 151(1)**,** 45-54.

Klein, M., Linnemann, D. & Rosenberg, J. 2011. Non-steroidal anti-inflammatory drug-induced colopathy. *BMJ Case Reports,* 2011, 3437 [4 pp.].

Kokue, E. I., Nakamura, T. & Hayama, T. 1977. Forestomach lesions induced by starvation in rat; its similarity with gastroesophogeal ulcer of swine. *Nihon Juigaku Zasshi,* 39**,** 337-341.

Krimsky, S. 2002. Environmental Ipacts of the Release of Genetically Modified Organisms. *In:* Pimentel, D. (ed.) *Encyclopedia of Pest Management.* New York: CRC Press.

Kroghsbo, S., Madsen, C., Poulsen, M., Schrøder, M., Kvist, P. H., Taylor, M., Gatehouse, A., Shu, Q. & Knudsen, I. 2008. Immunotoxicological studies of genetically modified rice expressing PHA-E lectin or Bt toxin in Wistar rats. *Toxicology,* 245**,** 24-34.

Kubo, M., Sasako, M., Gotoda, T., Ono, H., Fujishiro, M., Saito, D., Sano, T. & Katai, H. 2014. Endoscopic evaluation of the remnant stomach after gastrectomy: proposal for a new classification. *Gastric Cancer*, 5, 83-89.

Kuiper, H. A. & Kleter, G. A. 2003. The scientific basis for risk assessment and regulation of genetically modified foods. *Trends in Food Science and Technology,* 14**,** 277-293.

Kuiper, H. A., Kleter, G. A., Noteborn, H. P. J. M. & Kok, E. J. 2001. Assessment of the food safety issues related to genetically modified foods. *The Plant Journal,* 27**,** 503-528.

Lang, J., Price, A. B., Levi, A. J., Burke, M., Gumpel, J. M. & Bjarnason, I. 1988. Diaphragm disease: Pathology of disease of the small intestine induced by non-steroidal anti-inflammatory drugs. *Journal of Clinical Pathology,* 41**,** 516-526.

Laukoetter, M. G., Nava, P., Lee, W. Y., Severson, E. A., Capaldo, C. T., Babbin, B. A., Williams, I. R., Koval, M., Peatman, E., Campbell, J. A., Dermody, T. S., Nusrat, A. & Parkos, C. A. 2007. JAM-A regulates permeability and inflammation in the intestine in vivo. *The Journal of Experimental Medicine,* 204**,** 3067-3076.

Lee, F. D. 1993. Importance of apoptosis in the histopathology of drug related lesions in the large intestine. *Journal of Clinical Pathology,* 46**,** 118-122.

Lee, F. D. 1994. Drug-related pathological lesions of the intestinal tract. *Histopathology,* 25**,** 303-308.

Li, H. & Helander, H. 1996. Hypergastrinemia increases proliferation of gastroduodenal epithelium during gastric ulcer healing in rats. *Digestive Diseases and Sciences,* 41**,** 40-48.

Logan, R. M., Stringer, A. M., Bowen, J. M., Gibson, R. J., Sonis, S. T. & Keefe, D. M. 2009. Is the pathobiology of chemotherapy-induced alimentary tract mucositis influenced by the type of mucotoxic drug administered? *Cancer Chemotherapy and Pharmacology,* 63**,** 239-251.

Loogna, P., Franzen, L., Sipponen, P. & Domellof, L. 2002a. Cyclooxygenase-2 and Bcl-2 expression in the stomach mucosa of Wistar rats exposed to *Helicobacter pylori*, N'-methyl- N'-nitro- N-nitrosoguanidine and bile. *Virchows Archiv,* 441(1)**,** 77-84.

Ma, T. Y., Iwamoto, G. K., Hoa, N. T., Akotia, V., Pedram, A., Boivin, M. A. & Said, H. M. 2004. TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. *American Journal of Physiology. Gastrointestinal and Liver Physiology,* 286(3)**,** G367-G376.

Mankertz, J., Tavalali, S., Schmitz, H., Mankertz, A., Riecken, E. O., Fromm, M. & Schulzke, J. D. 2000. Expression from the human occludin promoter is affected by tumor necrosis factor alpha and interferon gamma. *Journal of Cell Science,* 113(Pt 11)**,** 2085-2090.

Markov, A., Veshnyakova, A., Fromm, M., Amasheh, M. & Amasheh, S. 2010. Segmental expression of claudin proteins correlates with tight junction barrier properties in rat intestine. *Journal of Comparative Physiology B,* 180**,** 591-598.

Marlow, S. L. & Blennerhassett, M. G. 2006. Deficient innervation characterizes intestinal strictures in a rat model of colitis. *Experimental and Molecular Pathology,* 80, 54-66.

Martìn-Padura, I., Lostaglio, S., Schneemann, M., Williams, L., Romano, M., Fruscella, P., Panzeri, C., Stoppacciaro, A., Ruco, L., Villa, A., Simmons, D. & Dejana, E. 1998. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *The Journal of Cell Biology,* 142, 117-127.

Martin, G. R. & Wallace, J. L. 2006. Gastrointestinal inflammation: A central component of mucosal defense and repair. *Experimental Biology and Medicine,* 231, 130-137.

Mason, F., Pascotto, E., Zanfi, C. & Spanghero, M. 2013. Effect of dietary inclusion of whole ear corn silage on stomach development and gastric mucosa integrity of heavy pigs at slaughter. *The Veterinary Journal*, 198, 717-719.

Menozzi, A. & Ossiprandi, M. C. 2010. Assessment of enteral bacteria. *Current Protocols in Toxicology,* Chapter 21, Unit 21.3.

Mensah, P. K., Palmer, C. G. & Muller, W. J. 2014. Lethal and sublethal effects of pesti-cides on aquatic organisms: The case of a freshwater shrimp exposure to Roundup®. *Pesticides - Toxic Aspects*. Rijeka: InTech Publications.

Mensah, P. K., Palmer, C. G. & Odume, O. N. 2015. Ecotoxicology of glyphosate and glyphosate-based herbicides - Toxicity to wildlife and humans. *Toxicity and Hazard of Agrochemicals*. Rijeka: InTech Publications.

Meyer, R. A., McGinley, D. & Posalaky, Z. 1986. Effects of aspirin on tight junction structure of the canine gastric mucosa. *Gastroenterology,* 91, 351-359.

Mohr, U. 1997. *International Classification of Rodent Tumors. The Rat*, Lyon, Springer Science & Business Media.

Morini, G. & Grandi, D. 2010. Methods to measure gastric mucosal lesions in the rat. *Current Protocols in Toxicology,* Chapter 21, Unit 21.2.

Morita, K., Furuse, M., Fujimoto, K. & Tsukita, S. 1999. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. *Proceedings of the National Academy of Sciences,* 96, 511-516.

Mowat, A. M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nature Reviews Immunology,* 3(4), 331-341.

Naoki, I., Araki, K., Kobayashi, A., Yamasaki, Y. & Ogata, T. 1998. Electron cytochemical observations on membrane redistribution in rat and human parietal cells after maximal acid secretion. *Medical Electron Microscopy*, 31, 128-134.

Nightingale, P. 2013. The GM slow motion train wreck [Online]. *The Guardian*. Available: http://www.theguardian.com/science/political-science/2013/jun/21/genetics-agriculture [Accessed 14 December 2015].

Niimi, C., Goto, H., Ohmiya, N., Niwa, Y., Hayakawa, T., Nagasaka, T. & Nakashima, N. 2002. Usefulness of p53 and Ki-67 immunohistochemical analysis for preoperative diagnosis of extremely well-differentiated gastric adenocarcinoma. *American Journal of Clinical Pathology,* 118(5), 683-692.

Noteborn, H. P. J. M., Bienenmann-Ploum, M. E., van den Berg, J. H. J., Alink, G. M., Zolla, L., Reynaerts, A., Pensa, M. & Kuiper, H. A. 1995. Safety Assessment of the *Bacillus thuringiensis* Insecticidal Crystal Protein CRYIAB(b) Expressed in Transgenic Tomatoes. *In:* Engel, K.-H., Takeoka, G. R. & Teranishi, R. (eds.) *Genetically Modified Foods: Safety Issues.* Washington: American Chemical Society.

Nusrat, A., Turner, J. R. & Madara, J. L. 2000. IV. Regulation of tight junctions by extracellular stimuli: Nutrients, cytokines, and immune cells. *American Journal of Physiology - Gastrointestinal and Liver Physiology,* 279, G851-G857.

Nusrat, A., Von Eichel-Streiber, C., Turner, J. R., Verkade, P., Madara, J. L. & Parkos, C. A. 2001. *Clostridium difficile* toxins disrupt epithelial barrier function by altering membrane microdomain localization of tight junction proteins. *Infection and Immunity,* 69(3), 1329-1336.

OECD 1981. *OECD Guidelines 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents, Guideline for the Testing of Chemicals*. Paris: OECD.

OECD 1998. *OECD Guideline 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents* [Online]. Available: http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en [Accessed 8 March 2013]

OECD 2008a. *OECD Guidelines 451: Carcinogenicity Studies, Guideline for the Testing of Chemicals.* Paris: OECD.

OECD 2008b. *OECD Guideline 452: Chronic Toxicity Studies, Guideline for the Testing of Chemicals*. Paris: OECD.

OECD 2012. *Guidance Document 116 on the Conduct and Design of Chronic Toxicity and Carcinogenicity Studies, Supporting Test Guidelines 451, 452 and 453.* 2nd edition. Paris: OECD.

Ojetti, V., Gigante, G., Ainora, M. E., Fiore, F., Barbaro, F. & Gasbarrini, A. 2009. Microflora imbalance and gastrointestinal diseases. *Digestive and Liver Disease Supplements,* 3**,** 35-39.

Ogata, T. & Yamasaki, Y. 2000. Scanning EM of resting gastric parietal cells reveals a network of cytoplasmic tubules and cisternae connected to the intracellular canaliculus. The Anatomical Record, 258, 15-24.

OGTR. 2016. *Approved GMOs* [Online]. Office of the Gene Technology Regulator. Available: http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/section-approved-gmos [Accessed 20 October 2016].

Oraby, H., Kandil, M., Shaffie, N. & Ghaly, I. 2015. Biological impact of feeding rats with a genetically modified-based diet. *Turkish Journal of Biology,* 39(2), 265-275.

Oshima, T. & Miwa, H. 2016. Gastrointestinal mucosal barrier function and diseases. *Journal of Gastroenterology*, 51, 768-778.

Owen, D. A. 2003. Gastritis and carditis. *Modern Pathology,* 16(4)**,** 325-341.

Padgette, S. R., Kolacz, K. H., Delannay, D. B., Lavallee, B. J., Tinius, C. N., Rhodes, W. K., Otero, Y. I., Barry, G. F., Eichholtz, D. A., Peschke, V. M., Nida, D. L., Taylor, N. B. & Kishore, G. M. 1995. Development, Identification, and Characterization of Glyphosate-Tolerant Soybean Line. *Crop Science,* 35**,** 1451-1461.

Paimela, H., Goddard, P. J. & Silen, W. 1995. Present views on restitution of gastrointestinal epithelium. *Digestive Diseases and Sciences,* 40(11)**,** 2495-2496.

Paparini, A. & Romano-Spica, V. 2006. Gene transfer and cauliflower mosaic virus promoter 35S activity in mammalian cells. *Journal of Environmental Science and Health. Part. B, Pesticides, Food Contaminants, and Agricultural Wastes,* 41(4)**,** 437-449.

Parfitt, J. R. & Driman, D. K. 2007. Pathological effects of drugs on the gastrointestinal tract: a review. *Human Pathology,* 38**,** 527-536.

Peckham, J. C. 2002. Animal Histopathology. *In:* Derelanko, M. J. & Hollinger, M. A. (eds.) *Handbook of Toxicology.* 2nd ed. Boca Raton: CRC Press.

Percy, D. H. & Barthold, S. W. 2008. Rat. *Pathology of Laboratory Rodents and Rabbits.* Blackwell Publishing Professional. Available: http://dx.doi.org/10.1002/9780470344613.ch2 [Accessed 7 September 2012]

Piastowska-Ciesielska, A. W. & Gralak, M. A. 2010. Influence of a low dose of dietary soybean on bone properties and mineral status in young rats. *BioFactors,* 36**,** 451-458.

Pigott, C. R. & Ellar, D. J. 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiology and Molecular Biology Reviews,* 71**,** 255-281.

Pitman, R. S. & Blumberg, R. S. 2000. First line of defense: The role of the intestinal epithelium as an active component of the mucosal immune system. *Journal of Gastroenterology,* 35**,** 805-814.

Poel, W. E. 1963. The alimentary tract: A route for carcinogenic exposure. *Journal of Occupational and Environmental Medicine,* 5**,** 22-33.

Polya, R. 1999. Genetically Modified Foods - Are We Worried Yet? *Current Issues Brief* [Online], No. 12 1998-99. Available: http://www.aph.gov.au/About_Parliament/Parliamentary_ Departments/Parliamentary_ Library/Publications_Archive/CIB/cib9899/99cib12

[Accessed 14 December 2015].
Powell, D. W. 1984. Physiological Concepts of Epithelial Barriers. *In:* Allen, A., Flemstrom, G., Garner, A., Silen, W. & Turnberg, L. A. (eds.) *Mechanisms of Mucosal Protection in the Upper Gastrointestinal Tract* New York: Raven Press.
Pozharisski, K. M. 1975. Morphology and morphogenesis of experimental epithelial tumors of the intestine. *Journal of the National Cancer Institute*, 54, 1115-1135.
Price, A. B. 2003. Pathology of drug-associated gastrointestinal disease. *British Journal of Clinical Pharmacology,* 56**,** 477-482.
Pusztai, A., Bardocz, S. & Ewen, S. W. 2003. Genetically Modified Foods: Potential Human Health Effects. *In:* D'Mello, J. P. F. (ed.) *Food Safety: Contaminants and Toxins.* Wallingford: CABI Publishing.
Qi, X., He, X., Luo, Y., Li, S., Zou, S., Cao, S., Tang, M., Delaney, B., Xu, W. & Huang, K. 2012. Subchronic feeding study of stacked trait genetically-modified soybean (3Ø5423 × 40-3-2) in Sprague–Dawley rats. *Food and Chemical Toxicology,* 50**,** 3256-3263.
Rausell, C., Munoz-Garay, C., Miranda-CassoLuengo, R., Gomez, I., Rudino-Pinera, E., Soberon, M. & Bravo, A. 2004. Tryptophan spectroscopy studies and black lipid bilayer analysis indicate that the oligomeric structure of Cry1Ab toxin from *Bacillus thuringiensis* is the membrane-insertion intermediate. *Biochemistry,* 43**,** 166-174.
Ridley, W. P., Sidhu, R. S., Pyla, P. D., Nemeth, M. A., Breeze, M. L. & Astwood, J. D. 2002. Comparison of the nutritional profile of glyphosate-tolerant corn event NK603 with that of conventional corn (Zea mays L.). *Journal of Agricultural and Food Chemistry,* 50(25)**,** 7235-7243.
Saitou, M., Furuse, M., Sasaki, H., Schulzke, J. D., Fromm, M., Takano, H., Noda, T. & Tsukita, S. 2000. Complex phenotype of mice lacking occludin, a component of tight junction strands. *Molecular Biology of the Cell,* 11(12)**,** 4131-4142.
Sakamoto, Y., Tada, Y., Fukumori, N., Tayama, K., Ando, H., Takahashi, H., Kubo, Y., Nagasawa, A., Yano, N., Yuzawa, K. & Ogata, A. 2008. [A 104-week feeding study of genetically modified soybeans in F344 rats]. *Shokuhin Eiseigaku Zasshi,* 49**,** 272-282.
Sakamoto, Y., Tada, Y., Fukumori, N., Tayama, K., Ando, H., Takahashi, H., Kubo, Y., Nagasawa, A., Yano, N., Yuzawa, K., Ogata, A. & Kamimura, H. 2007. [A 52-week feeding study of genetically modified soybeans in F344 rats]. *Shokuhin Eiseigaku Zasshi,* 48**,** 41-50.
Samonte, V. A., Goto, M., Ravindranath, T. M., Fazal, N., Holloway, V. M., Goyal, A., Gamelli, R. L. & Sayeed, M. M. 2004. Exacerbation of intestinal permeability in rats after a two-hit injury: burn and Enterococcus faecalis infection. *Critical Care Medicine,* 32(11)**,** 2267-2273.
Samsel, A. & Seneff, S. 2013. Glyphosate's suppression of cytochrome P450 enzymes and amino acid biosynthesis by the gut microbiome: Pathways to modern diseases. *Entropy,* 15**,** 1416-1463.
Sawada, N., Murata, M., Kikuchi, K., Osanai, M., Tobioka, H., Kojima, T. & Chiba, H. 2003. Tight junctions and human diseases. *Medical Electron Eicroscopy,* 36(3)**,** 147-156.
Saxena, D. & Stotzky, G. 2000. Insecticidal toxin from *Bacillus thuringiensis* is released from roots of transgenic Bt corn in vitro and in situ. *FEMS Microbiology Ecoogyl,* 33(1)**,** 35-39.
Schilter, B. & Constable, A. 2002. Regulatory control of genetically modified (GM) foods: Likely developments. *Toxicology Letters,* 127**,** 341-349.
Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R. & Dean, D. H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews,* 62(3)**,** 775-806.
Schrøder, M., Poulsen, M., Wilcks, A., Kroghsbo, S., Miller, A., Frenzel, T., Danier, J., Rychlik, M., Emami, K., Gatehouse, A., Shu, Q., Engel, K.-H., Altosaar, I. & Knudsen, I. 2007. A 90-day safety study of genetically modified rice expressing Cry1Ab protein (*Bacillus thuringiensis* toxin) in Wistar rats. *Food and Chemical Toxicology,* 45**,** 339-349.
Schulze, K. 2006. Imaging and modelling of digestion in the stomach and the duodenum. *Neurogastroenterology and Motility,* 18(3)**,** 172-183.
Şener, G., Paskaloglu, K., Kapucu, C., Cetinel, S., Contuk, G. & Ayanoğlu-Dülger, G. 2004. Octreotide ameliorates alendronate-induced gastric injury. *Peptides,* 25**,** 115-121.

Seralini, G. E., Cellier, D. & De Vendomois, J. S. 2007. New analysis of a rat feeding study with a genetically modified maize reveals signs of hepatorenal toxicity. *Archives of Environmental Contamination and Toxicology,* 52**,** 596-602.

Seralini, G. E., Clair, E., Mesnage, R., Gress, S., Defarge, N., Malatesta, M., Hennequin, D. & De Vendomois, J. S. 2012. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. *Food and Chemical Toxicology,* 50(11)**,** 4221-4231. *Retracted and republished as:* Séralini, G.-E., Clair, E., Mesnage, R., Gress, S., Defarge, N., Malatesta, M., Hennequin, D., & de Vendômois, J. S. 2014. Republished study: long-term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. *Environmental Sciences Europe*, 26, 1-14.

Shen, L., Weber, C. R., Raleigh, D. R., Yu, D. & Turner, J. R. 2011. Tight junction pore and leak pathways: A dynamic duo. *Annual Review of Physiology,* 73**,** 283-309.

Shimada, N., Miyamoto, K., Kanda, K. & Murata, H. 2006. Binding of Cry1Ab toxin, a *Bacillus thuringiensis* insecticidal toxin, to proteins of the bovine intestinal epithelial cell: An in vitro study. *Applied Entomology and Zoology,* 41**,** 295-301.

Siglin, J. C. & Baker, W. H. 2002. Laboratory Animal Management. In: Derelanko, M. J. & Hollinger, M. A. (eds.) *Handbook of Toxicology*. 2nd ed. Boca Raton: CRC Press.

Silen, W., & Ito, S. 1985. Mechanisms for rapid re-epithelialization of the gastric mucosal surface. *Annual Review of Physiology*, 47, 217-229.

Sissener, N., Bakke, A. M., Gu, J., Penn, M. H., Eie, E., Krogdahl, Å., Sanden, M. & Hemre, G. 2009. An assessment of organ and intestinal histomorphology and cellular stress response in Atlantic salmon (Salmo salar L.) fed genetically modified Roundup Ready® soy. *Aquaculture,* 298**,** 101-110.

Snell, C., Bernheim, A., Berge, J.-B., Kuntz, M., Pascal, G., Paris, A. & Agnes, E. R. 2012. Assessment of the health impact of GM plant diets in long-term and multigenerational animal feeding trials: A literature review. *Food and Chemical Toxicology.* 50**,** 1134-1148.

Soberon, M., Pardo-Lopez, L., Lopez, I., Gomez, I., Tabashnik, B. E. & Bravo, A. 2007. Engineering modified Bt toxins to counter insect resistance. *Science,* 318**,** 1640-1642.

Soberón, M., Pardo, L., Muñóz-Garay, C., Sánchez, J., Gómez, I., Porta, H. & Bravo, A. 2010. Pore Formation by Cry Toxins. *In:* Anderluh, G. & Lakey, J. (eds.) *Proteins: Membrane Binding and Pore Formation.* Austin: Landes Bioscience and Springer Science+Business Media.

Soler, A. P., Miller, R. D., Laughlin, K. V., Carp, N. Z., Klurfeld, D. M. & Mullin, J. M. 1999. Increased tight junctional permeability is associated with the development of colon cancer. Carcinogenesis, 20, 1425-1431.

Soler, A. P., Mullin, J. M., Knudsen, K. A. & Marano, C. W. 1996. Tissue remodeling during tumor necrosis factor-induced apoptosis in LLC-PK1 renal epithelial cells. *American Journal of Physiology - Renal Physiology*, 270, F869-F879.

Song, H., He, X., Zou, S., Zhang, T., Luo, Y., Huang, K., Zhu, Z. & Xu, W. 2015. A 90-day subchronic feeding study of genetically modified rice expressing Cry1Ab protein in Sprague-Dawley rats. *Transgenic Research,* 24(2)**,** 295-308.

*South Australian Prevention of Cruelty to Animals Act* 1985. Government of South Australia, Adelaide Available: https://www.legislation.sa.gov.au/LZ/C/A/Animal%20Welfare%20Act%201985.aspx

Staibano, S., Rocco, A., Mezza, E., De Rosa, G., Budillon, G. & Nardone, G. 2002. Diagnosis of chronic atrophic gastritis by morphometric image analysis. A new method to overcome the confounding effect of the inflammatory infiltrate. *The Journal of Pathology,* 198**,** 47-54.

Stefanov, S. B. 1985. [Visual classification during quantitative comparison of images]. *Arkhiv Anatomii, Gistologii i Embriologii*, 88, 78-83.

Stolte, M. & Meining, A. 2001. The updated Sydney system: Classification and grading of gastritis as the basis of diagnosis and treatment. *Canadian Journal of Gastroenterology,* 15(9)**,** 591-598.

Strzalkowski, A. K., Godlewski, M. M., Hallay, N., Kulasek, G., Gajewski, Z. & Zabielski, R. 2007. The effect of suplementing sow with bioactive substances on neonatal small intestinal epithelium. *Journal of Physiology and Pharmacology,* 58**,** 115-122.

Su, L., Shen, L., Clayburgh, D. R., Nalle, S. C., Sullivan, E. A., Meddings, J. B., Abraham, C. & Turner, J. R. 2009. Targeted epithelial tight junction dysfunction causes immune activation and contributes to development of experimental colitis. *Gastroenterology,* 136**,** 551-563.

Sukhotnik, I., Shehadeh, N., Coran, A. G., Mogilner, J. G., Karry, R., Shamian, B., Ure, B. M. & Shamir, R. 2008. Oral insulin enhances cell proliferation and decreases enterocyte apoptosis during methotrexate-induced mucositis in the rat. *Journal of Pediatric Gastroenterology and Nutrition,* 47**,** 115-122.

Takeda, H. 2004. Disease of the Small Intestine. *Japan Medical Association Journal* 47**,** 462-467.

Takeuchi, T., Miura, S., Wang, L., Uehara, K., Mizumori, M., Kishikawa, H., Hokari, R., Higuchi, H., Adachi, M., Nakamizo, H. & Ishii, H. 2002. Nuclear factor-kappaB and TNF-alpha mediate gastric ulceration induced by phorbol myristate acetate. *Digestive Diseases and Sciences,* 47(9)**,** 2070-2078.

Tang, M., Xie, T., Cheng, W., Qian, L., Yang, S., Yang, D., Cui, W. & Li, K. 2012. A 90-day safety study of genetically modified rice expressing rhIGF-1 protein in C57BL/6J rats. *Transgenic Research,* 21**,** 499-510.

Tarnawski, A., Douglass, T. G., Stachura, J. & Krause, W. J. 1991. Quality of gastric ulcer healing: Histological and ultrastructural assessment. *Alimentary Pharmacology and Therapeutics,* 5 Suppl 1**,** 79-90.

Teshima, R., Akiyama, H., Okunuki, H., Sakushima, J.-I., Goda, Y., Onodera, H., Sawada, J.-I. & Toyoda, M. 2000. Effect of GM and non-GM soybeans on the immune system of BN rats and B10A mice. *Journal of the Food Hygienic Society of Japan,* 41**,** 188-193.

Thompson, C. M., Proctor, D. M., Suh, M., Haws, L. C., Kirman, C. R. & Harris, M. A. 2013. Assessment of the mode of action underlying development of rodent small intestinal tumors following oral exposure to hexavalent chromium and relevance to humans. *Critical Reviews in Toxicology,* 43(3), 244-274.

Trojan, V., Vyhnánek, T., Štastník, O., Mrkvicová, E., Mareš, J. & Havel, L. 2016. Detection of DNA fragments from wheat in blood of animals. *Journal für Verbraucherschutz und Lebensmittelsicherheit,* 11, 259-264.

Tsukita, S. & Furuse, M. 1999. Occludin and claudins in tight-junction strands: Leading or supporting players? *Trends in Cell Biology,* 9**,** 268-273.

Tsukita, S., Furuse, M. & Itoh, M. 2001. Multifunctional strands in tight junctions. *Nature Reviews. Molecular Cell Biology,* 2**,** 285-293.

Tudek, B. & Speina, E. B. 2012. Oxidatively damaged DNA and its repair in colon carcinogenesis. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis,* 736**,** 82-92.

Tutel'ian, V. A., Gapparov, M. G., Avren'eva, L. I., Guseva, G. V., Zhminchenko, V. M., Kravchenko, L. V., Pashorina, V. A., Saprykin, V. P., Seliaskin, K. E. & Tyshko, N. V. 2010. [Medical and biological safety assessment of genetically modified soybean event MON 89788. Report 1. Toxicologo-hygienic examinations]. *Vopr Pitan,* 79**,** 4-12.

Tutel'ian, V. A., Gapparov, M. M., Avren'eva, L. I., Aksiuk, I. N., Guseva, G. V., Kravchenko, L. V., L'vova L, S., Saprykin, V. P., Tyshko, N. V. & Chernysheva, O. N. 2008. [Medical and biological safety assessment of genetically modified maize event MON 88017. Report 1. Toxicologo-hygienic examinations]. *Vopr Pitan,* 77**,** 4-12.

Tutel'ian, V. A., Kravchenko, L. V., Lashneva, N. V., Avren'eva, L. I., Guseva, G. V., Sorokina, E. & Chernysheva, O. N. 1999. [Medical and biological evaluation of safety of protein concentrate from genetically-modified soybeans. Biochemical studies]. *Vopr Pitan,* 68**,** 9-12.

Tutel'ian, V. A., Kravchenko, L. V., Sorokina, E., Korolev, A. A., Avren'eva, L. I., Guseva, G. V., Chernysheva, O. N. & Tyshko, N. V. 2001. [Medical and biological assessment of the safety of genetically modified corn lines MON 810 and GA 21: A toxicological-biochemical study]. *Vopr Pitan,* 70**,** 28-31.

Tyshko, N. V., Britsina, M. V., Gmoshinskii, I. V., Zakharova, N. S., Zorin, S. N., Mazo, V. K., Ozeretskovskaia, M. N. & Seliaskin, K. E. 2010. [Medical and biological safety assessment of genetically modified soybean event MON 89788. Report 2. Genotoxicologic, immunologic and allergologic examinations]. *Vopr Pitan,* 79**,** 13-17.

Tyshko, N. V., Britsina, M. V., Gmoshinskii, I. V., Zhanataev, A. K., Zakharova, N. S., Zorin, S. N., Mazo, V. K. & Semenov, B. F. 2008. [Medical and biological safety assessment of genetically modified maize event MON 88017. Report 2. Genotoxicologic, immunologic and allergologic examinations]. *Vopr Pitan,* 77**,** 13-17.

Vaeck, M., Reynaerts, A., Hofte, H., Jansens, S., De Beuckeleer, M., Dean, C., Zabeau, M., Van Montagu, M. & Leemans, J. 1987. Transgenic plants protected from insect attack. *Nature,* 328**,** 33-37.

Van Itallie, C. M. & Anderson, J. M. 1997. Occludin confers adhesiveness when expressed in fibroblasts. *Journal of Cell Science,* 110**,** 1113-1121.

Vazquez-Padron, R. I., Gonzales-Cabrera, J., Garcia-Tovar, C., Neri-Bazan, L., Lopez-Revilla, R., Hernandez, 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. *Biochemical and Biophysical Research Communications,* 271**,** 54-58.

Velimirov, A., Binter, C., Zentek, J., Cyran, N., Gülly, C., Handl, S., Hofstätter, G., Meyer, F., Skalicky, M., & Steinborn, R. 2008. Biological effects of transgenic maize NK603xMON810 fed in long term reproduction studies in mice. *Unpublished report: Institute fur Ernahrung,* Vienna.

Walsh, M. C., Buzoianu, S. G., Gardiner, G. E., Rea, M. C., Ross, R. P., Cassidy, J. P. & Lawlor, P. G. 2012. Effects of short-term feeding of Bt MON810 maize on growth performance, organ morphology and function in pigs. *British Journal of Nutrition*, 107, 364-371.

Wang, E. H., Yu, Z., Hu, J. & Xu, H. B. 2013a. Effects of 90-day feeding of transgenic Bt rice TT51 on the reproductive system in male rats. *Food and Chemical Toxicology,* 62**,** 390-396.

Wang, Y., Wei, B., Tian, Y., Wang, Z., Tian, Y., Tan, S., Dong, S. & Song, Q. 2013b. Evaluation of the potential effect of transgenic rice expressing Cry1Ab on the hematology and enzyme activity in organs of female Swiss rats. *PLoS One,* 8**,** e80424 [9 pp.].

Wang, Z.-H., Wang, Y., Cui, H.-R., Xia, Y.-W., Altosaar, I. & Shu, Q.-Y. 2002. Toxicological evaluation of transgenic rice flour with a synthetic cry1Ab gene from *Bacillus thuringiensis*. *Journal of the Science of Food and Agriculture*, 82(7), 738-744.

Wardill, H., Bowen, J. & Gibson, R. 2012. Chemotherapy-induced gut toxicity: Are alterations to intestinal tight junctions pivotal? *Cancer Chemotherapy and Pharmacology,* 70**,** 627-635.

Weber, C. R., Nalle, S. C., Tretiakova, M., Rubin, D. T. & Turner, J. R. 2008. Claudin-1 and claudin-2 expression is elevated in inflammatory bowel disease and may contribute to early neoplastic transformation. *Laboratory Investigation,* 88**,** 1110-1120.

Weibel, E. 1990. Morphometry: Stereological Theory and Practical Methods. *In:* Gil, J. (ed.) *Models of Lung Disease.* New York: Dekker.

Whiteley, L. O., Anver, M. R., Botts, S. & Jokinen, M. P. 1996. Proliferative Lesions of the Intestine, Salivary Glands, Oral Cavity, and Esophagus in Rats. *Guides for Toxicologic Pathology.* Washington: STP/ARP/AFIP.

WHO 1999. *Bacillus thuringiensis* (Environmental Health Criteria; 217). Geneva: WHO.

Wilson, A. K., Latham, J. R. & Steinbrecher, R. A. 2006. Transformation-induced mutations in transgenic plants: Analysis and biosafety implications. *Biotechnology and Genetic Engineering Reviews,* 23**,** 209-237.

Windels, P., Taverniers, I., Depicker, A., Van Bockstaele, E. & De Loose, M. 2001. Characterisation of the Roundup Ready soybean insert. *European Food Research and Technology* 213**,** 107-112.

Wittchen, E., Haskins, J. & Stevenson, B. 1999. Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. *The Journal of Biological Chemistry*, 274, 35179-35185.

Wittchen, E. S., Haskins, J. & Stevenson, B. R. 2000. Exogenous Expression of the Amino-Terminal Half of the Tight Junction Protein Zo-3 Perturbs Junctional Complex Assembly. *The Journal of Cell Biology*, 151, 825-836.

Wong, A. Y.-T. & Chan, A. W.-K. 2016. Genetically modified foods in China and the United States: A primer of regulation and intellectual property protection. *Food Science and Human Wellness*, 5, 124-140.

Yoshida, S. H. 2000. The safety of genetically modified soybeans: Evidence and regulation. *Food and Drug Law Journal,* 55(2), 193-208.

Yu, J., Tang, B. D., Leung, W. K., To, K. F., Bai, A. H. C., Zeng, Z. R., Ma, P. K., Go, M. Y. Y., Hu, P. J. & Sung, J. J. Y. 2005. Different cell kinetic changes in rat stomach cancer after treatment with celecoxib or indomethacin: Implications on chemoprevention. *World Journal of Gastroenterology,* 11, 41-45.

Yuan, Y., Xu, W., Luo, Y., Liu, H., Lu, J., Su, C. & Huang, K. 2011. Effects of genetically modified T2A-1 rice on faecal microflora of rats during 90 day supplementation. *Journal of the Science of Food and Agriculture,* 91(11), 2066-2072.

Zdziarski, I. M., Edwards, J. W., Carman, J. A. & Haynes, J. I. 2014. GM crops and the rat digestive tract: A critical review. *Environment International,* 73, 423-433.

Zeljenkova, D., Ambrusova, K., Bartusova, M., Kebis, A., Kovriznych, J., Krivosikova, Z., Kuricova, M., Liskova, A., Rollerova, E., Spustova, V., Szabova, E., Tulinska, J., Wimmerova, S., Levkut, M., Revajova, V., Sevcikova, Z., Schmidt, K., Schmidtke, J., La Paz, J. L., Corujo, M., Pla, M., Kleter, G. A., Kok, E. J., Sharbati, J., Hanisch, C., Einspanier, R., Adel-Patient, K., Wal, J. M., Spok, A., Poting, A., Kohl, C., Wilhelm, R., Schiemann, J. & Steinberg, P. 2014. Ninety-day oral toxicity studies on two genetically modified maize MON810 varieties in Wistar Han RCC rats (EU 7th Framework Programme project GRACE). *Archives of Toxicology,* 88, 2289-2314.

Zhang, X., Candas, M., Griko, N. B., Taussig, R. & Bulla, L. A., Jr. 2006. A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. *Proceedings of the National Academy of Sciences of the United States of America* 103, 9897-9902.

Zhang, X., Griko, N. B., Corona, S. K. & Bulla, L. A., Jr. 2008. Enhanced exocytosis of the receptor BT-R(1) induced by the Cry1Ab toxin of *Bacillus thuringiensis* directly correlates to the execution of cell death. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology* 149, 581-588.

Zhu, Y., Li, D., Wang, F., Yin, J. & Jin, H. 2004. Nutritional assessment and fate of DNA of soybean meal from roundup ready or conventional soybeans using rats. *Archives of Animal Nutrition,* 58(4), 295-310.

Zlatkina, A. R., Belousova, E. A., Vinitsky, L. I., Avtandilov, G. G. & Chervonnaya, L. V. 1990. Cyclic nucleotide concentrations of rectal mucosa in ulcerative colitis. *Scandinavian Journal of Gastroenterology,* 25(4), 341-344.

## Publications

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Review

# GM crops and the rat digestive tract: A critical review

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ABSTRACT

The aim of this review is to examine the relationship between genetically modified (GM) crops and health, based on histopathological investigations of the digestive tract in rats. We reviewed published long-term feeding studies of crops containing one or more of three specific traits: herbicide tolerance via the *EPSPS* gene and insect resistance via *cry1Ab* or *cry3Bb1* genes. These genes are commonly found in commercialised GM crops. Our search found 21 studies for nine (19%) out of the 47 crops approved for human and/or animal consumption. We could find no studies on the other 38 (81%) approved crops. Fourteen out of the 21 studies (67%) were general health assessments of the GM crop on rat health. Most of these studies (76%) were performed after the crop had been approved for human and/or animal consumption, with half of these being published at least nine years after approval. Our review also discovered an inconsistency in methodology and a lack of defined criteria for outcomes that would be considered toxicologically or pathologically significant. In addition, there was a lack of transparency in the methods and results, which made comparisons between the studies difficult. The evidence reviewed here demonstrates an incomplete picture regarding the toxicity (and safety) of GM products consumed by humans and animals. Therefore, each GM product should be assessed on merit, with appropriate studies performed to indicate the level of safety associated with them. Detailed guidelines should be developed which will allow for the generation of comparable and reproducible studies. This will establish a foundation for evidence-based guidelines, to better determine if GM food is safe for human and animal consumption.

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**Contents**

1. Introduction . . . 424
   1.1. Background . . . 424
   1.2. Unintended effects and the need for animal feeding studies . . . 424
   1.3. The importance of studying the gastrointestinal tract . . . 424
2. Methods . . . 424
3. Results . . . 424
4. Discussion . . . 425
   4.1. Review of the methods . . . 425
   4.2. Selection of endpoints . . . 425
   4.3. Lack of transparency in results . . . 427
   4.4. Relevance of OECD guidelines in the evaluation of the safety of consuming GM crops . . . 430
   4.5. Have enough studies been conducted to adequately state that GM crops are safe for human and animal consumption? . . . 430
5. Conclusions . . . 432
Acknowledgements . . . 432
References . . . 432

*Abbreviations:* EFSA, European Food Safety Agency; FAO, Food and Agricultural Organisation; FSANZ, Food Standards Australia New Zealand; GI, gastrointestinal; GM, genetically modified; H&E, haematoxylin and eosin; LM, light microscopy; OECD, Organisation for Economic Cooperation and Development; RR, Roundup Ready; TEM, transmission electron microscopy; WHO, World Health Organisation.

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## 1. Introduction

### 1.1. Background

Genetically modified (GM) or transgenic crops have been grown for human and animal consumption since the 1990s (Clive and Krattiger, 1996). There are currently over 200 different GM crops with various traits approved for human and animal consumption in many countries (ISAAA, 2013). Despite this, feeding studies examining the effects of GM crops on animal and human health are relatively scarce (Domingo, 2000; Domingo and Bordonaba, 2011; Snell et al., 2012).

### 1.2. Unintended effects and the need for animal feeding studies

The two most common methods of producing GM crops are through *Agrobacterium*-mediated transformation and microparticle bombardment (also known as microparticle acceleration or biolistics) (Wilson et al., 2006). A common criticism is that these processes are imprecise. In both processes, the insertion site of the new DNA is random (Altpeter et al., 2005; Wilson et al., 2006) and more than one copy of the DNA fragment may be inserted into the target genome (Christou, 1992; Gasson, 2003). This can affect gene expression in a positive or negative manner, for example, by causing gene suppression or gene silencing (Altpeter et al., 2005; Dai et al., 2001). In microparticle bombardment, the extra copies of the inserted DNA can be scrambled, inverted or incomplete (Altpeter et al., 2005). In addition, in microparticle bombardment, the site of insertion may undergo further recombination (Altpeter et al., 2005; Christou et al., 1988; Windels et al., 2001). For these reasons, the toxicity or nutritional value of the GM crop should be assessed as a whole.

Transgenic crops are produced through the insertion of a gene cassette, which consists of the desired trait genes, as well as several other genes such as viral promoter and marker genes. These genes tend to be truncated or shortened versions, which may even have gene sequence changes (ISAAA, 2013; Padgette et al., 1995; Vaeck et al., 1987). The effect of these genes acting together is not often determined or even required (FAO/WHO, 2000; FSANZ, 2007).

At present, establishing substantial equivalence is the only generally required safety assessment (FAO/WHO, 2000; FSANZ, 2007). Substantial equivalence relies on the premise that the safety of GM food can be assessed through a comparison with compounds or organisms of known safety. The purpose of the test for substantial equivalence is to identify possible hazard areas, which become the focus of further assessment (FSANZ, 2007; König et al., 2004). The test for substantial equivalence examines the individual characters and not the GM crop as a whole. For example, it assesses the toxicity of the new protein the plant has been designed to produce, such as an insecticidal protein or a protein conferring herbicide tolerance. Based on the safe history of consumption of that protein in its wild-type form, the protein is deemed safe (Kuiper et al., 2001). If the test for substantial equivalence shows no differences outside what could be obtained through natural variation, then food regulators may not require further examinations (Schilter and Constable, 2002). This type of general safety assessment does not consider that the genes present in the novel food may be additional or different from what is anticipated (Padgette et al., 1995; Vaeck et al., 1987; Wilson et al., 2006). It does not take into account the alteration of the protein gene sequence prior to insertion or the possibility that the protein gene sequence may have been altered due to the transformation process, although the latter has recently been incorporated into the European Food Safety Agency (EFSA) assessment processes (EFSA, 2008). Hence, we argue that GM crops should undergo thorough safety evaluations that do not simply consider the GM food as being composed of several substances of known safety, but as a novel entity, the safety of which needs to be evaluated as a whole.

Double- or multi-trait stacked crops are becoming more and more common (Clive, 2013). These are obtained either through more than one trait being inserted into one crop, or through cross-breeding of two or more GM crops (ISAAA, 2013). Many food regulators do not require any studies to be done on crops containing several stacked genes if all the genes in the stack have previously been individually approved for use in the same kind of plant (EFSA, 2010; FSANZ, 2010). However, the effect of two or more traits acting together is unknown. For example, two insecticidal proteins, when ingested together, may have a potentiating or synergistic effect (Schnepf et al., 1998). In real-life scenarios, animals and humans most probably consume GM material and products of various traits in a single meal. Therefore, it is suggested that long-term animal feeding studies be performed to investigate the toxicity of crops possessing more than one trait to investigate the toxicity of feed containing more than one GM component.

### 1.3. The importance of studying the gastrointestinal tract

The digestive tract is the first site of contact for any ingested compound. It follows that if a compound is toxic, the first signs of toxicity may be visible in the gastrointestinal (GI) tract. Furthermore, since the stomach and the intestines are the sites of longest residence for any ingested product, these should become the most important sites for the evaluation of an ingested compound's toxicity. It is difficult to assess damage to the digestive tract purely on macroscopic grounds (Morini and Grandi, 2010), therefore a histopathological analysis should be part of the investigation.

## 2. Methods

The purpose of this literature review was to examine the relationship between GM crops and histopathological observations in rats. The search only included crops possessing one or more of three specific traits which are commonly found in commercialised GM crops: herbicide tolerance via the *EPSPS* gene, and insect resistance via *cry1Ab* or *cry3Bb1* genes. A list of crop event names was first generated (Table 1) based on GM approval databases (CERA, 2012; FSANZ, 2011b; ISAAA, 2013) and publications, such as literature reviews (Domingo, 2007; Domingo and Bordonaba, 2011; Magaña-Gómez and De La Barca, 2009; Pusztai et al., 2003; Snell et al., 2012). The search used PubMed, Google Scholar and Embase to find studies that were published before April 2013. The search was restricted to published studies. Reports, such as EFSA reports, were not included since they do not contain detailed histopathological results. The keywords used were rat, rats, *rattus* and the specific crop event line name (Table 1). To make results comparable with each other, the search was limited to long-term rat feeding studies of no less than 90 days duration. The search excluded multigenerational studies, unless there was a histopathological investigation in the first generation of rats. No language limit was set. For non-English publications, help was obtained with their translation and accurate understanding.

## 3. Results

The search yielded 21 published studies (Table 2) with an additional two re-analyses of raw data of some of these studies (de Vendomois et al., 2009; Seralini et al., 2007). The re-analyses concentrated only on the blood, serum and urine test results. (These publications are not counted nor listed in the tables or figures since they are not original feeding studies). Eighteen (86%) out of the 21 studies investigated crops that have been approved for human and/or animal consumption somewhere in the world (Table 1). These 18 studies investigated only nine out of the 47 approved GM crops (19%) known to possess at least one of the traits of interest. No published rat-feeding studies could be found for the remaining 38 (81%) approved crops. Of all the 21 studies found, 12 (57%) generally assessed the long-term effect of GM feed on rat health (Hammond et al., 2004, 2006a,b; Healy et al., 2008; Qi et al., 2012; Sakamoto et al., 2007, 2008; Schrøder et al., 2007; Seralini et al., 2012; Tutel'ian et al., 2008, 2010; Wang et al., 2002), whilst seven

(33%) examined specific outcomes — signs of allergic or immunological reactions (Kroghsbo et al., 2008; Teshima et al., 2000), effects of a GM diet on the blood, urine and liver (Tutel'ian et al., 1999, 2001), fate of the inserted DNA (Zhu et al., 2004), comparison of GM soy versus conventional soy and its nutritional impact (Daleprane et al., 2009), and the impact of a soy diet, be it GM or non-GM, on aortic wall remodelling (Daleprane et al., 2010).

The majority of the studies found were published in the last decade (Figs. 1 and 2). The earliest study was published in 1995, which was of a GM tomato that was probably never commercially grown (Noteborn et al., 1995). The study investigated the effect of the insecticidal protein cry1Ab, on its own or in the GM tomato, on various mammalian digestive systems. However, at the time of publication, the researchers had not yet performed a histopathological analysis of the effect of the GM crop on rat health.

The earliest published study on an approved crop was in 1999 (Tutel'ian et al., 1999) (Fig. 2), which was four years after that crop had been approved for human and animal consumption. This study only investigated the blood, urea and the liver of animals fed GM soy. The first study that generally assessed the long-term effect of GM feed on rat health was in 2002 (Wang et al., 2002). It investigated a GM rice (KMD1) that is approved for commercial use only in China. This approval was granted seven years after the Wang et al. (2002) study was published (Chen et al., 2011). Two other studies also investigated this crop (Kroghsbo et al., 2008; Schrøder et al., 2007), both of which were published prior to the approval. The remaining 16 (76%) published studies found in this review were published after the crops had been approved for human and/or animal consumption. Half of these were performed at least nine years after the approval was granted.

Five studies based their methodology on the Organisation for Economic Cooperation and Development (OECD) guidelines for the testing of chemicals — OECD Guideline 408: repeated dose 90 day oral toxicity study (OECD, 1981, 1998). Fourteen studies indicated that the digestive tract was investigated histopathologically, but no details were given as to what analyses were performed. The only details most often provided were that tissue samples were processed, paraffin embedded, and sections were cut and stained with haematoxylin and eosin (H&E). Sections were then assessed using light microscopy (LM). Seralini et al. (2012) indicated that sections were stained with HES, but failed to specify whether this abbreviation meant haematoxylin and eosin, haematoxylin eosin safran/saffron or haematoxylin erythrosine saffron stain. Seralini et al. (2012) also indicated that if any tumours were observed, they were processed for transmission electron microscopy (TEM). There was no mention if tumours were observed in the GI tract.

Six of the studies indicate that a pathologist or veterinary pathologist performed the histopathological analysis. Five studies provided some form of results of their analyses, whilst most limited their results section to a statement that overall there were no treatment-related or diagnostically-significant observations.

Overall, all the studies examining the GI tract concluded that there were no toxicological or pathological changes observed that could be related to feeding GM crops to rats.

## 4. Discussion

The digestive tract is the first site of contact with the body of any ingested food. Therefore, if a novel food is toxic to the body, signs of toxicity may be present in the GI tract. Often these changes may only be detectable by histopathological analysis and not macroscopic observations (Morini and Grandi, 2010).

### 4.1. Review of the methods

Whilst 14 out of the 21 studies reviewed (67%) indicated that organs of the digestive tract were collected for histopathological examination, none of the methods sections in these publications included any details as to the nature of the histopathological examination. Several of the studies (Hammond et al., 2004, 2006a,b; Healy et al., 2008; Qi et al., 2012; Zhu et al., 2004) simply stated that a pathologist or veterinary pathologist performed the analysis, but no mention was given as to what these analyses entailed, for example what pathological parameters were used or what was measured and why. The exception appears to be a study by Teshima et al. (2000) who stated that the morphology of the small intestine mucosa was assessed, in particular the composition of goblet cells and intraepithelial lymphocytes. According to the authors, the analysis was based on a chapter in an immunotoxicology textbook (Kawabata, 1996). However, that chapter did not mention the purpose or even how the investigation of the small intestine should appear. In particular, it did not include the definition of what constitutes abnormal or diseased, such as, what changes in goblet cell population would indicate a pathology.

A paper that appears to be well-structured and thorough was the Tutel'ian et al. (2008) study published in Russian. The methods section clearly stated that the morphometric analysis of the internal organs was conducted according to textbook guidelines (Avtandilov, 1982, 1990) and results were compared according to guidelines set out by Stefanov (1985). The two Russian textbooks (Avtandilov, 1982, 1990) are manuals on how to conduct quantitative research to obtain a meaningful assessment of morphological changes. In other words, the Tutel'ian et al. (2008) study appears to be thorough and well set out, especially since detailed results are provided for the analyses. However, the publication lacks basic information. It does not specify the number of rats used in the study and it does not list which organs were collected for the histopathological analyses. Results seem to imply that the ileum was the only section of the GI tract to be analysed. A more thorough study would have investigated other sections of the GI tract to more accurately ensure that the GM crop did not have any adverse effects.

Another Russian study (Tutel'ian et al., 2010) also appears to be properly conducted. Its safety assessment is based on the Tutel'ian et al. (2008) study, which implies that the same rigorous morphometric analysis was also utilised. However, even this publication lacks key information. For example, the paper indicated that the morphometric analysis was conducted on the small intestine and colon, but results were only reported for the small intestine. In addition, the publication does not specify which section of the small intestine these results pertain to. This lack of detail in both Russian papers makes it difficult to determine the veracity of the results. It also makes it difficult to reproduce and further the study or to compare these studies to others. Indeed, in all the published papers, a lack of uniformity in the analytical approach as well as documentation of the methods and results makes any comparison or assessment of adequacy or inadequacy of the studies difficult.

### 4.2. Selection of endpoints

A major flaw in all the studies reviewed was the lack of any definition of toxicity or signs of pathology. Of all the studies generally assessing rat health on a GM diet, not one explained how the study would adequately show that the crop is safe for human and/or animal consumption. Furthermore, all the studies reviewed failed to justify or give reason for the choice of methods used. Yet, most studies concluded that the investigation did not reveal any meaningful differences between animals fed the GM or non-GM feed. One study even stated that "since no meaningful differences were observed, no further microscopic examinations were deemed necessary" (Hammond et al., 2004). However, the absence of meaningful differences in a preliminary investigation does not mean that further analysis would not find meaningful differences. In addition, the authors did not support this statement with proof since they provided few details as to what their microscopic examinations entailed or found. Therefore, they give very little evidence that their study adequately assessed the safety of consuming the GM crop.

Another common remark in these publications was that all changes observed were not diagnostically significant, were within the normal range, or are common to this strain and age of rat. The six studies that made this remark gave little evidence to support this conclusion (Hammond et al., 2004, 2006a,b; Healy et al., 2008; Qi et al., 2012; Teshima et al., 2000). Most gave no evidence at all. For example, Qi et al.

**Table 1**
Literature search: list of GM crop event names that were used in the search for published studies. Year of approval for animal and/or human consumption of each event name and number of publications found per event.

| Crop type | Event name (other name and/or code name)[a,b] | Number of published long-term rat feeding studies | Year approved for food and/or feed[c] |
|---|---|---|---|
| Roundup Ready (RR) or glyphosate-tolerant crops: | | | |
| *Crops containing EPSPS genes* | | | |
| Alfalfa/Lucerne | J101 (MON-00101-8) | 0 | 2004 |
| | J163 (MON-00163-7) | 0 | 2004 |
| Canola | GT200 (RT200, MON89249-2) | 0 | 1997 |
| | GT73 (RT73, MON737) | 0 | 1994 |
| | MON88302 (MON-88302-9) | 0 | 2012 |
| | ZSR500 (ZSR500 x GT73) | 0 | 1997 |
| | ZSR502 (ZSR502 x GT73) | 0 | 1997 |
| | ZSR503 (ZSR503 x GT73) | 0 | 1997 |
| Corn/Maize | GA21 (MON00021-9) | 1[d] | 2000 |
| | HCEM485 | 0 | 2012 |
| | NK603 (MON-00603-6) | 2 | 2000 |
| | MON832 | 0 | 1996 |
| | MON87427 (MON-87427-7) | 0 | 2012 |
| Cotton | GHB614 (BCS-GH205) | 0 | 2008 |
| | MON1445 (MON1445-2) | 0 | 1995 |
| | MON1698 (MON89383-1) | 0 | 1995 |
| | MON88913 (MON88913-8) | 0 | 2005 |
| Creeping bentgrass | ASR368 (SGM-36800-2)[e] | 0 | 2003 |
| Potato | RBMT22-082 (RBMT22-82, NMK-89896-6) | 0 | 1998 |
| | RBMT22-186 | 0 | 1998 |
| | RBMT22-238 | 0 | 1998 |
| | RBMT22-262 | 0 | 1998 |
| Soybean | 40-3-2 (GTS 40-3-2, MON04032-6) | 4 | 1995 |
| | FG-72 (MST-FG072-2) | 0 | 2012 |
| | MON87705 (MON87705-6) | 0 | 2011 |
| | MON87708 (MON87708-9) | 0 | 2011 |
| | MON87769 | 0 | 2011 |
| | MON89788 (MON89788-1) | 1[f] | 2007 |
| | Glyphosate-tolerant [g] | 3[g] | NA |
| | 305423 x 40-3-2 (DP305423 x GTS40-3-2, DP-305423-1 x MON-04032-6)[b] | 1 | 2010 |
| Sugar beet | GTS B77 (T9100152, SY-GTSB-77-8) | 0 | 1998 |
| | H7-1 (KM71-4) | 0 | 2003 |
| Wheat | MON71800 (MON-71800-3) | 0 | 2004 |
| Insect resistant or *Bt* crops: | | | |
| *Crops containing Cry3Bb1 and EPSPS genes* | | | |
| Corn/Maize | MON88017 (MON-88017-3)[h] | 2[h] | 1996 |
| *Crops containing Cry3Bb1 genes* | | | |
| Corn/Maize | MON863 (MON-00863-5) | 1 | 2001 |
| *Crops containing Cry1Ab genes* | | | |
| Corn/Maize | 5307 (SYN-05307-1) | 0 | 2012 |
| | Bt10 | 0 | 1995 |
| | Bt11 (x 4334CBR, x 4634CBR, SYN-Bt011-1) | 0 | 1996 |
| | Bt176 (176, SYN-EV176-9) | 0 | 1995 |
| Cotton | COT67B (IR67B, SYN-IR67B-1,) | 0 | 2009 |
| | GFM Cry1A (GTL-GFM311-7) | 0 | Cultivation only (2006) |
| | GK12 | 0 | Cultivation only (1997) |
| | T303-3 (BCS-GH003-6) | 0 | Cultivation only (2012) |
| | T304-40 (BCS-GH004-7) | 0 | 2010 |
| Rice | Tarom molaii + cry1ab | 0 | 2004 |
| | KMD 1 rice (Kemingdao 1, TR30) | 3 | 2009 |
| | GM Shanyou 63 | 0 | 2009 |
| | Hauhui-1/TT51-1 | 0 | Cultivation only (2009) |
| Tomato | RLE13-0009[i] | 1 | No approval info.[i] |
| | RLE6-1000[i] | 0 | No approval info.[i] |
| *Crops containing Cry1Ab and EPSPS genes* | | | |
| Corn/Maize | MON801 (MON80100) | 0 | 1996 |
| | MON802 (MON-80200-7) | 0 | 1997 |
| | MON809 (PH-MON-809) | 0 | 1996 |
| | MON810 (MON-00810-6) | 2[d] | 1996 |
| | Number of GM crop event lines studied | 10[f,h] | 9 approved |
| | Total number of GM crop event lines | 53[f,h] | 47 approved |
| | Total number of published studies found | 21 | 19 approved |

P6

(2012) referenced a study by Tang et al. (2012) to support their notion that "microscopic observations occurred spontaneously in Sprague–Dawley rats of this age." However, the referenced study made no mention of microscopic observations occurring spontaneously and the study did not even use Sprague–Dawley rats.

A very common statement found in the reviewed studies was that since the lesions or changes were observed in both groups, they were not deemed to be diet-related (Healy et al., 2008; Sakamoto et al., 2007, 2008; Wang et al., 2002). For example, in two studies (Hammond et al., 2006b; Sakamoto et al., 2007), there was a brief mention of gastric gland dilatations being observed in both the GM and non-GM fed groups. Gland dilatations can occur in aged rats (Frantz et al., 1991), but they can also be a pathological occurrence for example in alendronate-induced injury (Şener et al., 2004), ulcer healing (Tarnawski et al., 1991) or underlying neoplastic lesions (Frantz et al., 1991). In these pathologies, the dilatations are large, they may sometimes extend into the submucosa and they may become dysplastic (Kikuchi et al., 2010). In the two publications (Hammond et al., 2006b; Sakamoto et al., 2007), no specific details are mentioned, for example, the size of these dilatations, whether the treatment group had larger dilatations than the other group, whether the affected area was more widespread in one group than the other, or if the cells lining the dilatations had a specific staining property or shape common only to one group. If a pathology is seen, regardless of whether it occurs in both groups, further analysis should be performed to determine the nature of the occurrence and to completely rule-out disease. Furthermore, whilst the incidence of a pathology may be equal in both groups, the degree or severity may vary. Therefore, it is always important to record and report the severity of a pathology. For example, an animal may be prone to a certain pathology (e.g. Sprague–Dawley rats are known to spontaneously develop certain neoplastic lesions) (Chandra et al., 1992; Kaspareit and Rittinghausen, 1999), but it is possible that the GM component may increase the severity or risk of this development. In addition, the type of crop fed may cause a pathology. For example, soy is known to have adverse effects on bone and the digestive tract (Godlewski et al., 2006; Piastowska-Ciesielska and Gralak, 2010). Therefore, feeding soy would naturally cause changes to the gut, but the GM component may increase the severity of these changes. Hence, detailed histopathological and morphometric analyses are needed to completely rule out the GM crops' involvement in the development of the lesion or pathological condition. In other words, it is not sufficient to say that the GM food is safe if incidences of a pathology or lesion are equal in both groups. Further testing should be carried out to completely rule out the GM component's involvement in the development of the pathological incidence(s).

Another common conclusion made was that no changes were seen that could be considered treatment, test-article, or test-substance related, or toxicologically relevant. However, the six studies that made this conclusion did not define treatment-related or toxicologically relevant. (Hammond et al., 2006a,b; Healy et al., 2008; Qi et al., 2012; Wang et al., 2002; Zhu et al., 2004). Therefore, they did not provide clearly defined criteria by which to judge if a given tissue was normal or not, and if abnormal, whether the abnormality was toxicologically relevant and/or treatment-related. Some food regulators, such as Food Standards Australia New Zealand (FSANZ, 2007) describe GM food as novel food. In other words, they recognise that no definition yet exists for toxicologically relevant or test-substance related changes. However, by applying the test for substantial equivalence, food regulators argue that an existing compound or plant of known toxicity can be used to evaluate or predict the action of a novel compound or food such as a GM crop (FSANZ, 2007; König et al., 2004; Kuiper and Kleter, 2003). If so, defining what is toxicologically relevant or test-substance related should be a simple task. Consequently, the published studies should have been able to supply the definition and evidence that the results showed no treatment-related or toxicologically relevant changes.

If an existing compound can't predict the action of a GM crop on animal health, further investigation would be necessary. Known toxicity of single components of the GM crop may not define an overall toxicity of the entire crop. It is not clear whether the test for substantial equivalence is sufficient because it does not take into account the changes that could arise from the transformation process: (1) through the random insertion of the genes, (2) through the genetic alterations made to the transferred genes as a result of the transformation process, (3) through the genetic alterations made to the plant as a result of the transformation process (Wilson et al., 2006), (4) through the insertion of several traits or genes into one crop or (5) through the alteration made to the genes encoding the desired trait prior to the transformation.

### 4.3. *Lack of transparency in results*

Several of the reviewed publications do not adequately report their results. Some do not even provide any results (Table 2). For example, the paper by Zhu et al. (2004) not only lacks a detailed methods section, but also limits its histopathological results to a simple statement that "although some slight lesions (such as slightly dilated alveolus cavity, pelvic dilation of the kidneys, slight disconnection of myocardial fibre and collapse of jejunum villi) occurred in rats examined, they were not treatment related." Such a statement could imply that other changes may have been observed, but are not reported. Furthermore, this study does not mention the incidence or severity of any histopathological changes, including whether they occurred in the treatment or non-treatment group. For example, they do not state how many rats showed collapsed jejunum villi and whether these were more prevalent in one group or whether the collapsed villi were more severe in one group. A lack of transparency in results does not allow other researchers to judge whether a certain finding is pathologically relevant. Another paper (Tutel'ian et al., 2010) indicated that they had performed a morphometric analysis of the small and large intestines, but they did not report the colon results. A lack of transparency is also evident in two other studies: 1) Hammond et al. (2004) report the findings from "only those tissues with an incidence of 2 or more findings"; and 2) Healy et al. (2008) state that "findings in

Notes to Table 1

[a] Each line contains one crop. Succeeding names are the other names given to the crop event name and/or the crop's code name.

[b] GM crop hybrids are not listed in the table, unless the crop is listed in databases as a single event name. An exception is the GM soybean line 305423 × 40-3-2, since a feeding study publication was found during the search for publications.

[c] The year that the crop was first approved somewhere in the world for human and/or animal consumption.

[d] One publication contained results for two feeding studies – one on MON810 corn and the other on GA21 corn. As these are two separate feeding studies they have been counted as two published studies.

[e] ASR368 creeping bentgrass is solely intended for the production of turfgrass in golf courses, but it can be used as livestock feed (CERA, 2012).

[f] One study generating two published reports. The first reported the results for the analysis of morphological, haematological, and biochemical parameters and system biomarkers (Tutel'ian et al., 2010). The second reported the allergenic potential and immunoreactivity, as well as looked for signs of genotoxicity (Tyshko et al., 2010). Since the reports are of the same study, they have been counted as one published study.

[g] The GM crop or event name was not listed in three publications. The GM crop studied contained the *EPSPS* gene, which confers glyphosate tolerance. This was not counted as a separate event line in the final number of GM crop event lines studied nor in the total number of GM crop event lines.

[h] One study generating two published reports. The first reported the results for the analysis of morphological, haematological, and biochemical parameters and system biomarkers (Tutel'ian et al., 2008). The second reported the allergenic potential and immunoreactivity, as well as looked for signs of genotoxicity (Tyshko et al., 2008). Since the reports are of the same study, they have been counted as one published study.

[i] The Bt tomatoes may never have been released. The feeding study by Noteborn et al. (1995) looked at the effect of only RLE13-0009 on the rat. The effect of RLE6-10001 was not investigated in the rat, but in other laboratory animals.

**Table 2**
Summary of published studies in order of trait and publication date.

| Study | GM component (event or crop name) | Duration | Purpose of study | No. of rats/ treatment group | Histopathology performed and analysis of GI tract | Histopathological results for GI tract | Feeding study guidelines |
|---|---|---|---|---|---|---|---|
| *EPSPS* | | | | | | | |
| Tutel'ian et al. (1999) | 1.25 g/rat/day of GM soy (RR soy) | 5 months | To investigate the blood, urea and liver of animals fed GM soy | Not stated | No histopathology performed | NA | |
| Teshima et al. (2000) | 30% GM soy | 15 weeks | Study of the immune system of rats and mice | 5 | Payer's patches collected for histopathology, specific area of the small intestine not mentioned. Histopathological examination of H&E stained sections assessed structure of crypt and composition of cells (especially goblet cells and intraepithelial lymphocytes) according to Kawabata (1996). However, Kawabata did not contain guidelines as to how this assessment should be performed and what observations would be considered to be abnormal. No other details were provided as to how the histopath. assessment/analysis was performed. | No difference of crypt structure or goblet cell frequency. Results for intraepithelial lymphocytes were not stated. No actual data of any analyses were shown. Conclusion: No diagnostically significant abnormalities observed in the mucosa of the small intestine. | |
| Tutel'ian et al. (2001) | 3 g/rat/day of GM corn (GA21) | 6 months | To investigate the blood, urea and liver of animals fed GM corn GA21 or GM corn MON810 | Not stated | No histopathology performed | NA | |
| Zhu et al. (2004) | 30–90% GM soy (RR soy) | 13 weeks | Nutritional assessment and fate of DNA | 10 | Stomach and intestine collected for histopathology. Sections stained with H&E and examined by board-certified pathologist using LM. However, no details were given as to what histopath. analyses were performed. | Collapse of jejunum villi was observed, but actual incidence, including incidence in treatment and/or non-treatment group is not reported. No actual data of any analyses were shown. Conclusion: No treatment related differences seen. | |
| Hammond et al. (2004) | 11–33% GM corn (NK603) | 13 weeks | General study to assess the effect of the GM corn on rat health | 20 | Stomach, duodenum, jejunum, ileum, colon, and rectum collected for histopathology. Sections stained with H&E and examined by a board-certified pathologist using LM. However, no details were given as to what histopath. analyses were performed. | Incidence of microscopic findings was only listed for those tissues with an incidence of 2 or more findings. No mention of any GI tract observations. Conclusion: According to the examining pathologist, appearance of tissue was within normal limits. Microscopic changes observed were those that are typically seen in rats of this age and strain. | Modified from OECD 408 (1981) |
| Sakamoto et al. (2007) | 30% GM soy (RR soy) | 26 and 52 weeks | General study to assess the effect of the GM soy on rat health | 10 | Stomach and intestines collected for histopathology. Sections stained with H&E. However, no details were given as to what histopath. analyses were performed. The method used for the eosinophil and goblet cell counts in the jejunum was described in a table caption. | Pathological findings showed no meaningful differences between rats fed GM or non-GM soybeans. Relatively detailed results given for histological findings including eosinophil and goblet cell counts for jejunum. Gastric gland, dilatation of slight grade was observed in all groups. Conclusion: No obvious differences observed between GM and non-GM fed rats. | |
| Sakamoto et al. (2008) | 30% GM soy (RR soy) | 52 and 104 weeks | General study to assess the effect of the GM soy on rat health | 50 | Stomach and intestines collected for histopathology. Sections stained with H&E. However, no details were given as to what histopath. analyses were performed. | Detailed results given of the incidence of neoplastic and non-neoplastic lesions observed. No incidence or increase in incidence of any specific type of neoplastic or non-neoplastic lesions in GM fed group in both genders and there were no lesions reported in the GI tract. Conclusion: No meaningful differences between rats fed GM and non-GM soy. | |
| Daleprane et al. (2009) | 10% GM soy | 15 months (455 days) | Nutritional study – study of growth and haematology of rats on GM soy, non-GM soy or conventional diet | 10 | No histology performed | NA | |

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| Daleprane et al. (2010) | 10% GM soy | 15 months (455 days) | Health of the aorta of rats on GM soy, non-GM soy or conventional diet | 10 | Histology of the aorta | NA | |
| Tutel'ian et al. (2010) | 38% GM soy (MON89788) | 30 and 180 days | General study to assess the effect of the GM soy on rat health | 50 | Safety of the GM crop was examined as in Tutel'ian et al. (2008). Review of macro- and microscopic examinations according to textbook guidelines (Lillie, 1969). Morphometric analysis of the small intestine and colon performed with the aid of computer programme AxioVision. No other information was provided as to what other organs were collected and what histopath. analyses were performed. | Detailed results were given of morphometric analysis of the small intestine, but which section of the small intestine these results pertain to, was not mentioned. No results given for morphometric analysis of the colon.<br>Conclusion: Morphological analysis did not reveal toxic effect of GM soy. | |
| Seralini et al. (2012) | 11%, 22% and 30% GM corn (NK603) | 2 years | General study to assess the effect of the GM corn on rat health | 10 | Oesophagus, stomach, duodenum, jejunum, ileum, Payer's patches, and colon collected for histopathology. Sections stained with HES. However, no details of what histopath. analyses were performed. | Results for histopathological analysis of GI tract were not provided. | |
| Qi et al. (2012) | 7.5%, 15% and 30% GM soy (305423 × 40-3-2) | 90 days | General study to assess the effect of the GM soy on rat health | 10 | Stomach, duodenum, jejunum, and ileum collected for histopathology. Sections stained with H&E and examined by a pathologist from the Chinese Academy of Medical Sciences. However, no details of what histopath. analyses were performed. | No observations or results listed for GI tract.<br>Conclusion: No test-substance related observations. | |
| *Cry3Bb1 and EPSPS* | | | | | | | |
| Healy et al. (2008) | 11–33% GM corn (MON88017) | 13 weeks | General study to assess the effect of the GM corn on rat health | 20 | Stomach, duodenum, jejunum, ileum, colon, and rectum collected for histopathology. Sections stained with H&E and examined by board-certified veterinary pathologist using LM. However, no details of what histopath. analyses were performed. | Results table provided with microscopic findings, however findings for tissues that had an incidence of 1/20 were not reported. No mention of any GI tract results/observations.<br>Conclusion: No test-article related lesions. | Modified from OECD 408 (1998) |
| Tutel'ian et al. (2008) | 11 g/rat/day of GM corn (MON88017) | 30 and 180 days | General study to assess the effect of the GM corn on rat health | Not stated | Review of macro- and microscopic examinations according to textbook guidelines (Lillie, 1969) with the morphometric analyses performed on the internal organs according to Avtandilov (1982, 1990) and Stefanov (1985). List of organs collected for histopath. analysis was not provided. | Detailed results are given of morphometric analysis of ileum. No mention of results for any other area of the GI tract.<br>Conclusion: Morphological analysis did not confirm any toxic effect of GM corn. | |
| *Cry3Bb1* | | | | | | | |
| Hammond et al. (2006b) | 11–33% GM corn (MON863) | 90 days | General study to assess the effect of the GM corn on rat health | 20 | Stomach, duodenum, jejunum, ileum, colon, and rectum collected for histopathology. Histopath. examination was performed by a pathologist at Covance laboratories. However, no details were provided as to what histopath. methods were used including what stains were used and what analyses were performed. | Parasitism was observed in the rectum and glandular dilatation was observed in the stomach of animals in both GM and non-GM groups. The severity/degree of the parasitism or glandular dilatation was not mentioned.<br>Conclusion: Differences if seen were not considered to be test article related. | Modified from OECD 408 (1981) |
| *Cry1Ab* | | | | | | | |
| Noteborn et al. (1995) | 10% GM tomato (RLE13-0009) | 91 days | Study to see if the Cry1Ab protein on its own or in the GM tomato acts on mammals in a similar way as on target insect guts | 12 | Histological analysis was still in progress at the time of publication. | NA | |
| Tutel'ian et al. (2001) | 3 g/rat/day GM corn (MON810) | 6 months | To investigate the blood, urea and liver of animals fed GM corn GA21 or GM corn MON810 | Not stated | No histopathology performed | NA | |
| Wang et al. (2002) | 19–64% GM rice (KMD1) | 14 weeks (90 days) | General study to assess the effect of the GM rice on rat health | 20 | Stomach, duodenum, jejunum, ileum, colon, and rectum collected for histopathology. Sections were stained with H&E. However, no details of what histopath. analyses were performed. | No mention of any GI tract observations.<br>Conclusion: No toxicologically relevant changes. | |

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| Hammond et al. (2006a) | 11–33% GM corn (MON810) | 90 days | General study to assess the effect of the GM rice on rat health | 20 | Methods section indicates that histopathological examination was performed in the same manner as Hammond et al. (2004). No other details provided. | No mention of any GI tract observations. Conclusion: No treatment-related observations. | Modified from OECD 408 (1981) |
| Schrøder et al. (2007) | 60% GM rice (KMD1) | 90 days | General study to assess the effect of the GM rice on rat health | 16 (10 used in histopath. examination) | Stomach (fore and glandular), duodenum, jejunum, ileum, caecum, colon, and rectum collected for histopathology. Sections stained with H&E. "Main focus of histopathological examination was on intestinal tract and related organs", but no specific details given as to the type of analyses performed. | No mention of any pathological findings in the GI tract. Conclusion: No dose-related changes were observed in the intestinal tract and related organs. | OECD 408 (1981) with modifications[a] |
| Kroghsbo et al. (2008) | 60% GM rice (KMD1) | 28 and 90 day | Immuno study | 10 | No histopathology performed | NA | |

[a] Modifications from Consultation Meeting of Experts on Sub-chronic and Chronic Toxicity Testing (1995).

other tissues with an incidence of 1/20 are not reported." Neither of these papers provided a full account of pathologies present. Furthermore, Hammond et al. (2004) do not clearly state whether "incidence" pertains to two incidences per tissue or per rat. Such a lack of information does not ensure that the study and its results are reproducible or even comparable.

### 4.4. Relevance of OECD guidelines in the evaluation of the safety of consuming GM crops

Five of the published studies indicate that OECD 408 guidelines were used to assist in planning the study (Table 2). The OECD 408 guidelines are designed to test for carcinogenicity of compounds. The guidelines provide details on how such a feeding study should be conducted, including information on sample size, duration etc. However, the guidelines do not specify the histopathological analysis that should be performed. For example, what histopathological parameters should be used to detect or measure the carcinogenicity of a compound. Whilst it's our view that histopathological methods to determine carcinogenicity are well established in the scientific community, the effect of GM feed on animal health is not. In addition, the carcinogenic potential of a GM crop is not, and should not be, the only pathology investigated. Therefore, there is a question as to whether these OECD guidelines are relevant to investigation of the safety of consuming GM crops. Whilst they may be used as a starting point, it is our view that guidelines should be established specifically for GM crops. Since GM food is considered to be a novel food, the guidelines should list details for a thorough investigation that includes a histopathological analysis of the gut and other organs. In other models of GI tract damage, such as mucositis (Howarth et al., 1996; Logan et al., 2009; Sukhotnik et al., 2008), neonatal adjustment of piglets to normal diet (Godlewski et al., 2009; Strzalkowski et al., 2007), or in gastric biopsies (Fenoglio-Preiser, 1998; Staibano et al., 2002), the analytical method is detailed and specific, listing the changes that need to be investigated and the microscopic techniques and morphometric analyses that need to be used. For example, mitosis, apoptosis and autophagy are known to be good indicators of mucosal regeneration in the small intestine following injury. Therefore, immunohistochemistry with in-tissue cytometry looking at the expression of markers for mitosis (Ki67), apoptosis (caspase 3) and autophagy (MAP I LC3) can be used to assess mucosal regeneration (Godlewski et al., 2009). In mucositis-induced models, the investigation of the degree of damage regularly requires not only detailed quantitative histological analyses to be conducted (Howarth et al., 1996; Logan et al., 2009; Sukhotnik et al., 2008), but also immunohistochemistry for markers of apoptosis (caspase 3), cell proliferation (BrdU) (Sukhotnik et al., 2008), and pro-inflammatory cytokines (such as TNF, IL-1β and IL-6) (Logan et al., 2009). Such vigorous analyses allow for a more precise assessment of possible pathological changes, whilst at the same time decreasing the chance of subtle changes being overlooked. Therefore, it is our view that in the investigation of the safety of GM crops on animal and human health, such a vigorous and in-depth approach should also be implemented.

### 4.5. Have enough studies been conducted to adequately state that GM crops are safe for human and animal consumption?

Genetically modified crops have been approved for human and animal consumption for nearly 20 years (Clive and Krattiger, 1996) yet the debate about their safety continues. Fifty-three crops are known to possess at least one of the genes investigated in this review (herbicide tolerance via the *EPSPS* gene and insect resistance via the *cry1Ab* or *cry3Bb1* genes). Forty-seven of these crops have been approved for animal and/or human consumption, yet published toxicity studies could be found for only nine of these crops (19%) (Table 1). Of greater concern is that for eight of these crops, publications appeared after the crop had been approved for human and/or animal consumption. We understand that other studies may exist that are commercial in confidence, but these

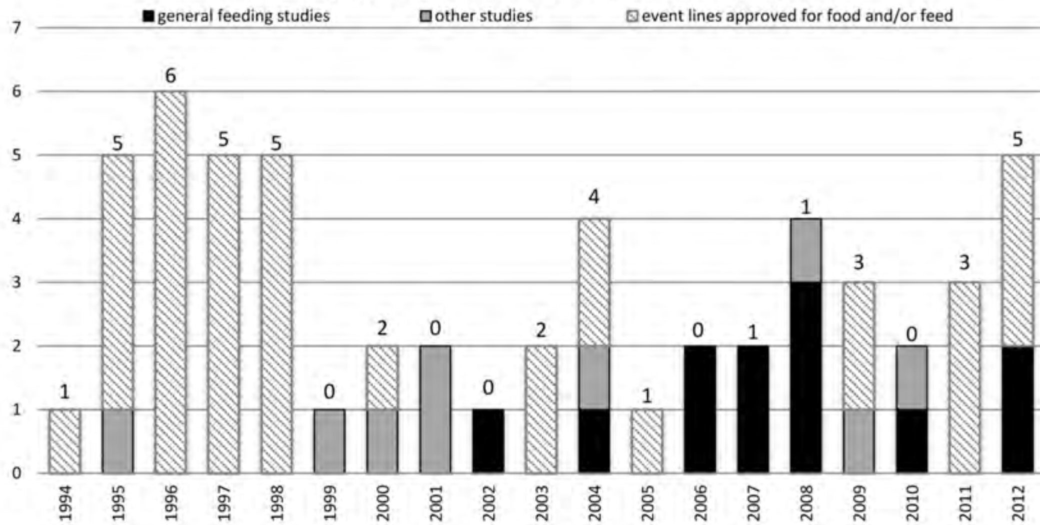


**Fig. 1.** Number of publications looking at the effects of feeding rats GM crops long-term and number of event lines approved each year for human and/or animal consumption. Studies researched the effects of ingesting GM crops that possessed the traits for herbicide tolerance (via the *EPSPS* gene) and insect resistance (via the *cry1Ab* or *cry3Bb1* genes). Studies investigating the general long-term effect of GM feed on rat health are indicated in black. Other studies investigating certain specific effect of GM feed on rats are indicated in grey. Striped bars indicate the number of GM crop event lines possessing the traits for herbicide tolerance (via the *EPSPS* gene) and insect resistance (via the *cry1Ab* or *cry3Bb1* genes) that were approved each year somewhere in the world for human and/or animal consumption.. The number of approvals per year is shown as the number above the bar. The year of approval for each event line is only counted once, being the first time the crop was approved somewhere in the world.

studies are not accessible to the scientific community. Other than the few studies mentioned in the EFSA reports, where histopathological results were not reported, our review of the published literature wasn't able to identify or locate any reported safety evaluations performed on rats on these eight crops prior to their approval. Our literature review also did not identify or locate published reports on rats for the remaining 38 crops.

The present review limited the search to only include feeding studies done on rats so that the results may be comparable. It is possible that more studies may be found if the search were to be extended to other

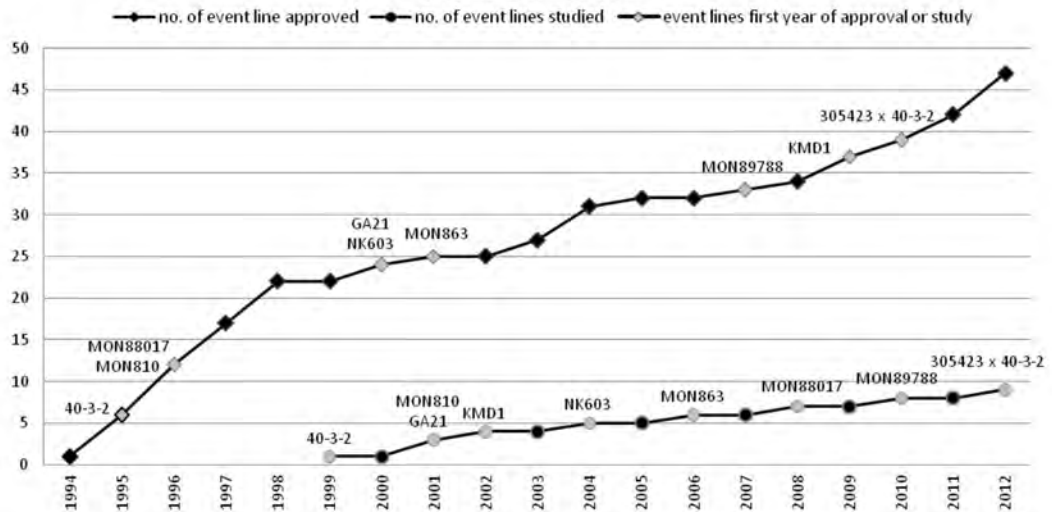


**Fig. 2.** The number of approved GM crops in the world for human and/or animal consumption (◆) and the number of approved GM crop lines with published studies investigating the effects of long-term feeding of these crops (●). Each crop was counted once when the first study appeared investigating that crop. The year of approval is the year that the crop was first approved somewhere in the world.

animals. However, based on what has been found for rat studies, it is unlikely that any additional studies would involve a thorough safety investigation and a detailed report of all of the 47 approved GM crops possessing one or more of the three traits. Moreover, the rat model is the accepted OECD standard for toxicological studies of this type.

Whilst the safety of a GM crop is primarily and sometimes solely evaluated by government food regulators using the test for substantial equivalence, this is likely to be inadequate to fully assess the safety of the crop for reasons stated above. Animal feeding studies provide a more thorough method of investigating the unintended effects of the GM process or the unintended effects of ingesting GM crop components. Animal feeding studies can identify target organs as well as predict the chronic toxic effect of an ingested compound (OECD, 2008).

## 5. Conclusions

The evidence reviewed here demonstrates an incomplete picture regarding the toxicity (and safety) of GM crops consumed by humans and animals. The majority of studies reviewed lacked a unified approach and transparency in their methodology and results, making it impossible to properly review or repeat these studies. Furthermore, such lack of detail makes it difficult to generate evidence-based guidelines to aid in the delivery of an optimum safety assessment process for GM crops for animal and human consumption.

When considering how a better risk assessment could be done, it is important to consider systems established for other novel substances that may generate unintended effects. For example, the registration of pharmaceutical products requires an examination of both benefits and risks associated with their use and a complete assessment of those benefits and risks to establish whether the products are appropriate for general use at a range of doses. We argue that each GM crop should be assessed using similar methods, where a GM crop is tested in the form and at the rates it will be consumed by animals and people.

Whilst this provides for an effective general approach, there are additional issues for assessing GM crops that need to be taken into account. For example, the process of developing GM crops may generate unintended effects. Furthermore, the plant developed is a novel entity with genes, regulatory sequences and proteins that interact in complex ways. Therefore, the resultant plant should be assessed as a whole so that any pleiotropic effects can also be assessed. As a result, long-term animal feeding studies should be included in risk assessments of GM crops, together with thorough histopathological investigations using a variety of methods to better detect subtle changes or the beginning or presence of pathologies. Such robust and detailed studies will then make it possible to put evidence-based guidelines in place, which will substantially help to determine the safety of GM crops for human and animal consumption.

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## References

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, et al. Particle bombardment and the genetic enhancement of crops: myths and realities. Mol Breed 2005;15:305–27.

Avtandilov GG. [Introduction to quantifying pathological morphology] Medicine, Moscow; 1982 (in Russian).

Avtandilov GG. [Medical morphometry. Manual] Medicine, Moscow; 1990 (in Russian).

CERA. GM crop database. Washington D.C.: Center for Environmental Risk Assessment (CERA). ILSI Research Foundation; 2012 [http://cera-gmc.org/index.php?action=gm_crop_database].

Chandra M, Riley MI, Johnson D. Spontaneous neoplasms in aged Sprague–Dawley rats. Arch Toxicol 1992;66:496–502.

Chen M, Shelton A, Ye GY. Insect-resistant genetically modified rice in China: from research to commercialization. Annu Rev Entomol 2011;56:81–101.

Christou P. Genetic transformation of crop plants using microprojectile bombardment. Plant J 1992;2:275–81.

Christou P, McCabe DE, Swain WF. Stable transformation of soybean callus by DNA-coated gold particles. Plant Physiol 1988;87:671–4.

Clive J. Global status of commercialized biotech/GM crops: 2013. ISAAA brief No. 46. Ithaca: ISAAA; 2013.

Clive J, Krattiger AF. Global review of the field testing and commercialization of transgenic plants, 1986 to 1995: the first decade of crop biotechnology. Ithaca: ISAAA; 1996.

Dai S, Zheng P, Marmey P, Zhang S, Tian W, Chen S, et al. Comparative analysis of transgenic rice plants obtained by *Agrobacterium*-mediated transformation and particle bombardment. Mol Breed 2001;7:25–33.

Daleprane JB, Feijó TS, Boaventura GT. Organic and genetically modified soybean diets: consequences in growth and in hematological indicators of aged rats. Plant Foods Hum Nutr 2009;64:1–5.

Daleprane JB, Chagas MA, Vellarde GC, Ramos CF, Boaventura GT. The impact of non- and genetically modified soybean diets in aorta wall remodeling. J Food Sci 2010;75: T126–31.

de Vendomois JS, Roullier F, Cellier D, Seralini GE. A comparison of the effects of three GM corn varieties on mammalian health. Int J Biol Sci 2009;5:706–26.

Domingo JL. Health risk of GM foods: many options but few data. Science 2000;288:1748–9.

Domingo JL. Toxicity studies of genetically modified plants: a review of the published literature. Crit Rev Food Sci Nutr 2007;47:721–33.

Domingo JL, Bordonaba JG. A literature review on the safety assessment of genetically modified plants. Environ Int 2011;37:734–42.

EFSA (European Food Safety Agency). Opinion of the scientific panel on genetically modified organisms on application (reference EFSA-GMO-NL-2006-36) for the placing on the market of the glyphosate-tolerant genetically modified soybean MON89788, for food and feed uses, import and processing under regulation (EC) no 1829/2003 from Monsanto. EFSA J 2008;758:1–23.

EFSA (European Food Safety Agency). Scientific opinion on application (EFSA-GMO-CZ-2008-62) for the placing on the market of insect resistant and herbicide tolerant genetically modified maize MON 89034 × 1507 × MON 88017 × 59122 and all subcombinations of the individual events as present in its segregating progeny, for food and feed uses, import and processing under regulation (EC) no 1829/2003 from Dow AgroSciences and Monsanto. EFSA J 2010;8(1781). [1737 pp.].

FAO/WHO (Food and Agricultural Organisation of the United Nations/World Health Organisation). Safety aspects of genetically modified foods of plant origin. Report of a joint FAO/WHO expert consultation on foods derived from biotechnology (29 May–2 June, 2000). Geneva; 2000.

Fenoglio-Preiser C. Creating a framework for diagnosing the benign gastric biopsy. Curr Diagn Pathol 1998;5:2–6.

Frantz J, Betton G, Cartwright M, Crissman J, Macklin A, Maronpot R. Proliferative lesions of the non-glandular and glandular stomach in rats. Guides for toxicologic pathology. Washington: STP/ARP/AFIP; 1991.

FSANZ (Food Standards Australia New Zealand). The role of animal feeding studies in the safety assessment of genetically modified foods. Report of a workshop hosted by Food Standards Australia New Zealand (Friday 15th June 2007). Canberra: FSANZ; 2007.

FSANZ (Food Standards Australia New Zealand). Food derived from GM plants containing stacked genes. http://www.foodstandards.gov.au/consumer/gmfood/stackedgene/Pages/default.aspx, 2010.

FSANZ (Food Standards Australia New Zealand). GM current applications and approvals. Genetically modified (GM) foods. http://www.foodstandards.gov.au/consumerinformation/gmfoods/gmcurrentapplication1030.cfm, 2011.

Gasson M. The safety evaluation of genetically modified foods. In: D'Mello JPF, editor. Food safety: contaminants and toxins. Wallingford: CABI Publishing; 2003.

Godlewski MM, Slazak P, Zabielski R, Piastowska A, Garlak MA. Quantitative study of soybean-induced changes in proliferation and programmed cell death in the intestinal mucosa of young rats. J Physiol Pharmacol 2006;57(Suppl. 7):125–33.

Godlewski MM, Bierla JB, Strzalkowski A, Martinez-Puig D, Pajak B, Kotunia A, et al. A novel cytometric approach to study intestinal mucosa rebuilding in weaned pigs fed with dietary nucleotides. Livest Sci 2009;123:215–20.

Hammond BG, Dudek R, Lemen JK, Nemeth M. Results of a 13 week safety assurance study with rats fed grain from glyphosate tolerant corn. Food Chem Toxicol 2004; 42:1003–14.

Hammond B, Dudek R, Lemen JK, Nemeth MA. Results of a 90-day safety assurance study with rats fed grain from corn borer-protected corn. Food Chem Toxicol 2006a;44: 1092–9.

Hammond B, Lemen J, Dudek R, Ward D, Jiang C, Nemeth M, et al. Results of a 90-day safety assurance study with rats fed grain from corn rootworm-protected corn. Food Chem Toxicol 2006b;44:147–60.

Healy C, Hammond B, Kirkpatrick J. Results of a 13-week safety assurance study with rats fed grain from corn rootworm-protected, glyphosate-tolerant MON 88017 corn. Food Chem Toxicol 2008;46:2517–24.

Howarth GS, Francis GL, Cool JC, Xu X, Byard RW, Read LC. Milk growth factors enriched from cheese whey ameliorate intestinal damage by methotrexate when administered orally to rats. J Nutr 1996;126:2519–30.

ISAAA. ISAAA's GM approval database. http://www.isaaa.org/gmapprovaldatabase/, 2013.

Kaspareit J, Rittinghausen S. Spontaneous neoplastic lesions in Harlan Sprague–Dawley rats. Exp Toxicol Pathol 1999;51:105–7.

Kawabata TK. Immunotoxicology of the gastrointestinal tract. In: Holsapple MP, Smialowicz RJ, editors. Experimental immunotoxicology. CRC Press; 1996. p. 143–55.

Kikuchi M, Nagata H, Watanabe N, Watanabe H, Tatemichi M, Hibi T. Altered expression of a putative progenitor cell marker DCAMKL1 in the rat gastric mucosa in regeneration, metaplasia and dysplasia. BMC Gastroenterol 2010;10:65.

König A, Cockburn A, Crevel RWR, Debruyne E, Grafstroem E, Hammerling U, et al. Assessment of the safety of foods derived from genetically modified (GM) crops. Food Chem Toxicol 2004;42:1047–88.

Kroghsbo S, Madsen C, Poulsen M, Schrøder M, Kvist PH, Taylor M, et al. Immunotoxicological studies of genetically modified rice expressing PHA-E lectin or Bt toxin in Wistar rats. Toxicology 2008;245:24–34.

Kuiper HA, Kleter GA. The scientific basis for risk assessment and regulation of genetically modified foods. TIFS 2003;14:277–93.

Kuiper HA, Kleter GA, Noteborn HPJM, Kok EJ. Assessment of the food safety issues related to genetically modified foods. Plant J 2001;27:503–28.

Lillie RD. Histopathological techniques and practical histochemistry. Moscow: Mir; 1969.

Logan RM, Stringer AM, Bowen JM, Gibson RJ, Sonis ST, Keefe DM. Is the pathobiology of chemotherapy-induced alimentary tract mucositis influenced by the type of mucotoxic drug administered? Cancer Chemother Pharmacol 2009;63:239–51.

Magaña-Gómez JA, De La Barca AM. Risk assessment of genetically modified crops for nutrition and health. Nutr Rev 2009;67(1):1–16.

Morini G, Grandi D. Methods to measure gastric mucosal lesions in the rat. Curr Protoc Toxicol 2010. [Chapter 21: Unit 21.2.1-15].

Noteborn HPJM, Bienenmann-Ploum ME, van den Berg JHJ, Alink GM, Zolla L, Reynaerts A, et al. Safety assessment of the *Bacillus thuringiensis* insecticidal crystal protein CRYIAB(b) expressed in transgenic tomatoes. In: Engel K-H, Takeoka GR, Teranishi R, editors. Genetically modified foods: safety issues. Washington: American Chemical Society; 1995.

OECD (Organisation for Economic Co-operation and Development). Test no. 408: repeated dose 90-day oral toxicity study in rodents, guideline for the testing of chemicals. Paris: OECD; 1981.

OECD (Organisation for Economic Co-operation and Development). Test no. 408: repeated dose 90-day oral toxicity study in rodents, guideline for the testing of chemicals. Paris: OECD; 1998.

OECD (Organisation for Economic Co-operation and Development). Test no. 452: chronic toxicity studies. Guideline for the testing of chemicals. Paris: OECD; 2008.

Padgette SR, Kolacz KH, Delannay DB, LaVallee BJ, Tinius CN, Rhodes WK, et al. Development, identification, and characterization of glyphosate-tolerant soybean line. Crop Sci 1995;35:1451–61.

Piastowska-Ciesielska AW, Gralak MA. Influence of a low dose of dietary soybean on bone properties and mineral status in young rats. Biofactors 2010;36:451–8.

Pusztai A, Bardocz S, Ewen SW. Genetically modified foods: potential human health effects. In: D'Mello JPF, editor. Food safety: contaminants and toxins. Wallingford: CABI Publishing; 2003.

Qi X, He X, Luo Y, Li S, Zou S, Cao S, et al. Subchronic feeding study of stacked trait genetically-modified soybean (3Ø5423 × 40-3-2) in Sprague–Dawley rats. Food Chem Toxicol 2012;50:3256–63.

Sakamoto Y, Tada Y, Fukumori N, Tayama K, Ando H, Takahashi H, et al. A 52-week feeding study of genetically modified soybeans in F344 rats. J Food Hyg Soc Jpn 2007;48:41–50. [in Japanese].

Sakamoto Y, Tada Y, Fukumori N, Tayama K, Ando H, Takahashi H, et al. A 104-week feeding study of genetically modified soybeans in F344 rats. J Food Hyg Soc Jpn 2008;49:272–82. [in Japanese].

Schilter B, Constable A. Regulatory control of genetically modified (GM) foods: likely developments. Toxicol Lett 2002;127:341–9.

Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, et al. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 1998;62:775–806.

Schrøder M, Poulsen M, Wilcks A, Kroghsbo S, Miller A, Frenzel T, et al. A 90-day safety study of genetically modified rice expressing Cry1Ab protein (*Bacillus thuringiensis* toxin) in Wistar rats. Food Chem Toxicol 2007;45:339–49.

Şener G, Paskaloglu K, Kapucu C, Cetinel S, Contuk G, Ayanoğlu-Dülger G. Octreotide ameliorates alendronate-induced gastric injury. Peptides 2004;25:115–21.

Seralini GE, Cellier D, de Vendomois JS. New analysis of a rat feeding study with a genetically modified maize reveals signs of hepatorenal toxicity. Arch Environ Contam Toxicol 2007;52:596–602.

Seralini GE, Clair E, Mesnage R, Gress S, Defarge N, Malatesta M, et al. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food Chem Toxicol 2012;50:4221–31. [Retracted and republished as: Séralini, G.E.; Clair, E.; Mesnage, R.; Gress, S.; Defarge, N.; Malatesta, M., et al., 2014. Republished study: long-term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Environ. Sci. Eur. 26 (1): 14].

Snell C, Bernheim A, Berge J-B, Kuntz M, Pascal G, Paris A, et al. Assessment of the health impact of GM plant diets in long-term and multigenerational animal feeding trials: a literature review. Food Chem Toxicol 2012;50:1134–48.

Staibano S, Rocco A, Mezza E, De Rosa G, Budillon G, Nardone G. Diagnosis of chronic atrophic gastritis by morphometric image analysis. A new method to overcome the confounding effect of the inflammatory infiltrate. J Pathol 2002;198:47–54.

Stefanov SB. Visual classification during quantitative comparison of images. Arkh Anat Gistol Embriol 1985;88:78–83. [in Russian].

Strzalkowski AK, Godlewski MM, Hallay N, Kulasek G, Gajewski Z, Zabielski R. The effect of supplementing sow with bioactive substances on neonatal small intestinal epithelium. J Physiol Pharmacol 2007;58:115–22.

Sukhotnik I, Shehadeh N, Coran AG, Mogilner JG, Karry R, Shamian B, et al. Oral insulin enhances cell proliferation and decreases enterocyte apoptosis during methotrexate-induced mucositis in the rat. JPGN 2008;47:115–22.

Tang M, Xie T, Cheng W, Qian L, Yang S, Yang D, et al. A 90-day safety study of genetically modified rice expressing rhIGF-1 protein in C57BL/6J rats. Transgenic Res 2012;21:499–510.

Tarnawski A, Douglass TG, Stachura J, Krause WJ. Quality of gastric ulcer healing: histological and ultrastructural assessment. Aliment Pharmacol Ther 1991;5(Suppl. 1):79–90.

Teshima R, Akiyama H, Okunuki H, Sakushima J-i, Goda Y, Onodera H, et al. Effect of GM and non-GM soybeans on the immune system of BN rats and B10A mice. J Food Hyg Soc Jpn 2000;41:188–93.

Tutel'ian VA, Kravchenko LV, Lashneva NV, Avren'eva LI, Guseva GV, Sorokina E, et al. Medical and biological evaluation of safety of protein concentrate from genetically-modified soybeans. Biochemical studies. Vopr Pitan 1999;68:9–12. [in Russian].

Tutel'ian VA, Kravchenko LV, Sorokina E, Korolev AA, Avren'eva LI, Guseva GV, et al. Medical and biological assessment of the safety of genetically modified corn lines MON 810 and GA 21: a toxicological–biochemical study. Vopr Pitan 2001;70:28–31. [in Russian].

Tutel'ian VA, Gapparov MM, Avren'eva LI, Aksiuk IN, Guseva GV, Kravchenko LV, et al. Medical and biological safety assessment of genetically modified maize event MON 88017. Report 1. Toxicologo-hygienic examinations. Vopr Pitan 2008;77:4–12. [in Russian].

Tutel'ian VA, Gapparov MG, Avren'eva LI, Guseva GV, Zhminchenko VM, Kravchenko LV, et al. Medical and biological safety assessment of genetically modified soybean event MON 89788. Report 1. Toxicologo-hygienic examinations. Vopr Pitan 2010;79:4–12. [in Russian].

Tyshko NV, Britsina MV, Gmoshinskii IV, Zhanataev AK, Zakharova NS, Zorin SN, et al. Medical and biological safety assessment of genetically modified maize event MON 88017. Report 2. Genotoxicologic, immunologic and allergologic examinations. Vopr Pitan 2008;77:13–7. [in Russian].

Tyshko NV, Britsina MV, Gmoshinskii IV, Zakharova NS, Zorin SN, Mazo VK, et al. Medical and biological safety assessment of genetically modified soybean event MON 89788. Report 2. Genotoxicologic, immunologic and allergologic examinations. Vopr Pitan 2010;79:13–7. [in Russian].

Vaeck M, Reynaerts A, Hofte H, Jansens S, De Beuckeleer M, Dean C, et al. Transgenic plants protected from insect attack. Nature 1987;328:33–7.

Wang Z-h, Wang Y, Cui H-r, Xia Y-w, Altosaar I, Shu Q-y. Toxicological evaluation of transgenic rice flour with a synthetic cry1Ab gene from *Bacillus thuringiensis*. JSFA 2002;82:738–44.

Wilson AK, Latham JR, Steinbrecher RA. Transformation-induced mutations in transgenic plants: analysis and biosafety implications. Biotechnol Genet Eng Rev 2006;23:209–37.

Windels P, Taverniers I, Depicker A, Van Bockstaele E, De Loose M. Characterisation of the Roundup Ready soybean insert. Eur Food Res Technol 2001;213:107–12.

Zhu Y, Li D, Wang F, Yin J, Jin H. Nutritional assessment and fate of DNA of soybean meal from roundup ready or conventional soybeans using rats. Arch Anim Nutr 2004;58:295–310.