Fate of transgenic DNA and protein in pigs fed genetically modified Bt maize and effects on growth and health

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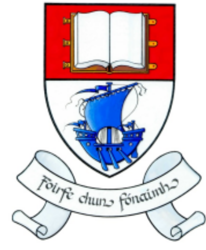

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Table of Contents

Declaration ... 5

List of tables ... 6

List of figures ... 9

Abstract ... 11

1. Literature review ... 12

1.1. Introduction ... 13

1.2. GMO definition and technology used to create GMOs ... 14

1.2.1. GMO history and present status ... 15

1.2.2. Bt MON810 maize ... 16

1.2.3. Risk assessment ... 18

1.2.4. Consumer acceptance of GMOs ... 19

1.2.5. Post-market monitoring ... 20

1.3. Assessment of safety of GM maize in animal feeding trials ... 20

1.3.1. Nutritional equivalence and presence of contaminants ... 21

1.3.2. Effects on growth performance and slaughter characteristics ... 23

1.4. Effects of GM maize on immunological parameters ... 28

1.4.1. Overview of the immune system ... 28

1.4.2. Allergenicity ... 34

1.5. Allergen testing of GMOs ... 37

1.6. Effect of GM maize on intestinal microbiota ... 39

1.6.1. The role of the intestinal microbiota ... 39

1.6.2. Composition of the porcine gastrointestinal microbiota ... 40

1.6.3. Effect of GM maize on intestinal microbiota ... 43

1.7. Effect of GM maize on tissue histology ... 44

1.7.1. Intestinal histology ... 45

1.7.2. Effect of GM maize on intestinal histology ... 48

1.7.3. Tissue histology ... 48

1.7.4. Effect of GM maize on tissue histology ... 49

1.8. Effect of GM maize on haematology and clinical biochemistry ... 50

1.9. Transgene and GM protein transfer ... 53

1.9.1. Transfer of transgene and GM protein to animal tissues ... 54

1.9.2. Gene transfer to bacteria ... 56

1.10. Conclusions 59
1.11. References 60
Overall objectives 75
2. Effects of short-term feeding of Bt MON810 maize on growth performance, organ morphology and function in pigs 76
2.1. Abstract 77
2.2. Introduction 78
2.3. Materials and methods 79
2.4. Results 83
2.5. Discussion 84
2.6. References 96
3. Fate of transgenic DNA from orally administered Bt MON810 maize and effects on immune response and growth in pigs 101
3.1. Abstract 102
3.2. Introduction 103
3.3. Materials and methods 104
3.4. Results 111
3.5. Discussion 114
3.6. References 135
4. High throughput sequence-based analysis of the intestinal microbiota of weanling pigs fed genetically modified Bt MON810 maize for 31 days 140
4.1. Abstract 141
4.2. Introduction 142
4.3. Materials and methods 144
4.4. Results 147
4.5. Discussion 150
4.6. References 169
5. Effect of feeding genetically modified Bt MON810 maize to ~40 day old pigs for 110 days on growth and health indicators 175
5.1. Abstract 176
5.2. Introduction 177
5.3. Materials and methods 178
5.4. Results 182
5.5. Discussion 185

5.6. References 204
6. Effects of feeding Bt MON810 maize to pigs for 110 days on peripheral immune response and digestive fate of the *cry1Ab* gene and truncated Bt toxin........................209
6.1. Abstract 210
6.2. Introduction 212
6.3. Materials and methods 213
6.4. Results 218
6.5. Discussion 221
6.6. References 245
7. The effect of feeding Bt MON810 maize to pigs for 110 days on intestinal microbiota 249
7.1. Abstract 250
7.2. Introduction 251
7.3. Materials and methods 252
7.4. Results 256
7.5. Discussion 258
7.6. References 281
Overall conclusions 287
8. Overall discussion 288
8.1. References 299
Future research 305
Implications 306
Acknowledgements 307

Declaration

No element of the work described in this thesis, unless otherwise stated, has been previously submitted for a degree at this or any other institution. The work in this thesis has been performed entirely by the author.

Signature________________________

Date____________________________

List of tables

Literature review

Table 1.1. Effects of feeding GM Bt maize on farm animal and poultry growth performance and slaughter characteristics…………………………………………......24

Table 1.2. Immune cells involved in innate immunity……………………………......29

Table 1.3. Immune cells involved in adaptive immunity……………………………..30

Table 1.4. Cytokines involved in allergic response…………………………………..36

Table 1.5. Biochemical parameters indicative of liver and kidney function…………51

Experimental

Table 2.1. Composition of acclimatization starter diet and experimental diets……….88

Table 2.2. Proximate, carbohydrate and amino acid analysis of Bt (MON810) maize and isogenic parent line maize…………………………………………………………90

Table 2.3. Histological indicators used for the identification of cell and organ dysfunction in the liver, heart, spleen, kidneys and muscle of weanling pigs fed GM or non-GM maize for 31 days……………………………………………………………..91

Table 2.4. Effects of feeding GM or non-GM maize for 30 days on weanling pig growth performance…………………………………………………………………….92

Table 2.5. Effects of feeding GM maize or non-GM maize for 31 days on organ weights and organo-somatic indices of weanling pigs…………………………………93

Table 2.6. Effects of feeding GM maize or non-GM maize for 31 days on small intestinal histology of weanling pigs……………………………………………………...94

Table 2.7. Effects of feeding GM maize or non-GM maize for 31 days on serum concentrations of enzymes and other parameters to assess the liver and kidney function of weanling pigs……………………………………………………………………...95

Table 3.1. Composition of acclimatization starter diet and experimental diets…….. 119

Table 3.2. Primers used in PCR reactions for the detection of three target sequences in porcine organ, white blood cell and digesta samples…………………………..……122

Table 3.3. Effects of feeding GM maize or non-GM maize with or without mitogenic stimulation on cytokine production from porcine isolated peripheral blood mononuclear cells, splenocytes and *lamina propria* and intraepithelial lymphocytes ……………………………...……………………………………………………124

Table 3.4. Detection of endogenous maize and porcine genes and transgenic *cry1Ab* gene in tissue and white blood cells of pigs fed GM maize versus pigs fed a non-GM maize diet for 31 days …………….……………………………………………..126
Table 3.5. Detection of endogenous maize and porcine genes and transgenic *cry1Ab* gene in stomach, ileal and caecal digesta and colon samples of pigs fed GM maize versus pigs fed a non-GM maize diet for 31 days...…………………………………..127
Table 3.6. Detection of the transgenic Cry1Ab protein in tissue, plasma and gastrointestinal digesta of pigs fed GM and non-GM maize…………………………128
Table 3.7. Effects of feeding GM or non-GM maize for 35 days on weanling pig growth performance …………….…………….……………………………………129
Table 4.1. Chemical composition of maize lines included in pig diets……………...155
Table 4.2. Composition of experimental diets…………………………………......156
Table 4.3. Effect of feeding a Bt maize-based diet for 31 days on faecal and intestinal microbial counts (log_{10} CFU/g) in weanling pigs……………………………………..158
Table 4.4. Estimations of bacterial diversity at 97% similarity within the caecum of weanling pigs fed isogenic and Bt maize-based diets……………………………...159
Table 4.5. Effect of feeding a Bt maize-based diet for 31 days on the relative abundance of caecal bacterial taxa in weanling pigs…………………………………160
Table 4.6. Relative abundance of bacterial taxa in the caecum of weanling pigs fed isogenic or Bt maize-based diets for 31 days…………………………………………161
Table 5.1. Composition of experimental diets………………………………………190
Table 5.2. Effect of feeding GM maize to pigs for 110 days on growth and on organ and carcass weights at day 110……………………………………………………..193
Table 5.3. Effect of feeding GM maize to pigs for 110 days on intestinal histology..196
Table 5.4. Effect of feeding GM maize to pigs for 110 days on serum biochemical parameters indicative of liver function……………………………………………..198
Table 6.1. Composition of experimental diets………………………………………227
Table 6.2. Primers and PCR conditions used for the detection of target genes in porcine organ, white blood cell and digesta samples…………………………………………230
Table 6.3. Cytokine production by resting PBMC from pigs fed diets containing Bt and isogenic maize…………………………………………………………………...231
Table 6.4. Cytokine production by mitogen stimulated PBMC from pigs fed diets containing Bt and isogenic maize……………………………………………………..233

Table 6.5. Effects of feeding Bt and isogenic maize on immune cell phenotypes of peripheral blood mononuclear cells……………………………………………………...235
Table 6.6. Effects of feeding of Bt and isogenic maize on immune cell counts in growing pigs………………………………………………………………………..237
Table 6.7. Effects of feeding of Bt and isogenic maize on haematological parameters in growing pigs………………………………………………………………………..239
Table 6.8. Detection of endogenous maize and porcine genes and transgenic *cry1Ab* gene in the organs and blood of pigs fed Bt and isogenic maize……………………...242
Table 6.9. Detection of endogenous maize and porcine genes and transgenic *cry1Ab* gene in stomach, ileal, caecal and colon digesta of pigs fed Bt and isogenic maize diets...………………………………………………………………………………….243
Table 6.10. Mean Cry1Ab protein concentrations in gastrointestinal digesta of pigs fed Bt and isogenic maize……………………………………………………………….244
Table 7.1. Effect of feeding isogenic or Bt maize-based diets to pigs from 12 days post-weaning for 110 days on faecal bacterial counts………………………………...262
Table 7.2. Effect of feeding isogenic or Bt maize-based diets to pigs from 12 days post-weaning for 110 days on caecal and ileal bacterial counts………………………264
Table 7.3. Estimation of bacterial diversity at 97% similarity in the caecum of pigs fed isogenic or Bt maize-based diets……………………………………………………...265
Table 7.4. Effect of feeding isogenic or Bt maize-based diet to pigs from 12 days post weaning for 110 days on the relative abundance of caecal bacterial taxa in pigs…….266
Table 7.5. Bacterial taxa detected in the caecum of ~150 day-old pigs……………..267
Table 7.6. Individual molecular identifiers used for PCR amplification of the 16S rRNA gene fragments from porcine caecal samples………………………………….271

List of figures

Literature review

Figure 1.1. Plasmid used to produce the MON810 maize.…………………………....17

Figure 1.2. Cells and mediators involved in the allergic response.…………………...37

Figure 1.3. Microbiota in the porcine GIT……………………………………………42

Figure 1.4. Schematic representation of intestinal structures………………………....46

Figure 1.5. Overview of the intestinal epithelial cell types.…………………………..46

Figure 1.6. Means of horizontal gene transfer………………………………………...57

Experimental

Figure 3.1. Fate of transgenic DNA from orally administered Bt MON810 maize in pigs……………………………………………………………………………….130

Figure 3.2. Effects of feeding non-GM maize, GM maize or non-maize feed to pigs for 31 days on plasma concentration of Cry1Ab specific IgG antibody (Experiment 1)…131

Figure 3.3. Effects of feeding non-GM maize, GM maize or non-maize feed to pigs for 31 days on plasma concentration of Cry1Ab specific IgA antibody (Experiment 1)....132

Figure 3.4. Changes in the proportion of different leukocyte populations in the ileum of pigs fed non-GM or GM maize for a period of 35 days (Experiment 2)…………..133

Figure 3.5. Changes in the proportion of different leukocytes populations in the spleen of pigs fed non-GM or GM maize for a period of 35 days (Experiment 2).…………134

Figure 4.1. Effect of feeding a Bt or isogenic maize-based diet for 31 days on the mean relative abundance of the major phyla, families and genera in the caecum of weanling pigs……………………………………………………………………………166

Figure 4.2. Rarefaction curves for caecal bacteria of pigs fed an isogenic maize-based diet or a Bt maize-based diet for 31 days.…………………………………………167

Figure 4.3. Unweighted beta diversity in the caecum of pigs fed an isogenic maize-based diet or a Bt maize-based diet for 31 days.…………………...…………………168

Figure 5.1. Effect of feeding GM maize to pigs on serum AST concentration………200

Figure 5.2. Effect of feeding GM maize to pigs for 110 days on serum creatinine concentration……………………………………………………………………….201

Figure 5.3. Effect of feeding GM maize to pigs for 110 days on serum total protein concentration……………………………………………………………………….202

Figure 5.4. Effect of feeding GM maize to pigs for 110 days on serum urea concentration……………………………………………………………………….203
Figure 7.1. Effect of feeding Bt maize to pigs on relative abundance of major caecal bacterial phyla…………………………………………………………………………273
Figure 7.2. Effect of feeding Bt maize to pigs on relative abundance of major caecal bacterial families………………………………………………………………………274
Figure 7.3. Effect of feeding Bt maize to pigs on relative abundance of major caecal bacterial genera……………………………………………………………………..275
Figure 7.4. Bacterial alpha diversity at 97% similarity in the caecum of ~150 day-old pigs………………………………………………………………………………….276
Figure 7.5. Bacterial alpha diversity at 97% similarity in the caecum of ~150 day-old pigs…………………………………………………………………………………277
Figure 7.6. Bacterial alpha diversity at 97% similarity in the caecum of ~150 day-old pigs…………………………………………………………………………………278
Figure 7.7. Bacterial alpha diversity at 97% similarity in the caecum of ~150 day-old pigs…………………………………………………………………………………279
Figure 7.8. Unweighted bacterial beta diversity in the caecum of ~150 day-old pigs…………………………………………………………………………………280

Fate of transgenic DNA and protein in pigs fed genetically modified Bt maize and effects on growth and health

by Stefan Gabriel Buzoianu

Abstract

The effects of short- and long-term feeding of genetically modified Bt MON810 maize on porcine growth performance and health were investigated in two studies. Bt maize expresses the Cry1Ab toxin from *Bacillus thuringiensis* which confers insect resistance. Fate of the *cry1Ab* transgene and Cry1Ab protein were also investigated. Short-term (31 days) feeding of Bt maize to weanling pigs resulted in higher feed intake but overall weight gain and feed conversion efficiency were unaffected. Organ function, as measured by blood biochemistry, organ weight and histology, was unaffected in the Bt group. Minor changes were observed in immune cell population distribution but these were not associated with local or systemic inflammation and there was no Cry1Ab-specific immune response. Bt maize consumption had minimal impact on caecal microbial community structure; the only populations affected were *Enterococcaceae*, *Erysipelotrichaceae*, *Blautia* and *Bifidobacterium*, which were found in low abundance and at low prevalence. The effect of long-term (110 days) feeding of Bt maize to pigs from 12 days post-weaning to slaughter was investigated in a second feeding trial which used a crossover of diets (from isogenic to Bt maize and *vice-versa*) after 30 days. There were no adverse effects on growth, slaughter parameters, immune response, intestinal histology or microbiology. Organ function was not adversely affected and no histopathology was present. In both short- and long-term studies the transgene was mostly degraded during digestion and neither the transgene nor protein were detected in the blood or organs. Overall, short- or long-term feeding of Bt maize to pigs did not adversely affect any of the growth or health parameters investigated. These findings should provide assurance to pig producers as well as the feed industry of the safety of Bt maize, one of the main ingredients of pig diets. They should also help assure consumers, as a similar lack of adverse effects should be expected in humans, for which pigs are considered an excellent model.

1. Literature review

1.1. Introduction

Genetically modified (GM) food and feed have long been a source of scientific controversy with one of the main concerns relating to potential health effects (EFSA, 2009). Other concerns are related to potential gene transfer from the GM plant to organs or to indigenous microbiota of humans and animals (Dona and Arvanitoyannis, 2009; EFSA, 2009). The fate of the novel compounds and transgenic DNA from GM crops in the environment is also a major concern as it is feared that the novel compounds may have deleterious effects on the ecosystem into which they are released. Furthermore, gene transfer from GM crops to weeds may lead to the development of herbicide tolerant weeds (Anthony *et al.*, 2003). In addition, gene transfer to soil bacteria may potentially spread antibiotic resistance in the environment (Nielsen *et al.*, 1998).

The presence of GM plants in animal feed causes two main concerns:

- Are GM plants as safe for animals as their conventional counterparts and is the same level of safety to be expected in humans?
- Do products from animals fed GM crops contain any transgenic compound which would render them unsafe for human consumption?

To address these concerns every GM plant that is released on the market is subjected to risk assessment (2001/18/EC). However as some of the effects of consuming GM plants may not be seen in short-term animal trials, further evaluation may be required. This need to ensure thorough safety testing of GM ingredients has led to sustained efforts by the scientific community and regulatory bodies to create a framework to assess GM components after they reach the market. As a result, the concept of post-market monitoring (PMM) has emerged (König *et al.*, 2004).

The creation of a PMM framework is neither an easy task nor a definitive process as genetic manipulations differ from plant to plant and the target species are diverse. Therefore, the framework needs to be rigid enough to ensure thorough monitoring of GM components, yet flexible enough to detect effects of GM components when consumed by a genetically diverse population, in a variety of situations, over a long period of time (Wal *et al.*, 2003).

This review will present the current knowledge on safety assessment of GM Bt maize in animal studies. The review will focus on pigs, as pig meat accounts for half of all meat consumed in the EU (FAO, 2012). Furthermore, pigs are used extensively as a

model for humans (Moughan *et al*., 1992; Kararli, 1995; Swindle, 2007; Patterson *et al*., 2008; Swindle *et al*., 2011). Only data relating to Bt maize will be reviewed, as maize is one of the most important ingredients in pig diets. In 2010 29% of the maize grown globally was genetically modified and most of it (24.6%) contained insect resistance genes (James, 2010), which has made sourcing non-GM maize increasingly difficult.

1.2. GMO definition and technology used to create GMOs

According to Directive 2001/18/EC of the European Parliament, a genetically modified organism (GMO) is "an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination" (2001/18/EC). The result of this genetic alteration is the expression of one or more foreign traits in the target organism. The main traits that are used in the engineering of GM plants are insect resistance and herbicide tolerance (James, 2010). In addition, plants that are resistant to disease or are able to grow in adverse conditions such as dry or salty soils have also been engineered and are expected to become available over the next few years (James, 2010). Genes coding for phytase production that improve phosphorus digestibility have also been inserted in maize and these lines of maize are proposed to reach the market by 2015 (James, 2009). The latest scientific research is focusing on creating plants that contain combinations of up to eight genes coding for herbicide and insect resistance (James, 2010). However, all individual traits need to be proven safe for consumption before they can be combined.

The technology used to produce GM plants is relatively new with the first successful experiments that yielded fertile transgenic plants being presented in 1983 at the Miami Winter Symposium (Vasil, 2008). GM plants are produced by one of the following procedures (Vasil, 2008):

- **Vector-based gene transfer**: DNA is delivered to target tissues using a bacterium (*Agrobacterium tumefaciens*). *A. tumefaciens* is a soil microorganism which causes development of tumours in plants through incorporation of bacterial DNA into the plant via the *Ti* plasmid (Vasil, 2008). This plasmid can be manipulated in the laboratory to engineer transgenic plants. The main disadvantage of this method was that *A. tumefaciens* could only infect certain plants. However, this was overcome by cultivating the microorganism with factors that exacerbated bacterial

virulence (Vasil, 2008) and currently, this is the preferred method for producing transgenic plants (Meyers *et al*., 2010) due to stability of the insert over generations (Shrawat *et al*., 2007).

- **DNA delivery into protoplasts** (plant cells without cell walls) by chemical or physical means (electroporation, irradiation, heat, osmotic shock, chemicals) (Davey *et al*., 2005; Meyers *et al*., 2010). This technique is commonly used to transform plants that are not suitable for *A. tumefaciens* transformation and it implies uptake of DNA by protoplasts in the form of plasmids (Davey *et al*., 2005).
- **Microprojectile bombardment of target tissues**: DNA is coated on microparticles (0.5 - 5 µm) which are then loaded into a particle accelerator gun and discharged on the target tissue. Acceleration of these particles is achieved by compressed gas, and the combination of high speed and small size makes the DNA coated particle pass through cell membranes, facilitating integration of the genetic material into the target cell. Gold particles are more frequently used due to their uniformity and because gold is a biologically inert element (Uchida *et al*., 2009). One of the main disadvantages of this procedure is reduction of target cell viability due to the high pressure of the gas (Uchida *et al*., 2009).

1.2.1. GMO history and present status

The first GM plant to reach the market was the Flavr Savr^{TM} tomato which was released in the US in 1996. This tomato was engineered to have a longer shelf life by inhibition of indigenous degrading enzymes (Bruening and Lyons, 2000). This was followed in the same year by transgenic disease resistant tobacco in China (Jan-Peter *et al*., 2003). The area of GM crops cultivated worldwide has grown exponentially from 1.7 million hectares in 1996 to reach 148 million hectares in 2010 with 45% of GM crops grown in the US, followed by Brazil with 17% and Argentina with 16% (James, 2010). The number of countries cultivating GM crops has also increased from six in 1996 to 29 in 2010. Within the European Union (EU), GM crops were grown by eight countries in 2010 with Spain cultivating the largest area (>80%) (James, 2010).

Of the 158 million hectares of maize grown globally in 2010, 24.6% contained insect resistance genes (James, 2010). In the EU, only insect resistant GM maize lines are currently grown (MON810 and Bt176), with MON810 the most cultivated (James,

2010). However, outside the EU, maize lines that express herbicide tolerance or both insect resistance and herbicide tolerance are used (James, 2010).

1.2.2. Bt MON810 maize

Bt MON810 maize was developed by Monsanto through micro projectile bombardment (EFSA, 2009) and was approved for use as food and feed in 1998 with approval for cultivation following in 2004 (James, 2010). It is engineered to produce a truncated version (65 – 91 kDa) of the Cry1Ab toxin (Andow and Hilbeck, 2004) from the bacterium *Bacillus thuringiensis* (Bt), var. kurstaki which confers protection to the European corn borer (*Ostrinia nubilalis*), the Southwestern corn borer (*Diatraea grandiosella*) and corn earworm (*Helicoverpa zea*) which are common pests of maize (Betz *et al*., 2000).

The Cry1Ab protein is produced as a protoxin (130 – 140 kDa) by *B. thuringiensis* and is dependant on proteases in the insect mid-gut to hydrolyse it to its active form (Andow and Hilbeck, 2004). However, in GM Bt crops, Cry1Ab is present in the active form, so it interacts with the insect's intestine immediately (Clark *et al*., 2005). This protein was approved in the US in 1961 and has been extensively used as a biopesticide, being the pesticide of choice for organic crop production (Betz *et al*., 2000).

The *cry1Ab* coding gene is 3470 base pairs (bp) in length (EFSA, 2009). The minimum functional unit is 1800 bp but DNA of this size is difficult to amplify even from animal feed (Mazza *et al*., 2005; Rossi *et al*., 2005). The plasmid construct used to incorporate *cry1Ab* into transgenic maize is comprised of elements that flank the *cry1Ab* gene such as the *CaMV 35s* promoter, the *Hsp70* intron and the *nos3'* terminator fragment (Figure 1.1). The plasmid also includes the *nptII* antibiotic resistance gene (EFSA, 2009) accompanied by the *ori-PUC* origin of replication and the *lac* promoter (Figure 1.1). The antibiotic resistance gene (*nptII*) was included as a marker gene that facilitated detection of plants expressing the transgene in the presence of kanamycin, neomycin, geneticin or paramomycin (Miki and McHugh, 2004). However, association to the *ori-PUC* origin of replication and *lac* promoter allowed deletion of the antibiotic resistance gene after the development stage.

Bt MON810 maize has been thoroughly tested by undergoing pre-market risk assessment and has received approval for release on the EU market (EFSA, 2009). The gene insert has also been thoroughly studied over several generations and has been found to be stable in maize (Aguilera *et al*., 2008; EFSA, 2009). The presence of the

cry1Ab gene has been confirmed in plant tissues, as well as absence of the *nptII* antibiotic resistance gene (EFSA, 2009). The EFSA Scientific Opinion of the Panel on GMOs (EFSA, 2009) also concluded that there is no substantial difference between Bt MON810 and conventional lines of maize, except for expression of the *cry1Ab* gene. *In silico*, studies have shown no homology between the newly expressed protein and any known allergen or toxin (EFSA, 2009).

Some studies have shown that the Cry1Ab protein is rapidly degraded in gastric fluid and degradation is markedly increased by heating (Okunuki, 2002) while others have found immunogenic fragments to persist following gastric digestion (Guimaraes *et al*., 2010) and even in faeces (Chowdhury *et al*., 2003). However, no allergic reaction was observed when serum from maize-allergic patients was exposed to the Cry1Ab protein from two GM maize lines (MON810 and Bt11) (Kim *et al*., 2009).

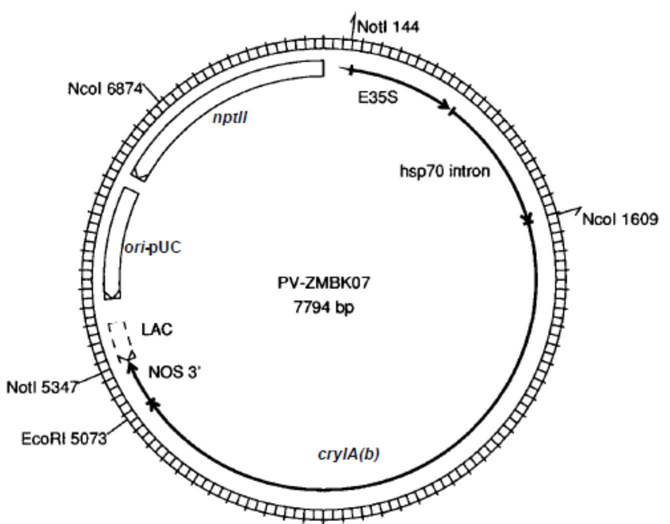

Figure 1.1. Plasmid used to produce the MON810 maize (EFSA, 2009). From http://cera-gmc.org/docs/decdocs/02-269-010.pdf

Expression of the insecticidal toxin in all parts of the plant for the entire growing season gives Bt maize a distinct advantage over conventional lines. The advantages of cultivating Bt maize over traditional lines were studied at farm level by Brookes (2008) in the seven EU countries planting GM crops from 1998 to 2006. The main advantages of planting GM maize were not only lower use of insecticides but also higher yields of up to 24% and the absence of pests or at least low infestation rates (Gomez-Barbero and Rodriguez-Cerezo, 2006; Brookes, 2008). Other benefits of GM technology include the so called "intangible impacts" related to lower energy consumption for insecticide

spraying and reduced insecticide exposure for farm workers. In addition, reduced use of pesticides on GM Bt maize allows a more stable community of beneficial insects to establish, thus encouraging biodiversity and further supplementing pest control (Betz *et al*., 2000).

1.2.3. Risk assessment

EU regulations require that GMOs undergo rigorous risk assessment prior to environmental release (2001/18/EC). In the case of GM plants, risk assessment is carried out in a comparative manner, using a conventional counterpart as a reference comparator based on the history of safe consumption of the conventional line (OECD, 2002; EFSA, 2006; Hartnell *et al*., 2007; EFSA, 2008; Hepburn *et al*., 2008; EFSA, 2011). The closest conventional safe counterpart of the GM plants is the parental line from which it was originally derived on the condition that the plants are grown under similar environmental conditions at the same time (OECD, 2002). Initially a thorough molecular characterization of the GM plant is performed to detect if, aside from the genetic modification there are any other differences from the isogenic counterpart (EFSA, 2011). Following this initial characterization, GM crop lines are compared to their isogenic counterparts and conventional lines in terms of agronomic traits, nutrient content, key toxins and anti-nutritional composition (EFSA, 2011). Compounds that play major roles in biochemical pathways are investigated and if the differences between the GMO and the conventional plant are significant, further tests are undertaken to detect any potential health risks (König *et al*., 2004; EFSA, 2006; EFSA, 2008). However, natural variability frequently occurs between conventional maize lines and it has been shown that environmental conditions have a greater influence on maize nutrient content than genetic modification (Barros *et al*., 2010). This has given rise to a new concept, that of bioequivalence (EFSA, 2008), which implies that variability between the isogenic and the GM plant is compared to the variability normally found between conventional lines (EFSA, 2011). If the composition of the GM plant is found to differ from the conventional counterpart and the difference is larger than differences between conventional lines or if there are reasons to foresee a higher risk of unintended effects, animal trials should be used to further investigate the GM plant.

Animal trials are used to determine any toxicological or allergenic effects and to confirm nutritional equivalence between GM and conventional plants (EFSA, 2008). The GM crop being tested should be included in animal diets at the highest possible

concentration that does not lead to nutritional imbalance. This testing approach allows evaluation of much higher concentrations of the crop than those normally consumed by humans. For example, the theoretical daily intake of maize for a person in Europe is estimated to be 23 g/person/day (0.32 g maize/kg for a 70 kg adult), whereas in animal risk assessment studies daily maize intake is often up to 100-fold greater (Betz *et al.*, 2000; EFSA, 2008).

Prior to market release of a GMO, EU legislation requires the company applying for the release to submit a dossier containing a detailed characterization of the GMO and risk assessment data to the national competent authority. The detailed characterization of the GMO must include information on the technology used for its creation, as well as data on expression and stability of the newly inserted traits. The risk assessment should include details regarding the identification and evaluation of any adverse effects on human/animal health or the environment that may arise from the genetic modification (2001/18/EC; EFSA, 2008).

However, at this stage the risk assessment is limited to an examination of the effects of the GMO on a sample population and responses in a larger genetically diverse population may be different. This issue can, however, be addressed with the implementation of a post-market monitoring (PMM) system (Wal *et al.*, 2003).

1.2.4. Consumer acceptance of GMOs

Although European consumers are familiar with gene technology and understand the reasons for its use and development, the acceptance of GM foods has dropped from 47% in 1996 to 29% in 2005 and 25% in 2010 (Gaskell, 2005; Gaskell *et al.*, 2010). However, European consumer attitudes towards the use of biotechnology for industrial and medical purposes are not as conservative as they are towards the use of gene technology in the food industry (Gaskell, 2005). The negative opinion towards GM foods is a combination of perceived risk of eating food that has been altered in the laboratory and a lack of confidence in EU regulatory bodies (Gaskell, 2005; Rollin *et al.*, 2011). Furthermore, most of the consumer concerns regarding GM foods were due to a perceived lack of safety (Gaskell *et al.*, 2010). European consumer opinions vary from country to country, with consumers from countries cultivating GM crops more supportive of this technology (Gaskell *et al.*, 2010; Rollin *et al.*, 2011). Furthermore, EU attitudes towards GM foods are radically different to those in the US and China, where GM crops are widely accepted (Gaskell, 2005; Huang *et al.*, 2006; Rollin *et al.*,

2011).

1.2.5. Post-market monitoring

During the pre-approval risk assessment stage of GM authorization, it is difficult to account for all of the factors that can influence an individual response to a food component and further evaluation may be needed (Wal *et al.*, 2003; König *et al.*, 2004). Therefore, PMM allows for confirmation that consumption of GM material is as anticipated by the pre-market risk assessment and allows screening for any immediate or delayed, direct or indirect adverse effects that may arise from large scale consumption for long periods of time by a genetically diverse population (Wal *et al.*, 2003; EFSA, 2008). Monitoring of the GMO after its release on the market enables a more definitive decision regarding safety of the GMO to be reached. All tests to determine the safety of a novel compound have limitations and a "case by case" approach is necessary for any safety assessment procedure, as is comprehensive testing and implementation of further quality assurance policies such as PMM (Wal and Pascal, 1998).

The focus of the next section will be on reviewing data from animal studies investigating the safety of Bt maize.

1.3. Assessment of safety of GM maize in animal feeding trials

As transgenic proteins expressed by plants following genetic engineering have the potential to undergo post-translational modification (Prescott and Hogan, 2006), rendering them toxic or allergenic, feeding experiments using the whole GM plant are a valuable tool for safety assessment. Growth performance trials reinforced by assessment of carcass characteristics are among the measurements recommended by the International Life Sciences Institute (ILSI) for testing GMOs (Hartnell *et al.*, 2007). To standardise animal trial methodology and measurements, ILSI issued a guide for designing and performing trials to assess the safety of GM crops (Hartnell *et al.*, 2007). This guide gives indications on sampling procedures for GMOs and feed, dietary treatments used, number of animals and replication as well as collection and interpretation of data (Hartnell *et al.*, 2007). Likewise, starting from ILSI recommendation, EFSA has also developed guidelines for GMO testing using animal trials (EFSA, 2008).

Testing of GMOs on the target species has distinct advantages, as a potential

species-specific response could lead to erroneous results. In farm animal species testing of the GM crop on the species of interest is and recommended (Hartnell *et al*., 2007), as an effect on production parameters would be of interest. However, testing of new crops on humans is not an option, therefore models for human physiology have to be employed. From this point of view, laboratory animals are not considered a suitable human model, due to differences in size, diet and practice of coprophagy in rodents (Dybing *et al*., 2002; Patterson *et al*., 2008). Furthermore, ruminants and birds are also not considered good human models due to differences in digestive physiology (Madigan *et al*., 2000). However, the pig is considered a suitable human model (Moughan *et al*., 1992; Kararli, 1995; Swindle, 2007; Patterson *et al*., 2008; Swindle *et al*., 2011) and is of great value in testing the safety of GMOs.

1.3.1. Nutritional equivalence and presence of contaminants

Nutritional equivalence is one of the key concepts when testing novel foods in general and GM food and feed in particular. International guidelines relating to this concept require that the novel food is compared to its closest safe counterpart (Hartnell *et al*., 2007; EFSA, 2008; EFSA, 2011).

Nutritional equivalence of different transgenic maize lines and their isogenic counterparts has been reviewed by Aumaitre *et al.* (2002). The results show only minor differences in proximate analysis, amino acid content, fat content and fatty acid proportion in both seeds and maize silage which are within the natural variability of maize lines established by the Organization for Economic Co-operation and Development (OECD, 2002). The next step in the assessment of nutritional equivalence is testing of the GM Bt maize in digestibility studies. Pig-feeding studies with GM maize have shown increased P and N intake in the control group but no other differences (Custodio *et al*., 2006). However, Aulrich *et al.* (2001) found no differences in metabolizable energy, nitrogen excretion and protein digestibility in poultry and pigs fed GM maize.

An extensive study investigating a broad range of micro and macronutrients, as well as energetic value of two maize lines (GM and parental) found the composition of the maize to be similar and within the normal values cited in the literature (OECD, 2002; Reuter *et al*., 2002). The same authors performed digestibility experiments with grower-finisher pigs and found no major differences between the maize lines (Reuter *et al*., 2002).

The presence of contaminants (pesticides, fungi, mycotoxins) also needs to be considered. Fungal contamination of grain is a major problem worldwide with enormous financial losses occurring due to contamination of stored cereals and health risks associated with consumption of mycotoxin contaminated grains. Contamination can occur both in the field and during storage and the main fungal contaminants of maize are *Fusarium*, *Aspergillus* and *Penicillium* (Munkvold and Desjardins, 1997; Bakan *et al.*, 2002; Khosravi *et al.*, 2007). The main mycotoxins produced by these fungi are fumonisins, zearalenone and aflatoxins (Bakan *et al.*, 2002) which are responsible for various illnesses in livestock and humans and are recognised as being carcinogenic (Bakan *et al.*, 2002).

Insect damage to maize tissues has been associated with increased fungal growth and higher mycotoxin levels (Bakan *et al.*, 2002). Insects not only damage plant tissue, facilitating fungal growth but also act as vectors for fungi. The insect that is most frequently associated with *Fusarium* contamination of maize is the European corn borer (ECB) (Munkvold and Desjardins, 1997). As GM Bt maize is resistant to damage by ECB, lower mycotoxin contamination might be expected. This was the case in a study conducted by Bakan *et al.* (2002) which found that in maize samples collected in Spain and France in 1999, *Fusarium* contamination was 2-3 times greater in non-GM maize compared to the GM (MON810) maize. The concentration of ergosterol, which is a fungal contamination indicator, was also 4-10 times lower in the GM maize. While levels of trichotecenes and zearalenone in both maize lines were below acceptable limits, the GM maize had lower concentrations than its conventional counterpart. Fumonisin levels in all GM maize samples tested were below acceptable limits; however, some of the conventional maize samples had levels that were high enough to preclude the maize from use as a food/feed ingredient (Bakan *et al.*, 2002).

Other studies performed by Munkvold *et al.* (1999) reported differences in fumonisin concentrations depending on the transgenic line used, the seed provider, grade of infestation and cultivation year. Overall, transgenic maize samples had less insect infestation and damage and lower fumonisin concentrations than the isogenic lines. The differences reported between the different transgenic lines are believed to occur due to different gene insertion technologies and different insecticidal protein concentrations present in plant tissues (Munkvold *et al.*, 1999). While laboratory tests provide valuable data on the nutritional value of GM maize and the presence of contaminants, animal feeding trials are needed to assess *in vivo* effects.

1.3.2. Effects on growth performance and slaughter characteristics

A number of animal trials have been conducted to assess the nutritional value of Bt maize in pigs, sheep, broilers and laying hens (Table 1.1). In general, there were no differences in growth and slaughter characteristics between animals fed Bt maize (5-80% dietary inclusion rate) and those fed control diets in studies ranging in duration from 35 days to 3 years (Table 1.1). There were however, some exceptions, as pigs fed Bt maize had poorer feed conversion efficiency (FCE), increased feed intake and differences in muscle colour were observed (Custodio *et al*., 2006). In addition, weaner pigs fed GM maize had higher daily gain and were heavier than controls, due to lower mycotoxin contamination of the Bt maize (Piva *et al*., 2001). Higher yields in poultry breast muscles as a result of feeding Bt maize were also reported (Brake and Vlachos, 1998; Taylor *et al*., 2003). These findings further indicate that Bt maize is at least as nutritious as conventional maize lines and that it does not adversely affect growth and slaughter performance.

Body composition and carcass composition of pigs are further indicators of nutrient assimilation and functionality of organs and metabolic pathways. However, determining body composition by dissection is a very laborious process which can only be performed on dead animals (Mitchell *et al*., 2001). Area bone mineral density, bone mineral content and fat percentage can be measured on live anaesthetised pigs with DXA (Dual Energy X-ray Absorptiometry) technology. Although, DXA has not yet been used for GMO safety assessment, this technology has the potential to provide valuable data regarding bone metabolism and fat deposition.

Table 1.1. Effects of feeding GM Bt maize on farm animal and poultry growth performance and slaughter characteristics.

Species (number)	Weight/age/stage (duration)	Maize line (trait)	% maize in diet	Parameters measured	Results (GM maize vs controls)	Reference
Pigs (64)	60 to 110 kg (~50 days)	Bt11 (IR × HT)	77-83	ADG, ADFI, FCE Digestibility Carcass characteristics Muscle quality	No differences in ADG, ADFI, carcass characteristics. Poorer FCE, lower P and N digestibility lower Hunter value (less intense yellow colouration of the *longissimus dorsi* muscle).	Custodio *et al.,* 2006
Pigs (120)	17 to 120 kg (~110 days)		70-76		No differences in ADG. Increased ADFI and poorer FCE. Lower Hunter value (more intense yellow colouration of the *longissimus dorsi* muscle).	
Pigs (48)	Weaner-finisher (91 days)	Bt176 (IR × HT)	70	ADG, ADFI Carcass characteristics	No significant differences	Reuter *et al.,* 2002

Species (number)	Weight/age/stage (duration)	Maize line (trait)	% maize in diet	Parameters measured	Results (GM maize vs controls)	Reference
Pigs (128)	Weaner (35 days)	MON810 (IR)	33	ADG, ADFI, FCE, BW	Higher ADG and BW No differences in ADFI and FCE	Piva *et al.*, 2001
Sheep (100)	Adult ewes and progeny from birth to weaning (3 years)	Bt176 (IR × HT)	5-24	Ewe BW and BCS Fertility and twin rate Lamb BW Lamb weight gain	No significant differences	Trabalza-Marinucci *et al.*, 2008
Cattle (40)	Birth to slaughter (246 days)	Not mentioned (IR)	Maize silage ~19 kg/day	ADG, ADFI Final BW Carcass characteristics	No significant differences	Flachowsky *et al.*, 2007
Cattle (12)	2 months of age (3 months)	Bt11 (IR × HT)	43	ADG	No significant differences	Shimada *et al.*, (2006)
Poultry (282)	Broilers 1 day old (39 days)	Bt176 (IR × HT)	60	ADG, ADFI, FCE BW Water consumption Meat composition	No significant differences	Aeschbacher *et al.*, 2005

Species (number)	Weight/age/stage (duration)	Maize line (trait)	% maize in diet	Parameters measured	Results (GM maize vs controls)	Reference
Poultry (36)	Hens 17 weeks of age (6 months)	Bt176 (IR × HT)	60	ADFI, FCE, BW Laying rate Egg composition	No significant differences	Aeschbacher *et al.*, 2005
Poultry (1280)	Broilers 1 day old (38 days)	Bt176 (IR × HT)	58-64	FCE, BW Mortality Carcass characteristics	Better FCE and increased weight of the *pectoralis minor* muscle No other significant differences	Brake and Vlachos, 1998
Poultry (100)	Broilers 1 day old (42 days)	DK551 (IR)	55-61	FCE, BW Mortality Carcass characteristics	Higher yields in breast muscle No other significant differences	Taylor *et al.*, 2003
Poultry (1600)	Broilers 1 day old (47 days)	Bt11 (IR × HT)	48-63	FCE, BW Mortality Carcass characteristics	No significant differences	Brake *et al.*, 2003
Poultry (432)	Broilers 1 day old (42 days)	MON810 (IR)	48-62	ADG, FCE, BW	No significant differences	Rossi *et al.*, 2005

Species (number)	Weight/age/stage (duration)	Maize line (trait)	% maize in diet	Parameters measured	Results (GM maize vs controls)	Reference
Poultry (120)	Broilers 1 day old (42 days)	MON810 (IR)	60	ADG, ADFI, FCE BW	No significant differences	Deaville and Maddison, 2005

IR = insect resistant; HT = herbicide tolerant; ADG = average daily gain; ADFI = average daily feed intake; FCE = feed conversion efficiency; BW = body weight; BCS = body condition score.

1.4. Effects of GM maize on immunological parameters

1.4.1. Overview of the immune system

The response of an organism to an external aggression is initiated and regulated by the immune system. According to Janeway *et al.* (2001), immunity can be differentiated into:

- **Innate immunity** - characterized by an immediate, non-specific response of the same intensity at every aggression and without immunological memory (Beutler, 2004).
- **Adaptive immunity** - a delayed but highly specific response with immunological memory i.e. able to respond immediately and with a greater amplitude and intensity at the next encounter with the same aggressor.

Table 1.2. Immune cells involved in innate immunity.
Images from
http://www.vetmed.vt.edu/education/curriculum/vm8054/labs/lab6/lab6.htm
http://www.wadsworth.org/chemheme/heme/microscope/celllist.htm

Cell type	Cell	Major role	Reference
Mononuclear	Monocyte/ macrophage	Antibacterial response	Janeway *et al.*, 2001 Beutler, 2004
	Dendritic cell	Antigen presentation to lymphocytes	
	Mast cell	Vascular changes in inflammatory response	
	Natural killer cell	Removing cells infected by intracellular pathogens	
Polymorphonuclear	Neutrophil	Phagocytic, first cells at an inflammation site	
	Eosinophil	Antiparasitic response and allergic reaction	
	Basophil	Inflammatory response	

Table 1.3. Immune cells involved in adaptive immunity.
Images from
http://www.vetmed.vt.edu/education/curriculum/vm8054/labs/lab6/lab6.htm
http://www.wadsworth.org/chemheme/heme/microscope/celllist.htm

<table>
<tr><th>Cell type</th><th>Cell</th><th>Major role</th><th>Reference</th></tr>
<tr><td rowspan="2">Mononuclear</td><td>B lymphocyte→plasma cell</td><td>When stimulated by T helper 2 cells, B cells differentiate into plasma cells able to secrete antibodies</td><td rowspan="2">Janeway et al., 2001
Gerner et al., 2009</td></tr>
<tr><td>T lymphocyte</td><td>Destroy cells infected by viruses, stimulate macrophages to elicit their antibacterial response and help B cells differentiate into plasma cells</td></tr>
</table>

Innate immunity relies on phagocytic cells that can engulf, digest and expose antigen to other cells (Beutler, 2004; Table 1.2). On the other hand, the adaptive response relies on B lymphocytes to secrete antibodies and T lymphocytes to destroy infected cells and stimulate B lymphocytes (Table 1.3; Janeway *et al*., 2001).

Activated B lymphocytes respond to the presence of antigens through the production of antibodies. Antibodies, by their nature and function, target extracellular antigens. In contrast, T cell subsets are designed to detect intracellular pathogens through recognition of antigens presented on the surface of other immune cells and they in turn activate B cells to produce antibodies (Table 1.3). All of these cells have a common precursor, the pluripotent stem cell, which originates in the bone marrow (Janeway *et al*., 2001).

Nevertheless, the two types of immune responses are interlinked. White blood

cells (leukocytes) are the main component of both the adaptive and innate immune response and the types of cells involved in the immune response differ as follows:

- **Innate immunity**
  - **Mononuclear cells**
    - Monocytes/macrophages - play a major role in antibacterial responses. They are phagocytic cells that possess receptors which recognise and bind to common constituents of the bacterial cell wall. These cells engulf and process bacteria and present the antigen on their surface. Simultaneously, they release compounds named cytokines that influence the behaviour of other cells. Cytokines secreted by monocytes/macrophages recruit and stimulate other immune cells they also responsible for inflammation (Table 1.4). Monocytes are found in the blood and they migrate to the tissues, where they differentiate and become macrophages (Beutler, 2004).
    - Dendritic cells - found throughout the organism in large numbers. They migrate from the bone marrow to peripheral tissues where they are activated following encounters with antigens. Their role is to continuously sample tissues for antigens. Upon activation, they engulf and process antigens and migrate to the lymph nodes, where they present them to lymphocytes (Janeway *et al*., 2001).
    - Mast cells - located near blood vessels throughout the organism. They play an important role in vascular changes during an inflammatory response. They are also involved in allergic symptomatology through secretion of inflammation mediators.
    - Natural killer cells – play a role in recognition and destruction of cells infected by intracellular pathogens, as they are capable of recognizing cells with altered receptor patterns (due to intracellular pathogens) (Janeway *et al*., 2001).
  - **Polymorphonuclear cells (granulocytes)**
    - Neutrophils - the first cells to appear at an inflammation site. They are phagocytic cells, which, similar to macrophages, possess surface receptors for common bacterial components.
    - Eosinophils - play a role in response to parasitic infestations and in allergies. Some are found in circulation but most reside in the

tissue underlining the mucosae. They destroy pathogens by means of cytotoxic granules and free radicals and also secrete compounds that recruit other immune cells.

♦ Basophils – involved in the inflammatory response with a similar role to that of eosinophils (Janeway *et al*., 2001).

● **Adaptive immunity** – is comprised only of mononuclear cells which can be either circulating or found within specialized lymphatic structures.

■ **Lymphocytes** – produced in the bone marrow but have different maturation sites.

♦ B cells – remain in the bone marrow for maturation. Upon activation, they proliferate into plasma cells which are capable of secreting antibodies (immunoglobulins; Ig). Antibodies are proteins which bind antigens with high specificity forming a complex which is eliminated, thus rendering the antigens harmless. Such complexes are recognised and destroyed by phagocytic cells. A plasma cell can produce antibodies against any antigen due to gene rearrangement. Antibodies are divided into five classes according to their structure and function (Butler *et al*., 2009):

– **IgG** - dominant antibody in blood and extracellular fluid. Has has a role in binding pathogens for destruction by specialised phagocytic cells.

– **IgM** - first antibody to be secreted in the adaptive immune response. Present in the blood and has an important role in complement activation.

– **IgA** - secreted by lymphocytes found at mucosal sites and transported to the luminal surface. Has a role in the prevention of pathogen binding to mucosal cells.

– **IgE** - binds to and remains on mast cells until pathogen encounter and has a major role in allergic response.

– **IgD** - role in B cell maturation.

♦ T cells – produced in the bone marrow and migrate to the thymus where they differentiate into three distinct types of T cells (Janeway *et al*., 2001):

– **T cytotoxic** – express CD8 cell surface marker which recognize MHC class I on the surface of antigen presenting cells. They are able to detect and destroy cells infected by intracellular pathogens, as they contain granules which can induce cell death through pore formation and DNA denaturation.

– **T helper (Th)** – express CD4 cell surface marker which recognize MHC class II on the surface of antigen presenting cells. They have an important role to play in the antibacterial response. They can further differentiate into:

→ **Th1 cells** – stimulate macrophages to elicit their antibacterial response when invaded by intracellular pathogens.

→ **Th2 cells** – activate B cells to secrete antibodies in response to extracellular pathogens.

→ **Th17 cells** - involved in clearance of extracellular pathogens that are not efficiently cleared by other cell types. They are highly pro-inflammatory and have a major involvement in autoimmunity (Bettelli *et al.*, 2007).

– **Regulatory T cells** - involved in down-regulation of the immune response in order to maintain immune self-tolerance (Gerner *et al.*, 2009; Käser *et al.*, 2011)

Aside from immune cells, the immune system is able to fight bacterial aggression through other means, such as the complement system. This system is comprised of inactive circulating molecules which are activated in response to bacterial antigens or by circulating antibodies. Once activated, the complement system develops a “cascade” reaction which leads to the destruction of bacteria either by formation of pores in the cell wall or by coating the bacterial wall (opsonisation), thus signalling phagocytes to destroy the cell (Janeway *et al.*, 2001).

The immune system is comprised of primary lymphoid tissue which includes the thymus and bone marrow and from these sites immune cells develop from progenitor cells. Secondary lymphoid tissues which include the spleen and lymph nodes are the sites at which the immune response is initiated (Janeway *et al.*, 2001; Bailey and Haverson, 2006). Lymph nodes are rich in immune cells and are located at key points

of the lymphatic system and filter the lymph, trapping any antigens that are transported from peripheral tissues. The spleen has a similar role to the lymph nodes except that antigens from the blood and not lymph are filtered.

Changes in immune cell populations can be used as signs of potential interactions between GM feed ingredients and the immune system. As mononuclear cells form an important portion of the overall blood cell population, an immune response elicited against ingested GMOs should be visible through changes in these immune cells. Therefore, analysis of peripheral blood mononuclear cell populations (PBMC) may be a useful tool for safety assessment (Finamore *et al*., 2008). However, the immune response may be discrete and localised at the mucosal site of absorption. As the mucosal associated lymphoid tissue (MALT) is a major component of the immune system, containing half of the total number of lymphocytes (Janeway *et al*., 2001), changes within the immune cell populations at this site should be also investigated as part of the safety assessment of the GMOs.

1.4.2. Allergenicity

Immune cells within the intestine are exposed daily to large quantities of antigen, however, an allergic or inflammatory response rarely occurs. This is due to regulatory pathways which can distinguish between normal and pathogenic (Bailey and Haverson, 2006) and an immune mechanism referred to as "immune tolerance". Studies have shown that injecting an antigen after feeding the same antigen does not lead to a systemic immune response but to a local state of activation (Bailey and Haverson, 2006; Bailey, 2009). This demonstrates the regulatory function of the gut associated lymphoid tissue (GALT).

The barrier function of the intestine, together with the selective passage of intestinal antigens through specialised sites within the GALT, plays a major role in immune tolerance. It has been shown that individuals with impaired intestinal barrier function are predisposed to allergic reactions (Bailey, 2009). In pigs, the GALT is comprised of ileal and jejunal Peyers patches in the small intestine and isolated lymphoid follicles and patches in the large intestine (Liebler-Tenorio and Pabst, 2006). Continuous contact with the luminal contents at these sites is facilitated by the villous architecture and the structure of the lymphoid follicles. Luminal antigen is processed by dendritic cells located beneath the epithelium and is supplied to these by either dying enterocytes (cells of the intestinal epithelium) on the intestinal villi or by M cells on the

lymphoid follicles (Bailey, 2009). Antigen uptake by M cells is facilitated by the lack of a glycocalix and the absence of mucus secretion.

Tolerance to food antigens requires a reduction in T cell stimulation. This is achieved either by deletion of the specific T cell population in response to very high quantities of antigen, by absence of co-stimulatory signals or by differentiation of regulatory T cells (Janeway *et al*., 2001). Differentiation of these regulatory T cells is stimulated by dendritic cells which are in close contact with enterocytes (Fritz *et al*., 2008).

However, there are situations when the immune system malfunctions and reacts to harmless antigens. Such reactions are referred to as allergic reactions or allergies and the compounds that trigger them are allergens. Low presentation of antigenic molecules by antigen presenting cells stimulates differentiation of T lymphocytes into the Th2 class (Janeway *et al*., 2001). These lymphocytes, through secretion of cytokines, such as IL-4 and IL-13 stimulate IgE production by activated B lymphocytes (Broide, 2001). Th2 cells also secrete IL-5 which stimulates development of eosinophils (Broide, 2001) and IL-10 which inhibits the development of Th1 cells (Janeway *et al*., 2001). IgE binds to mast cell membranes and on contact with the antigen induces degranulation of mast cells. Amines and leukotrienes released from mast cells upon activation are responsible for slowing blood flow, increasing blood vessel permeability, mucus secretion and contraction of smooth muscle (Broide, 2001). The acute allergic response initiated by mast cells through degranulation is followed by a more persistent inflammatory response involving other cell types (lymphocytes, eosinophils, basophils) and immunoglobulins (Vojdani, 2009). An immune response and activation of certain cell types could be detected by measurement of major cytokines involved in the allergic response (Table 1.4) and by detection of immunoglobulins specific to the allergen in question. Cytokine measurements accompanied by immunoglobulin detection can also be used when assessing allergenicity of GMOs.

Table 1.4. Cytokines involved in the allergic response.

Name	Producing cell	Role	Involved in	Reference
IL-4	Th2 lymphocytes, mast cells	Stimulation of IgE production	Humoral immunity	Broide, 2001 Janeway *et al.*, 2001
IL-5	Th2 lymphocytes	Differentiation and migration of eosinophils to the site of inflammation	Humoral immunity / allergy	Janeway *et al.*, 2001 Broide, 2001
IL-6	Macrophages	Stimulation of antibody production through activation of lymphocytes	Humoral immunity	Janeway *et al.*, 2001
IL-8	Eosinophils, macrophages	Direction of leukocytes and neutrophils at the site of allergic inflammation	Acute phase response	Janeway *et al.*, 2001 Raymond and Wilkie, 2004 Murtaugh *et al.*, 1996
IL-10	Th2 lymphocytes	Inhibition of the development of Th1 cells	Humoral immunity	Janeway *et al.*, 2001
IL-12	Macrophages	Stimulation of the development of NK and Th1 cells	Cellular immunity	Janeway *et al.*, 2001
IL-13	Th2 lymphocytes, mast cells	Stimulation of IgE production, inhibition of macrophage activity	Humoral immunity	Broide, 2001 Janeway *et al.*, 2001
IFN-γ	Th1 cells	Inhibits Th2 cells	Cellular immunity	Janeway *et al.*, 2001 Murtaugh *et al.*, 2009
TNF-α	Macrophages Activated mast cells	Vascular inflammatory response	Allergy	Janeway *et al.*, 2001 Murtaugh *et al.*, 1996

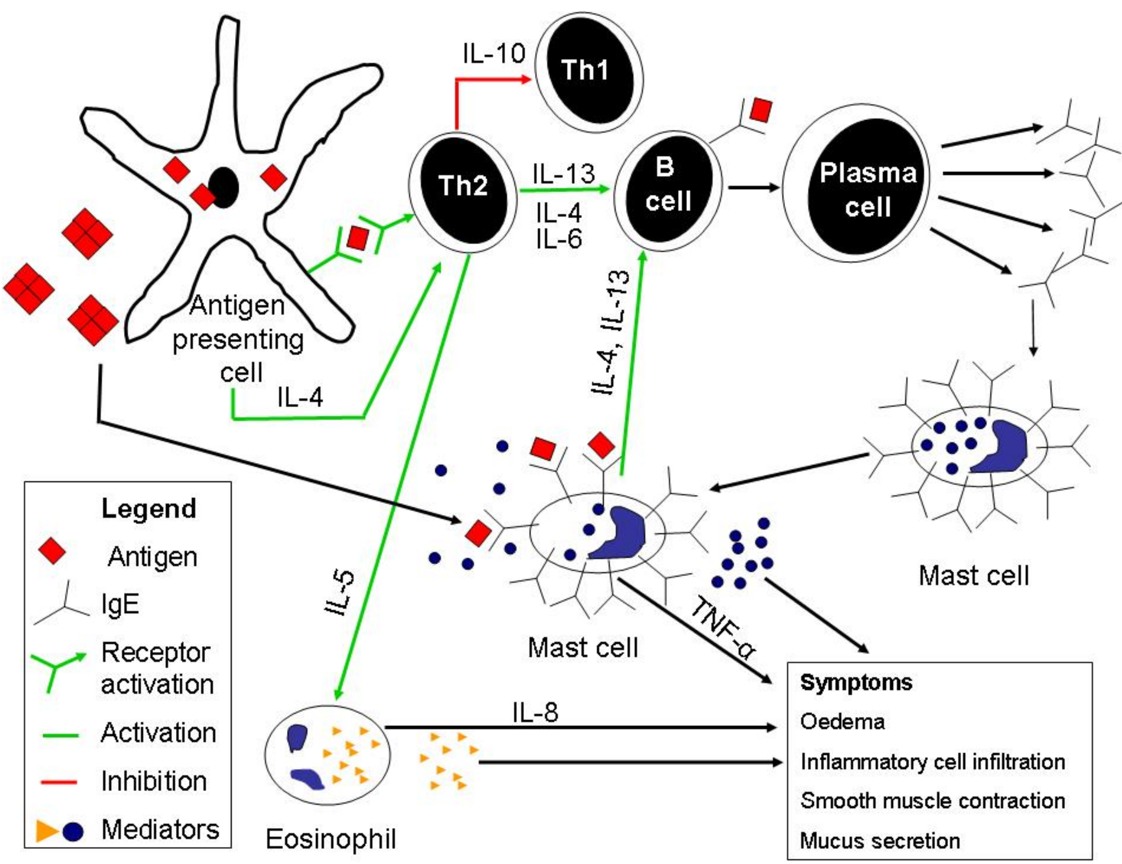

Figure 1.2. Cells and mediators involved in the allergic response (Janeway *et al*., 2001; Broide, 2001)

1.5. Allergen testing of GMOs

One of the requirements for the authorization of GMOs is that the structure, function, bioavailability, specificity and potential allergenicity of the newly expressed protein are elucidated (EFSA, 2006). If the newly expressed protein comes from a source that is known to be allergenic, cross-reactivity tests are undertaken. Cross-reactivity tests involve testing serum from individuals allergic to the initial source against the protein expressed in the GMO (EFSA, 2011). However, if the protein comes from a source that is not known to be allergenic, homology of the protein to any known allergens is initially investigated (EFSA, 2011). Oligopeptides with less than 6-8 amino acids are not recognized by specialized antigen sampling mechanisms in the intestine (Untersmayr and Jensen-Jarolim, 2006). Therefore, if there are fragments of 6-8 or more contiguous amino acids identical between the tested protein and a known allergen, then cross-reactivity testing is conducted (König *et al*., 2004; EFSA, 2008). However, amino acid profiling of the test protein is not the definitive answer. The absence of 6-8

contiguous amino acids does not necessarily mean that the protein is non-allergenic. The three-dimensional structure of the protein could possibly bring eight or more amino acids together to form a conformational epitope which could initiate an allergic response (König *et al.*, 2004; EFSA, 2006).

As proteins that are easily digested in the gastrointestinal tract (GIT) are less likely to be allergenic (König *et al.*, 2004), sensitivity to simulated gastrointestinal conditions is also determined during safety testing. On the other hand, some proteins require digestion to elicit an immunogenic response. Therefore, immunoreactivity of digested fragments of protein fragments is a useful tool for GMO safety assessment (Wal and Pascal, 1998). As mentioned in Section 1.2.2, MON810 maize has been tested during the risk assessment procedure and found to lack similarity to known allergens. It was also shown to be to be degraded in intestinal simulations (Okunuki, 2002; EFSA, 2009). However, studies have shown that food antigens which are known to cause allergies are digested in a simulated digestive environment (Untersmayr and Jensen-Jarolim, 2006); therefore, decision making regarding GM plant safety should not rely solely on such assays and requires further assessment *in vivo*.

Animal models are also useful for allergenicity testing of GM proteins provided they are carefully selected for the type of response anticipated and that ethical considerations are met (Lehrer and McClain, 2009). Similarity of patho-physiological pathways between humans and pigs has made them a suitable candidate as an animal model for allergy studies (Helm *et al.*, 2002).

However, no studies to date have investigated the immune response of pigs fed GM maize. In weanling mice, increased cytokine production (IL-6, IL-13, IL-12p70 and MIP1β) was observed following 30 days of feeding Bt MON810 maize. These changes in cytokine production were correlated with increased T cell and decreased B cell proportions in the GALT, increased B cells, $CD8^+$ cells and γδ T cells and decreased $CD4^+$ cells in spleen (Finamore *et al.*, 2008). Likewise, a decrease in blood B cells, $CD8^+$ cells and αβ T cells were also observed following 30 days of feeding of Bt MON810 maize to weanling mice (Finamore *et al.*, 2008). Following 90 days of feeding Bt MON810 maize, B cell populations were increased in the GALT and in blood of weaned mice and were decreased in the GALT and blood of elderly mice (Finamore *et al.*, 2008). In elderly mice a decrease in $CD4^+$ and an increase in γδ T cells was also observed in GALT associated with an increase in $CD4^+$ and a decrease in $CD8^+$ cells(Finamore *et al.*, 2008). Adel-Patient *et al.* (2011) found no antibody

response in mice sensitised to the Cry1Ab protein extracted from MON810. However, the same study found higher antibody production in mice sensitised to bacterial Cry1Ab by the intra-peritoneal route compared to controls. This demonstrates differences in response to the truncated Cry1Ab protein present in MON810 maize compared to the bacterially-produced Cry1Ab protein. Feeding Bt maize was shown to have no effect on blood immune cell numbers in rats after 90 days of feeding (Hammond *et al*., 2006) and in calves after 12 weeks of feeding (Shimada *et al*., 2006). Following a three year period of feeding Bt maize to sheep, no differences were seen between treatments in monocyte/macrophage activity or in the response to non-specific immune stimulation (Trabalza-Marinucci *et al*., 2008).

1.6. Effect of GM maize on intestinal microbiota

1.6.1. The role of the intestinal microbiota

Microbial numbers in the mammalian GIT exceed the number of host cells by a factor of 10 (Montalto *et al*., 2009; Sekirov *et al*., 2010). The intestinal microbiota is usually well tolerated by the host (Hooper, 2004) and is, in fact, considered necessary for host health. With the emergence of new, high-throughput molecular biology techniques, the microbial ecology of the GIT has become a major research target and it is becoming more obvious that GIT microbiota are simultaneously symbiotic, commensal and pathogenic (Sekirov *et al*., 2010). Microbial colonization of the GIT by microorganisms after birth plays a crucial role in the development of intestinal immunity. The interaction between microorganisms and intestinal structures helps maintain the normal state of low level activation of the GALT (Cebra, 1999; Hooper, 2004). Studies in germ-free pigs have shown that they have lower serum antibody concentrations and that antibodies have delayed maturation and responsiveness compared to conventional pigs (Cukrowska *et al*., 2001; Tlaskalová-Hogenová *et al*., 2004; Butler *et al*., 2006). Likewise, germ-free pigs exhibit an extensive hyper-activation of the immune system and development of autoimmune disease following exposure to viruses (Butler *et al*., 2009). The intestinal microbiota also induces production of secretory IgA and development of intraepithelial lymphocytes (Hooper, 2004). Studies in germ-free animals have underlined the importance of the intestinal microbiota in the development of the protective intestinal mucus layer (Deplancke and Gaskins, 2001). Development of the vascular network in the intestine is also greatly

influenced by microbial colonization (Hooper, 2004). The indigenous gut microbiota also prevents colonization of the intestine by pathogens through competition for binding sites and secretion of antimicrobial compounds (Sekirov *et al*., 2010).

Furthermore, the nutritional role of the intestinal microbiota should not be underestimated, considering their role in digestion of compounds that are indigestible by the host (Hooper, 2004) and production of beneficial compounds, such as vitamins B and K (Canny and McCormick, 2008). Bacterial fermentation of otherwise indigestible fibre provides a double advantage for the host; firstly it increases the host digestive capacity and produces compounds available for absorption and use by the host. Secondly, through the production of volatile fatty acids, it reduces intestinal pH which inhibits growth of potentially pathogenic bacteria (Russell and Diez-Gonzalez, 1998). Likewise, intestinal bacteria can utilise compounds that diffuse from the blood into the gut lumen such as urea thereby playing a potentially therapeutic role in kidney failure (Younes *et al*., 1995). In addition, intestinal microbiota stimulate the absorptive capacity of enterocytes and intestinal motility (Willing and Van Kessel, 2010).

However, due to the fact that the intestinal microbiota also comprises pathogens or opportunistic pathogens, harbouring such a large bacterial load in the intestine does not come without risks. Bacterial metabolism produces some compounds that may have detrimental effects on the host, including ammonia, hydrogen sulphide, amines, phenols and indoles (Lewis and Southern, 2001). Furthermore, some compounds that are rendered harmless by conjugation in the liver can be processed by bacteria and made available for reabsorption (Montalto *et al*., 2009). Intestinal microbiota also compete with the host for nutrients and through degradation of bile acids, they interact with the host's ability to process fat (Lewis and Southern, 2001). In addition, mucolytic bacteria degrade the mucus layer coating the intestine and can predispose the host to bacterial infections (Lewis and Southern, 2001). Furthermore, due to the presence of intestinal bacteria, the host immune system is in a constant state of activation, producing mediators that inhibit growth (Jacobi *et al*., 2006). As a result, resources are directed to maintaining this state of immune stimulation. This is particularly important in farm animals, as it renders the host unable to reach its genetic potential for growth (Spurlock, 1997).

1.6.2. Composition of the porcine gastrointestinal microbiota

The adult pig intestine is colonised by >100 bacterial genera and >350 bacterial

species (Leser *et al*., 2002; Vahjen *et al*., 2010; Kim *et al*., 2011), with the highest density in the large intestine (Jensen and Jorgensen, 1994). However, the density of the intestinal microbiota throughout the GIT is highly variable, depending on the health status, digesta flow and properties, nutrient availability and immune reactivity of the host. The most abundantly represented phyla in the intestine of pigs are *Firmicutes* (30 - 98%), *Bacteroidetes* (2 – 55%) and *Proteobacteria* (0.9 – 5%) (Poroyko *et al*., 2010; Vahjen *et al*., 2010; Kim *et al*., 2011).

Furthermore, microbiota in the GIT are not uniformly distributed, with distinct numbers and populations colonizing each gastrointestinal segment (Figure 1.3). Bacterial numbers in the stomach and proximal small intestine are lower due to rapid digesta transit, toxicity of bile salts in the small intestine (Walter, 2008) and HCl production in the stomach, which maintains the pH at values below 4. Microbial populations in the stomach and proximal small intestine are comprised mostly of acid tolerant lactobacilli and streptococci (Mackie *et al*., 1999; Montagne *et al*., 2003) and are present in relatively low numbers (~10^3-10^8 CFU/g of digesta) (van Winsen *et al*., 2001; Castillo *et al*., 2006). The large intestine is the site at which most bacterial activity takes place, a situation which is reflected by the large numbers of bacteria present in the caecum and colon (10^{10}-10^{11} CFU/g of digesta) (Jensen and Jorgensen, 1994; van Winsen *et al*., 2001) (Figure 1.3). These high counts are as a result of increased residence time and an increase in pH relative to the proximal GIT (Varel and Yen, 1997). The anaerobic environment in the large intestine promotes the growth of anaerobic bacteria. The bacterial populations resident in the pig large intestine are largely dependent on available substrates with members of the genera *Prevotella*, *Clostridium, Megasphaera, Faecalibacterium, Acidaminococcus*, and *Succinivibrio* most frequently encountered (Leser *et al*., 2002; Piva *et al*., 2003; Guo *et al*., 2008; Kim *et al*., 2011; Lamendella *et al*., 2011).

The most commonly used microbial indicators of intestinal health are *Enterobacteriaceae*, *Lactobacillus* and total anaerobes, with the *Lactobacillus*: *Enterobacteriaceae* ratio reported to be similar when measured using either traditional or molecular techniques (Castillo *et al*., 2006). *Enterobacteriaceae* are indicators of pathogenic bacteria, as the family includes species such as *Escherichia coli* and *Salmonella* which can cause disease in humans and animals. *Lactobacillus* are considered beneficial. For example, lactic acid produced by *Lactobacillus* as an end product of carbohydrate metabolism, lowers the intestinal pH acting as an antimicrobial

barrier (Barrow *et al*., 1977; Lewis and Southern, 2001; van Winsen *et al*., 2001). Furthermore, in suckling piglets in which the gastric secretion of HCl is not yet fully developed, the production of lactic acid from lactose in milk by lactobacilli is the only factor that maintains a low stomach pH (Lewis and Southern, 2001). Other bacteria such as members of the *Bacteroides-Prevotella* group, through degradation of dietary fibre and subsequent production of organic acids, have been associated with beneficial effects in pigs (Varel and Yen, 1997; Flint, 2004).

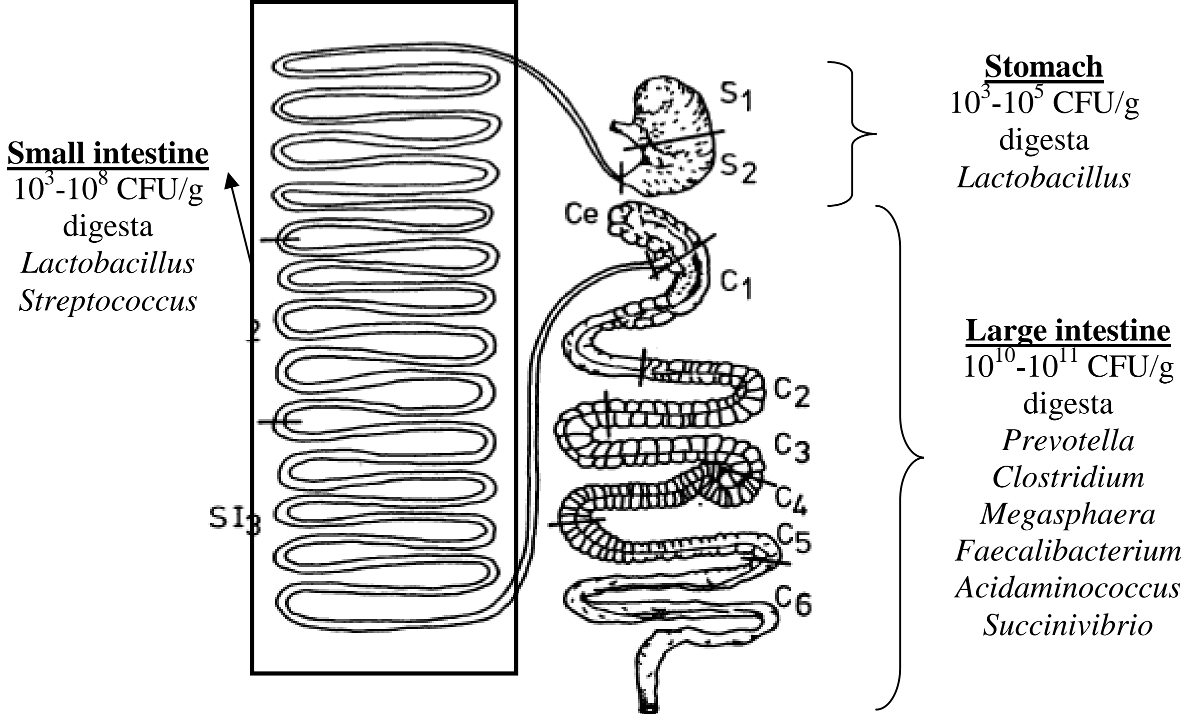

Figure 1.3. Microbiota in the porcine GIT.
Total counts (CFU = colony forming unit) as well as principal bacterial genera are indicated. Modified from Jensen and Jorgensen, 1994.

Traditionally, microbial numbers in the GIT were determined by cultivation on microbiological media. However, this technique is time consuming and laborious and will only allow for the detection of live bacteria. Furthermore, as many members of the gastrointestinal microbiota are unculturable (Suau *et al*., 1999) traditional methods fail to provide an accurate representation of the intestinal microbiota. However, this has been overcome by the development and standardization of molecular techniques, such as real-time-PCR and gene sequencing. These techniques provide a more accurate insight into intestinal microbial populations, as they can supply information about

unculturable as well as culturable bacterial populations. They also have the added advantage that samples can be stored for subsequent analysis (Konstantinov *et al*., 2004; Castillo *et al*., 2006; Murphy *et al*., 2010).

However, molecular methods can overestimate bacterial numbers, due to the inability to distinguish between dead bacterial cells or free DNA and live bacteria. On the other hand, as bacteria may adhere to particulate material during serial dilutions, traditional plate culturing may overlook some of the bacteria present (Castillo *et al*., 2006).

The 16S rRNA gene is one of the most commonly used genes in bacterial taxonomy studies because of its ubiquitous presence and stability and because it is large enough for bioinformatics purposes, yet small enough to allow low-cost sequencing (Janda and Abbott, 2007). An alternative to the 16S rRNA gene would be the chaperonin 60 gene; however it is less widely used than the former (Hill *et al*., 2005). Gene sequencing-based approaches give a much broader view of the intestinal microbiota. Furthermore, automatization of the sequencing process has resulted in increased accuracy and has increased the amount of data available for comparison. While gene sequencing technology has been available since the 1970s, the methods used were time-consuming, required expensive equipment ad were labour-intensive (Mardis, 2008). However, next-generation approaches to gene sequencing such as 454 sequencing which have been recently developed offer distinct advantages such as shorter sequencing times, much larger volumes of data obtained and usage of less resources (Mardis, 2008; Rothberg and Leamon, 2008). The 454 sequencing approach has been recently used to characterise the gut microbiota of pigs (Poroyko *et al*., 2010; Vahjen *et al*., 2010; Kim *et al*., 2011). However, sequencing may not always yield a definitive answer as to the identity of a particular bacterial species. Studies have shown the existence of bacterial groups with high DNA sequence similarity, which differ phenotypically as well as bacterial groups which have similar phenotypic characteristics but less DNA similarity (Janda and Abbott, 2007).

1.6.3. Effect of GM maize on intestinal microbiota

The Cry1Ab protein has been shown to possess *in vitro* antimicrobial activity against *Clostridium butyricum* and *Clostridium acetobutylicum* and an *Archaea*, *Metanosarcina barkeri* (Yudina *et al*., 2007). However, a more detailed *in vitro* study showed no effect of Cry1Ab against gram negative intestinal bacterial species such as

Proteus spp., Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, and other Gram negative bacteria such as *Pseudomonas aeruginosa, Agrobacterium radiobacter*, as well as Gram positives (*Arthrobacter globiformis, Bacillus subtilis and Bacillus cereus*) (Koskella and Stotzky, 2002). Although important to the overall safety assessment of Bt maize, such studies only provide preliminary data and should be replicated *in vivo*. However, to our knowledge, no *in vivo* studies have investigated the effect of Bt maize on the porcine gastrointestinal microbiota. An 11 day study in using four cows showed no effect of feeding Bt maize silage on six ruminal strains using real-time PCR analysis (Wiedemann *et al*., 2007). Likewise, a five-week feeding study with four cows revealed no differences in ruminal bacterial community structure using ribosomal intergenic spacer analysis (Brusetti *et al*., 2011). However, such low numbers of replicates per treatment, may yield misleading results due to well recognised inter-animal variability. Another four week study on cows also found no effect of GM maize silage on rumen microbiota as investigated by 16S rRNA gene sequencing (Einspanier *et al*., 2004). However, as the microbiota needs up to six weeks to adapt to food physical structure and chemical composition (Castillo *et al*., 2007), short-term studies may not be adequate to provide a conclusive answer as to the effect of GM maize on the intestinal microbiota. In a longer-term (3-year) study Trabalza-Marinucci *et al.* (2008) did not find any differences in culturable ruminal bacterial populations in sheep fed Bt maize silage. Furthermore, as there are substantial differences between the intestinal microbiota and digestive physiology of ruminants and monogastrics, ruminants are unsuitable as a model for humans when for studying response to GM plant consumption.

1.7. Effect of GM maize on tissue histology

When investigating the effects of GM plants on animal and human health, there are two major issues that need to be addressed. One is the effects and fate of the transgenic DNA and the second is the the effects and fate of the newly expressed transgenic protein (Cry1Ab protein in Bt maize). The fate of the transgene and transgenic protein are discussed in detail in section 1.9. Degradation of dietary DNA begins in the upper digestive tract with mastication, continues in the stomach due to the action of low gastric pH and is finalised in the small and large intestine under the influence of mammalian and bacterial endonucleases (Jonas *et al*., 2001; Lewis and Southern, 2001). Fragmented DNA is absorbed as nucleotides and recycled (Jonas *et*

al., 2001). Therefore, the most likely to result in a histologically detectable lesion would be the newly expressed transgenic protein. However, if DNA were to remain intact during intestinal transit, its incorporation into bacteria and/or host tissues could also lead to metabolic changes in these cells that may translate into production of harmful compounds, leading to an indirect pathogenic effect. Therefore, any signs of pathology as a result of GM ingredient consumption should be thoroughly investigated and interpreted in conjunction with other health indicators to identify the exact cause.

As Cry1Ab is a protein, protein metabolism should be kept in mind when searching for adverse effects. Protein degradation begins in the stomach, due to the action of low pH and gastric enzymes. It is further intensified in the small intestine, where pancreatic and epithelial proteolytic enzymes cause further digestion. This continues in the large intestine under the influence of bacterial proteases. Proteins are either recycled in the enteric epithelium or enter the portal circulation and are transported to the liver (Lewis and Southern, 2001) for degradation and recycling or inactivation (in the case of toxic compounds). After passage through the liver, compounds are released into the circulation, and pass into tissues or are eliminated. Therefore, the first tissues/organs to be examined when evaluating possible adverse effects of the Cry1Ab protein should be the intestinal lining, the associated lymphoid tissue, the liver and kidneys.

1.7.1. Intestinal histology

The intestine constitutes a semi-permeable barrier which allows the transport of nutrients from the lumen to absorptive cells and at the same time prevents bacteria and toxic bacterial metabolites from being absorbed. The ability of the intestine to act as a protective barrier is due to its complex structure and the interactions between the underlying immune system, mucus and host antibacterial compounds on one side and bacterial exclusion on the other side (Lewis and Southern, 2001).

The intestinal mucosa consists of the epithelium, the *muscularis mucosa* and *lamina propria* (Figure 1.4).

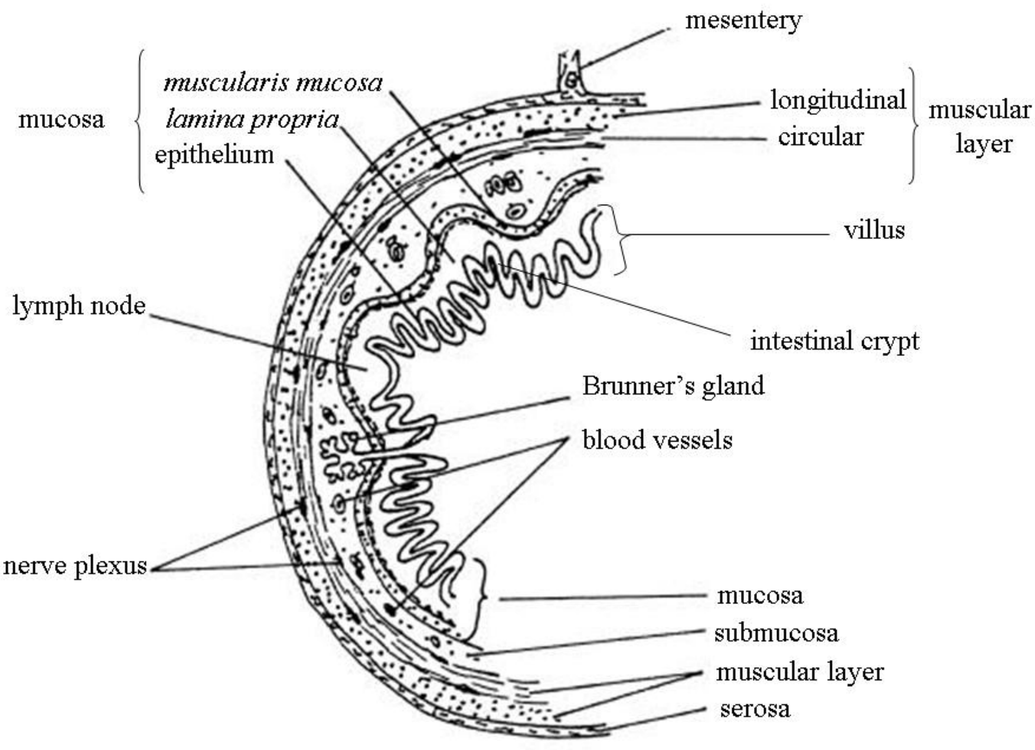

Figure 1.4. Schematic representation of intestinal structures (Lewis and Southern, 2001).

The epithelium is a dynamic, metabolically active single cell layer which lines the intestinal villi. The villi are finger-like projections of the mucosa, which increase the absorptive surface considerably and help maintain mechanical properties of the intestine.

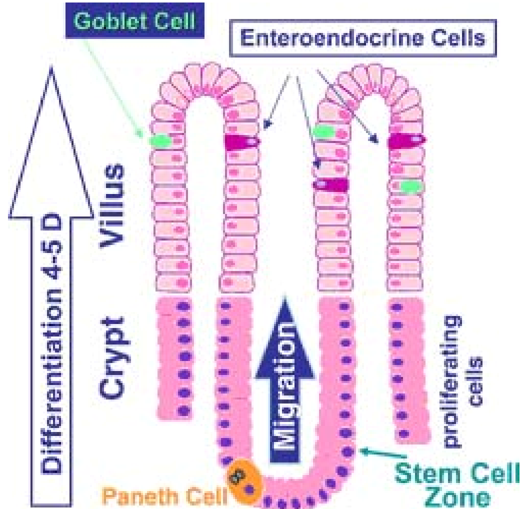

Figure 1.5. Overview of the intestinal epithelial cell types.

From http://www.umassmed.edu/gastro/labs/leiterlab.aspx

The epithelium is composed of enterocytes, goblet cells, Paneth cells and endocrine cells (Lewis and Southern, 2001) (Figure 1.5). Most of the cells lining the villus are enterocytes responsible for the absorptive and secretory capacity of the epithelium. Goblet cells are responsible for producing the mucus layer that lubricates and protects the intestine (see below) (Deplancke and Gaskins, 2001) and account for approximately 5% of the cells lining the villus. Mucus is also produced in the small intestine by Brunner's glands (Delmann and Eurell, 1998). The endocrine cells are responsible for hormone secretion within the intestine and are found in low numbers (~1%) (Lewis and Southern, 2001). Intestinal crypts are spaces between the villi populated with stem and Paneth cells (Figures 1.4 and 1.5). Stem cells migrate from the crypts towards the tip of the villus and differentiate along the way into cells lining the villus or Paneth cells. Paneth cells are secretory cells which protect the gut via secretion of several antibacterial compounds (e.g. lysozyme and defensins) and promote intestinal development by stimulating angiogenesis within the villi (Hooper, 2004; Motyl and Zabielski, 2005; Deheragoda and Wright, 2006). The intestine is a highly active environment, with the epithelium totally replaced every 2-20 days and old cells disposed of either by sloughing off into the lumen of the intestine, phagocytosis, apoptosis or autophagy (Lewis and Southern, 2001; Motyl and Zabielski, 2005).

The other components of the mucosa, the *muscularis mucosa* and the *lamina propria* provide support for movement of the villi (*muscularis mucosa*), blood supply, contain nerve endings and growth factors that regulate differentiation and proliferation of the stem cells (Deheragoda and Wright, 2006).

The mucus layer which is secreted by the goblet cells and covers the intestinal mucosa provides protection against pathogens, lubrication for intestinal contents and acts as a medium to transport nutrients across the intestinal lumen to the epithelial cells. The mucus layer is composed of mucins which are normally secreted at a baseline rate but in the case of an aggression, bioactive compounds stimulate the goblet cells to increase mucin production (Deplancke and Gaskins, 2001). The intestinal microbiota and their secretions have been shown to influence mucus quantity and structure either directly or by stimulating host cytokines such as TNF-α and IL-6 (Deplancke and Gaskins, 2001). Th2 type cytokines, involved in the allergic and antiparasitic IgE response also stimulate mucus production by goblet cells (Deplancke and Gaskins, 2001). Nutrition also plays a role and post-weaning deprivation of amino acids such as threonine leads to reduced mucus production throughout the porcine intestine (Lalles *et*

al., 2004). The mucus layer increases in thickness from the small intestine towards the rectum. The small intestine has a thin mucus layer which is discontinuous over the Peyer's patches allowing these immune structures to continuously sample the intestinal lumen.

Health and functionality of the intestine are closely related to tissue architecture (Skrzypek *et al*., 2007). Light microscopy evaluation of the size, shape and density of the villi, as well as depth of the intestinal crypts and density of mucus-producing cells can provide important information about the functional status of the intestine. An intestinal immune response due to dietary allergenic proteins is translated into shortened villi, increased eosinophil concentration in the *lamina propria* (Dreau *et al*., 1994), increased number of goblet cells per villus, higher mucus production and increased crypt depths (Pluske *et al*., 1997). The same changes have been shown to occur in pigs during the post-weaning period as a result of low feed consumption.

1.7.2. Effect of GM maize on intestinal histology

To our knowledge, no studies to date have investigated the effect of feeding Bt maize on intestinal histology of pigs. Previous studies with rats have shown a lack of effect of feeding Bt maize over three generations on stomach and duodenal histology (Kilic and Akay, 2008). Similarly, rats fed the Cry1Ab purified protein for 2 weeks showed no signs of gastrointestinal histopathology (Onose *et al*., 2008). However, Fares and El-Sayed (1998), described histological alterations in the ileum of mice fed Bt delta-endotoxin for 2 weeks but not in those fed GM potatoes expressing the toxin. No effects on intestinal histology were observed following eight months of feeding Bt maize to salmon (Sanden *et al*., 2005). Likewise, feeding Bt maize to sheep for three years did not influence on tissue architecture of the rumen, abomasum, duodenum or caecal appendix (Trabalza-Marinucci *et al*., 2008). However, the same study showed proliferative activation of ruminal basal cells of sheep fed Bt maize, indicating an increased epithelial turnover rate.

1.7.3. Tissue histology

Most tissues share a remarkable adaptability in relation to a changing environment. However, upon aggression beyond their ability to adapt, tissues lose their ability to function normally, leading to metabolic changes which in turn result in structural changes observable via microscopy. The most common structural changes are

(Stevens *et al*., 2002):

- **Cloudy swelling** - the cellular organelles become filled with fluid
- **Hydropic degeneration** - appearance of cytoplasmic vacuoles
- **Fatty change** - due to impaired cellular lipid metabolism, fat accumulates in cells

The normal tissue response to an aggression is inflammation. The histological characteristics of inflammation are increased blood flow (congestion) which results in accumulation of fluid and inflammatory cells in the tissue. The outcomes of inflammation are:

- **Resolution** - the destroyed tissue is replaced with normal, functional tissue
- **Fibrosis** - destroyed tissue is replaced by connective, fibrous tissue which has no other role than to fill the structural gap
- **Progression** to chronic inflammation

All stages of inflammation are common to most tissues and can be observed microscopically. Examining tissues for inflammation can be a useful tool when assessing the effects of GM ingredient consumption.

1.7.4. Effect of GM maize on tissue histology

Normal rats and rats with gastrointestinal impairment fed purified Cry1Ab protein for 2 weeks showed no significant differences in the histology of the brain, thymus, lungs, heart, spleen, liver, adrenals, kidneys, testes, skin, mammary gland, sternum with marrow, femur with marrow, submandibular and mesenteric lymph nodes, salivary glands, aorta, trachea, tongue, oesophagus, stomach, small and large intestines, pancreas, urinary bladder, epididymides, seminal vesicles, prostate, bulbourethral gland, pituitary, thyroids, parathyroids, spinal cord, trigeminal nerve, sciatic nerve, nasal cavity, Harderian gland, eyes and thigh muscle compared to the control group (Onose *et al*., 2008). However, the short duration of this study is unlikely to predict the outcome of long-term consumption.

Differences in tissue histopathology were, however, found in another study in rats after ~3 months of feeding Bt maize (20% of the diet). Liver congestion and kidney tissue alterations were found but were thought not to be clinically significant or life threatening (Kilic and Akay, 2008). Minor alterations in hepatic and pancreatic cell nuclei have also been found in sheep following 3 years of Bt maize consumption

(Trabalza-Marinucci *et al.*, 2008). To our knowledge, no studies to date have investigated the effect of Bt maize on tissue/organ histology in pigs.

1.8. Effect of GM maize on haematology and clinical biochemistry

Serum/plasma and urine biochemistry are extensively used as indicators of health status and closely reflect changes in metabolic pathways and disease status. As all cells have a metabolic role, they possess a large arsenal of enzymes which may escape into the intracellular spaces and therefore into blood in the event of cell damage. Some of these enzymes are tissue-specific; however, many are widely distributed (Kaneko, 1980; Giannini *et al.*, 2005).

As the liver and kidneys are the main detoxifying organs in the body, assessment of their function is of crucial importance for the safety assessment of GM ingredients. The most frequently used biochemical indicators of liver and kidney dysfunction are summarized in Table 1.5 (Kaneko, 1980; Boone *et al.*, 2005).

Table 1.5. Biochemical parameters indicative of liver and kidney function.

Name	Normal range in pigs	Change with dysfunction	Reference
Liver - indicators in serum			
Aspartate aminotransferase (AST)	29 - 84 U/L	2.5 - 41 fold increase	Kaneko, 1980 Casteel *et al.,* 1993 Radostits *et al.,* 2007 Boone *et al*., 2005 Giannini *et al*., 2005 Fernandez and Kidney, 2007 Kaneko *et al*, 2008 Evans, 2009 Egeli *et al*., 1998
Alanine aminotransferase (ALT)	31 - 58 U/L	5 - 10 fold increase	
Alkaline phosphatase (ALP)	118 - 395 U/L	2.3 - 9.6 fold increase	
Total protein	35 - 89 g/L	Decrease	
Gamma glutamyl transferase (GGT)	10 - 60 U/L	2.9 - 5.7 fold increase	
Kidney			
Indicators in serum			
Creatinine	88 - 239 µmol/L	> 2 fold increase	Evans, 2009 Radostits *et al*., 2007 Stonard, 1990 Xin *et al*., 2004 Baum *et al*., 1975
Urea	1.67 – 10.7 mmol/L	> 2 fold increase	
Indicators in urine			
Creatinine	No data available	Decrease	Stonard, 1990 Evans, 2009
Protein	No data available	Increase	

Liver associated enzymes, such as AST and ALT are widely recognised as indicators of hepatocellular damage (Boone *et al*., 2005; Giannini *et al*., 2005; Kaneko *et al*., 2008). During hepatic injury, the hepatocyte membrane loses its integrity, allowing cellular components to escape into the blood. Due to their localization in the hepatocyte, AST and ALT can yield information on the severity of the injury and the stage of recovery, as follows. AST is mostly found in mitochondria while ALT is found mostly in cytoplasm of hepatocytes (Giannini *et al*., 2005; Kaneko *et al*., 2008). Therefore, an aggression results in an initial loss of cytoplasmic enzymes (ALT). If the aggression continues, hepatocyte destruction reaches cellular organelles, such as mitochondria, releasing AST. However, differential localization of these enzymes within the liver parenchyma may also indicate the nature of the dysfunction. For example AST is more concentrated in hepatocytes which are closer to the portal circulation and receiving blood directly from the intestine. Therefore, a nutritional deficiency or lack of oxygen which would preferentially damage this part of the liver may result in a much greater release of AST from hepatocytes into the blood stream (Delmann and Eurell, 1998; Giannini *et al*., 2005).

Likewise, a rise in serum ALP and GGT would be indicative of bile retention, which is common in liver disease as a result of biliary spasm and inability to excrete bile (Kaneko *et al*., 2008).

Total serum protein can also be lower as a result of malnutrition or when liver damage impairs hepatic protein secretion and turnover. As serum proteins are not normally excreted in urine, a rise in urinary protein is indicative of kidney damage and demonstrates the loss of the kidneys' ability to act as a biological filter (Kaneko *et al*., 2008).

Creatinine and urea have been widely used as indicators of kidney dysfunction. As creatinine is secreted by the kidney, kidney damage resulting in an impaired secretory function leads to a rise in serum creatinine and a decrease in urinary creatinine (Kaneko *et al*., 2008). Factors reducing kidney blood flow or impairing urine excretion lead to renal urea reabsorption thus increasing serum urea (Kaneko *et al*., 2008).

Haematological examinations are also useful in detection of any adverse effects of GM ingredient consumption. For example, determination of numbers of blood cells and distribution of the white cell component can reveal details of an inflammatory response and provide clues as to the nature of the event. Determination of haemoglobin and erythrocyte numbers can give valuable information pertaining to iron metabolism.

Furthermore, coagulation factors, such as platelets can be an indicator of toxic effects (Feldman *et al*., 2006).

To our knowledge, to date, no studies investigating the effect of GM maize on clinical chemistry or haematology have been conducted in pigs. Feeding Bt maize to rats over three generations resulted in lower total protein and higher creatinine (Kilic and Akay, 2008). However, another study in rats investigating the effects of purified Cry1Ab protein for 2 weeks (Onose *et al*., 2008) and a 90-day rat study feeding Bt MON810 maize (Hammond *et al*., 2006) showed no significant differences in haematology and serum biochemistry between the treatment and control groups.

Sheep fed Bt maize were found to have higher serum GGT, but this increase was not associated with any haematological changes or other change in liver integrity indicators (Trabalza-Marinucci *et al*., 2008). However, the authors did not assess pesticide, herbicide or mycotoxin contamination of the diets. In contrast, a study in calves, where mycotoxin contamination was not detected in diets found no difference in blood biochemistry parameters (Shimada *et al*., 2006).

1.9. Transgene and GM protein transfer

One of the greatest public concerns regarding GM food and feed is the possible transfer of genetic material from transgenic plants to other plants, or to human or animal tissues and possible interference with the DNA of the latter (EFSA, 2008). Concerns are further fuelled by the fact that upon particle acceleration multiple transgenic DNA fragments may be inserted at random into the host plant genome and that it may behave differently than anticipated. Such a scenario could potentially disrupt physiological processes or lead to expression of truncated versions of the intended product with different effects (Rosati *et al*., 2008). However, transgenic DNA does not differ in structure from the DNA normally present in animal or plant cells and spontaneous gene rearrangement has already been documented for conventional maize lines (Jonas *et al*., 2001; Rosati *et al*., 2008). The only difference is the arrangement of the coding sequences, so there is no reason to suspect why transgenic DNA should act differently to normal plant DNA upon ingestion (Jonas *et al*., 2001).

As outlined in section 1.2.2, production of transgenic plants requires introduction of so called “selectable marker” genes. These genes code for resistance to certain compounds which can then be used for identification of the plants expressing the newly introduced trait. The most commonly used marker genes encode for antibiotic (either

kanamycin or hygromycin) resistance or herbicide resistance (Miki and McHugh, 2004). The use of antibiotic resistance genes in transgenic plants has lead to consumer concerns regarding the possibility of antibiotic resistance transfer to indigenous microbiota (EFSA, 2008; Dona and Arvanitoyannis, 2009). However, as a result of this, the use of antibiotic resistance genes in GM plants has been reduced and alternatives, such as genes encoding for compounds detectable by chemical reactions are being considered (Miki and McHugh, 2004).

The *nptII* gene used in the engineering of MON810 maize (EFSA, 2009) encodes for resistance to kanamycin, neomycin, geneticin and paramomycin (Miki and McHugh, 2004). However, due to their toxicity, these antibiotics are rarely used in human therapy (Miki and McHugh, 2004). Furthermore, the construct expressed in the MON810 transgenic maize was shown to be stable and to lack the *nptII* marker gene (EFSA, 2009).

1.9.1. Transfer of transgene and GM protein to animal tissues

As outlined in section 1.2.2, the minimum functional unit of the *cry1Ab* gene is 1800 bp and DNA of this size is difficult to amplify even from processed animal feed (Mazza *et al*., 2005; Rossi *et al*., 2005). Recovery of a 519 bp *cry1Ab* gene fragment from piglet blood and tissues was reported by Mazza *et al.* (2005). This study found the highest recovery rate (30%) from the blood of piglets fed MON810 maize at 50% of the diet for 35 days. For other tissues (spleen, liver, kidney), the recovery rate was ~10%, the lowest being in the thigh muscle (~5%).

In contrast, Chowdhury *et al.* (2003) did not detect gene fragment in the blood of 40 kg pigs. However, 110 bp fragments of the gene were detected in the stomach, small intestine, caecum and rectum of pigs. Larger fragments (437 bp) were found in the stomach of all animals, in the small intestine and caecum of only three out of five pigs and in the rectum of one out of five pigs. Maize-specific gene fragments were also found in most digesta samples from the same pigs (Chowdhury *et al*., 2003). Similarly, Reuter *et al.* (2003) found a 211 bp fragment of the *cry1Ab* gene at various locations along the GIT of pigs within 48 h of feeding and maize-specific genes were detected in digesta of all pigs, up to 72 h after feeding. However, no transgenic (211 bp) or maize-specific (226 bp) DNA was found in blood, liver, lymph nodes, spleen, kidney, various muscles and ovaries. Further searches for the plant-specific multicopy *rubisco* gene with a primer specific for a shorter sequence (140 bp) revealed plant DNA fragments in

different organs. The highest concentration was in the blood and organs with a high blood flow (ovaries, muscle and liver) (Reuter and Aulrich, 2003). A study in wild boars revealed the presence of the *rubisco* gene in the digesta but not in the organs. However, a 211 bp and a 420 bp *cry1Ab* gene fragments were only detected in stomach of 40% of the animals and not further down the GIT or organ samples, while a 727 bp fragment was not detected in any of the samples (Wiedemann *et al*., 2009). Fragments of the *cry1Ab* gene (189 bp) along with plant-specific DNA (199 bp) were amplified from duodenal juice from cattle but no such fragments were detected in faeces or blood (Einspanier *et al*., 2001).

Guertler *et al.* (2010) reported the presence of conventional maize DNA and the absence of transgenic DNA fragment (206 bp) in the blood, urine, milk and faeces of cows fed Bt maize for 25 months.

In poultry, Deaville *et al.* (2005) found that a 203 bp fragment of the *cry1Ab* gene were mostly present in the gizzard digesta (15% of samples) and less frequently in the small or large intestine (1 and 3%, respectively). Similar length fragments of maize-specific genes were also found in the GIT. However, these genes were amplified more often (49-99% of samples). No fragments of the transgene were found in any of the blood or organ samples. In contrast, maize-specific gene fragments were found in all of the organs investigated, with higher recovery in the gizzard, bursa and kidney (Deaville and Maddison, 2005). The absence of *cry1Ab* (189 bp) fragment detection in poultry organs was also reported by Einspanier *et al.* (2001). However, plant-specific DNA was present in all tissues sampled (Einspanier *et al*., 2001). Successful amplification of an 1800 bp transgenic fragment, corresponding to the *cry1Ab* minimum functional unit was reported by Rossi *et al.* (2005) from the crop and gizzard digesta of broilers. However, no fragment of this size was found in the blood or intestinal digesta. In the same study, plant-specific DNA was isolated from all segments of the GIT, as well as from blood (Rossi *et al*., 2005).

The Cry1Ab protein has been shown to persist in the intestinal tract of cattle in quantities ranging from 1.4 to 5.1 ng/g of digesta. Immunoblotting revealed that the 62 kDa protein present in maize was fragmented into 17 and 34 kDa fragments (Lutz *et al*., 2005). Within the GIT of pigs, the protein has been found in digesta from all segments, with no adverse effects reported (Chowdhury *et al*., 2003). The Cry1Ab protein was not detected in the blood (Vijay *et al*., 2008) or organs (Chowdhury *et al*., 2003) of cattle fed Bt maize.

From the literature reviewed, it is clear that *cry1Ab* transgene fragments are present with variable frequency in blood, digesta and organs of farm animals. Concurrent, plant-specific DNA was also amplified from the respective samples, demonstrating that transgenic DNA does not differ from plant-specific DNA. However, no adverse health effects were linked to the presence of transgenic or inherent plant DNA. This is perhaps because the presence of genetic material in the blood and tissue of animals does not guarantee incorporation into the host genome or any adverse effects in the host. Furthermore, the Cry1Ab protein has been detected in intestinal digesta but does not seem to frequently transfer to blood or organs.

1.9.2. Gene transfer to bacteria

Studies have shown that horizontal gene transfer from plants to bacteria is rare, hindered by a number of factors and likely to happen over much longer periods of time than anticipated (Nap *et al*., 1992). Uptake of genetic material by intestinal bacteria may occur in three ways; 1) by integration of free DNA from the environment (transformation); 2) it may be mediated by viruses capable of infecting bacteria (transduction) or 3) through plasmid transfer between two adjacent cells (conjugation) (Lorenz and Wackernagel, 1994; Nielsen *et al*., 1998; Madigan *et al*., 2000) (Figure 1.6).

Transformation is the most likely mode of uptake of transgenes from transgenic plants to intestinal bacteria.

Although studies have demonstrated the presence of transgenic DNA in the environment, in the vicinity of bacteria, this DNA is not necessarily incorporated into the bacterial genome (Nielsen *et al*., 1998). To avoid the incorporation of random DNA into the genome, bacteria possess an arsenal of DNA restriction enzymes which degrade DNA into fragments of about 250 bp (Nielsen *et al*., 1998).

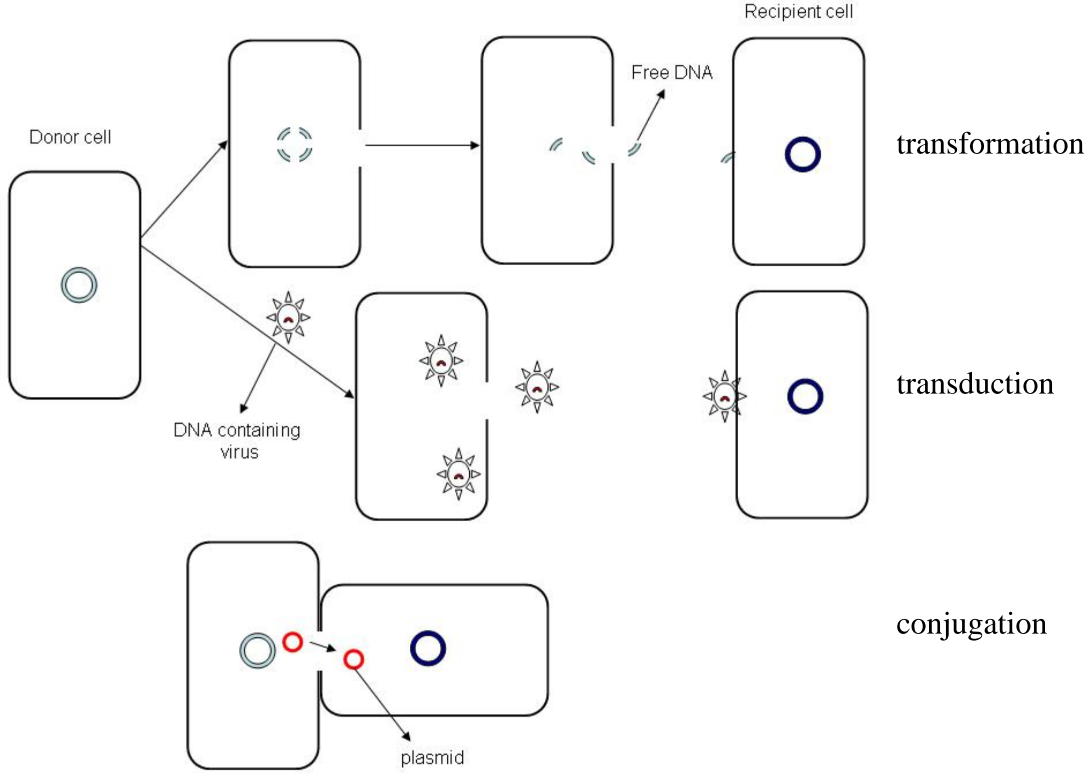

Figure 1.6. Means of horizontal gene transfer. Modified from Madigan *et al*., 2000

For bacteria to incorporate genetic material the following conditions must be met:

- **Quantity and quality** – the gene must be of sufficient quality and present in large enough quantities. Before it reaches the intestinal lumen, dietary DNA is subjected to various processes that fragment it into lengths unlikely to be of any transcriptional value. DNA degradation begins with milling of the food/feed ingredients, pH alterations and heat treatment during food/feed processing. Following processing, DNA incorporated into the whole food/feed is then protected by the "matrix effect" i.e. DNA adsorbed to or incorporated into solid particles is more resistant to degradation (Lorenz and Wackernagel, 1994; Jonas *et al*., 2001). On consumption, DNA is further degraded by mechanical means (mastication), through DNase activity of salivary, pancreatic, intestinal and microbial enzymes (Takeshita *et al*., 2000) and the low pH in the stomach (Jonas *et al*., 2001).
- **Bacterial competence** – the target bacteria have to be capable of DNA uptake (competent) (Lorenz and Wackernagel, 1994; Nielsen *et al*., 1998; Madigan *et al*., 2000; Jonas *et al*., 2001) and naturally competent cells represent a small fraction of bacteria. Studies have shown that the transforming frequency of bacterial species of

clinical importance is very low, ranging from 1.1×10^{-2} to 5.5×10^{-6} chromosomal marker transformants/viable cell, while in other species of bacteria, it ranges from 1×10^{-2} to 1.9×10^{-9} chromosomal marker transformants/viable cell (Lorenz and Wackernagel, 1994). To be successfully incorporated (Jonas *et al*., 2001), DNA needs to be of a certain length and this differs with the bacterial species involved. Development of bacterial competence is greatly influenced by temperature and pH. DNA uptake is also dependent on environmental conditions, therefore, the presence of ions such as Mg^{2+}, Na^{+}, K^{+}, NH_4^{+} (Lorenz and Wackernagel, 1994) will greatly influence DNA uptake by bacterial cells. As the intestinal environment is normally stable in each segment, competence should remain stable. However, when the gut environment changes, as happens during antibiotic treatment, stress at weaning and in immuno-compromised individuals, the number of competent bacterial cells may increase.

- **DNA homology** – bacterial DNA uptake is correlated with sequence homology between the free DNA and the target cell genome. Therefore, DNA transfer is more likely to take place between cells that are closely related, (Lorenz and Wackernagel, 1994). In the case of antibiotic resistance, if the cell population does not already possess the gene within their genome, transfer is highly unlikely. However, in some bacterial species, non-specific uptake has been demonstrated (Lorenz and Wackernagel, 1994).

- **Expression and stability** – gene expression and persistence of the gene within the microbiota in any host is subject to selective pressure. In the case of herbicide resistance genes, the risk is not incorporation into the bacterial genome but the development of "superweeds". However, the search for alternate marker genes and careful assessment of the ecosystem in which the GM plant will be grown are likely to lower this risk. Antibiotic resistance genes may being incorporated into the bacterial genome but in this case, the selective pressure required for successful expression is missing. In addition, occurrence of the antibiotic resistance genes in natural habitats is already high (Nielsen *et al*., 1998), therefore, transfer of these genes from GM plants to bacteria is unlikely to be of practical significance.

DNA transfer and incorporation into bacterial genomes is not easy to demonstrate and to date very few studies have investigated it. A soybean transgene fragment has been detected in the small intestine of human lacking the large intestine (ileostomists) and incorporation of the gene fragment into small intestinal bacteria was demonstrated

(Netherwood *et al*., 2004). Furthermore, the gene was never detected in the faeces of non-ileostomists. However, the physiology of ileostomists differs from that of healthy individuals and consequently the intestinal microbiota is also likely to differ. Furthermore, as the transgene was detected with a very low frequency even in bacteria grown in an enrichment medium the bacteria that have incorporated the gene are likely to make up a small proportion of the intestinal microbiota. Therefore, additional research needs to be conducted to fully interpret such findings.

A 184 bp fragment of the *cry1Ab* gene was undetectable in ruminal amylolytic and cellulolytic bacteria in sheep fed Bt maize for three years (Trabalza-Marinucci *et al*., 2008). However, the gene fragment was also undetectable in the ruminal fluid. No studies to date have investigated gene transfer from GM plants to the porcine intestinal microbiota.

1.10. Conclusions

To date, no clear pattern of adverse health effects following Bt maize consumption has emerged. Minor changes in serum biochemistry and histology of rats were observed. However, data should be carefully interpreted and statistically significant differences correlated with clinical findings in order to provide a clearer picture. Furthermore, some biochemical parameters have been shown to differ between farm and laboratory animals, due to differences in physiology, size and dietary habits. Therefore, extrapolation of findings from laboratory animals to humans should be done cautiously. Blood biochemistry, haematology, and tissue histology do not have great individual merit when viewed alone but when interpreted in combination, they provide a highly accurate picture of the impact of a GM feed/food on animal or human health.

Each public concern regarding GM ingredients needs to be addressed with the utmost care and impartiality, so that the public has a clear picture of the progress of research. This is necessary to increase public confidence in testing procedures. In addition, PMM should be implemented so that any adverse effects of GM ingredients in diverse populations are rapidly detected and proper measures taken.

1.11. References

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Overall objectives

1. To investigate the effect of short-term (31 days) feeding of genetically modified (GM) Bt maize to weanling pigs on growth performance, organ structure and function.
2. To investigate the effect of short-term (31 days) feeding of Bt maize to weanling pigs on their immune response.
3. To assess transfer of the *cry1Ab* transgene and Cry1Ab protein to animal tissues and disappearance from the digestive tract following 31 days of feeding Bt maize.
4. To investigate the effect of short-term feeding of Bt maize to pigs on intestinal microbiota.
5. To investigate the effect of long-term (110 days) feeding of Bt maize to pigs on growth performance, slaughter characteristics, body composition, organ structure and function and intestinal structure.
6. To investigate the effect of long-term (110 days) feeding of Bt maize to pigs on their immune response.
7. To assess transfer of the *cry1Ab* transgene and Cry1Ab protein to animal tissues and disappearance from the digestive tract of pigs following 110 days of feeding Bt maize.
8. To assess a potential age-specific response to feeding Bt maize and to examine if such a response is reversible.
9. To investigate the effect of long-term feeding of Bt maize to pigs on intestinal microbiota.

2. Effects of short-term feeding of Bt MON810 maize on growth performance, organ morphology and function in pigs

Walsh, M. C., Buzoianu, S. G., Gardiner, G. E., Cassidy, J. P., Rea, M. C., Ross, R. P. and Lawlor, P. G. (2012). Effects of short-term feeding of Bt MON810 maize on growth performance, organ morphology and function in pigs. Br. J. Nutr. 107: 364-371.

2.1. Abstract

Male weanling pigs (n=32) with a mean initial body weight of 7.5 kg and a mean weaning age of 28 d were used in a 31 d study to investigate the effects of feeding genetically modified (GM; Bt MON810) maize on growth performance, intestinal histology and organ weight and function. At weaning, pigs were fed a non-GM starter diet during a 6 day acclimatization period. Pigs were then blocked by weight and litter ancestry and assigned to diets containing 38.9% GM (Bt MON810) or non-GM isogenic parent line maize for 31 days. Body weight and feed disappearance were recorded on a weekly basis (n=16/treatment) and pigs (n=10/treatment) were sacrificed on d 31 for collection of organ, tissue and blood samples. GM maize-fed pigs consumed more feed than control pigs during the 31 day study ($P < 0.05$) and were less efficient at converting feed to gain during d 14-30 ($P < 0.01$). Kidneys of pigs fed GM maize tended to be heavier than those of control pigs ($P = 0.06$); however, no histopathological changes or alterations in blood biochemistry were evident. Small intestinal morphology was not different between treatments. However, duodenal villi of GM fed pigs tended to have less goblet cells/µm of villus compared to control pigs ($P = 0.10$). In conclusion, short-term feeding of Bt (MON810) maize to weaned pigs resulted in increased feed consumption, less efficient conversion of feed to gain and a decrease in goblet cells/µm of duodenal villus. There was also a tendency for an increase in kidney weight but this was not associated with changes in histopathology or blood biochemistry. The biological significance of these findings is currently being clarified in long-term exposure studies in pigs.

2.2. Introduction

The inclusion of GM plants in animal feed and for human consumption has increased consistently over the past 15 years since they were first cultivated in 1996 (James, 2010). During this time, the cultivation area of GM crops has increased 87-fold reaching 148 million hectares worldwide in 2010, making the procurement of exclusively non-GM crops more difficult and expensive. GM maize is the second most important biotech crop after GM soybeans (James, 2010) and the first one to have a wider variety of genetic modifications than glyphosate-tolerant soybean.

Since the introduction of GM crops, much debate, both within and outside the scientific community has centred on issues related to safety for consumption. The most dominant agronomic traits introduced by genetic engineering include herbicide tolerance, insect resistance (Bt) and stacking of both of these traits (James, 2010). These genetically engineered traits offer the possibility of higher agronomic productivity in times of insect infestation without the use of insecticides and/or the use of less expensive broad spectrum herbicides for weed control (Bertoni and Marsan, 2005). However, the increased usage of GM crops for direct human consumption and feeding to meat- and milk-producing animals has lead to public concern (Paparini and Romano-Spica, 2004). These concerns however, do not relate to the GM technology itself but to any unintended consequences that may arise from its use. Consumer concerns are mostly related to a perceived risk to health, development of toxicity, allergenicity of the transgenic proteins or the transfer of antibiotic resistance from the plant to bacteria residing in the human gastrointestinal tract (GIT) (Bertoni and Marsan, 2005). Other concerns are associated with environmental issues, such as gene transfer from GM crops to indigenous plants, reducing biodiversity and influence of the GM crops on non-target species (Moses, 1999; Malarkey, 2003; Paparini and Romano-Spica, 2004; Hug, 2008).

Bt (MON810) maize is engineered to express the truncated Cry1Ab toxin from *Bacillus thuringiensis* which confers resistance to the European corn borer. This toxin interacts with the target larvae's intestinal cells disrupting the intestinal lining leading to death (Schnepf *et al*., 1998; Crickmore, 2005; Broderick *et al*., 2009). However, the toxin is believed to be non-toxic to mammals, birds, reptiles and amphibians due to a lack of specific receptors in the intestinal tract (Schnepf *et al*., 1998). The Cry1Ab protein has no homology to any allergenic proteins and was successfully degraded in simulated gastric conditions (EFSA, 2008). Trials conducted with Bt maize in pigs,

cattle, and poultry have shown no significant differences in performance or demonstrated any adverse health effects (Reuter *et al*., 2002; Custodio *et al*., 2006; Shimada *et al*., 2006; Flachowsky *et al*., 2007). However, some studies found subtle histopathological changes in rats (Kilic and Akay, 2008) and altered immune responses in mice (Finamore *et al*., 2008) and fish (Sagstad *et al*., 2007) fed Bt maize. Currently, GMOs receive EU authorization for use following pre-market risk assessment which focuses on the risk that untoward responses will arise from GMO exposure. The main concern with GMOs is that unintended responses may not be evident until a genetically diverse population has been exposed to them for a long period of time. Post-market monitoring of GMOs may reveal any long-term effects of GM exposure not identified during the short-term pre-market risk assessment (EFSA, 2009).

In the present study, we evaluated the effects in pigs of 31 days of feeding Bt (MON810) maize compared to an isogenic parent line maize. Particular attention was afforded to changes in growth performance, intestinal morphology and organ pathology and function in an attempt to identify any changes which may serve as an early warning sign for a biological effect. To our knowledge, this is the first experiment in pigs that has simultaneously evaluated the safety of Bt (MON810) maize over such a wide range of physiological processes.

2.3. Materials and methods

Experimental design and diets

The pig trial complied with European Union Council Directive 91/630/EEC (outlines minimum standards for the protection of pigs) and European Union Council Directive 98/58/EC (concerns the protection of animals kept for farming purposes) and was approved by, and a license obtained from, the Irish Department of Health and Children. Ethical approval was obtained from the Teagasc and Waterford Institute of Technology ethics committees. Thirty-two crossbred (Large White × Landrace) weanling pigs (entire males) were weaned at approximately 28 days of age and were blocked by weight and litter, and randomly assigned to one of two dietary treatments; (1) non-GM isogenic parent line of maize (Pioneer PR34N43) and (2) GM maize (Pioneer PR34N44 event MON810). Planted seeds derived from MON810 and its parental control maize (PR34N44 and PR34N43 varieties, respectively) were grown simultaneously side by side in Valtierra, Navarra, Spain. A non-GM starter diet was fed *ad libitum* for the first 6 days post-weaning during a baseline acclimatization period and

either the non-GM or GM maize experimental diets were fed for the remaining 31 days. Diets were manufactured in the Moorepark feed mill and were formulated to meet or exceed the NRC (NRC, 1998) requirements for weanling pigs (Table 2.1). Stringent quality control measures were employed to avoid cross contamination of non-GM with GM diets. Carryover in the feed manufacturing system was minimized by flushing the system with non-GM ingredients and the preparation of non-GM diets prior to diets containing the GM-maize. In addition, non-GM soybean meal was used in the manufacture of all diets. Cereals were ground by hammer mill through a 3 mm screen before mixing. Diets were pelleted to 5 mm diameter after steam conditioning at 50-55ºC. Proximate analysis (FBA Laboratories, Waterford, Ireland) and amino acid analysis (Sciantec Analytical Services Ltd., Cawood, UK) was performed on all diets. Representative samples of feed were taken from each treatment prior to feeding according to Hartnell *et al.* (2007). Prior to analysis, samples were ground through a 2 mm screen in a Christy Norris hammer mill. Dry matter was determined by oven drying for 4 h at 103ºC. Ash was determined by incineration in a muffle furnace (Gallenkamp, London, UK) at 550ºC overnight. Crude protein was determined as N x 6.25 using a LECO FP - 2000 analyser (Leco Instruments Ltd., Stockport, Cheshire, UK). Fat was determined by extraction with perchlorethylene in a Foss Let 15300 (A/S N. Foss Electric, Hillerod, Denmark) according to the method described by Usher *et al.* (1973). Crude fibre was measured by a Fibretec semi-automatic system (Tecator, Hoganas, Sweden). Analysis of the carbohydrate fractions, amino acid profile (Sciantec Analytical Services Ltd., Cawood, UK) and proximate composition (FBA Laboratories, Waterford, Ireland) of the GM and non-GM maize was also performed (Table 2.2). The carbohydrate fractions quantified included starch, sugar as sucrose, acid detergent fibre, neutral detergent fibre, water soluble carbohydrate, enzyme resistant starch and acid detergent lignin. The quality of the manufactured pellets was also assessed, including pellet durability, diameter and hardness, as previously described by McMahon and Payne (1991) (Table 2.1). The GM and non-GM maize were tested for the presence of the *cry1Ab* gene by MON810 event-specific PCR (IdentiGen, Dublin, Ireland). The GM and non-GM maize were analysed for aflatoxin (B1, B2, G1, G2), ochratoxin, zearalenone, vomitoxin, T2 toxin, and fumonisin by ELISA (Oldcastle Laboratories Ltd., Meath, Ireland). Both types of maize were also analysed for pesticide residues by testing against a panel of 355 different active substances (Pesticide Control Service, Department of Agriculture, Backweston, Kildare, Ireland).

Animal housing and management

Pigs were housed individually in a total of four rooms with eight pigs per room (16 pigs/treatment). Each treatment group was represented in each room to avoid an effect of room. Pigs were individually penned in fully slatted pens (1.07 m × 0.6 m) with plastic slats (Faroex, Manitoba, Canada). Pigs had unlimited access to water and feed through a single bowl drinker fitted in each pen and a door-mounted stainless steel feed trough (410 mm long) with centre divider, respectively. Heat was provided by a wall mounted electric bar heater (Irish Dairy Services, Portlaoise, Ireland) and thermostatically controlled. The rooms were naturally ventilated with an air inlet in the door and exhaust by way of a roof mounted chimney. Temperature was maintained at 28-30ºC in the first week and reduced by 2ºC per week to 22ºC in the fourth week. Pigs were observed closely at least three times daily. Any pigs showing signs of ill health were treated as appropriate. All veterinary treatments were recorded including identity of pig, symptom, medication used and dosage. Individual body weight and feed disappearance were recorded on day -6, 0, 7, 14, 21, 28 and 31 of the study.

Intestinal, organ and blood sampling

On day 31, 10 pigs/treatment were sacrificed by captive bolt stunning followed by exsanguination. The last meal was administered 3 h prior to sacrifice. The heart, liver, spleen, kidneys and a sample of *semitendinosus* muscle were removed first, to prevent contamination with digesta, followed by the entire GIT. Samples were also taken from the duodenum (15 cm caudal from the pyloric junction), jejunum (midway between the pyloric junction and ileo-caecal junction), and ileum (15 cm cranial from the ileo-caecal junction) for histological analysis (as outlined below). Blood samples were taken from the anterior vena cava of 10 pigs per treatment at slaughter (day 31) for plasma collection. Plasma was stored at -20ºC for subsequent blood biochemical analysis. The heart, liver, kidneys and spleen were removed, trimmed of any superficial fat or blood clots and weighed. Samples were then taken from the liver (distal end centre of central lobe), kidney (cortex and medulla), spleen (anterior end of spleen), heart (left ventricle wall) and *semitendinosus* muscle for histological examination.

Intestinal and organ histology

Small intestinal (duodenal, jejunal and ileal) and organ (heart, liver, kidney, spleen

and muscle) samples were rinsed in PBS and placed in No-Tox fixative (Scientific Device Lab, Des Plaines, IL) on a shaker for a minimum of 48 h. Samples were then dehydrated through a graded alcohol series, cleared with Sub-X clearing agent (Surgipath, Richmond, IL) and embedded in paraffin. Tissue samples were sliced using a microtome (Leica RM2235, Wetzlar, Germany), mounted on a microscope slide and stained with hematoxylin and eosin (Sigma-Aldrich) for light microscope examination. Determination of gross morphological parameters of intestinal structure (villus height and crypt depth) was conducted according to Applegate and Lilburn (1999) and Gao *et al.* (2000). Ten villi and crypts were measured on three-four fields of view for each pig and the means were utilized for statistical analysis. Goblet cell number was determined by periodic acid-Schiff staining according to Thompson and Applegate (2006). Positively stained periodic acid-Schiff cells were enumerated on 10 villi/sample, and the means were utilized for statistical analysis. Organ samples were examined for any histological indicators of organ dysfunction by an experienced histopathologist and characterised based on the indicators of cell and organ dysfunction listed in Table 2.3.

Blood biochemistry

Liver and kidney function were assessed by measuring the concentrations of aspartate aminotransferase, alanine aminotransferase, gamma glutamyltransferase, creatinine, urea and total protein in plasma taken at slaughter (day 31). Plasma samples were prepared using appropriate kits according to the manufacturer's instructions and analysed using an Alfa Wassermann ACE Clinical Chemistry system (Alfa Wassermann BV, Woerden, The Netherlands).

Statistical analysis

All data were analysed as a complete randomized block design using the GLM procedures of SAS (SAS, 2010). For all response criteria, the individual pig was the experimental unit. Treatment effect was tested against residual error term with initial bodyweight as a blocking factor. Growth performance, intestinal histology and blood biochemistry data were analysed as a one-factor analysis of variance (ANOVA) using the GLM procedure of SAS. Organ weights were also analysed as a one-factor analysis of variance using the GLM procedure of SAS using final body weight (day 31) as a covariate in the model. The level of significance for all tests was $P < 0.05$. Trends were reported up to $P = 0.10$.

2.4. Results

Analysis of GM and non-GM maize for the *cry1Ab* gene, mycotoxins, pesticide residues and carbohydrate fraction

The GM maize was found to have > 5% event-specific *cry1Ab* gene insert. However, the non-GM maize was also found to have event-specific *cry1Ab* gene insert, but only 0.2%. The non-GM maize was also positive for the *cp4epsp* gene from Round-up Ready Soybean; however this transgene could not be quantified due to the absence of a reference sample. The levels of all mycotoxins detected in the non-GM and GM maize were below the maximum allowable levels as outlined in EU legislation (Commission Regulation (EC) no 1881/2006). The non-GM and GM maize were also negative for all pesticide residues tested. The GM maize was found to have 2.2% greater starch content, 1.03% greater water soluble carbohydrate content, 0.55% lower acid detergent fibre, 0.96% greater sucrose content and 2.35% less enzyme resistant starch than the non-GM maize (Table 2.2). Neutral detergent fibre, acid detergent lignin and digestible energy content were similar in each of the two maize types. The non-GM and GM maize were also similar in chemical composition and amino acid concentration (Table 2.2).

Effect of feeding diets containing GM or non-GM maize on health, body weight and growth performance

Two weeks after the beginning of the study, two pigs (one from each treatment) were observed to have nasal discharge, difficulties in breathing and a lack of appetite. The pigs were administered with injectable Enrofloxacin (2.5 mg/kg body weight) for three days. By the end of the treatment period, both pigs were no longer displaying symptoms of ill health. No other pigs displayed signs of ill health throughout the trial.

There were no differences in feed consumption, average daily gain (ADG) and feed conversion efficiency (FCE) between treatment groups during the first 14 days. During day 14 to 30, pigs fed the GM maize diet consumed more feed (Table 2.4; $P < 0.05$) and had poorer FCE than pigs fed the non-GM maize diet ($P < 0.05$). Overall, pigs fed the GM maize diet had higher daily feed consumption ($P < 0.05$), and had numerically greater ADG and heavier body weight on day 30 compared to pigs fed the non-GM maize diet, but this was not statistically significant.

Effect of short-term feeding of GM and non-GM maize diets on organ weights

The weights of the heart, liver and spleen did not differ between treatments. However, pigs fed GM maize diets tended to have heavier kidneys than control pigs (161.0 vs 145.2 g, respectively; P = 0.06; Table 2.5). There were no differences in organ weights between treatment groups when expressed as organo-somatic indices (Table 2.5).

Effect of feeding GM and non-GM maize diets on small intestinal and organ histology

Short-term feeding of GM maize to weanling pigs had no effect on duodenal, jejunal or ileal villus height, crypt depth or villus height:crypt depth ratio (Table 2.6). The number of goblet cells per villus in the duodenum, jejunum and ileum of weaned pigs did not differ between treatment groups following a 31 day feeding period. However, there was a tendency (P = 0.10) for a decrease in the number of goblet cells per µm of duodenal villus in pigs fed GM maize compared to control pigs (Table 2.6). There were no histopathological indicators of organ dysfunction identified in the samples examined (heart, liver, kidney, spleen and muscle).

Effect of feeding GM and non-GM maize diets on liver and kidney function

Short-term feeding of GM maize had no effect on plasma total protein or creatinine and urea concentrations, the latter two being indicators of kidney function (Table 2.7). Likewise, there was no effect of treatment on plasma concentrations of the liver enzymes, alanine aminotransferase, aspartate aminotransferase and gamma glutamyl transferase. In addition, all parameters were within the normal range of values for pigs of this age (Table 2.7).

2.5. Discussion

To date, research documenting the effect of feeding Bt (MON810) maize to pigs has focused primarily on changes in growth response. To our knowledge, this is the first experiment in pigs that assesses the effects of feeding Bt (MON810) maize on kidney and liver function, intestinal histology and growth performance. Changes in physiological processes in response to GM maize exposure have previously been investigated in other species such as rats, mice, poultry and fish. However, research in

this area in pigs has been somewhat limited.

No consistent effects on feed intake and ADG have been reported in the numerous pig-feeding trials that have compared GM maize with conventional maize varieties (Bressener *et al*., 2002; Fischer *et al*., 2002; Hyun *et al*., 2004). However, Custodio *et al.* (2006) reported an increase in average daily feed intake in grow-finish (17-120 kg) pigs fed transgenic Bt11 maize but not in pigs fed Bt11 maize from 60 to 120 kg. In a similar study with MON810 maize, pigs fed GM maize were reported to have increased ADG (Piva *et al*., 2001). This was, however, attributed to lower concentrations of the mycotoxin, fumonisin B in the GM maize. Likewise, higher weight in zebrafish fed MON810 maize compared to fish fed an isogenic parent line maize was attributed to a lower level of mycotoxin in the Bt (MON810) maize (Sissener *et al*., 2010). In the current study, while there was a significant increase in feed intake following Bt (MON810) maize consumption, growth rates and body weight of these pigs were only numerically higher than control pigs suggesting a minimal effect of GM maize on growth parameters measured. Also, the levels of mycotoxin detected in both the GM and non-GM maize were below maximum allowable levels as outlined by EU legislation (Commission Regulation (EC) No 1881/2006) and, as such, cannot explain the difference in feed intake observed between treatments. Analysis of the carbohydrate fractions of the GM and non-GM maize highlighted some differences between the two types of maize. Willis *et al.* (2009) found that not all types of fibre influence satiety equally and that resistant starch had the greatest impact on satiety. The GM maize fed to pigs in the current study had lower levels of resistant starch than the non-GM maize. This may account for the higher feed intake observed in GM maize-fed pigs compared to pigs fed non-GM maize. However, an effect of feeding GM maize on pig growth may not become evident until the pig has been exposed for a longer period of time. With this in mind, we are currently conducting a longer-term pig-feeding study examining the effect of feeding GM maize to pigs from 12 days post-weaning to slaughter (110 days).

In this 31 day study, we reported no effect of maize type on the absolute weights of the heart, liver and spleen, but found a slight increase in the kidney weights of pigs fed the Bt (MON810) maize, indicating possible renal toxicity. However, there was no evidence of any histopathological changes in the kidney or other organs examined. Furthermore, kidney and liver function were unaffected, as evidenced by blood biochemistry data. Similarly, no changes in organ weights were found in rats fed a GM

rice expressing the Cry1Ab protein for 90 days (Schroder *et al*., 2007) or in rats fed MON810 maize at inclusion rates of 11 or 33% (Hammond *et al*., 2006). While some research has raised questions as to whether GM maize is responsible for signs of hepatorenal toxicity (Seralini *et al*., 2007), a three generation study with rats using modified Bt maize reported no significant differences in relative organ weights and only minimal histopathological changes in the liver and kidneys which were independent of diet (Kilic and Akay, 2008). The increase in kidney weight observed in GM maize-fed pigs in the present study may have arisen as a result of an adaptive phenomenon known as hyperfiltration, which can increase kidney weight in response to elevated serum urea (Martin *et al*., 2005). Fermentable carbohydrates in the diet have been shown to enhance microbial fermentation in the caecum and colon, thus increasing the demand for urea. This increased demand for urea has been shown to be satisfied through urea diffusion from the blood into the caecum and colon, which in turn lowers the urea load to be filtered by the kidneys (Younes *et al*., 1995; Younes *et al*., 1995). The GM maize used in this experiment was found to be lower in enzyme resistant starch than the non-GM variety. Therefore, we hypothesise that there was less hindgut microbial fermentation in the GM maize-fed pigs and less urea therefore diffused from the blood into the hindgut. As a result, more serum urea was excreted by the kidneys which resulted in hypertrophy. While there were no differences in serum urea concentrations between treatments in this experiment, subsequent work by our group has reported an increase in serum urea concentration in GM maize-fed pigs after 30 days of exposure (Chapter 5).

The present study demonstrated a lack of effect of Bt (MON810) maize on the small intestinal histology of weanling pigs. Similar findings were reported in studies with Atlantic salmon (Sanden *et al*., 2005), where, although changes were observed in the intestines of Atlantic salmon parr, they could not be ascribed to dietary inclusion of GM plant material. There was however, a slight decrease in the number of goblet cells per μm of duodenal villus of GM maize-fed pigs in the current study compared to control pigs. Ganessunker *et al.* (1999) previously observed an increase in goblet cell number in the small intestine of pigs as a result of total parenteral nutrition which was correlated with a change in gut microbiota, enhanced inflammation, and decreased integrity of the mucosal barrier. Our results show that feeding GM maize does not compromise the small intestinal mucosal barrier but the alterations in goblet cell number observed in the duodenum may be attributed to changes in gut microbial

populations observed in these pigs. Preliminary data from an investigation of the gut microbiota of the GM maize-fed pigs would suggest differences in relative abundance of certain intestinal microbial populations relative to the control group (Chapter 4). Changes in intestinal microbiota have been linked with alterations in goblet cell number in other studies (Mair *et al*., 2010) and intestinal commensal microbiota have been reported to impact directly on intestinal epithelial functions including those of goblet cells (Kim and Ho, 2010) by producing mucin degrading enzymes (Macfarlane *et al*., 2005) or by stimulation of mucin gene expression (Dohrman *et al*., 1998).

In conclusion, results obtained from the short-term feeding of Bt (MON810) maize to weanling pigs have demonstrated no adverse effects on growth performance or intestinal morphology. There were no changes in organ weights, with the exception of a tendency for an increase in kidney weight; however, this was not associated with histopathological or blood biochemical changes. Therefore, based on the parameters investigated in this study we can conclude that short-term feeding of Bt (MON810) maize to pigs is safe. However, long-term studies are required to fully evaluate safety. Furthermore, since the pig is considered to be an excellent model for humans due to similarities in gastrointestinal anatomy and physiology (Moughan *et al*., 1992), similar responses to Bt (MON810) maize consumption could be expected in humans. Therefore, the findings of this study offer at least some assurance to consumers as to the safety of short-term exposure to GM food and feed ingredients and give a greater insight into expected responses in pigs to short-term Bt (MON810) maize exposure. However, to comprehensively evaluate the effects of feeding Bt (MON810) maize to pigs, long-term feeding studies are necessary and are currently ongoing.

Table 2.1. Composition of acclimatization starter diet and experimental diets (fresh weight basis, %).

	Baseline (day -6 to -1)	Experimental (day 0 to 31)	
Ingredient (%)	**Non-GM**	**Non-GM**	**GM**
Maize (non-GM)	27.33	38.88	-
Maize (GM – MON810)	-	-	38.88
Soybean meal (non-GM)	24.00	25.00	25.00
Lactofeed 70[1]	25.00	20.00	20.00
Immunopro 35[2]	12.50	9.00	9.00
Soybean oil	8.00	4.00	4.00
L-Lysine HCl	0.30	0.30	0.30
DL-Methionine	0.25	0.20	0.20
L-Threonine (98)	0.12	0.12	0.12
L-Tryptophan	0.10	0.10	0.10
Vitamin and mineral premix[3]	0.30	0.30	0.30
Mycosorb[4]	0.20	0.20	0.20
Salt	0.30	0.30	0.30
Dicalcium Phosphate	0.50	0.50	0.50
Limestone flour	1.10	1.10	1.10
Analysed Chemical Composition (%)			
Dry matter	91.3	89.4	89.2
Crude protein	20.9	20.9	21.1
Fat	9.6	6.1	5.9
Crude fibre	1.7	2.1	1.9
Ash	6.3	5.5	5.6
Lysine	1.55[5]	1.42	1.42
Ca[5]	0.83	0.78	0.78
P[5]	0.61	0.59	0.59
Digestible energy, MJ of DE/kg[5]	16.33	15.38	15.38
Pellet durability, g	-[6]	96.35	96.73
Pellet diameter, mm	-[6]	5.08	5.11
Pellet hardness, kg	-[6]	4.30	3.70

[1]Lactofeed 70 contains 70% lactose, 11.5% protein, 0.5% oil, 7.5% ash and 0.5% fibre (Volac, Cambridge, UK).

[2]Immunopro 35 is a whey protein powder product containing 35% protein (Volac, Cambridge, UK).

[3]Premix provided per kg of complete diet: Cu, 155 mg; Fe, 90 mg; Mn, 47 mg; Zn, 120 mg, I, 0.6 mg; Se, 0.3 mg; vitamin A, 6000 IU; vitamin $D_{3,}$ 1000 IU; vitamin E, 100 IU; vitamin K, 4 mg; vitamin $B_{12,}$ 15 µg; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; choline chloride, 250 mg; vitamin $B_{1,}$ 2 mg; vitamin $B_{6,}$ 3 mg.

[4]Mycosorb is an organic mycotoxin absorbent (Alltech Inc. Dunboyne, Ireland).

[5]Calculated values.

[6]Baseline non-GM maize diet was fed in the form of meal.

Table 2.2. Proximate, carbohydrate and amino acid analysis of Bt (MON810) maize and isogenic parent line maize (fresh weight basis).

	Non-GM maize	**GM maize**
%		
DM	88.1	87.4
Crude protein	7.4	7.7
Fat	3.8	3.3
Crude fibre	2.6	2.0
Ash	1.2	1.6
Starch	61.9	64.1
Sugar (sucrose)	1.20	2.16
ADF	4.03	3.48
NDF	11.50	11.20
ADL	1.01	1.01
Enzyme resistant starch	5.93	3.58
Water soluble carbohydrate	2.13	3.16
Digestible energy, MJ of DE/kg [1]	15.31	15.10
Net energy, MJ of NE/kg[2]	12.12	12.12
Lysine	0.32	0.31
Methionine	0.16	0.15
Cysteine	0.22	0.22
Threonine	0.34	0.32
Tryptophan	0.10	0.10

DM = dry matter; ADF = acid detergent fibre; NDF = neutral detergent fibre; ADL = acid detergent lignin; DE = digestible energy; NE = net energy.

[1]Calculated from equation number 24 in Noblet and Perez (1993) using analysed values on a fresh weight basis.

[2]Calculated from equation number 11 in Noblet *et al.* (1994) using analysed values on a fresh weight-basis.

Table 2.3. Histological indicators used for the identification of cell and organ dysfunction in the liver, heart, spleen, kidneys and muscle of weanling pigs fed GM or non-GM maize for 31 days.

Indicators of organ dysfunction
Cell swelling
Cytoplasmic vacuolar development
Cytoplasmic vacuolar development due to fatty accumulation (fatty change)
Cytoplasmic/nuclear shrinkage/fragmentation
Cell apoptosis/degeneration/necrosis
Haemorrhage/oedema/inflammation/fibrosis
Tissue hyperplasia/metaplasia
Cell pigmentation
Alteration to blood vessel structure/integrity

Table 2.4. Effects of feeding GM or non-GM maize for 30 days on weanling pig growth performance[1].

	Non-GM maize	**GM maize**	**SEM**	***P*-value**
ADG (g/d)				
Days				
0 - 14	391	427	19.1	0.25
14 - 30	738	790	23.1	0.19
0 - 30	576	620	18.2	0.11
ADFI (g/d)				
Days				
0 - 14	475	484	19.0	0.70
14 - 30	893	1021	30.1	0.02
0 - 30	697	770	22.9	0.03
FCE				
Days				
0 - 14	1.21	1.13	0.032	0.13
14 - 30	1.21	1.29	0.016	0.01
0 - 30	1.22	1.24	0.015	0.28
Body weight (kg)				
Day				
30	24.7	26.0	0.56	0.11

ADG = average daily gain; ADFI = average daily feed intake; FCE = feed conversion efficiency; SEM = standard error of the mean.

[1]*n* = 16 pigs in control group and 16 pigs in the GM maize-fed group.

Table 2.5. Effects of feeding GM maize or non-GM maize for 31 days on organ weights and organo-somatic indices of weanling pigs[1].

	Non-GM maize	**GM maize**	**SEM**	***P*-value**
Organ weights (g)[2]				
Kidneys	145.2	161.0	4.52	0.06
Spleen	47.5	54.3	2.71	0.14
Liver	690.0	665.3	17.98	0.38
Heart	133.3	142.2	3.96	0.18
Organo-somatic indices[3]				
Kidneys	0.0058	0.0061	0.00017	0.21
Spleen	0.0019	0.0021	0.00009	0.16
Liver	0.0270	0.0258	0.00065	0.29
Heart	0.0052	0.0055	0.00014	0.14

SEM = standard error of the mean.

[1]*n* = 10 pigs in control group and 10 pigs in the GM maize-fed group.

[2]Organ weights were calculated using final body weight on day 30 as a covariate.

[3]Organo-somatic indices were calculated by expressing the organ weights as a fraction of the body weight on day 30.

Table 2.6. Effects of feeding GM maize or non-GM maize for 31 days on small intestinal histology of weanling pigs[1].

	Non-GM maize	GM maize	SEM	*P*-value
Villus height (μm)				
Duodenum	787	774	85.0	0.91
Jejunum	524	612	54.6	0.30
Ileum	517	567	20.1	0.13
Crypt depth (μm)				
Duodenum	517	543	45.3	0.71
Jejunum	456	483	18.8	0.37
Ileum	378	381	32.3	0.96
Villus height:crypt depth ratio				
Duodenum	1.60	1.43	0.238	0.63
Jejunum	1.20	1.31	0.158	0.64
Ileum	1.43	1.57	0.112	0.41
Number of goblet cells/villus				
Duodenum	16.5	13.4	2.11	0.29
Jejunum	8.2	9.0	1.06	0.62
Ileum	8.7	10.5	1.96	0.42
Number of goblet cells/μm of villus				
Duodenum	0.027	0.019	0.0026	0.10
Jejunum	0.018	0.016	0.0021	0.52
Ileum	0.017	0.018	0.0030	0.81

SEM = standard error of the mean.

[1]*n* = 10 pigs in control group and 10 pigs in the GM maize-fed group. Ten villi and crypts were measured for each pig and the means were utilized for statistical analysis.

Table 2.7. Effects of feeding GM maize or non-GM maize for 31 days on serum concentrations of enzymes and other parameters to assess the liver and kidney function of weanling pigs[1].

	Non-GM maize	GM maize	SEM	*P*-value	Normal range for pigs	Reference
Total protein (g/L)	55.1	57.9	1.08	0.12	35 - 60	Radostits *et al.*, 2007
Creatinine (µmol/L)	109.5	103.0	3.74	0.27	90 - 240	Radostits *et al.*, 2007
Urea (mmol/L)	5.9	5.3	0.51	0.45	3.6 – 10.7	Kaneko, 1980
Liver enzymes (U/L)						
Alanine aminotransferase	41.1	44.8	3.57	0.49	31 - 58	Kaneko, 1980
Aspartate aminotransferase	54.7	56.3	2.96	0.74	32 - 84	Kaneko, 1980
Gamma glutamyl transferase	43.6	38.1	7.63	0.64	10 - 60	Kaneko, 1980

SEM = standard error of the mean.

[1] *n* = 10 pigs in control group and 10 pigs in the GM maize-fed group.

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3. Fate of transgenic DNA from orally administered Bt MON810 maize and effects on immune response and growth in pigs

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

3.1. Abstract

We assessed the effect of short-term feeding of genetically modified (GM: Bt MON810) maize on immune responses and growth in weanling pigs and determined the fate of the transgenic DNA and protein *in vivo*. Pigs were fed a diet containing 38.9% GM or non-GM isogenic parent line maize for 31 days. We observed that IL-12 and IFNγ production from mitogenic stimulated peripheral blood mononuclear cells decreased ($P < 0.10$) following 31 days of GM maize exposure. While Cry1Ab-specific IgG and IgA were not detected in the plasma of GM maize-fed pigs, the detection of the *cry1Ab* gene and protein was limited to the gastrointestinal digesta and was not found in the kidneys, liver, spleen, muscle, heart or blood. Feeding GM maize to weanling pigs had no effect on growth performance or body weight. IL-6 and IL-4 production from isolated splenocytes was increased ($P < 0.05$) in response to feeding GM maize while the proportion of $CD4^+$ T cells in the spleen decreased. In the ileum, the proportion of B cells and macrophages decreased while the proportion of $CD4^+$ T cells increased in GM maize-fed pigs. IL-8 and IL-4 production from isolated intraepithelial and *lamina propria* lymphocytes were also increased ($P < 0.05$) in response to feeding GM maize. In conclusion, there was no evidence of *cry1Ab* gene or protein translocation to the organs and blood of weaning pigs. The growth of pigs was not affected by feeding GM maize. Alterations in immune responses were detected; however their biological relevance is questionable.

3.2. Introduction

Worldwide, the inclusion of genetically modified (GM) plants in animal feed and for human consumption has consistently increased over the past fifteen years since they were first cultivated in 1996 (Schnepf *et al*., 1998). The increase in demand for GM ingredients has coincided with an 87-fold increase in cultivation area of GM crops reaching 148 million hectares worldwide in 2010 thus making the procurement of exclusively non-GM crops more difficult and expensive. In 2007, GM maize became the second most important biotech crop after GM soybeans (James, 2010) and the first one to have a wider variety of genetic modifications than glyphosate-tolerant soybean.

GM plants are designed to provide more nutritious food and to enhance agronomic productivity without the use of pesticides (Ye *et al*., 2000; Shimada *et al*., 2006). However, the increased usage of GM crops for direct human consumption and feeding to meat- and milk-producing animals has lead to public concern. Consumer concerns are mostly related to a perceived risk to health, allergenicity of the transgenic proteins or the transfer of recombinant DNA from feed to livestock and livestock derived products that are consumed by humans (Bertoni and Marsan, 2005). Other concerns are associated with environmental issues such as gene transfer from GM crops to indigenous plants, reducing biodiversity and influence of the GM crops on non-target insect species (Moses, 1999; Malarkey, 2003; Paparini and Romano-Spica, 2004; Hug, 2008). Adoption of GM technology has received varying degrees of support worldwide. However, much greater resistance to food biotechnology exists in Europe compared to other parts of the world (Ganiere *et al*., 2006).

The entry of GM plants into the food chain is highly regulated, particularly within the European Union where rigorous pre-market risk assessment is untaken to ensure the safety of GM plants for animal and human consumption. Numerous animal studies have focused on evaluating the risks of feeding Bt maize on health and growth parameters and no abnormalities have been identified (Reuter *et al*., 2002; Sanden *et al*., 2005; Custodio *et al*., 2006; Sanden *et al*., 2006; Shimada *et al*., 2006; Flachowsky *et al*., 2007; Trabalza-Marinucci *et al*., 2008). However, some studies have found alterations in the immune response of mice fed Bt maize (Finamore *et al*., 2008) and peas expressing the bean α-amylase inhibitor (Prescott *et al*., 2005). To date, the Cry1Ab protein has been proven safe in most animal studies. The transgenic protein has no homology to any allergenic proteins and was successfully degraded in simulated gastric conditions (EFSA, 2009).

To fully address safety concerns related to GM feed ingredients, studies to determine the fate of ingested recombinant DNA fragments in animals have also been conducted. Many of these animal studies have failed to observe translocation of recombinant DNA fragments outside the GIT (Deaville and Maddison, 2005; Alexander *et al*., 2006; Yonemochi *et al*., 2010) although in some studies, low levels of recombinant DNA have been documented in the organs of pigs (Mazza *et al*., 2005; Sharma *et al*., 2006).

The objectives of the experiments outlined in this paper were to evaluate both the intestinal and peripheral immune response in pigs in response to short-term GM maize exposure previously only conducted in mice. A further objective was to determine the fate of ingested recombinant DNA and protein in pigs thus allowing a clearer assessment of the safety of GM maize to be made.

3.3. Materials and methods

Ethics statement

The pig experiments described below complied with European Union Council Directive 91/630/EEC (outlines minimum standards for the protection of pigs) and European Union Council Directives 98/58/EC (concerns the protection of animals kept for farming purposes) and was approved by, and a license obtained from, the Irish Department of Health and Children (licence number B100/4147). Ethical approval was obtained from the Teagasc and Waterford Institute of Technology ethics committees.

Genetically modified maize

Seeds derived from MON810 and its parental control maize (PR34N44 and PR34N43 varieties, respectively: Pioneer Hi-Bred, Sevilla, Spain) were grown simultaneously side by side in Valtierra, Navarra, Spain by independent tillage farmers. The GM and isogenic control maize were purchased by the authors from the tillage farmers for use in these animal studies.

Animal housing, diets and management

Two experiments were conducted to assess the effect of short-term feeding of Bt (MON810) maize on the peripheral and systemic immune response in weanling pigs and to determine the fate of transgenic DNA *in vivo*.

Experiment 1.

Thirty-two crossbred (Large White × Landrace) weanling pigs (entire males) were weaned at approximately 28 days of age and were blocked by weight and litter, and randomly assigned to one of two experimental treatments; (1) non-GM isogenic parent line of maize (Pioneer PR34N43) and (2) GM maize (Pioneer PR34N44 event MON810). A non-GM starter diet was fed *ad libitum* for the first 6 days post-weaning during an acclimatization period and either the non-GM or GM maize experimental diets were fed for the remaining 31 days. Diets were manufactured in the Moorepark feed mill and were formulated to meet or exceed the NRC (NRC, 1998) requirements for weanling pigs (Table 3.1). Stringent quality control measures were employed to avoid cross contamination of non-GM with GM diets. Carryover in the feed manufacturing system was minimized by flushing the system with non-GM ingredients and the preparation of non-GM diets prior to diets containing the GM maize. In addition non-GM soybean meal was used in the manufacture of all diets. Cereals were ground by hammer mill through a 3 mm screen before mixing. Diets were pelleted to 5 mm diameter after steam conditioning to 50-55ºC. The GM and non-GM maize were tested for the presence of the *cry1Ab* gene, pesticide contaminants and mycotoxins as described in Chapter 2. Proximate, (FBA Laboratories, Waterford, Ireland) amino acid and carbohydrate analysis (Sciantec Analytical Services Ltd., Cawood, UK) of experimental diets was performed (Table 3.1).

Pigs were housed individually in a total of four rooms with eight pigs per room (16 pigs/treatment). Pigs were individually penned in fully slatted pens (1.07 m × 0.6 m) with plastic slats (Faroex, Manitoba, Canada). Pigs had unlimited access to water and feed through a single bowl drinker fitted in each pen and a door-mounted stainless steel feed trough (410 mm long) with centre divider, respectively. Heat was provided by a wall mounted thermostatically controlled electric bar heater (Irish Dairy Services, Portlaoise, Ireland). The rooms were naturally ventilated with an air inlet in the door and exhaust by way of a roof mounted chimney. Temperature was maintained at 28-30ºC in the first week and reduced by 2ºC per week to 22ºC in the fourth week. Lighting was provided by tubular fluorescent lights from 0830 h to 1630 h. Pigs were observed closely at least three times daily. Any pigs showing signs of ill health were treated as appropriate.

Experiment 2.

A second experiment was conducted to examine the effects of short-term exposure

to GM maize on local immune response of weanling pigs. Pigs (n = 20) were weaned at approximately 28 days of age and were blocked by weight and litter, and randomly assigned to one of two experimental treatments; (1) non-GM isogenic parent line of maize (Pioneer PR34N43) and (2) GM maize (Pioneer PR34N44 event MON810) similar to pigs in Experiment 1. Pigs were fed experimental starter diets from day 0 to 7 post-weaning and experimental link diets from day 7 to 35 post-weaning (Table 3.1). Pigs were penned individually in the same room for the duration of the experiment (35 days). Pens were fully slatted (1.2 m × 0.9 m) with plastic slats (Faroex, Manitoba, Canada) and plastic dividers between pens. Water was available *ad libitum* from one nipple-in-bowl drinker (BALP, Charleville-Mezieres, Cedex, France) per pen. Feed was also available *ad libitum* from a single stainless steel feeder 30 cm wide (O'Donovan Engineering, Coachford, Co. Cork). Environmental condition control and management of pigs was conducted in a same manner as outlined for Experiment 1.

Intestinal, organ and blood sampling

Experiment 1.

On day 31, 10 pigs/treatment were sacrificed by captive bolt stunning followed by exsanguination. The last meal was administered 3 h prior to sacrifice. During the sampling procedure, special care was taken to prevent any cross contamination between the GM and non-GM maize-fed pigs. All non-GM maize-fed pigs were sacrificed first followed by the GM maize-fed pigs. All surgical instruments were cleaned with a 70% ethanol solution between animals. During the sampling procedure, all assistants wore single-use gloves that were replaced after each sample was taken. The heart, liver, spleen, kidneys and a sample of the *semitendinosus* muscle were removed first, to prevent contamination with digesta contents, followed by the entire GIT. Whole blood samples were taken from the anterior vena cava of 10 pigs per treatment and collected in heparinised blood collection tubes (BD Vacutainer Systems, Franklin Lakes, NJ) on day 0 and 29. Samples were stored at room temperature and peripheral blood mononuclear cells (PBMC) were isolated and assayed within 30 h. Blood samples were also taken at slaughter (day 31) and collected in EDTA-containing tubes (BD Vacutainer Systems) and immediately placed on ice for transport to the laboratory. Blood samples were centrifuged at 2500 × g for 20 min after which the buffy coat of white blood cells was removed and stored at -20ºC for subsequent analysis for the *cry1Ab* gene. Plasma from these samples was stored at -20ºC for subsequent Cry1Ab-

specific Ig analysis. The heart, liver, kidneys and spleen were removed, trimmed of any superficial fat or blood clots. The outermost layer of each tissue was removed to ensure that samples were taken from interior sections to prevent any residues of feed causing contamination of the samples. Samples were taken from the liver (distal end centre of central lobe), kidney (middle of the kidney cortex and medulla), spleen (anterior end of spleen), heart (left ventricle wall) and *semitendinosus* muscle and snap frozen in liquid N and stored at -20ºC for subsequent analysis of the Cry1Ab protein and gene. Digesta samples from the stomach, ileum, caecum and caecal samples were stored at -20ºC for subsequent analysis of the *cry1Ab* gene and protein.

Experiment 2.

On day 35, 10 pigs/treatment were sacrificed (as outlined in Experiment 1) and spleen samples were taken (anterior end) and placed on ice in Hank's balanced salt solution (HBSS; Sigma-Aldrich, St. Louis, MO) for splenocyte isolation. Ileal samples (15 cm cranial to the ileo-caecal junction) were taken and placed on ice in HBSS (Sigma-Aldrich) for subsequent *lamina propria* and intraepithelial lymphocyte isolation.

Growth

Experiment 2.

Individual body weight and feed disappearance were recorded on day 0, and 30 of the experiment for calculation of growth performance.

Evaluation of the immune response to oral administration of Bt MON810 maize in pigs

Isolation and stimulation of PBMC and cytokine measurement

Isolation of PBMC from whole blood was conducted as described by Walsh *et al.* (2008). Stimulation of PBMC was performed with phosphate buffered saline (PBS), or a combination of 25 ng/μL phorbol myristate acetate (PMA; Sigma-Aldrich) and 2 mg/mL ionomycin (I; Sigma-Aldrich) for 18 h at 37ºC in a 5% (v/v) CO_2 humidified atmosphere. Following stimulation, the cell culture supernatant was collected and stored at -80ºC. Concentrations of IL-4, IL-6, IL-10, IL-12, TNFα and IFNγ were subsequently determined in these supernatants using porcine-specific cytokine ELISA kits (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's

instructions. Samples were analysed in duplicate on each plate. Duplicate samples with intra-assay precision (CV%) of greater than 10% underwent repeat analysis.

Isolation and stimulation of lamina propria and intraepithelial lymphocytes and splenocytes and measurement of cytokine production

Lamina propria lymphocytes (LPL) and intraepithelial lymphocytes (IEL) were isolated from porcine ileum tissue samples collected at sacrifice on day 35, as described for human LPL and IEL (Schwarz *et al*., 2004). For isolation of splenocytes, ~10 g of spleen were pressed through a 50-mesh screen (Sigma-Aldrich). Remaining cells were washed through with HBSS containing 2% heat inactivated fetal bovine serum (HBSS-FBS; Invitrogen, Paisley, UK). The cells were pelleted at 200 × g for 10 min and re-suspended in HBSS-FBS. Erythrocytes were lysed with lysing buffer (BD Biosciences, Devon, UK) according to manufacturer's instructions. Cells were pelleted at 200 × g for 10 min and re-suspended in HBSS-FBS. The cell suspension was filtered through a sterile 70 µm nylon cell strainer (BD Biosciences) and centrifuged for 10 min at 275 × g. The cell pellet was re-suspended in 30% Percoll (Sigma-Aldrich; diluted with 0.9% NaCl) and layered over a 70% Percoll solution. The above gradient separation solution following centrifugation for 20 min at 1230 × g yielded a population of mononuclear cells at its interface. Cells were recovered and washed twice in HBSS-FBS by centrifugation at 360 × g for 10 min. Both LPL/IEL and splenocytes were counted and re-suspended in complete medium [IMDM + Glutamax (Invitrogen), 20% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin (Invitrogen) at a concentration of 1×10^6 cells/mL and dispensed into 24 well plates (Sarstedt, Numbrecht, Germany). Stimulation of LPL, IEL and splenocytes was performed and the cell supernatants collected and stored as outlined above for PBMC. Concentrations of IL-4, IL-6, IL-8, and TNFα were subsequently determined in the cell supernatants using multiplex porcine-specific cytokine ELISA kits (Meso Scale Discovery, Gaithersburg, Maryland) in accordance with the manufacturer's instructions. Samples were analysed in duplicate on each plate. Duplicate samples with intra-assay precision (CV%) of greater than 10% underwent repeat analysis.

Immune cell phenotyping

Following stimulation, LPL/IEL and splenocytes were resuspended at ~1×10^6 cells/mL in PBS containing 2% FBS (PBS-FBS). Primary and secondary antibodies

were added at concentrations determined by titration and incubated in the dark at room temperature for 15 min. Cells were washed and re-suspended in PBS-FBS and acquired using a BD FACSCanto II^{TM} flow cytometer. Antibodies used included anti-porcine CD3 PE/Cy5 (Abcam, Cambridge, UK), anti-porcine CD4 fluorescein isothiocyanate (FITC), anti-porcine CD8 phycoerythrin (PE), anti-porcine macrophage FITC (Abd Serotec, Kidlington, UK), anti-porcine CD3 (Abcam), anti-mouse IgG1 peridinin chorolphyll protein (PerCP; Santa Cruz Biotechnology, Santa Cruz, CA), anti-porcine CD45 (Abd Serotec), anti-porcine B cell marker PE (Abcam), anti-mouse IgG1 allophycocyanin (APC), anti-porcine γδ T cell, anti-rat IgG2a APC, anti-rat CD16/CD32 and anti-mouse CD32 (all antibodies were obtained from BD Biosciences, Devon, UK unless otherwise stated). Antibodies were used according to manufacturer's recommendations. The percentages of T and B lymphocytes and macrophages were calculated on leukocyte ($CD45^+$) gate, whereas $CD4^+$, $CD8^+$, $CD4^+/CD8^+$ and γδ T cell subsets were calculated on $CD3^+$ gate. At least 50,000 events were acquired and analysed. Data was analysed using FACSDIVA software (BD Biosciences).

Cry1Ab-specific antibody response

For detection of Cry1Ab specific IgA and IgG in pig plasma, 96-well plates were coated overnight at 4ºC with 1 μg/mL of purified Cry1Ab toxin in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Plates were blocked for 1 h at 37ºC with 0.01 M phosphate buffered saline (PBS), pH 7.4, containing 1% gelatine (Sigma-Aldrich). PBS alone or serial dilutions (from 1:5 to 1:640) of pig plasma from GM maize, non-GM maize or non-maize (control) exposed pigs were added to the plate and incubated for 1 h at 37ºC. Plates were then incubated with horse radish peroxidase (HRP)-labelled anti-pig IgA or IgG (1:5000; Bethyl laboratories, Montgomery, TX) antibodies for 1 h at 37ºC followed by the addition of H_2O_2/o-phenylenediamine containing PPD Fast substrate solution pH 5 (Sigma-Aldrich). The plates were developed for 5 min in the dark and the enzyme reaction was stopped by the addition of 4 M H_2SO_4. The absorbance was read at 492/630 nm (Dynatech MR 7000, Dynatech Laboratories Ltd., UK). Plasma from non-GM maize exposed pigs was used as a negative control. Plasma from non-maize fed pigs was used as a control to ensure no cross-reactivity with other maize proteins. Samples were analysed in duplicate and the washing procedure repeated after each step involved manually washing the plates twice with PBS containing 1% gelatine and decanting.

Tracking of the Cry1Ab protein and gene in feed and porcine digesta, organs and blood

Cry1Ab protein quantification

Digesta samples were centrifuged for 15 min at 540 × g and 10 µL of 10 mM phenylmethylsulfonyl fluoride (PMSF) was added per mL of supernatant and samples were centrifuged for 20 min at 9390 × g. Ten microlitres of 10 mM PMSF and 10 µL of 1% sodium azide were added per mL of supernatant, followed by 50 µL of bovine serum albumin (BSA) 15 min later. The samples were centrifuged for 20 min at 9390 × g and the resultant supernatant was analysed for the Cry1Ab protein using a QuantiPlate kit for Cry1Ab/Cry1Ac (Envirologix, Maines, USA) according to the manufacturer's instructions.

DNA extraction

<u>Feed and digesta samples.</u> Milled feed or digesta (250 mg) was incubated in 1000 µL TRIS-EDTA-SDS extraction buffer (pH 8.0) for 1 h at 65°C. Following incubation, the suspension was cooled and 60 mg polyvinylpyrrolidone (PVP) and 500 µL 7.5 M ammonium acetate were added. This was incubated for 30 min on ice followed by centrifugation for 10 min at 14430 × g. The supernatant was collected, combined with 1 mL isopropanol and incubated for 30 min on ice. Samples were centrifuged (10 min at 14430 × g) and the supernatant was discarded. The remaining DNA pellet was washed with 70% ethanol and was resuspended in 50 µL TE buffer.

<u>Animal tissue.</u> DNA was extracted from animal tissue as described by Meyer *et al.* (Meyer *et al*., 1994). The supernatant (500 µL) was purified using the Wizard® DNA Clean-up system (Promega, Madison, WI) according to the manufacturer's instructions. Total DNA was quantified using a spectrophotometer (UV-1601 spectrophotometer, Shimadzu) at OD260nm and purity was assessed by determining the OD260nm:OD280nm.

<u>White blood cells.</u> DNA was extracted from 10 µL of buffy coat isolated from blood samples using an Extract-N-Amp Blood PCR kit (Sigma-Aldrich) according to the manufacturer's instructions.

PCR

A preliminary cross-dilution assay was performed to determine the detection limit

of the *cry1Ab*-specific PCR and the possible inhibitory effect of porcine DNA. Three primer pairs targeting an endogenous maize gene, the *cry1Ab* gene and a porcine growth hormone gene, respectively (Table 3.2) were obtained from Invitrogen (Paisley, UK). Two microlitres of extracted DNA was used in PCR amplifications which were performed in a final volume of 50 µL. Each PCR reaction contained 25 µL of either REDTaq ReadyMix PCR Reaction Mix containing $MgCl_2$ (Sigma-Aldrich) (for white blood cells) or DreamTaq Green PCR Master Mix (Fermentas, Ontario, Canada) (for tissue samples and digesta), as well as 0.01, 0.004 or 0.006 µM of the SW, *cry1Ab* or Sh2 primers, respectively and 2 µL of extracted DNA. PCR reactions were performed in a GeneAmp 2400 or 2700 thermal cycler (Applied Biosystems, Foster City, CA). The PCR conditions used are outlined in Table 3.2. Each set of PCR reactions included a positive control (DNA from MON810 maize), DNA from isogenic non-GM maize, contamination controls without template DNA, and a negative extraction control (DNA from normal pig meat). PCR products were analysed on 10% polyacrylamide gels run at 200 V for 50 min and visualized by SYBR Green-staining.

Statistical analysis

All data were analysed as a complete randomized block design using the GLM procedures of SAS (SAS, 2010). For all response criteria, the individual pig was the experimental unit. Treatment effect was tested against residual error term with initial bodyweight as a blocking factor. Growth performance data were analysed as a one-factor analysis of variance (ANOVA) using the GLM procedure of SAS. Cytokine production data was analysed as a two-factor ANOVA with interactions to determine if differences in cytokine production varied with diet and mitogenic stimulation. Baseline cytokine levels (day 0) were also used as covariates in the model. The level of significance for all tests was $P < 0.05$. Trends were reported up to $P = 0.10$.

3.4. Results

Analysis of GM and non-GM maize for the *cry1Ab* gene, mycotoxins and pesticide residues

The GM maize was found to have > 5% event-specific *cry1Ab* gene insert. However, the non-GM maize was also found to have 0.20% event specific *cry1Ab* gene insert. The *cry1Ab* gene was also detected in the GM maize but was not found in the non-GM maize analysed (Figure 3.1) which indicates that the level of *cry1Ab* gene

contamination of the non-GM maize was too low to be detected by non-quantitative PCR. Conventional feed ingredients containing unintentional traces of genetically modified organisms below a threshold level of 0.9% of total DNA are not required to be labelled as GM (EFSA, 2009). The levels of all mycotoxins detected in the GM and non-GM maize were below the maximum allowable levels as outlined in EU legislation [Commission Regulation (EC) No 576/2006]. The GM and non-GM maize were also negative for all pesticide residues tested.

Experiment 1. Effect of short-term feeding of GM maize on the systemic immune response

Mitogenic stimulation resulted in a significant ($P < 0.05$) increase in the production of IL-10, IL-6, IL-4 and TNFα by PBMC (Table 3.3). There was a tendency for a treatment × PMA/I interaction for IL-12 ($P = 0.09$) and IFNγ ($P = 0.08$) production from isolated PBMC. Both IL-12 and IFNγ production by isolated PBMC tended to be reduced following PMA/I stimulation in pigs fed GM maize compared to non-GM maize-fed control pigs following 29 days of feeding. In addition, Cry1Ab-specific IgG (Figure 3.2) or IgA (Figure 3.3) were not detected in plasma taken from pigs fed either the GM or non-GM maize or non-maize feed (control) even at the lowest dilution used.

Detection of transgenic and endogenous plant genes in white blood cells, tissues and digesta of GM maize-fed pigs

Neither endogenous (*Sh2*) nor transgenic (*cry1Ab*) plant gene fragments were detected in the white blood cells or any of the tissues examined (Table 3.4). However, as expected all the white blood cells and tissue samples were positive for the endogenous (*SW*) porcine gene. PCR analysis of liver samples is shown in Figure 3.1 as an example. The endogenous porcine gene was also detected at a relatively high frequency in digesta along the length of the GIT in both the non-GM maize and GM maize-fed pigs (stomach, 100%; ileum, 80 and 70%; caecum, 60 and 70%; colon, 100 and 90%, respectively). The endogenous plant gene was detected in all stomach digesta samples taken from both the GM and non-GM maize-fed pigs, while the *cry1Ab* gene was only detected in the stomach digesta of pigs fed the GM maize diets (Table 3.5). Further down the GIT, the endogenous maize gene was detected in the ileal digesta of 50% of the non-GM maize-fed pigs and 20% of the GM maize-fed pigs, in the caecal

digesta of 10% of each treatment group and was undetectable in colon samples. The *cry1Ab* gene was also detected in the lower gastrointestinal tract of GM maize-fed pigs, but only in the ileal and caecal digesta (20% and 10%, respectively) and at a lower frequency than in the stomach digesta. The *cry1Ab* gene was not detected in colon samples from either GM maize or non-GM maize-fed pigs.

Transgenic Cry1Ab protein detection in plasma, tissue and digesta of GM maize-fed pigs

The Cry1Ab protein was not detected in the heart, liver, kidney, spleen, muscle or plasma of pigs fed either GM maize or non-GM maize diets (Table 3.6). Likewise, no transgenic protein was detected in the stomach, ileum, caecum or colon digesta of pigs fed non-GM maize diets. The Cry1Ab protein was, however, detected in the stomach and caecal digesta of 30% of the GM maize-fed pigs 3 h after the last GM maize meal was administered and in the colon and ileal digesta of 80% of these pigs. The concentration of Cry1Ab protein detected in the digesta samples of GM maize-fed pigs was very low and ranged from 2.41 – 2.74 ng/mL.

Experiment 2. Effect of feeding GM maize on body weight and growth performance

There was no effect of feeding GM maize to pigs on growth performance or body weight (Table 3.7).

Effect of short-term feeding of GM maize on the local immune response

Short-term feeding of GM maize to pigs resulted in increased IL-6 and IL-4 production from isolated splenocytes ($P < 0.05$) and increased IL-8 and IL-4 production from isolated LPL and IEL ($P < 0.05$; Table 3.3) compared to non-GM maize-fed pigs. There was no effect of treatment on TNFα production from splenocytes or LPL/IEL or on IL-8 production from splenocytes or IL-6 production from LPL/IEL. Phorbol myristate acetate/ionomycin stimulation resulted in a significant ($P < 0.05$) increase in production of IL-8, IL-6 and TNFα by splenocytes and LPL/IEL while IL-4 production was increased by mitogen stimulation in the spleen but not the ileum. Local immune responses were also assessed by phenotyping leukocytes isolated from spleen and ileum. Short-term feeding of GM maize to pigs resulted in a lower proportion of ileal B cells and macrophages than in non-GM maize-fed pigs ($P = 0.001$; Figure 3.4). There

was no effect of treatment on the proportion of $CD8^+$ T cells, $CD4^+CD8^+$ T cells or γδ T cells isolated from the ileum. However, the proportion of $CD4^+$ T cells increased in response to feeding GM maize (P = 0.01). The number of $CD4^+$ T cells as a proportion of the total splenocyte population tended to decrease (P = 0.06; Figure 3.5) in response to short-term feeding of GM maize to pigs. There was no effect of feeding GM maize on the numbers of B cells, macrophages, $CD8^+$ T cells, double positive $CD4^+CD8^+$ T cells and γδ T cells isolated from the spleen.

3.5. Discussion

Previously, a study with mice reported alterations in both the local and systemic immune systems in response to feeding Bt (MON810) maize for 30 days post-weaning (Finamore *et al*., 2008). Several disturbances in lymphocyte subsets at gut and peripheral sites were documented in this study. We observed a similar decrease in $CD4^+$ T cells and B cells in the spleen and ileum, respectively, when Bt (MON810) maize was feed to weanling pigs for 35 days post-weaning (Experiment 2). However, an increase in $CD4^+$ T cell subsets within the porcine ileal lymphocyte population in Experiment 2 was contrary to findings in mice (Finamore *et al*., 2008) and unlike the results with mice, we also found a reduction in porcine ileal macrophages in response to feeding Bt (MON810) maize. The implications of the alterations in $CD4^+$ T cells, B cells and macrophages in the ileum and $CD4^+$ T cell in the spleen have yet to be explained. However, work by Murtaugh *et al.* (2009) in pigs found evidence of an anti-proliferative effect of IL-4 on B-cells. Interleukin-4 production from cultured ileal lymphocytes was elevated in GM maize-fed pigs (Experiment 2) and this may account for the observed reduction in B-cells. While GM exposure also increased IL-4 production in the spleen in Experiment 2, there was no effect on B cell populations indicating a potential site-specific effect of IL-4. Previous work found that IL-6 and IL-4 play a major role in mediating B-cell activation and antibody production (Rincon *et al*., 1997). Interleukin-6 is also known to antagonize the IL-12/IFNγ mediated differentiation of naïve T cells towards a Th1 inflammatory type response in favour of the Th2 humoral immune response. In Experiment 2, the production of IL-6 and IL-4 from cultured splenocytes and IL-4 from ileal lymphocytes was increased in GM maize-fed pigs and results from Experiment 1 showed a decrease in IL-12 and IFNγ from cultured PBMC. Pro-inflammatory cytokines, IL-6 and IL-4 are known to be involved in allergic and inflammatory responses (Rincon *et al*., 1997). However, the increase in

antigen-specific IgA and IgG that accompanies a Th2-mediated allergic inflammatory response (Schiavi *et al.,* 2011) was notably absent in Experiment 1 which indicates that feeding GM maize did not elicit an allergenic response. Adel-Patient *et al.* (2011) also reported finding no specific anti-Cry1Ab antibody in serum from mice given MON810 maize after either i.g. or i.p. sensitization. The basal concentration of both IL-12 and IFNγ from resting PBMC (no PMA/I stimulation) isolated from non-GM maize-fed pigs was greater than similar cytokine concentrations in GM maize-fed pigs. By nature of the transgene insertion, GM maize is protected from insect damage and may as a result contain less endotoxins than its non-GM maize counterpart. The potentially greater exposure to endotoxins from feeding non-GM maize may account for the elevated Th1 profile of cytokines evident in both resting and stimulated PBMC isolated from pigs fed non-GM maize. Therefore, feeding GM maize to pigs may protect against a systemic inflammatory response characterized by an elevated Th1 cytokine profile.

The increase in cytokine production from cultured cells in Experiment 2, while statistically significant was numerically small and unlikely to be of biological relevance. These findings together with the lack of Cry1Ab-specific antibody production in blood make the development of a Th2-mediated allergic response highly unlikely. Overall, the findings from the two experiments suggest that some GM maize-induced systemic and local immune alterations are occurring in the weaned pig. The Cry1Ab protein, which was found in the majority of small intestinal digesta samples from GM maize-fed pigs (Experiment 1), has been shown to lack homology with known allergens and is not at risk of causing allergenic cross-reactivity (Randhawa *et al.*, 2011). The presence of the Cry1Ab protein in the GIT of GM maize-fed pigs is the only measured difference between these pigs and the control pigs. Therefore, the non-allergenic alterations observed in the immune response of GM maize-fed pigs most likely are attributed to the Cry1Ab protein and in some cases feeding GM maize may prevent a systemic Th1 inflammatory response.

One of the main consumer concerns with the use of GM foods is transfer of the transgenic DNA to human tissues or to animal products such as meat. Numerous animal studies have been conducted in which transgenic DNA has not been detected in food products derived from animals fed GM feed ingredients (Weber and Richert, 2001; Nemeth *et al.*, 2004; Deaville and Maddison, 2005; Alexander *et al.*, 2006; Yonemochi *et al.*, 2010). However, the transfer of endogenous plant DNA across the gut barrier is a natural phenomenon, as it has been detected in both animal tissue and products (Reuter

and Aulrich, 2003; Nemeth *et al*., 2004; Mazza *et al*., 2005). Previously, Sharma *et al.* (2006) detected a 278 bp fragment of the *cp4epsps* transgene found in Round-Up Ready canola in the liver and kidney of swine. However, only one liver and one kidney sample out of 36 samples tested were positive for the transgenic DNA fragment. Likewise, a 519 bp fragment of *cry1Ab* was detected in the blood, liver, kidney, and spleen of piglets following 35 days of administration of Bt (MON810) maize (Mazza *et al*., 2005). However, the intact *cry1Ab* gene (3470 bp) or the gene's smallest functional unit (1800 bp) was never detected. In Experiment 1, the target *cry1Ab* gene fragment (211 bp) was not detected in the white blood cells, heart, liver, spleen, kidney or muscle of pigs fed GM maize for 31 days. While much emphasis has been placed on minimizing potential cross-contamination between animals fed GM feed ingredients and controls in other studies, transgenes have been detected in tissue (Mazza *et al*., 2005; Sharma *et al*., 2006). However in Experiment 1, no cross-contamination occurred and the *cry1Ab* gene was not detected in tissues or blood and this may question the effectiveness of contamination preventive measures used in previous studies where transgenes were found in tissues. Furthermore, findings from Experiment 1 demonstrate a lack of transfer of endogenous plant DNA across the gut barrier into organs and blood. The frequency of gene detection is dependent on copy number of the genes ingested. In Experiment 1, the *Sh2* gene was used as a control to compare the behaviour of a single copy gene to that of the single copy *cry1Ab* gene. In some cases, the multiple copy *rubisco* or *zein* genes are used as indicators of endogenous plant DNA transfer (Nemeth *et al*., 2004; Mazza *et al*., 2005). These genes have a higher detection frequency than single copy genes such as *Sh2* which may explain the discrepancies between our findings and those that have detected endogenous plant DNA in animal tissue. Similar to previous findings Chowdhury *et al.* (2003), the detection of both the endogenous plant and *cry1Ab* genes in the digesta of GM maize-fed pigs decreased during passage through the GIT from 100% recovery in the stomach to undetectable in the feces. Chowdhury *et al.* (2003) found that a primer pair with a 110 bp PCR product detected *cry1Ab* in a greater number of pig digesta samples than a primer pair that detected a 437 bp fragment. Thus, the recombinant DNA appears to be degraded as it passes through the GIT. Consequently, the recombinant DNA may have been present in smaller fragments than were detectable using the *cry1Ab* primer pair used in the Experiment 1 (211 bp) accounting for the low frequency of detection in the distal GIT.

Similar to the findings documented by Yonemochi *et al.* (2010), transgenic

Cry1Ab protein was not detected in the plasma or organs of any pigs from Experiment 1. This is not surprising, considering that the transgene was also undetectable in plasma and organs. The Cry1Ab protein was only detected in 30% of stomach samples taken from GM maize-fed pigs and at low concentrations (2.74 ng/mL) even though the transgene was recovered from all of these samples. Chowdhury *et al.* (2003) reported much higher levels of Cry1Ab protein (300 ± 140 ng/g) in rectal digesta of pigs. However, these pigs were heavier and fed a diet containing 20% more maize than pigs in the current study. Numerous proteolytic cleavage sites for pepsin in particular, have been reported within the Cry1Ab protein (Kirouac *et al.*, 2006). Consequently, the Cry1Ab protein is likely to have undergone some degree of degradation by pepsin accounting from the lower detection frequency in the stomach. The lower detection frequency of the *cry1Ab* gene compared to the protein further down the GIT (small intestine, caecum and colon) found in Experiment 1 has been observed previously (Chowdhury *et al.*, 2003) when a 437 base pair primer was used for the detection of the *cry1Ab* gene in pig digesta. However, in the same study when a *cry1Ab* primer for a shorter fragment length (110 bp) was used, transgene detection frequency increased and mirrored that of the protein detection rates. Likewise, Einspanier *et al.* (2004) found that the use of primers with shorter expected fragment lengths increased the chance of detection of plant and maize DNA from cows. In Experiment 1, the use of a 211 bp primer instead of a primer with a shorter expected fragment length may account for the discrepancies observed in detection frequencies between the *cry1Ab* gene and protein at similar sites along the GIT.

No consistent effects on feed intake and ADG have been reported in the numerous pig-feeding studies that have compared GM maize with conventional maize varieties (Bressener *et al.*, 2002; Fischer *et al.*, 2002; Hyun *et al.*, 2004). In Chapter 2 we found that, similar to results from Experiment 2, feeding GM maize to weanling pigs had no effect on growth rate or body weight following 31 days of feeding. Although an increase in feed intake was observed in Chapter 2 in Bt (MON810) maize-fed pigs, feed intake was not affected in Experiment 2. Consequently, short-term feeding of GM maize is unlikely to effect the growth of weanling pigs.

In conclusion, data obtained from short-term feeding of GM maize to weanling pigs have demonstrated no adverse effects on growth performance. Maize-derived DNA, either of intrinsic or recombinant origin, was largely degraded in the GIT. There was no evidence of *cry1Ab* gene or protein translocation to organs or plasma.

Transgenic protein was detected in GIT digesta but at very low concentrations. Exposure to GM maize did induce some alterations in localized and peripheral immune responses in weanling pigs which require further investigation. The lack of Cry1Ab specific Ig production in plasma however suggests that the immune response was not allergenic and there is evidence to indicate that feeding GM maize may help to prevent an elevation in the inflammatory Th1 cytokine profile observed following non-GM maize consumption. Although the significance of the alterations in immune response have yet to be established, the lack of recombinant DNA or protein translocation to tissues or changes in growth should help to offer assurance to consumers as to the safety of GM feed ingredients. To further investigate the changes observed in this study, we are currently conducting a study to assess any immune responses that may arise from long-term feeding of GM maize to pigs.

Table 3.1. Composition of acclimatization starter diet and experimental diets (fresh weight basis, %) [1,2].

	Experiment 1			Experiment 2			
	Baseline (day -6 to 0)	Experimental (day 0 to 31)		Experimental (Starter, day 0 to 7)		Experimental (Link, day 7 to 35)	
Ingredient (%)	**Non-GM**	**Non-GM**	**GM**	**Non-GM**	**GM**	**Non-GM**	**GM**
Maize (non-GM)	27.33	38.88	-	27.33	-	38.88	-
Maize (GM – MON810)	-	-	38.88	-	27.33	-	38.88
Soybean meal (non-GM)	24.00	25.00	25.00	24.00	24.00	25.00	25.00
Lactofeed 70[3]	25.00	20.00	20.00	25.00	25.00	20.00	20.00
Immunopro 35[4]	12.50	9.00	9.00	12.50	12.50	9.00	9.00
Soybean oil	8.00	4.00	4.00	8.00	8.00	4.00	4.00
L-Lysine HCl	0.30	0.30	0.30	0.30	0.30	0.30	0.30
DL-Methionine	0.25	0.20	0.20	0.25	0.25	0.25	0.25
L-Threonine	0.12	0.12	0.12	0.12	0.12	0.12	0.12
L-Tryptophan	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin and mineral premix[5]	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Mycosorb[6]	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Dicalcium Phosphate	0.50	0.50	0.50	0.50	0.50	0.50	0.50

	Experiment 1			Experiment 2			
	Baseline (day -6 to 0)	Experimental (day 0 to 31)		Experimental (Starter, day 0 to 7)		Experimental (Link, day 7 to 35)	
Ingredient (%)	**Non-GM**	**Non-GM**	**GM**	**Non-GM**	**GM**	**Non-GM**	**GM**
Limestone flour	1.10	1.10	1.10	1.10	1.10	1.10	1.10
Analysed Chemical Composition (%)							
Dry matter	91.3	89.4	89.2	91.7	91.3	91.3	90.9
Crude protein	20.9	20.9	21.1	20.5	21.8	21.1	20.1
Fat	9.6	6.1	5.9	9.8	9.8	7.1	6.7
Crude fibre	1.7	2.1	1.9	1.6	1.5	1.6	1.5
Ash	6.3	5.5	5.6	5.8	6.4	5.7	5.8
Lysine	1.55[7]	1.42	1.42	1.48	1.39	1.43	1.50
Ca[7]	0.83	0.78	0.78	0.83	0.83	0.78	0.78
P[7]	0.61	0.59	0.59	0.61	0.61	0.59	0.59
Digestible energy, MJ/kg[7]	16.33	15.38	15.38	16.33	16.33	15.38	15.38

[1]Non-GM starter diet fed to pigs (Experiment 1) for 6 days post-weaning.

[2]Experimental GM and non-GM maize diets fed to pigs in Experiment 1 for 31 days and Experiment 2 for 35 days.

[3]Lactofeed 70 contains 70% lactose, 11.5% protein, 0.5% oil, 7.5% ash and 0.5% fibre (Volac, Cambridge, UK).

[4]Immunopro 35 is a whey protein powder product containing 35% protein (Volac, Cambridge, UK).

[5]Premix provided per kg of complete diet: Cu, 155 mg; Fe, 90 mg; Mn, 47 mg; Zn, 120 mg, I, 0.6 mg; Se, 0.3 mg; vitamin A, 6000 IU; vitamin $D_{3,}$ 1000 IU; vitamin E, 100 IU; vitamin K, 4 mg; vitamin $B_{12,}$ 15 μg; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; choline chloride, 250 mg; vitamin $B_{1,}$ 2 mg; vitamin $B_{6,}$ 3 mg.

[6]Mycosorb is organic mycotoxin absorbent (Alltech Inc. Dunboyne, Ireland).

[7]Calculated values.

Table 3.2. Primers used in PCR reactions for the detection of three target sequences in porcine organ, white blood cell and digesta samples.

Primer name	Sequence (5'-3')	Specificity	Target gene	Amplicon size (bp)	PCR conditions	Reference
Sh2 – F	TTC GGG AGG CAA GTG TGA TTT CG	Plant (endogenous)	ADP glucose pyrophosphorylase	213	94°C × 3 min 94°C × 30 s 55°C × 30 s } x 30 72°C × 45 s 72°C × 5 min	Jennings *et al.*, 2003
Sh2 – R	GTC GGC AAG AAT GGA GCA ATT C					
cry1Ab - F	CCT GGA GCG CGT CTG GGG CCC TGA TTC T	Plant (transgenic)	*cry1Ab*	211	94°C × 3 min 95°C × 30 s 64°C × 30 s } x 32 72°C × 30 s 72°C × 5 min	Jennings *et al.*, 2003
cry1Ab - R	GGC GCT GCC CCT GAA GCT ACC GTC GAA GTT CT					

Primer name	Sequence (5'-3')	Specificity	Target gene	Amplicon size (bp)	PCR conditions	Reference
SW - F *SW* - R	TCA GTT TAC ACT CAC CTG ATA GCA TCT GGG TGG TGG AGA GGG GTG AAT T	Animal (porcine)	Pig growth hormone	108	94°C × 1 min 94°C × 30 s 60°C × 30 s } x 30 72°C × 40 s 72°C × 3 min.	Meyer *et al*., 1994

Table 3.3. Effects of feeding GM maize or non-GM maize with or without mitogenic stimulation on cytokine production from porcine isolated peripheral blood mononuclear cells (**Experiment 1**), splenocytes and *lamina propria* and intraepithelial lymphocytes (**Experiment 2**)[1,2].

Treatments	**Non-GM maize**		**GM maize**			***P*-value**		
Cytokines, pg/mL	**- PMA/I[3]**	**+ PMA/I**	**- PMA/I**	**+ PMA/I**	**SEM**	**trt**	**PMA/I**	**trt×PMA/I**
Peripheral blood mononuclear cells[4]								
IL-10	9.6	89.8	12.7	106.5	7.6	0.17	0.001	0.29
IL-12	55.8	1292.3	9.0	333.4	308.5	0.07	0.03	0.09
IL-6	15.9	47.6	7.5	40.3	9.6	0.40	0.01	0.94
IL-4	46.3	301.9	9.7	181.4	75.3	0.24	0.02	0.49
TNFα	0	5586.1	0	3686.9	1546.7	0.26	0.01	0.28
IFNγ	555.9	3041.3	247.7	850.8	644.5	0.04	0.04	0.08
Splenocytes[5]								
IL-8	283.4	1144.3	67.1	1103.1	245.9	0.60	0.001	0.72
IL-6	1.5	4.2	2.7	6.4	0.8	0.04	0.001	0.56
IL-4	3.9	10.1	11.7	21.2	3.3	0.01	0.02	0.60
TNFα	22.3	274.0	3.3	383.3	41.7	0.28	0.001	0.12
***Lamina propria* and intraepithelial lymphocytes[5]**								
IL-8	7.6	43.8	12.3	82.2	10.8	0.05	0.001	0.13
IL-6	1.6	3.4	1.5	3.9	0.5	0.69	0.001	0.60

Treatments	Non-GM maize		GM maize			P-value		
Cytokines, pg/mL	- PMA/I[3]	+ PMA/I	- PMA/I	+ PMA/I	SEM	trt	PMA/I	trt×PMA/I
IL-4	2.7	2.1	13.2	21.9	3.3	0.001	0.22	0.17
TNFα	1.1	128.8	1.1	197.2	22.2	0.14	0.001	0.12

SEM = standard error of the mean.

[1]*n* = 10 pigs in control group and 10 pigs in the GM maize-fed group.

[2]Peripheral blood mononuclear cells (PBMC) were isolated from pigs fed treatments for 29 days (**Experiment 1**) and splenocytes and *lamina propria* & intraepithelial lymphocytes were isolated from pigs fed treatments for 35 days (**Experiment 2**).

[3]All isolated cells were stimulated with PBS (- PMA/I) or 25 ng/μL phorbol myristate acetate and 2 mg/mL ionomycin (+ PMA/I) for 18 h at 37ºC in a 5% (v/v) CO_2 humidified atmosphere.

[4]Data analysis was performed using two factor analysis of variance (ANOVA) with interactions to determine if differences in cytokine production varied by treatment and mitogen stimulation. Baseline values measured on day 0 were included as covariates in the model.

[5]Data analysis was performed using two factor analysis of variance (ANOVA) with interactions to determine if differences in cytokine production varied by treatment and mitogen stimulation.

Table 3.4. Detection of endogenous maize and porcine genes and transgenic *cry1Ab* gene in tissue and white blood cells of pigs fed GM maize versus pigs fed a non-GM maize diet for 31 days (**Experiment 1**)[1].

Fragment amplified	**Organ/Tissue**											
	Heart		**Liver**		**Spleen**		**Kidney**		**Muscle**		**White blood cells**	
GM treatment[2]	-	+	-	+	-	+	-	+	-	+	-	+
Endogenous												
Sh2 (maize)	0	0	0	0	0	0	0	0	0	0	0	0
SW (porcine)	10	10	10	10	10	10	10	10	10	10	10	10
Transgenic												
cry1Ab	0	0	0	0	0	0	0	0	0	0	0	0

[1]Number of samples that tested positive for the gene of interest out of 10 samples analysed. One sample was tested per pig (*n* =10 pigs per treatment).

[2]GM treatments; - denotes non-GM maize-fed pigs and + denotes GM maize-fed pigs.

Table 3.5. Detection of endogenous maize and porcine genes and transgenic *cry1Ab* gene in stomach, ileal and caecal digesta and colon samples of pigs fed GM maize versus pigs fed a non-GM maize diet for 31 days (**Experiment 1**)[1].

Fragment amplified	**Digesta**							
	Stomach		**Ileum**		**Caecum**		**Colon**	
GM treatment[2]	**-**	+	**-**	+	**-**	+	**-**	+
Endogenous								
Sh2 (maize)	10	10	5	2	1	1	0	0
SW (porcine)	10	10	8	7	6	7	10	9
Transgenic								
cry1Ab (maize)	0	10	0	2	0	1	0	0

[1]Number of samples that tested positive for the gene of interest out of 10 samples analysed. One sample was tested per pig (*n* =10 pigs per treatment).

[2]GM treatments; - denotes non-GM maize-fed pigs and + denotes GM maize-fed pigs.

Table 3.6. Detection of the transgenic Cry1Ab protein in tissue, plasma and gastrointestinal digesta of pigs fed GM and non-GM maize.

	Number of positive samples[1]		**Mean concentration (ng/mL)**	**Positive detection frequency in GM-maize fed pigs**[2]
	Non-GM maize	**GM maize**		
Heart	0	0	BLD[3]	0
Liver	0	0	BLD	0
Spleen	0	0	BLD	0
Kidney	0	0	BLD	0
Muscle	0	0	BLD	0
Plasma	0	0	BLD	0
Stomach digesta	0	3	2.74	30
Ileum digesta	0	8	2.45	80
Caecum digesta	0	3	2.41	30
Colon digesta	0	8	2.67	80

[1]Number of samples that tested positive for the Cry1Ab protein out of 10 samples analysed. One sample was tested per pig (n = 10 pigs per treatment).

[2]Percentage of samples positive for the Cry1Ab protein taken from GM maize fed pigs i.e. (number of positive samples/number of samples tested) × 100.

[3] BLD = below the limit of detection.

Table 3.7. Effects of feeding GM or non-GM maize for 35 days on weanling pig growth performance (**Experiment 2**)[1].

	Non-GM maize	**GM maize**	**SEM**	***P*-value**[2]
Overall, day 0 - 30				
ADG, g/d	321	355	37.5	0.53
ADFI, g/d	465	479	41.8	0.82
FCE	1.54	1.43	0.097	0.45
day 30 BW, kg	17.6	18.6	1.21	0.54

ADG = average daily gain; ADFI = average daily feed intake; FCE = feed conversion efficiency [calculated as feed intake (g)/body weight (g)]; BW = body weight; SEM = standard error of the mean.

[1] n = 12 pigs in control group and 11 pigs in the GM maize-fed group.

[2] Data analysis was performed using one factor analysis of variance (ANOVA) using the GLM procedure of SAS.

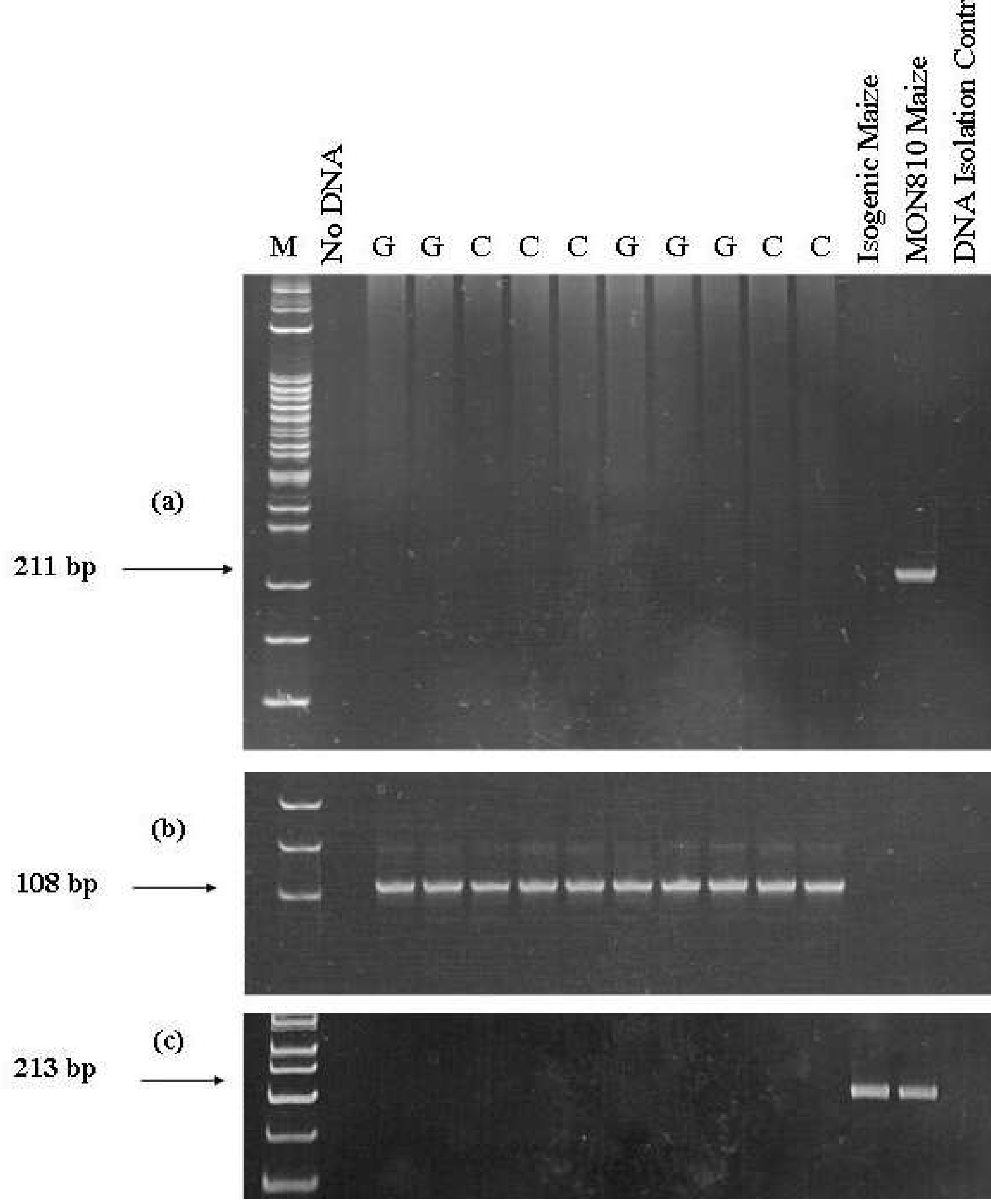

Figure 3.1. Fate of transgenic DNA from orally administered Bt MON810 maize in pigs. PCR products from liver samples of 10 pigs (Experiment 1) fed either GM maize (G) or control maize (C), for each of the target gene fragments; (a) *cry1Ab*, (b) *SW* (endogenous porcine) and (c) *Sh2* (endogenous maize). Arrows indicate the expected size of PCR products. Reactions without template DNA (no DNA) and with purified genomic DNA from MON810 maize and isogenic parent line maize were included as controls. A DNA isolation control which did not contain any sample material was also used. M = molecular weight marker.

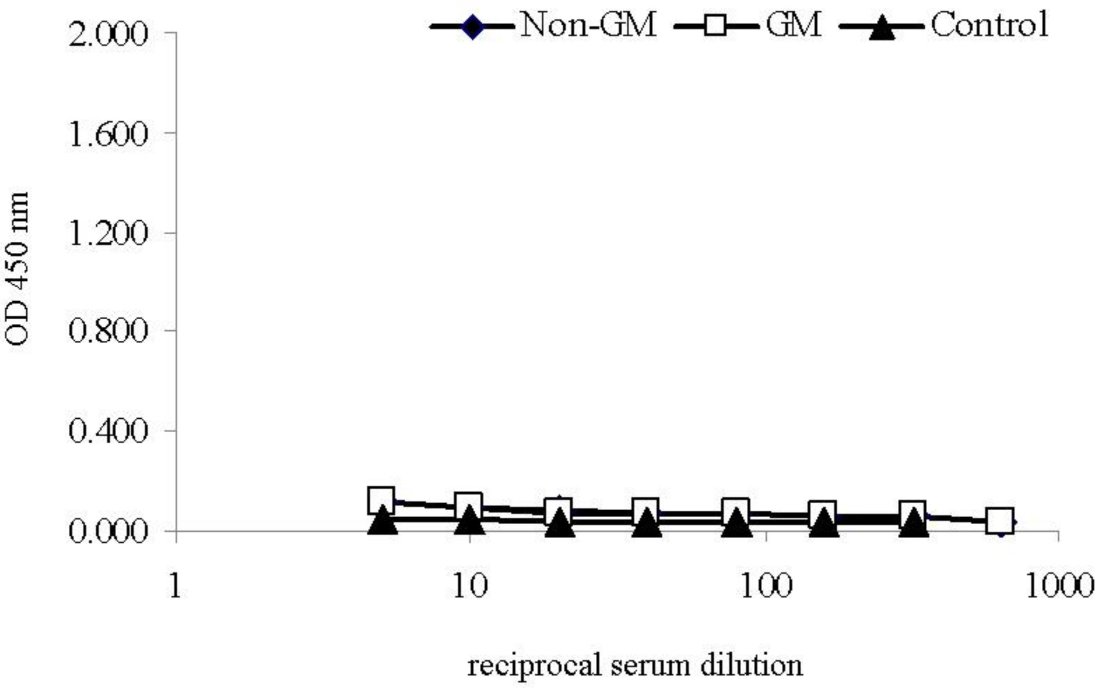

Figure 3.2. Effects of feeding non-GM maize (♦), GM maize (□) or non-maize feed (▲) to pigs for 31 days on plasma concentration of Cry1Ab specific IgG antibody (Experiment 1). Cry1Ab specific IgG antibody was not detected in any of the samples assayed.

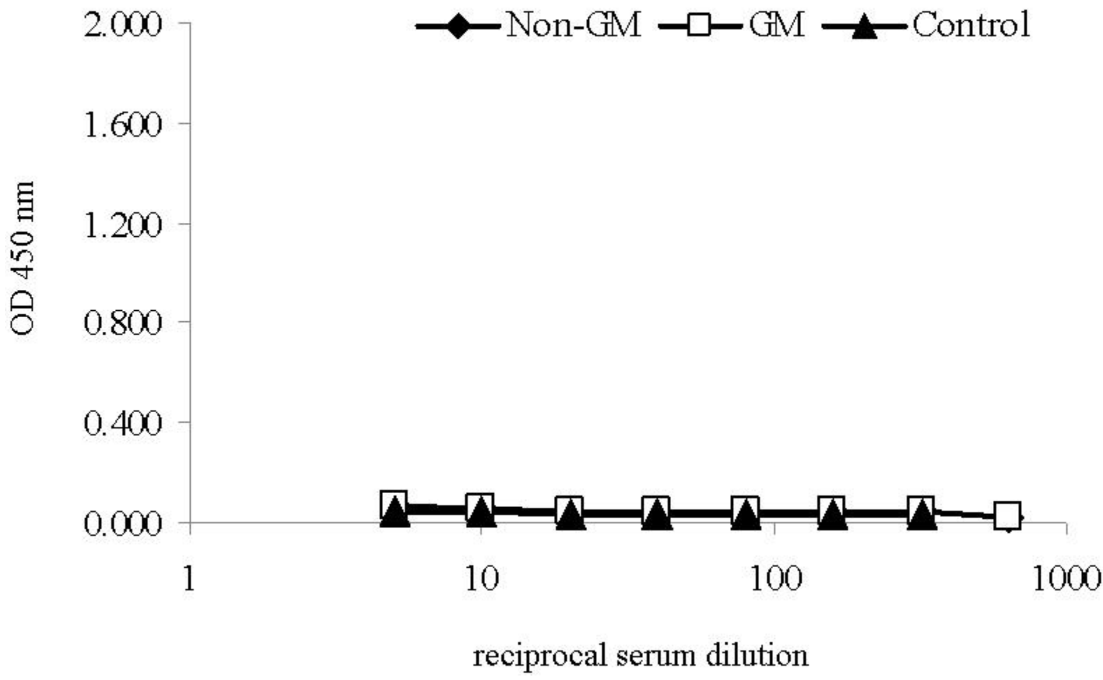

Figure 3.3. Effects of feeding non-GM maize (♦), GM maize (□) or non-maize feed (▲) to pigs for 31 days on plasma concentration of Cry1Ab specific IgA antibody (Experiment 1). Cry1Ab specific IgA antibody was not detected in any of the samples assayed.

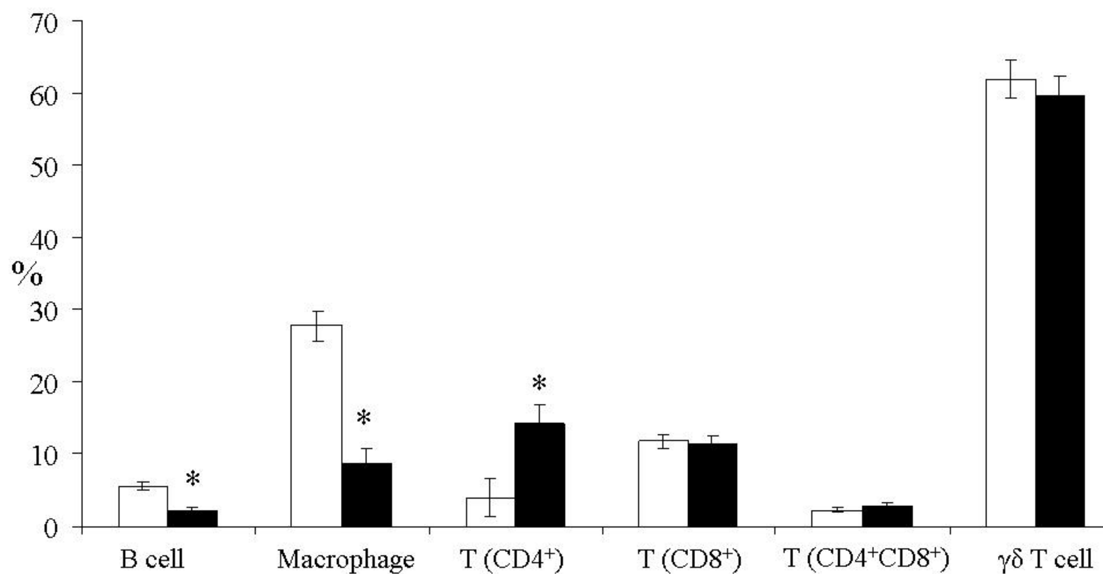

Figure 3.4. Changes in the proportion of different leukocyte populations in the ileum of pigs fed non-GM (□) or GM (■) maize for a period of 35 days (Experiment 2). Mean values ± SEM were calculated as a percentage of the total *lamina propria* and intra-epithelial lymphocyte populations. * Significance at $P < 0.01$

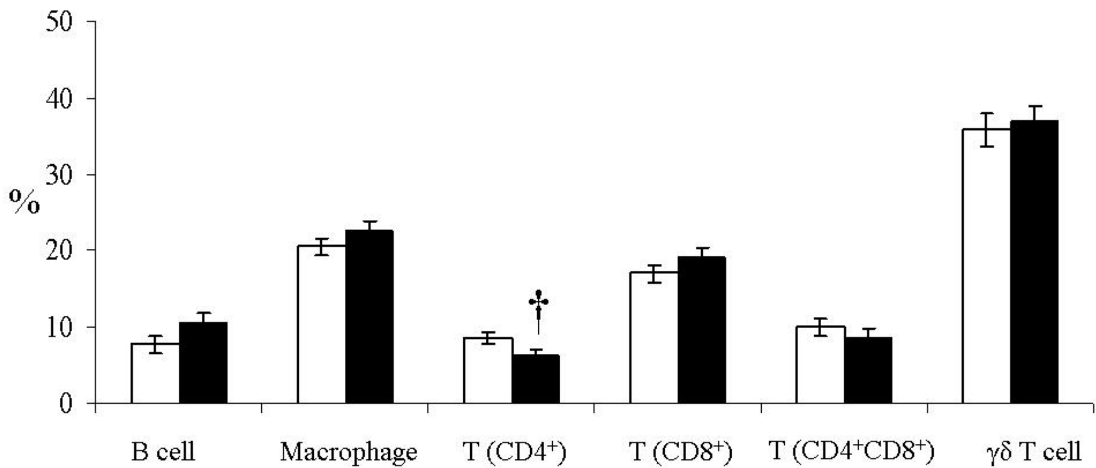

Figure 3.5. Changes in the proportion of different leukocytes populations in the spleen of pigs fed non-GM (□) or GM (■) maize for a period of 35 days (Experiment 2). Mean values ± SEM were calculated as a percentage of the total splenocyte population. †Tendency for a significance at $P < 0.10$.

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4. High throughput sequence-based analysis of the intestinal microbiota of weanling pigs fed genetically modified Bt MON810 maize for 31 days

Buzoianu, S. G., Walsh, M. C., Rea, M. C., O'Sullivan, O., Cotter, P. D., Ross, R. P., Gardiner, G. E. and Lawlor, P. G. (2012). High throughput sequence-based analysis of the intestinal microbiota of weanling pigs fed genetically modified Bt MON810 maize for 31 days. Appl. Environ. Microbiol. DOI: 10.1128/AEM.00307-12.

4.1. Abstract

The objective of this study was to investigate if feeding genetically modified (GM) Bt maize had any effects on the porcine intestinal microbiota. Eighteen pigs were weaned at ~28 days, and following a 6 day acclimatization period, were assigned to diets containing either GM (Bt MON810) or non-GM isogenic parent line maize for 31 days (n = 9/treatment). Effects on the porcine intestinal microbiota were assessed through culture-dependent and -independent approaches. Faecal, caecal and ileal counts of total anaerobes, *Enterobacteriaceae* and *Lactobacillus* were not significantly different between pigs fed the isogenic or Bt maize-based diets. Furthermore, high-throughput 16S rRNA gene sequencing revealed few differences in the composition of the caecal microbiota. The only differences were that pigs fed the Bt maize diet had higher caecal abundance of *Enterococcaceae* (0.06 vs 0%; $P < 0.05$), *Erysipelotrichaceae* (1.28 vs 1.17%; $P < 0.05$) and *Bifidobacterium* (0.04 vs 0%; $P < 0.05$) and lower abundance of *Blautia* (0.23 vs 0.40%; $P < 0.05$) compared to pigs fed the isogenic maize diet. A lower enzyme resistant starch content in the Bt maize, which is most likely a result of normal variation and not due to the genetic modification, may account for some of the differences observed within the caecal microbiota. These results indicate that Bt maize is well tolerated by the porcine intestinal microbiota and provide additional data for safety assessment of Bt maize. Furthermore, these data can potentially be extrapolated to humans, considering the suitability of pigs as a human model.

4.2. Introduction

Maize is one of the main nutrient sources for humans and animals worldwide (FAO, 2012). The application of gene technology to improve maize has proven successful, as genetically modified (GM) insect resistant maize accounted for 24.6% of global maize production in 2010, reflecting a rapid increase in its use over the last 15 years (James, 2010). Most of the GM maize cultivated worldwide is Bt maize, which is resistant to insect damage via expression of the Cry1Ab transgenic protein originally identified in *Bacillus thuringiensis*.

Although many benefits are associated with the presence of the Cry1Ab protein in maize (Brookes, 2008), its presence in human and animal diets has raised concerns. Concerns expressed by some consumers are mainly related to potential health risks. Alternatively, effects may be mediated by potential changes in intestinal bacterial populations following GM plant consumption. Research on bacterial-host interactions has revealed the enormous influence of the intestinal bacteria on host physiology (Sekirov *et al*., 2010). Therefore, any effect a GM plant may have on intestinal bacteria could potentially affect the host, especially immunocompromised individuals (Rotterdam and Tsang, 1994).

European Food Safety Authority (EFSA) guidelines for testing GM feeds recommend the investigation of effects on the host as well as on host bacterial populations (EFSA, 2008). However, most of the research to date examining the impact of Bt maize on bacteria has focused on soil microbiota and has found no effects of the Bt maize-derived Cry1Ab protein (Saxena and Stotzky, 2001; Oliveira *et al*., 2008).

An *in vitro* study has demonstrated antimicrobial activity of the Cry1Ab protein in both intact (130 kDa) and fragmented (~60 kDa) form against *Clostridium butyricum*, *Clostridium acetobutylicum* and *Methanosarcina barkeri* (Yudina *et al*., 2007). However, conflicting data were obtained by Koskella and Stotzky, (2002) who failed to observe activity *in vitro* against a range of Gram-positive and -negative bacteria, namely *Proteus spp., Pseudomonas aeruginosa, Enterobacter spp., Escherichia coli, Klebsiella, Agrobacterium radiobacter, Arthrobacter globiformis* and *Bacillus spp.* Sung *et al.* (Sung *et al*., 2006) also found that Bt maize had no effect on *Fibrobacter succinogenes* and *Streptococcus bovis* populations *in vitro* following a 12 or 24 hour period of exposure. Although no such studies have been conducted *in vivo* with Bt maize grain or feed, data from our group and others show that the protein is not completely degraded during intestinal transit when administered in feed (Chowdhury *et al*., 2003; Einspanier

et al., 2004; Chapter 3). Therefore, studies examining its effect on the intestinal microbiota *in vivo* are warranted.

However, to our knowledge, the only studies that have investigated the effects of Bt maize on intestinal bacterial populations *in vivo* have been conducted in ruminants. Wiedemann *et al.* (2007) employed real-time PCR analysis and found no effects of Bt maize on six ruminal bacterial strains following 11 days of feeding. Einspanier *et al.* (2004) found no effect of 28 days of feeding Bt maize to cows on ruminal bacterial communities using 16S rRNA gene sequencing. Similarly, ribosomal intergenic spacer analysis revealed no effects of feeding Bt 176 maize silage for 35 days on ruminal bacterial population structure in cows (Brusetti *et al.*, 2011). Likewise, Trabalza-Marinucci *et al.* (2008) reported no effect of feeding Bt maize for 36 months on the ruminal microbiota of sheep, as revealed by culturing. Using traditional culturing techniques, a study in rats found that *Bifidobacterium* counts were lower in the duodenum and coliforms were higher in the ileum following 90 days of feeding Bt rice (Schrøder *et al.*, 2007). However, to date, no studies have examined the effect of feeding Bt maize on the intestinal microbiota of pigs, a species whose intestinal tract more closely resembles that of humans (Moughan *et al.*, 1992). Furthermore, high-throughput DNA sequencing technologies, which have revolutionized our ability to investigate microbial community structure, have not yet been employed to investigate the effect of GM crops on porcine intestinal microbial populations.

The Organization for Economic Co-operation and Development (OECD), EFSA and the International Life Sciences Institute (ILSI) recommend that animal trials involving GM plants include as a comparator the parental plant from which the GM plant originated (OECD, 2002; Hartnell *et al.*, 2007; EFSA, 2008). Furthermore, the recommendations state that the plants compared should be grown under similar conditions (Hartnell *et al.*, 2007). However, while some of the published studies to date have provided information on the genetic background and growing conditions of the non-GM comparator plant used (Hyun *et al.*, 2005; Schrøder *et al.*, 2007; Wiedemann *et al.*, 2007), others have not (Trabalza-Marinucci *et al.*, 2008). This makes interpretation of results from the latter studies difficult, as environmental conditions and season can influence Bt maize composition more than the genetic modification itself (Barros *et al.*, 2010).

Therefore, our objective was to assess the effect of feeding a Bt maize-based diet for 31 days on intestinal bacteria, employing the pig as a model, and using the isogenic parent line maize grown under similar environmental conditions to the Bt maize as a

comparator. By employing culture-dependent and -independent approaches, we aimed to provide the most detailed investigation of the effect of Bt maize on intestinal microbial populations to date. By doing so, we hope to address consumer concerns regarding the safety of Bt maize and to provide additional data which can be used for further development and improvement of GM plant testing procedures.

4.3. Materials and methods

Pig feeding study

The pig study complied with EU regulations outlining minimum standards for the protection of pigs (91/630/EEC) and concerning the protection of animals kept for farming purposes (98/58/EC) and was approved by, and a license obtained from, the Irish Department of Health and Children. Ethical approval was obtained from both the Teagasc and Waterford Institute of Technology ethics committees.

Eighteen crossbred (Large White × Landrace) pigs (entire males, 7.5 ± 1.5 kg body weight) were weaned at approximately 28 days of age and allowed a 6 day adjustment period, during which they were provided *ad libitum* access to a non-GM starter diet (n = 9/treatment). Following the adjustment period, on day 0 of the study, pigs were blocked by weight and litter and randomly assigned to one of two dietary treatments; 1) non-GM isogenic parent line maize-based diet (Pioneer PR34N43) and 2) GM maize-based diet (Bt; Pioneer PR34N44 event MON810). Both treatments were fed for 31 days. Pigs were penned individually in a total of four rooms, with 4 – 5 pigs in each room and each treatment group represented in each room to avoid an effect of room. The temperature of the rooms was maintained at 28°C in the first week and reduced by 2°C per week to 22°C in the fourth week. For the duration of the study, pigs were allowed *ad libitum* access to feed and water and none of the pigs received antibiotic treatment.

Maize and diets

The MON810 and isogenic parent line maize (PR34N44 and PR34N43, respectively; Pioneer Hi-Bred, Sevilla, Spain) were grown simultaneously in neighboring plots in Valtierra, Navarra, Spain by independent tillage farmers over the 2007 season to ensure similar environmental conditions in accordance with EFSA and ILSI recommendations (Hartnell *et al*., 2007; EFSA, 2008). The isogenic and Bt maize were purchased by the authors from the tillage farmers for use in this animal study. Samples from the isogenic and Bt maize were tested for chemical, amino acid and

carbohydrate composition and for the presence of the *cry1Ab* gene, pesticide contaminants and mycotoxins, as previously described in Chapter 2. All dietary ingredients, with the exception of the Bt maize, were non-GM. Diets were formulated to exceed the National Research Council requirements for swine (NRC, 1998). For both diets, maize (either isogenic or Bt) was included at identical levels. Diets were manufactured and analysed as previously described in Chapter 2 . As a precaution, an organic mycotoxin absorbent (Mycosorb®; Alltech, Dunboyne, Co. Meath, Ireland) was included in all diets.

Sample collection

Individual faecal samples were collected in sterile containers following rectal stimulation prior to (day -1) and following 30 days (day 30) of isogenic or Bt maize consumption. On day 31, pigs were euthanized by captive bolt stunning followed by exsanguination. The last meal was administered 3 h prior to euthanasia. Immediately after euthanasia, terminal ileal (15 cm before the ileo-caecal junction) and caecal (from the terminal tip of the caecum) digesta were collected from all pigs. All faecal and digesta samples were stored in sterile containers at $4^{o}C$ in anaerobic jars containing Anaerocult A gas packs (Merck, Darmstadt, Germany) until analysis (within 12 hours).

Lactobacillus and *Enterobacteriaceae* counts were determined in individual faecal, ileal and caecal samples, as indicators of beneficial and pathogenic bacteria, respectively (Castillo *et al*., 2006). This was performed as previously described by Gardiner *et al.* (2004), with one modification; nystatin (50 U/mL; Sigma Aldrich Ireland Ltd., Wicklow, Ireland) was added to the *Lactobacillus* selective agar (Becton Dickinson, Cockeysville, MD) to inhibit the growth of yeasts and moulds. Total anaerobic bacterial counts were performed in individual faecal, ileal and caecal samples as described by Rea *et al.* (2007). To maintain anaerobiosis, all manipulations with these samples were performed in a Whitley A85 anaerobic workstation (DW Scientific, Shipley, UK) and the plates were also incubated anaerobically within the workstation. Caecal digesta samples were frozen at $-20^{o}C$ for subsequent 16S rRNA gene sequencing.

DNA extraction, PCR and 16S rRNA gene sequencing

Total DNA was extracted from individual caecal digesta samples using the QIAamp DNA stool mini kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions with some modifications, as follows; an initial bead beating

step was included and the lysis temperature was increased from the recommended 70 to 90°C to aid in the recovery of DNA from bacteria that are difficult to lyse.

The microbial composition of these samples was determined by sequencing of 16S rRNA tags (V4 region; 239 bp long). The V4 region was amplified using universal 16S primers predicted to bind to 94.6% of all 16S genes, as previously outlined by Murphy *et al.* (2010). The sequence of the forward primer was 5' AYTGGGYDTAAAGNG 3'. A mix of four reverse primers was used; R1 (5' TACCRGGGTHTCTAATCC 3'), R2 (5' TACCAGAGTATCTAATTC 3'), R3 (5' CTACDSRGGTMTCTAATC 3') and R4 (5' TACNVGGGTATCTAATC 3') (Murphy *et al.*, 2010). Each PCR reaction contained 2 µL of template DNA, 200 nM of forward primer, 50 nM of each of the four reverse primers and 25 µL Biomix Red (Bioline, London, UK) in a total volume of 50 µL. A negative control sample where template DNA was replaced with double distilled water and a positive control sample containing previously amplified caecal bacterial DNA were included. The PCR cycle began with initial denaturation at 94°C for 2 minutes, followed by 35 cycles of 1 minute denaturation at 94°C, annealing at 52°C for 1 minute followed by 1 minute elongation at 72°C. The final elongation step was performed at 72°C for 2 minutes. All PCR amplifications were performed in a G-Storm GS1 thermal cycler (G-Storm, Somerton, Somerset UK). The presence of the target amplicons was verified by visualization under UV light following electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.3 ng/µL). Amplicons were purified using the High Pure PCR product purification kit (Roche Applied Science, Mannheim, Germany). DNA was stained using the Quant-it Pico Green dsDNA kit (Invitrogen Ltd., Paisley, UK) according to the manufacturer's instructions and then quantified using a NanoDrop 3300 spectrophotometer (Fisher Scientific, DE, USA). Sequencing was performed on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd., Burgess Hill, West Sussex) according to 454 protocols. Sequencing reads were checked and assigned to NCBI taxonomies as previously described by Murphy *et al.* (2010). Likewise, de-noising was performed using traditional techniques within the RDP pyrosequencing pipeline. Reads with lengths below 150 bp for the V4 region and with quality scores below 40 as well as reads which did not have an exact match to the primer sequence were removed. MOTHUR software was used to perform clustering and to compute population indices (Schloss *et al.*, 2009). Trimmed FASTA sequences were then BLASTed (Altschul *et al.*, 1997) against a previously published 16S-specific database (Urich *et al.*, 2008) using default parameters. The resulting BLAST output was parsed using MEGAN (Huson *et al.*, 2007). MEGAN assigns reads to NCBI

taxonomies by employing the Lowest Common Ancestor algorithm which assigns each RNA-tag to the lowest common ancestor in the taxonomy from a subset of the best scoring matches in the BLAST result. Bit scores were used from within MEGAN for filtering results prior to tree construction and summarization (absolute cut-off: BLAST bitscore 86, relative cut-off: 10% of the top hit) (Urich *et al.*, 2008; Murphy *et al.*, 2010). The relative abundance of each bacterial taxonomic rank in the pig caecum was calculated by dividing the number of reads assigned to each rank by the total number of reads assigned at the highest rank i.e. the phylum. Therefore, relative abundance is presented as a ratio, with values ranging from 0 (0%) to 1 (100%).

Statistical analysis

For all analyses, the individual pig was considered the experimental unit. Bacterial counts and relative abundance data were log-transformed to the base 10 in an attempt to ensure normal distribution. Only data which were normally distributed and with equal variances (Schlotzhauer and Litell, 1997) were analysed as a one-factor analysis of variance (ANOVA) using the GLM procedure of SAS (SAS/STAT®9.22, SAS Inst. Inc., Cary, NC). Data which were non-normally distributed following log transformation or which had unequal variances were subjected to non-parametric analysis using the Kruskal-Wallis test within the NPAR1WAY procedure of SAS. For analysis of day 30 faecal bacterial counts, baseline (day -1) counts were included as a covariate in the model, thus accounting for any variability present in the data at the beginning of the study. The level of significance for all tests was $P \leq 0.05$. Tendencies were reported up to $P \leq 0.10$. Bacterial counts are presented as means ± SE of the log-transformed values while relative abundance are presented as medians with 5th-95th percentiles (Schlotzhauer and Litell, 1997).

4.4. Results

Maize and diets

The composition of maize lines and diets used in the present study are presented in Tables 4.1 and 4.2, respectively. No major differences were observed between the Bt and the isogenic maize and values were mostly within the normal range of variation for maize varieties (Table 4.1). The Bt maize was found to have a lower enzyme resistant starch content and a higher overall starch content compared to the isogenic maize, but the values remained within the natural variability for maize varieties cited in the

literature. Amino acid content was similar for the two maize lines. Both the Bt and isogenic maize diets had similar proximate composition and amino acid content (Table 4.2).

Culture-based investigation of the effect of feeding Bt maize on intestinal microbiota of weanling pigs.

Faecal counts of *Lactobacillus, Enterobacteriaceae* and total anaerobes did not differ between the isogenic and Bt maize groups at day -1 i.e. prior to administration of experimental diets (P >0.05; Table 4.3). A tendency towards decreased *Lactobacillus* was, however, observed on day -1 in the faeces of pigs assigned to the Bt treatment (P = 0.06) but this has been accounted for by inclusion of day -1 values as a covariate in the statistical model. Faecal counts of *Lactobacillus, Enterobacteriaceae* and total anaerobes were not affected by 30 days of Bt maize exposure (P >0.05; Table 4.3). Likewise, there were no differences in the counts of *Lactobacillus, Enterobacteriaceae* or total anaerobes in the ileum or caecum of pigs fed isogenic or Bt maize-based diets for 31 days (P >0.05).

High throughput 16S rRNA gene sequencing analysis of porcine caecal microbiota to evaluate impact of feeding Bt maize

A total of 332,888 V4 variable regions of the 16S rRNA gene (239 bp) were generated, corresponding to an average of 18,493 sequences per pig. Of the total number of sequences, 321,476 (96.6%) were assigned to the phylum level, 200,679 (60.3%) to the family level and 146,344 (44%) to the genus level. Genus richness, coverage, and diversity estimations were calculated for each pig (Table 4.4). At the 97% similarity level, the Shannon index, a metric for community diversity, revealed a similar level of overall biodiversity for both treatments, with values ranging from 5.1 to 6.8. The Good's coverage at the 97% similarity level ranged between 92 and 96. Rarefaction curves showed a similar caecal bacterial diversity between treatments (Figure 4.2 a and b). Beta diversity analysis using the unweighted option did not reveal a split between treatments (Figure 4.3).

A full outline of the relative abundance of all bacterial taxa in the porcine caecum is available in Table 4.6. A total of 15 different bacterial phyla were detected in the porcine caecum. However, 93% of the sequence reads classified at the phylum level were derived from three phyla; *Firmicutes* (75% of total), *Bacteroidetes* (12% of total)

and *Proteobacteria* (6% of total), with the remaining 12 phyla accounting for only 7% of the sequence reads (Figure 4.1a and Table 4.6). No significant differences were observed with respect to the relative abundance of bacterial phyla in the caecum of pigs fed the Bt versus the isogenic maize. A low prevalence was observed for *Fusobacteria* which were detected in the caecum of only one of nine pigs fed the isogenic maize-based diet and four of nine pigs fed the Bt maize-based diet. This resulted in a tendency for higher abundance of *Fusobacteria* in pigs fed the Bt maize-based diet compared to pigs fed the isogenic maize-based diet (P = 0.08; Table 4.5). Similarly, a low prevalence was observed for *Tenericutes,* which were detected in only three pigs fed the Bt maize-based diet and in none of the pigs fed the isogenic maize-based diet, leading to a tendency for the relative abundance of *Tenericutes* to be greater in pigs fed the Bt maize-based diet (P = 0.07; Table 4.5).

A total of 39 bacterial families were identified in the weanling pig caecum (Table 4.6). The most abundant in pigs on both treatments were *Veillonellaceae* (overall average of 13.6%), *Prevotellaceae* (9.0%), *Clostridiaceae* (8.9%)*, Ruminococcaceae* (6.3%) and *Succinivibrionaceae* (1.7%; Figure 4.1b). There were no significant differences between treatments in the relative abundance of any of these major families (Figure 4.1b). A greater abundance of *Enterococcaceae* and *Erysipelotrichaceae* (P < 0.05) was observed in the caecum of pigs fed the Bt maize-based diet compared to pigs fed the isogenic maize-based diet (Table 4.5). No other family differed with respect to caecal abundance between pigs fed the isogenic and the Bt maize-based diets (Table 4.5 and 5.6). However, caecal abundance of *Succinivibrionaceae* tended to be lower for pigs fed the Bt treatment compared to pigs fed the isogenic treatment (0.17% versus 1.80%; P = 0.08; Figure 4.1b). A low prevalence was observed for the *Bifidobacteriaceae* family which was detected in the caecum of only two pigs from the isogenic treatment and five pigs from the Bt treatment. This resulted in a tendency towards higher *Bifidobacteriaceae* abundance in the caecum of pigs fed Bt maize-diets compared to pigs fed isogenic maize diets (P = 0.06; Table 4.5)

A total of 54 genera were identified in the caecum of pigs in the present study (Table 4.6). Figure 4.1c summarizes the six most abundant genera identified in the weanling pig caecum, which included *Prevotella* (overall average of 7.1%), *Clostridium* (7.5%), *Succinivibrio* (1.4%), *Faecalibacterium* (3.4%), *Acidaminococcus* (2.3%) and *Megasphaera* (1.6%). There were no differences between treatments in the relative abundance of any of these major genera (P > 0.05; Figure 4.1c). However, caecal abundance of *Blautia* (P < 0.05; Table 4.5) was lower for pigs fed the Bt maize diet

compared to pigs fed the isogenic maize diet. Similar to its corresponding family (*Bifidobacteriaceae*), *Bifidobacterium* prevalence in the caecum of pigs was low, as this genus was detected in only one of nine pigs from the isogenic treatment and five of nine pigs from the Bt treatment. This resulted in a higher relative abundance of *Bifidobacterium* in pigs fed the Bt maize compared to pigs fed the isogenic counterpart ($P < 0.05$; Table 4.5). No other genera differed significantly in relative caecal abundance between pigs fed the Bt or isogenic maize-based diets (Table 4.6). The genus *Succinivibrio* tended to be less abundant in the caecum of pigs fed the Bt treatment compared to pigs fed the isogenic treatment ($P = 0.10$; Figure 4.1c), similar to findings for the corresponding family, *Succinivibrionaceae*. Caecal *Mitsuokella* also tended to be lower in pigs fed the Bt maize-based diet compared to pigs fed the isogenic maize-based diet ($P = 0.06$; Table 4.5).

4.5. Discussion

To date, research investigating the effect of feeding Bt maize on gastrointestinal bacterial communities has been limited to studies in ruminants (Einspanier *et al*., 2004; Wiedemann *et al*., 2007; Trabalza-Marinucci *et al*., 2008). To our knowledge, the present study is the first to characterize porcine intestinal microbiota composition following Bt maize consumption. A culture-independent high-throughput DNA sequencing-based approach was employed together with traditional culture-based methods to profile intestinal bacterial communities. This study is also one of the few to employ deep sequencing to evaluate community-wide relative abundance of various bacterial taxa in the porcine intestinal tract and uses the largest number of independent samples to date (18 pigs).

Culturable faecal, caecal and ileal counts of total anaerobes, *Enterobacteriaceae* (indicator of pathogenic bacteria) and *Lactobacillus* (indicator of beneficial bacteria) did not differ between pigs fed an isogenic or Bt maize-based diet. These findings are in agreement with those of Schrøder *et al.* (2007), who reported that Bt rice administration for 90 days had no effects on faecal or small intestinal counts of *Lactobacillus* in rats. They did, however, report lower duodenal bifidobacteria and higher ileal coliform counts. While such studies provide valuable safety data for GM dietary ingredients and provide some indication as to effects on intestinal bacterial populations, care should be taken when extrapolating results from rodents to humans, due to differences in size (Dybing *et al*., 2002), physiology, feed intake and diet (Patterson *et al*., 2008) and the practice of coprophagy. In this respect, the pig is a more suitable model for humans,

especially in terms of gastrointestinal physiology and microbiology (Moughan *et al.*, 1992; Guilloteau *et al.*, 2010).

Sequence-based compositional analysis of the caecal microbiota revealed no significant differences in relative abundance of bacterial phyla between the Bt and isogenic maize-fed pigs, indicating that the Bt maize is well tolerated by the host and intestinal microbiota at the phylum level. This deep 16S rRNA gene sequencing approach detected 15 different bacterial phyla in 65 day old pigs, with *Firmicutes* dominating, followed by *Bacteroidetes* and *Proteobacteria*. The relative distribution is in agreement with that previously observed in the large intestine of humans and pigs using 16S rRNA gene sequencing (Leser *et al.*, 2002; Mariat *et al.*, 2009; Paliy *et al.*, 2009; Lamendella *et al.*, 2011). Similarly, Poroyko *et al.* detected 11 phyla in the caecum of 21 day old pigs but found that *Bacteroidetes* dominated, followed by *Firmicutes* and *Proteobacteria* (Poroyko *et al.*, 2010). However, Vahjen *et al.* detected only five phyla in the ileum of 42 day old pigs but in agreement with our findings, observed that *Firmicutes* dominated (Vahjen *et al.*, 2010).

The presence of *Enterococcaceae* at low abundance as well as at low prevalence in the porcine intestine has previously been reported (Leser *et al.*, 2002, Vahjen *et al.*, 2010). However, their role in the porcine intestine is unclear. Some members of the family are considered beneficial, as they produce bacteriocins and others are used as probiotics (Fisher and Phillips, 2009). On the other hand, enterococci are able to translocate across the intestinal epithelium, leading to bacteremia or localized infections (Fisher and Phillips, 2009). However, although in the present study, *Enterococcaceae* were more abundant in the caecum of Bt maize-fed pigs, histological examination of intestinal tissue and mesenteric lymph nodes from these pigs did not reveal any signs of intestinal damage or inflammation (Chapter 2).

The increase in *Erysipelotrichaceae* in pigs fed Bt maize may have occurred as a result of the higher feed intake in these pigs (Chapter 2). We hypothesized that the higher feed intake was due to the lower enzyme resistant starch content of Bt maize, which may have reduced satiety in these pigs (Chapter 2). An increase in colonic *Erysipelotrichaceae* has recently been associated with increased dietary fat intake, body weight and fat deposition and decreased faecal short chain fatty acid (SCFA) concentrations in mice (Fleissner *et al.*, 2010). Although intestinal SCFA concentrations were not measured in the present study, Bt maize has been shown not to affect the production of volatile fatty acids in the rumen of sheep fed Bt maize for three years (Trabalza-Marinucci *et al.*, 2008). However, in the present study, no differences

were observed in relative abundance of caecal microbiota with known fibre-degrading activity which would increase SCFA concentrations. Measurement of key microbial metabolites in combination with functional metagenomic studies will enable a complete exploration of the metabolic profile of the intestinal microbiota of animals fed Bt maize.

Caecal *Bifidobacterium* and the family to which it belongs (*Bifidobacteriaceae*) were increased in the present study as a result of Bt maize consumption. However, these differences are not likely to have a detrimental effect on the host. In fact, the opposite may be true, as intestinal *Bifidobacterium* is associated with beneficial effects, at least in humans (Sekirov *et al*., 2010). The role of bifidobacteria in the porcine intestine has not yet been fully elucidated and may not be important, considering that they are not numerically dominant, as demonstrated in this and other studies (Mikkelsen *et al*., 2003; Kim *et al*., 2011).

Members of the genus *Blautia* are known to utilize hydrogen and to produce acetate in the intestine (Rey *et al*., 2010). The lower abundance of *Blautia* in the caecum of pigs fed Bt maize may also be as a result of the lower enzyme resistant starch content of the Bt maize and a potentially reduced fibre-fermenting capacity of the intestinal microbiota. This is because lower fibre fermentation may have lead to lower hydrogen concentrations in the intestine, thereby creating a less suitable environment for *Blautia* to thrive.

Overall, the biological relevance of the statistically significant but numerically small differences in abundance of certain bacterial taxa observed in the present study as a result of Bt maize consumption remains to be established, especially where prevalence of these bacterial populations is low. In any case, the differences in relative abundance of certain caecal bacterial taxa observed in the present study were not associated with any adverse health effects, as small intestinal morphology was unaffected and no histological or biochemical indications of organ dysfunction were observed in a range of samples obtained from the same animals (Chapter 2).

Previous studies in cows failed to demonstrate any effects of Bt maize silage on ruminal bacteria (Einspanier *et al*., 2004; Wiedemann *et al*., 2007). However, Wiedemann *et al.* (2007) used only four cows and studied only six ruminal species, making assessment of a community-wide effect difficult. Trabalza-Marinucci *et al.* (2008) also found no effect of Bt maize on culturable amylolytic and cellulolytic bacteria in the rumen of ewes fed Bt maize silage for 36 months. Furthermore, the ruminal microbiota differs considerably from that found in monogastrics, such as pigs and humans (Madigan *et al*., 2000).

Although the Cry1Ab protein has previously been shown to have antibacterial activity against *Clostridium* spp. *in vitro* (Yudina *et al*., 2007), the present study did not reveal any anti-clostridial effects within the porcine caecum on administration of the Cry1Ab-containing Bt maize. Similarly, another *in vitro* study found that the active form of the Cry1Ab protein had no effect on a range of Gram-positive and -negative bacterial strains (Koskella and Stotzky, 2002). However, the concentration of Cry1Ab protein detected in the caecal digesta of pigs in the present study was 2.41 ng/ml, which was 90 times lower than that in the feed (Chapter 3) and ~4000 times lower than the concentrations used by Koskella and Stotzky (2002) and Yudina *et al.* (2007) in their *in vitro* studies. This may account for the lack of an antibacterial response within the intestinal microbiota on feeding Bt maize.

Differences observed in the taxonomic distribution of caecal bacteria in pigs fed the Bt diet in the present study are believed, at least in part, to be due to nutrient differences between the Bt maize and its isogenic counterpart and are not necessarily linked to the Cry1Ab transgenic protein *per se*. Minor differences in maize composition were also found in previous studies comparing GM and non-GM maize (Aumaitre *et al*., 2002; Trabalza-Marinucci *et al*., 2008). Although nutrient differences between the Bt and isogenic maize may be a result of the *cry1Ab* gene insertion, natural variation in nutrient composition is frequently observed between non-GM maize varieties (OECD, 2002; Gajda *et al*., 2005; Hernot *et al*., 2008; García-Rosas *et al*., 2009; ILSI, 2010; Zilic *et al*., 2011; FAO, 2012). Furthermore, it has been concluded in a study by Barros *et al.* (2010) that environmental factors can affect maize composition more than the genetic modification itself. In any case, the composition of the isogenic and Bt maize used in the present study falls, for the most part, within the normal variation reported in the literature (OECD, 2002; Gajda *et al*., 2005; Hernot *et al*., 2008; García-Rosas *et al*., 2009; ILSI, 2010; Zilic *et al*., 2011; FAO, 2012).

In conclusion, 31 days of Bt maize consumption had only minimal impact on microbial community structure in the caecum of pigs, resulting in statistically significant differences in abundance of only two of 39 bacterial families and two of 54 genera detected. Nutrient differences in the Bt maize, which are most likely a result of natural variation, may account for some of the observed differences in intestinal microbiota, highlighting the plasticity of the mammalian intestinal microbiota in response to diet. However, the low abundance and frequency of detection of some taxa, as well as the lack of information on their role within the intestine, makes interpretation of some of the data difficult. Nonetheless, results from the present study indicate that

dietary Bt maize is well tolerated at the level of the intestinal microbiota following 31 days of exposure, as the differences observed are not believed to be of major biological importance and were not associated with any adverse health effects. These data can potentially be extrapolated to the human host, considering the suitability of pigs as a model for humans. However, as we enumerated a limited number of culturable bacterial groups and only investigated taxonomic distribution, additional analyses, using, for example, quantitative PCR and functional metagenomics, are needed to fully elucidate the effects of Bt maize consumption on functional capacity of intestinal bacterial populations and consequently host health.

Table 4.1. Chemical composition of maize lines included in pig diets.

	Isogenic	Bt	Normal values[1] (Reference)
DM (% of fresh weight)	88.1	87.4	85.6 - 90.6 (OECD, 2002)
% of DM			
Crude protein	8.40	8.81	6.00 -12.70 (OECD, 2002)
Fat	4.31	3.78	1.65 – 6.02 (ILSI, 2010)
			3.10 - 5.80 (OECD, 2002)
Crude fibre	2.95	2.29	0.35 – 3.24 (ILSI, 2010)
Ash	1.36	1.83	0.62 – 6.02 (ILSI, 2010)
			1.27 – 1.52 (OECD, 2002)
Starch	70.26	73.34	25.6 – 75.4 (ILSI, 2010)
			54.6 – 69.9 (Zilic *et al*., 2011)
Sugar (sucrose)	1.36	2.47	1.81 (FAO, 2012)
			2.39 – 4.25 (Zilic *et al*., 2011)
ADF	4.57	3.98	3.00 – 4.30 (OECD, 2002)
NDF	13.05	12.81	8.30 – 11.90 (OECD, 2002)
ADL	1.15	1.16	0.29 – 0.80 (Zilic *et al*., 2011)
Enzyme resistant starch	6.73	4.10	3.59 - 3.90[2] (García-Rosas *et al*., 2009)
			16.3[3] (Hernot *et al*., 2008)
			25.2[3] (Bednar *et al*., 2001)
			34.9 (Gajda *et al*., 2005)
Water soluble carbohydrate	2.42	3.62	NA
Lysine	0.36	0.35	0.20 – 0.38 (OECD, 2002)
Methionine	0.18	0.17	0.10 – 0.28 (OECD, 2002)
Cystine	0.25	0.25	0.12 – 0.27 (OECD, 2002)
Threonine	0.39	0.37	0.27 – 0.49 (OECD, 2002)
Tryptophan	0.11	0.11	0.05 – 0.12 (OECD, 2002)

DM = dry matter; ADF = acid detergent fibre; NDF = neutral detergent fibre; ADL = acid detergent lignin; NA = not available.

[1]From OECD Consensus Document on compositional considerations for new varieties of maize, International Life Sciences Institute Crop Composition Database, Food and Agriculture Organization and published studies, as indicated.

[2]Calculated at a DM content of 88%.

[3]No information about maize line is provided.

Table 4.2. Composition of experimental diets (fresh weight basis, %).

	Baseline (day -6 to -1)	Experimental (day 0 to 31)	
	Isogenic	**Isogenic**	**Bt**
Ingredient (%)			
Maize (Isogenic)	27.33	38.88	-
Maize (Bt – MON810)	-	-	38.88
Soybean meal (non-GM)	24.00	25.00	25.00
Lactofeed 70[1]	25.00	20.00	20.00
Immunopro 35[2]	12.50	9.00	9.00
Soybean oil	8.00	4.00	4.00
L-Lysine HCl	0.30	0.30	0.30
DL-Methionine	0.25	0.20	0.20
L-Threonine	0.12	0.12	0.12
L-Tryptophan	0.10	0.10	0.10
Vitamin and mineral premix[3]	0.30	0.30	0.30
Mycosorb[4]	0.20	0.20	0.20
Salt	0.30	0.30	0.30
Dicalcium Phosphate	0.50	0.50	0.50
Limestone flour	1.10	1.10	1.10
Analysed Chemical Composition (%)			
Dry matter	91.3	89.4	89.2
Crude protein	20.9	20.9	21.1
Fat	9.6	6.1	5.9
Crude fibre	1.7	2.1	1.9
Ash	6.3	5.5	5.6
Lysine	1.55[5]	1.42	1.42
Ca[5]	0.83	0.78	0.78
P[5]	0.61	0.59	0.59
DE, (MJ of DE/kg) [5]	16.33	15.38	15.38

DE = digestible energy.

[1]Lactofeed 70 contains 70% lactose, 11.5% protein, 0.5% oil, 7.5% ash and 0.5% fibre (Volac, Cambridge, UK).

[2]Immunopro 35 is a whey protein powder product containing 35% protein (Volac, Cambridge, UK).

[3]Premix provided per kg of complete diet: Cu, 155 mg; Fe, 90 mg; Mn, 47 mg; Zn, 120 mg, I, 0.6 mg; Se, 0.3 mg; vitamin A, 6000 IU; vitamin D_3, 1000 IU; vitamin E, 100 IU; vitamin K, 4 mg; vitamin B_{12}, 15 μg; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; choline chloride, 250 mg; vitamin B_1, 2 mg; vitamin B_6, 3 mg.
[4]Mycosorb is an organic mycotoxin adsorbent (Alltech, Dunboyne, Co. Meath, Ireland).
[5]Calculated values.

Table 4.3. Effect of feeding a Bt maize-based diet for 31 days on faecal and intestinal microbial counts (log_{10} CFU/g) in weanling pigs[1].

	Isogenic[2]	**Bt**[3]	**Pooled SE**[4]	***P*-value**[4]
Faeces, day -1[5]				
Enterobacteriaceae	7.97	7.77	0.093	0.26
Lactobacillus	7.94	6.98	0.236	0.06
Total anaerobes	9.37	9.48	0.091	0.48
Faeces, day 30				
Enterobacteriaceae	7.23	6.52	0.275	0.16
Lactobacillus	8.82	9.66	0.346	0.20
Total anaerobes	9.31	9.12	0.116	0.37
Ileal digesta, day 31				
Enterobacteriaceae	5.87	5.71	0.433	0.83
Lactobacillus	6.27	6.28	0.139	0.96
Total anaerobes	7.32	6.96	0.257	0.40
Caecal digesta, day 31				
Enterobacteriaceae	6.35	6.75	0.214	0.27
Lactobacillus	7.83	7.90	0.192	0.81
Total anaerobes	9.35	9.35	0.086	0.95

SE = Standard error of the mean.

[1]Mean of n = 9 pigs/treatment.

[2]Isogenic maize-based diet fed for 31 days.

[3]Bt maize-based diet fed for 31 days.

[4]*P*-value from the one-way ANOVA test.

[5]Variability present at day -1 has been accounted for by including these day -1 values as covariates in the statistical model.

Table 4.4. Estimations of bacterial diversity at 97% similarity within the caecum of weanling pigs fed isogenic and Bt maize-based diets[1].

	Isogenic[2]	Bt[3]
Chao 1 richness estimation	3904	3149
Shannon's index for diversity	6.2	6.1
Good's coverage	95%	96%

[1]Mean of n = 9 pigs/treatment.

[2]Isogenic maize-based diet fed for 31 days.

[3]Bt maize-based diet fed for 31 days.

Table 4.5. Effect of feeding a Bt maize-based diet for 31 days on the relative abundance of caecal bacterial taxa in weanling pigs[1].

Taxon	Isogenic[2] Relative abundance	Isogenic[2] 5th - 95th percentiles[4]	Bt[3] Relative abundance	Bt[3] 5th - 95th percentiles	*P*-value[5]	N[6]
Phylum						
Fusobacteria	0	0 - 0.0003	0	0 - 0.001	0.08†	1 vs 4
Tenericutes	0	0	0	0 - 0.0007	0.07†	0 vs 3
Family						
Enterococcaceae	0	0 - 0.0004	0.0006	0 - 0.0032	0.03†	1 vs 5
Erysipelotrichaceae	0.012	0.0023 - 0.0148	0.013	0.0016 - 0.0295	0.05*	9 vs 9
Bifidobacteriaceae	0	0 - 0.0004	0.0004	0 - 0.0126	0.06†	2 vs 5
Genus						
Mitsuokella	0.0006	0 - 0.0040	0.0005	0 - 0.0024	0.06*	7 vs 6
Blautia	0.0040	0.0005 - 0.0073	0.0023	0 - 0.0053	0.01*	9 vs 7
Bifidobacterium	0	0 - 0.0004	0.0004	0 - 0.0126	0.03†	1 vs 5

[1]Medians of n = 9 pigs/treatment. The individual pig was considered the experimental unit. A full outline of the relative abundance of all bacterial taxa in the porcine caecum is available in Table 4.6.

[2]Isogenic maize-based diet fed for 31 days.[3]Bt maize based-diet fed for 31 days.

[4]The 5th percentile is greater than 5% of the values and the 95th percentile is larger than 95% of the values.

[5]P-value from the one-way ANOVA test (*) or the Kruskal-Wallis non parametric test (†).

[6]Number of animals in which the bacterial taxon was present (Isogenic vs Bt).

Table 4.6. Relative abundance of bacterial taxa in the caecum of weanling pigs fed isogenic or Bt maize-based diets for 31 days[1].

	Isogenic[2]	**Bt[3]**
Phylum		
Proteobacteria	0.0407	0.0670
Bacteroidetes	0.1654	0.1071
Planctomycetes	0.0000	0.0000
Spirochaetes	0.0036	0.0085
Firmicutes	0.7081	0.7938
Verrucomicrobia	0.0000	0.0000
Lentisphaerae	0.0000	0.0000
Fusobacteria	0.0000	0.0000
Cyanobacteria	0.0003	0.0000
Deferribacteres	0.0000	0.0000
Fibrobacteres	0.0000	0.0000
Actinobacteria	0.0009	0.0007
Tenericutes	0.0000	0.0000
Chlamydiae	0.0000	0.0000
Streptophyta	0.0000	0.0000
Family		
Acetobacteraceae	0.0000	0.0000
Rhizobiaceae	0.0000	0.0000
Moraxellaceae	0.0000	0.0000
Enterobacteriaceae	0.0008	0.0022
Aeromonadaceae	0.0000	0.0000
Succinivibrionaceae	0.0180	0.0017
Pasteurellaceae	0.0062	0.0192
Desulfovibrionaceae	0.0039	0.0058
Helicobacteraceae	0.0000	0.0000
Campylobacteraceae	0.0006	0.0010
Oxalobacteraceae	0.0000	0.0000
Neisseriaceae	0.0000	0.0000

	Isogenic[2]	Bt[3]
Alcaligenaceae	0.0004	0.0006
Burkholderiaceae	0.0000	0.0000
Rhodocyclaceae	0.0000	0.0000
Prevotellaceae	0.1095	0.0733
Bacteroidaceae	0.0098	0.0130
Porphyromonadaceae	0.0000	0.0000
Spirochaetaceae	0.0036	0.0085
Leuconostocaceae	0.0000	0.0000
Planococcaceae	0.0000	0.0000
Enterococcaceae	0.0000	0.0006
Streptococcaceae	0.0009	0.0011
Lactobacillaceae	0.0095	0.0074
Lachnospiraceae	0.0062	0.0048
Veillonellaceae	0.1328	0.1460
Clostridiaceae	0.0850	0.1162
Eubacteriaceae	0.0077	0.0090
Peptococcaceae	0.0040	0.0032
Ruminococcaceae	0.0680	0.0410
Erysipelotrichaceae	0.0117	0.0128
Fusobacteriaceae	0.0000	0.0000
Fibrobacteraceae	0.0000	0.0000
Bifidobacteriaceae	0.0000	0.0004
Coriobacteriaceae	0.0006	0.0003
Verrucomicrobia subdivision 5	0.0000	0.0000
Anaeroplasmataceae	0.0000	0.0000
Chlamydiaceae	0.0000	0.0000
Poaceae	0.0000	0.0000
Genus		
Anaerobiospirillum	0.0008	0.0008
Acinetobacter	0.0000	0.0000
Succinivibrio	0.0158	0.0013

	Isogenic[2]	Bt[3]
Actinobacillus	0.0054	0.0172
Haemophilus	0.0000	0.0000
Desulfovibrio	0.0039	0.0058
Helicobacter	0.0000	0.0000
Acrobacter	0.0000	0.0000
Campylobacter	0.0006	0.0008
Azoarcus	0.0000	0.0000
Nitrosospira	0.0000	0.0000
Spirochaeta	0.0000	0.0000
Sutterella	0.0004	0.0006
Prevotella	0.0926	0.0408
Bacteroides	0.0000	0.0000
Odoribacter	0.0000	0.0000
Treponema	0.0033	0.0059
Leuconostoc	0.0000	0.0000
Weisella	0.0000	0.0000
Kurthia	0.0000	0.0000
Streptococcus	0.0007	0.0011
Enterococcus	0.0000	0.0000
Lactococcus	0.0000	0.0000
Lactobacillus	0.0095	0.0074
Acetitomaculum	0.0000	0.0000
Butyrivibrio	0.0000	0.0000
Lachnospira	0.0006	0.0000
Dorea	0.0016	0.0019
Anaerostipes	0.0000	0.0000
Selenomonas	0.0000	0.0000
Coprococcus	0.0008	0.0004
Megasphaera	0.0251	0.0124
Veillonella	0.0000	0.0000
Acidaminococcus	0.0220	0.0271

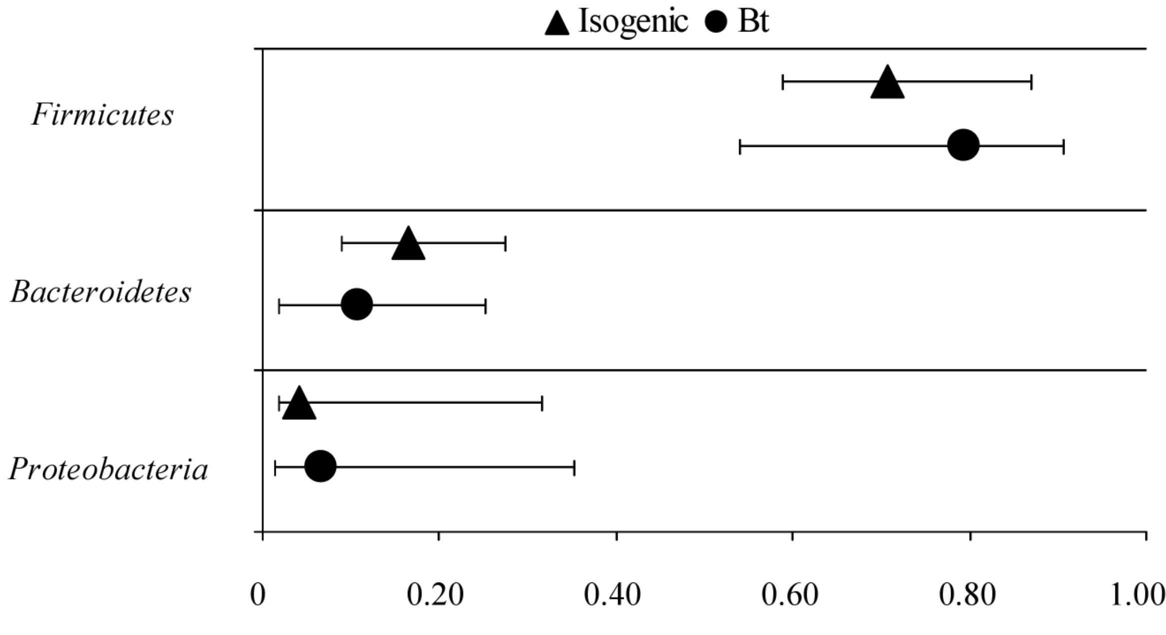
	Isogenic[2]	Bt[3]
Mitsuokella	0.0006	0.0005
Allisonella	0.0002	0.0000
Clostridium	0.0733	0.1135
Eubacterium	0.0077	0.0095
Blautia	0.0040	0.0023
Peptococcus	0.0040	0.0032
Ruminococcus	0.0091	0.0073
Anaerofilum	0.0011	0.0007
Oscillospira	0.0097	0.0042
Faecalibacterium	0.0461	0.0314
Turicibacter	0.0000	0.0004
Catenibacterium	0.0010	0.0053
Holdemania	0.0007	0.0026
Fibrobacter	0.0000	0.0000
Bifidobacterium	0.0000	0.0004
Spirulina	0.0000	0.0000
Olsenella	0.0000	0.0000
Anaeroplasma	0.0000	0.0000
Fusobacterium	0.0000	0.0000
Chlamydia	0.0000	0.0000

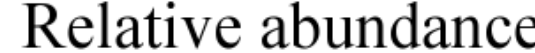
[1]Median of n = 9 pigs/treatment. Zero values correspond to taxa which were detected in a low number of samples per treatment with an abundance of < 0.001.

[2]Isogenic maize-based diet fed for 31 days.

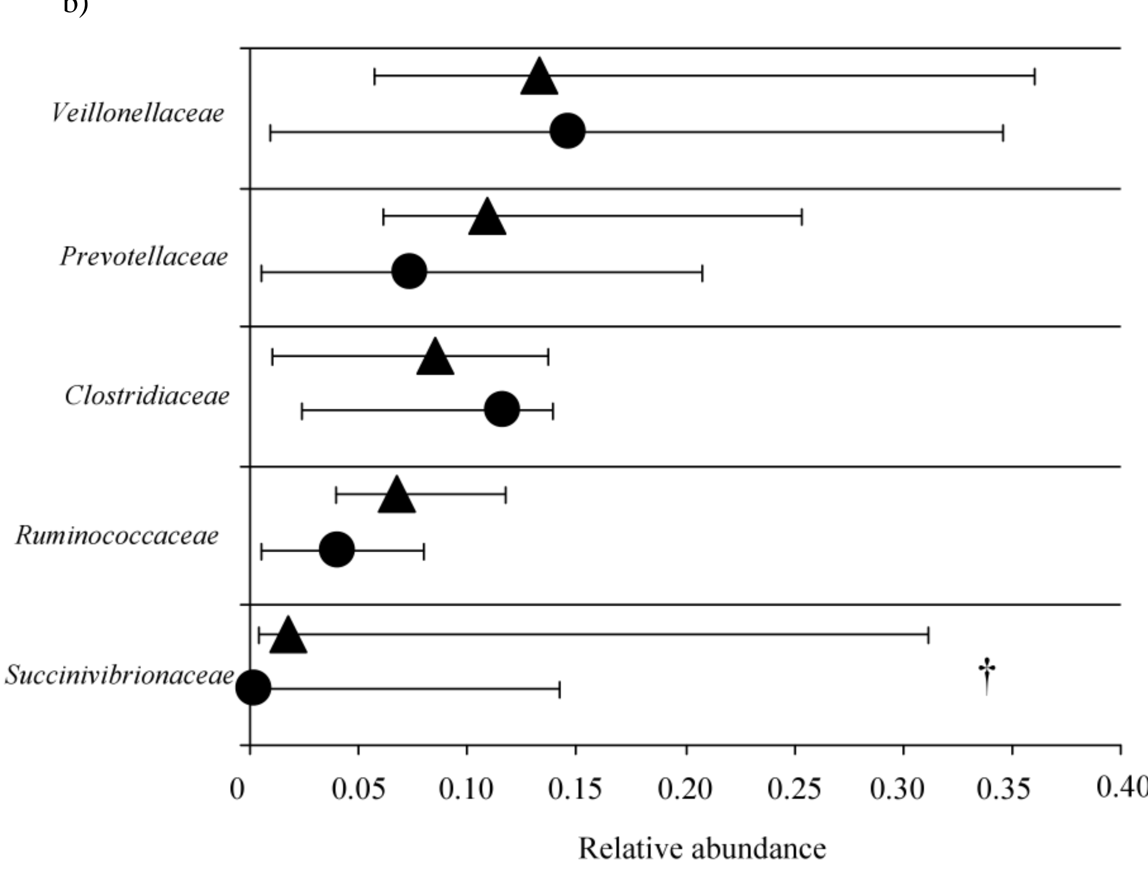
[3]Bt maize-based diet fed for 31 days.

a)





b)



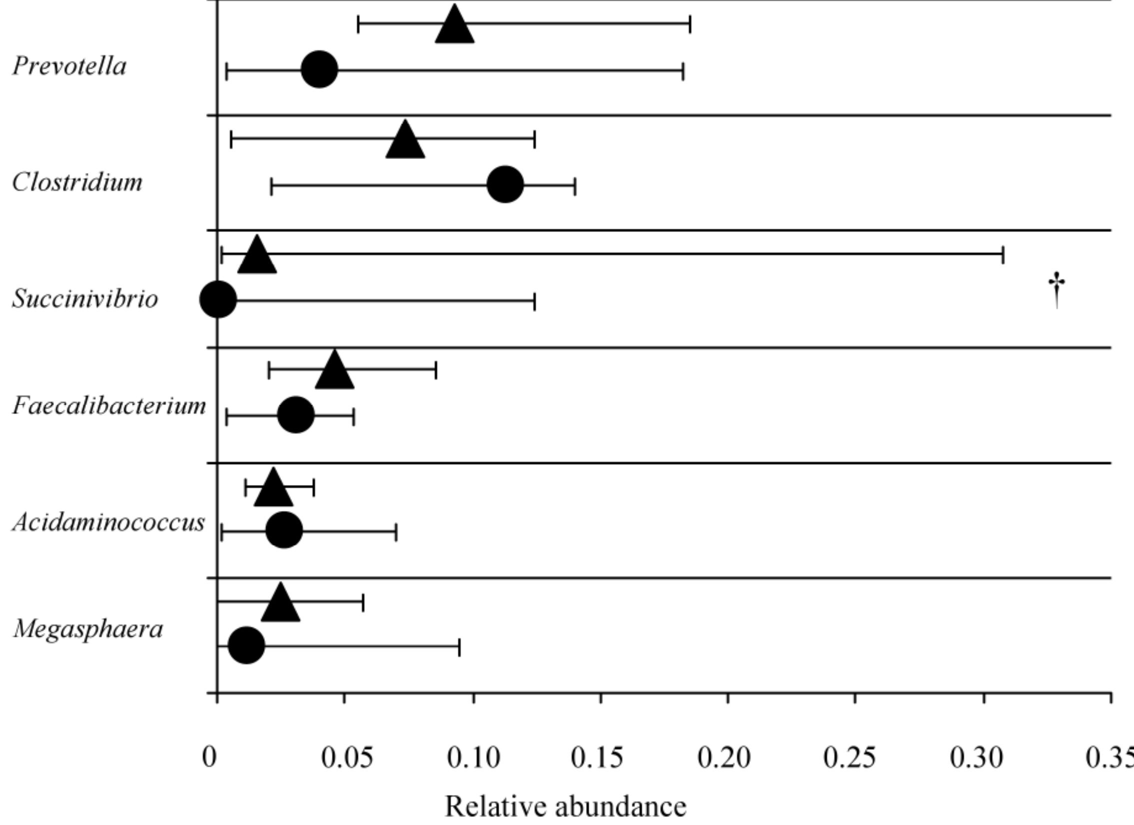
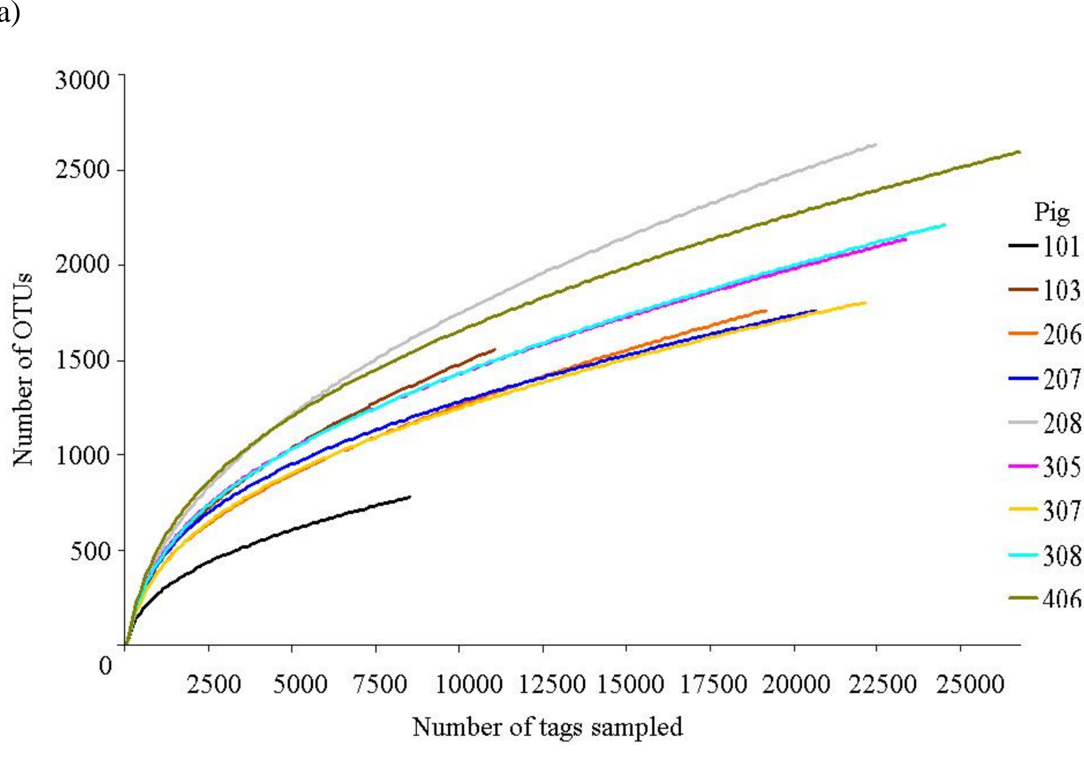


Figure 4.1. Effect of feeding a Bt or isogenic maize-based diet for 31 days on the mean relative abundance of the major phyla (a), families (b) and genera (c) in the caecum of weanling pigs. A full outline of the relative abundance of all bacterial taxa in the porcine caecum is available in Table 4.6. Data are presented as the medians from nine pigs per treatment. Whiskers on each bar represent the 5th and 95th percentiles (the 5th percentile is greater than 5% of the values and the 95th percentile is larger than 95% of the values). † $0.05 < P \leq 0.1$ computed using the Kruskal-Wallis non-parametric test.

a)



b)

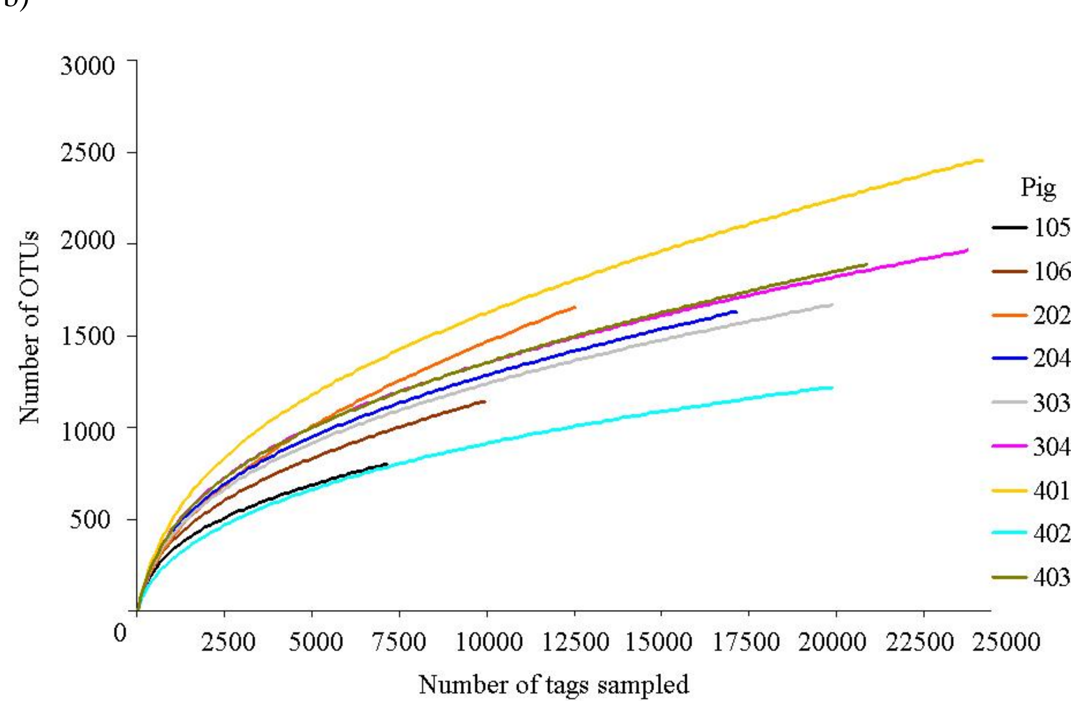


Figure 4.2. Rarefaction curves for caecal bacteria of pigs fed an isogenic maize-based diet (a) or a Bt maize-based diet (b) for 31 days.

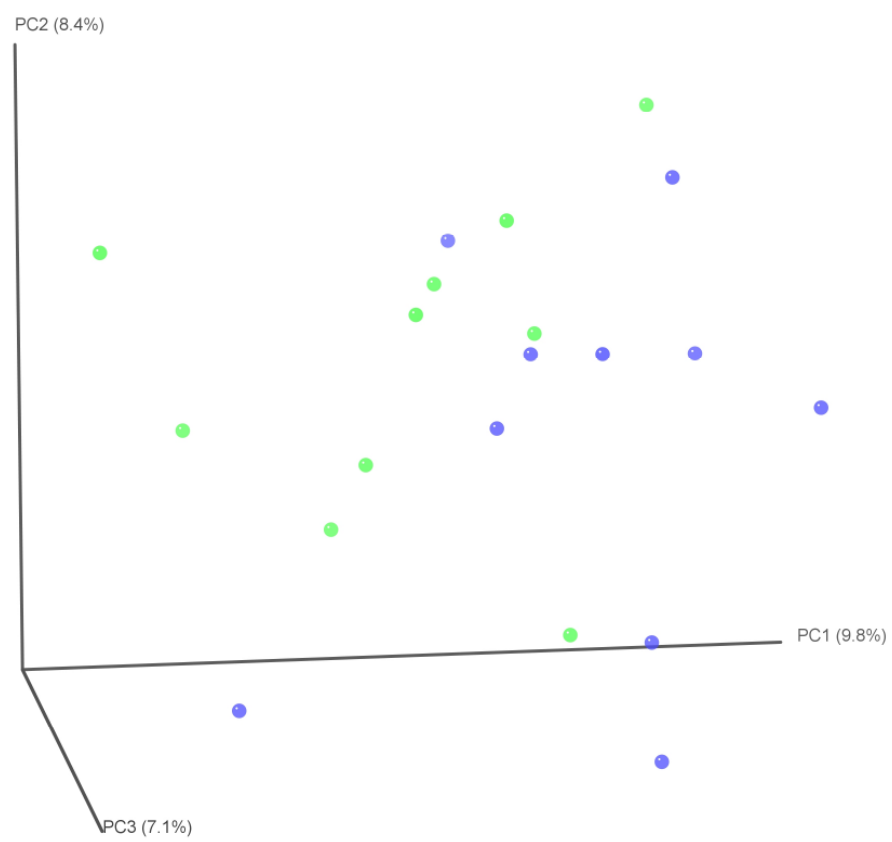


Figure 4.3. Unweighted beta diversity in the caecum of pigs fed an isogenic maize-based diet (blue) or a Bt maize-based diet (green) for 31 days.

4.6. References

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5. Effect of feeding genetically modified Bt MON810 maize to ~40 day old pigs for 110 days on growth and health indicators

Buzoianu, S. G., Walsh, M. C., Rea, M. C., Cassidy, J. P., Ross, R. P., Gardiner, G. E. and Lawlor, P. G. (2012). Effect of feeding genetically modified Bt MON810 maize to ~40 day old pigs for 110 days on growth and health indicators. Animal. DOI: 10.1017/S1751731112000249.

5.1. Abstract

Seventy-two male weaned pigs were used in a 110 day study to investigate the effect of feeding genetically modified (GM) Bt MON810 maize on selected growth and health indicators. It was hypothesized that in pigs fed Bt maize, growth and health are not impacted compared to pigs fed isogenic maize based diets. Following a 12 day basal period, pigs (10.7 ± 1.9 kg body weight; ~40 days old) were blocked by weight and ancestry and randomly assigned to treatments: 1) non-GM maize diet for 110 days (non-GM), 2) GM maize diet for 110 days (GM), 3) non-GM maize diet for 30 days followed by GM maize diet to day 110 (non-GM/GM) and 4) GM maize diet for 30 days followed by non-GM maize diet to day 110 (GM/non-GM). Body weight and daily feed intake were recorded on days 0, 30, 60 and 110 (n=15). Body composition was determined by dual energy X-ray absorptiometry (n=10) on day 80. Following slaughter on day 110, organs and intestines were weighed and sampled for histological analysis and urine was collected for biochemical analysis (n=10). Serum biochemistry analysis was performed on days 0, 30, 60, 100 and 110. Growth performance and serum biochemistry were analysed as repeated measures with time and treatment as main factors. The slice option of SAS was used to determine treatment differences at individual time points. There was no effect of feeding GM maize on overall growth, body composition, organ and intestinal weight and histology or serum biochemistry on day 60 and 100 and urine biochemistry on day 110. A treatment × time interaction was observed for serum urea (SU; $P < 0.05$), creatinine (SC; $P < 0.05$) and aspartate aminotransferase (AST; $P < 0.05$). On day 30, SU was lower for the non-GM/GM treatment compared to the non-GM, GM and GM/non-GM treatments ($P < 0.05$). On day 110, SC was higher for the non-GM/GM and GM/non-GM treatments compared to non-GM and GM treatments ($P < 0.05$). Overall, serum TP was lower for the GM/non-GM treatment compared to the non-GM/GM treatment ($P < 0.05$). The magnitude of change observed in some serum biochemical parameters did not indicate organ dysfunction and the changes were not accompanied by histological lesions. Long-term feeding of GM maize to pigs did not adversely affect growth or the selected health indicators investigated.

Implications

The presence of genetically modified (GM) ingredients in the human food chain has created consumer concerns regarding negative effects following consumption (Dona and Arvanitoyannis, 2009; Martinez-Poveda *et al*., 2009). The absence of negative effects on growth and health indicators in pigs fed GM maize for 110 days in the present study indicates that Bt maize is well tolerated by the animal during an exposure of 110 days. The results of the present study provide additional safety assessment data on Bt maize for consumers, producers and policy makers.

5.2. Introduction

An 87-fold increase in the adoption of genetically modified (GM) plants worldwide since their introduction in 1996 makes it the fastest technology adopted in agriculture in recent years (James, 2010). Insect resistant GM varieties make up 24.6% of the maize grown globally (James, 2010). Therefore, maize-based food and feed that are GM free have become difficult to source. The Bt MON810 maize expresses the bacterial *cry1Ab* gene and produces as a result a truncated Cry1Ab protein conferring resistance to a range of *Lepidoptera* insects (Schnepf *et al*., 1998). Mammals lack the intestinal receptors for the Cry1Ab protein (Schnepf *et al*., 1998) and are not believed to be affected by this toxin.

Consumer concerns regarding GM products relate mostly to unanticipated health effects that may arise from direct consumption of GM products or products from animals fed GM ingredients (Malarkey, 2003; Dona and Arvanitoyannis, 2009; Martinez-Poveda *et al*., 2009). Before market release in the European Union, GM products are subjected to comprehensive short-term risk assessment which cannot predict the consequences arising from long-term consumption by a genetically diverse population (Wal *et al*., 2003).

Several studies have found no detrimental effects of feeding GM maize on the growth of pigs (Reuter *et al*., 2002; Chowdhury *et al*., 2003; Custodio *et al*., 2006; Flachowsky *et al*., 2007), poultry (Brake *et al*., 2003; Aeschbacher *et al*., 2005) or ruminants (Aumaitre *et al*., 2002; Trabalza-Marinucci *et al*., 2008). Higher feed intake and a tendency for higher kidney weight were observed in weanling pigs after 30 days of feeding GM maize (Chapter 2). These findings highlight the need for longer-term studies investigating the effect of GM maize on pig health. Minor changes in liver and kidney histology and serum biochemical parameters have been reported in rats (Kilic and Akay, 2008) and mice (Finamore *et al*.,

2008) in response to feeding GM maize. To our knowledge, no studies to date have investigated effects of GM maize on organ and intestinal health indicators in pigs throughout their entire productive life. Pigs are an excellent model for humans when considering similarities in digestive, liver and kidney anatomy and physiology (Bollen *et al*., 2000; Lewis and Southern, 2001; Swindle, 2007). Therefore, as well as providing additional insight into possible physiological alterations in response to feeding GM maize to pigs, pig-feeding studies may also provide data relevant to human nutrition.

The hypothesis of the present study was that there would be no differences in growth performance and health between pigs fed Bt maize or isogenic maize diets. The objective of this study was to determine the effect of feeding GM maize to pigs from 12 days post-weaning (day 0) to slaughter (day 110) on health as assessed through measurement of growth rate, body composition, organ and intestinal weights and histology and clinical biochemistry. By alternating between GM and non-GM maize and vice versa after 30 days of feeding, the study was also designed to investigate any age-specific effect of feeding GM maize.

5.3. Materials and methods

The pig study complied with European Union Council Directives 91/630/EEC (outlines minimum standards for the protection of pigs) and 98/58/EC (concerns the protection of animals kept for farming purposes) and was approved by, and a license obtained from the Irish Department of Health and Children. Ethical approval was obtained from Teagasc and Waterford Institute of Technology ethics committees.

Animals and experimental design

Seventy-two crossbred (Large White × Landrace) entire male pigs were weaned at ~28 days of age and were provided *ad libitum* access to a non-GM starter diet during a 12 day basal period. On day 0, pigs were blocked by weight and litter ancestry and within block randomly assigned to one of four treatments (n=18 pigs/treatment) where day 0 was the first day experimental diets were fed. Dietary treatments were as follows; 1) non-GM maize-based diet (isogenic parent line; Pioneer PR34N43) fed to day 110 (non-GM); 2) GM maize-based diet (Bt; Pioneer PR34N44 event MON810) fed to day 110 (GM); 3) Non-GM maize-based diet fed for 30 days followed by GM maize fed to day 110 (non-

GM/GM); and 4) GM maize-based diet fed for 30 days followed by non-GM maize-based diet fed to day 110 (GM/non-GM). The duration of the study was 110 days.

Housing and management

From weaning to day 60 of the study, pigs were penned individually in one of three similar rooms each containing 24 pens. The pens were fully slatted (1.2 m × 0.9 m) with plastic slats (Faroex, Manitoba, Canada) and plastic dividers between pens. Temperature was controlled by a hot air heating system and an exhaust fan drawing air from under slat level, both connected to a Stienen PCS 8400 controller (Stienen BV, Nederweert, The Netherlands). The temperature was maintained at 28ºC in the first week post-weaning after which it was reduced by 2ºC per week to 22ºC.

On day 60 of the study, pigs were transferred to one of four similar finisher rooms containing 18 individual pens per room and remained there until day 110 of the study. Pens (1.81 m × 1.18 m) were fully slatted with plastic panelled partitions. Ventilation was by exhaust fans and air inlets connected to a Stienen PCS 8200 controller (Stienen BV). Temperature was maintained at 20 to 22ºC.

During the study, water was available *ad libitum* from one nipple-in-bowl drinker (BALP, Charleville-Mezieres, Cedex, France) per pen. Feed was available *ad libitum* from a single stainless steel 30 cm wide feeder per pen (O'Donovan Engineering, Coachford, Co. Cork, Ireland). Throughout the study, the dietary treatments were equally represented in each room to avoid additional variation due to environmental conditions. Pigs showing signs of ill health were treated as appropriate and all veterinary treatments were recorded.

Maize and diets

Seeds derived from GM Bt MON810 and non-GM parent line control maize (PR34N44 and PR34N43, respectively; Pioneer Hi-Bred, Sevilla, Spain) were grown simultaneously side by side in 2007 in Valtierra, Navarra, Spain by independent tillage farmers. The GM and non-GM control maize were purchased by the authors from the tillage farmers for use in this animal study. Samples from the GM and non-GM maize were tested for chemical, amino acid and carbohydrate composition as well as for presence of the *cry1Ab* gene, pesticide contaminants and mycotoxins as previously described in Chapter 2.

All diets were manufactured and analysed for chemical composition and amino acid concentration as previously described in Chapter 2. Sampling of the diets was conducted in

accordance with international guidelines (ILSI, 2007). All diets were formulated to meet or exceed the National Research Council requirements for pigs of given weights (NRC, 1998). To avoid any animal health parameter perturbations due to undetected mycotoxins in the feed, an organic mycotoxin adsorbent (Mycosorb®, Alltech, Dunboyne, Co. Meath, Ireland) was included in all diets used in the present study. Both non-GM and GM maize were offered in link diets fed from day 0 to 30, weaner diets fed from day 31 to 60, finisher 1 diets fed from day 61 to 100 and finisher 2 diets fed from day 101 to 110. Pellet hardness and durability were determined as described by Lawlor *et al.* (2003).

Growth, carcass characteristics and body composition

Body weight (BW) and daily feed intake were recorded on days 0, 30 and 60 and at the end of the study prior to slaughter (day 110) for the determination of growth performance (n=15/treatment). Feed conversion efficiency (FCE) was calculated as average daily feed intake (ADFI) divided by the average daily gain (ADG). Hot carcass weight was recorded immediately following slaughter and evisceration on day 110 and values were multiplied by 0.98 to obtain cold carcass weight (n=15/treatment). Dressing out percentage was calculated by expressing carcass weight as a fraction of live weight at slaughter. Body composition of anaesthetised pigs (n=10/treatment) was determined on day 80 of the study using dual energy X-ray absorptiometry (DXA) technology as described by Ryan *et al.* (2011).

Organ and intestinal sampling and histological analysis

On day 110, pigs were sacrificed by electrical stunning followed by exsanguination. The heart, kidneys, spleen and liver were removed, trimmed of any superficial fat or blood, blotted dry and weighed (n=10/treatment). Samples were then taken from the liver (centre of quadrate lobe), kidney (cortex and medulla), spleen (anterior end), heart (left ventricle wall), mesenteric lymph nodes (close to ileo-caecal junction) and *semitendinosus* muscle (n=10/treatment). Histological examination of these tissue samples was performed as outlined in Chapter 2. The stomach and small intestine were removed, emptied of contents, flushed with water, cleared of fat and connective tissue, blotted dry and weighed (n=10/treatment). Intestinal tissue sampling and determination of villus height, width, crypt depth, villus height/crypt depth ratio and number of goblet cells per villus and per µm

villus in the duodenum, jejunum and ileum (n=10/treatment) was performed as described in Chapter 2.

Blood and urine sampling and analysis

Blood samples were collected from 10 pigs/treatment on days 0, 30, 60, 100 and 110 for serum biochemistry analysis. Blood samples were collected from the anterior vena cava of pigs on days 0 and 30, from the external jugular vein on days 60 and 100 and during exsanguination on day 110. Blood samples for serum biochemistry analysis were collected in serum collection tubes (BD Vacutainer Systems, Franklin Lakes, NJ, USA) and allowed to clot at room temperature for 2 to 3 h prior to centrifugation (2500 × g for 20 minutes). Serum was collected and stored at -20ºC for subsequent biochemical analysis. Urine was collected in a sterile manner at slaughter by puncturing the bladder and stored at -20ºC for the analysis of creatinine and protein.

Serum samples were analysed using an ABX Pentra 400 clinical chemistry analyser (Horiba ABX, Northampton, UK) for aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), alkaline phosphatase (ALP), creatinine, urea and total protein (TP). Urinary protein and creatinine concentrations were also determined using an ABX Pentra 400 clinical chemistry analyser according to manufacturer's instructions. For both serum and urine, the biochemistry analyser was calibrated according to manufacturer's instructions. Analyser accuracy was maintained by analysis of samples of known concentration and all serum and urine samples were analysed in duplicate.

Urine collected on day 110 was tested using dipsticks (Omega Diagnostics Ltd., Wicklow, Ireland) in accordance with manufacturer's instructions. Dipstick analysis was performed for determination of glucose, bilirubin, ketones, specific gravity, blood, pH, protein, urobilinogen, nitrite and leukocytes.

Statistical analysis

All data with the exception of serum biochemistry and growth performance data (BW, ADG, ADFI and FCE) were analysed as a complete randomised block design using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). Organ weights were also analysed as a one-factor analysis of variance using the GLM procedure of SAS using body weight on day 110 as a covariate in the model. Treatment effect was tested against residual error

terms with initial body weight and litter ancestry as blocking factors. Duncan's multiple range test was used for means separation. Serum biochemistry and growth performance data were analysed as repeated measures using the MIXED procedure of SAS with sampling day as the repeated variable. Simple effects were obtained using the slice option in SAS to determine differences between treatments at individual time points. Means separation was performed using the Tukey-Kramer adjustment for multiple comparisons. Fixed effects included treatment and sampling day while block was included as a random effect in the model. For serum biochemistry day 0 values were used as a covariate in the model while for growth performance parameters weight on day 0 was used as a covariate in the statistical model. Urine dipstick analysis data were analysed using the Kruskal-Wallis non-parametric test within the NPAR1WAY procedure of SAS. For all tests, the level of significance was $P < 0.05$ and tendencies were reported for $0.05 < P < 0.10$. For all statistical analysis, the individual pig served as the experimental unit.

5.4. Results

Analysis of non-GM and GM diets

Similar chemical and amino acid composition were observed for the non-GM and the GM diets (Table 5.1).

Effects of feeding GM maize on health, growth performance, carcass characteristics and body composition

During the first 14 days of the study, 10 pigs died from oedema disease-like symptoms (three from the non-GM treatment, one from the GM treatment, three from the non-GM/GM treatment and three from the GM/non-GM treatment). As a preventive measure injectable Enrofloxacin (2.5 mg/kg body weight) was administered to all pigs between days 9 and 11 of the study. Between days 70 and 80 of the study, six pigs (two from the non-GM treatment, three from the GM treatment and one from the GM/non-GM treatment) were injected with Enrofloxacin (2.5 mg/kg body weight) for three days to treat clinical symptoms of diarrhoea. Following treatment all pigs made a complete recovery.

Growth performance of pigs was investigated over three time periods (day 0 to 30, 31 to 60 and 61 to 110) and overall (Table 5.2). No treatment × time interaction was observed

for any of the growth performance parameters (data not shown). There was no effect of feeding GM maize to pigs on ADFI, FCE or BW at any time point during the study. There was a tendency for ADG to be higher for pigs fed the non-GM/GM treatment compared to all other treatments between days 31 and 60 (P = 0.06; Table 5.2). Overall, ADG, ADFI, FCE and BW were not affected by feeding GM maize to pigs for 110 days from 12 days post-weaning to slaughter.

On day 80, pigs fed the non-GM, GM, non-GM/GM and GM/non-GM treatments had similar fat percentages (11.9, 13.2, 13.0 and 12.0%, respectively; s.e. = 0.54), bone mineral content (1.05, 1.14, 1.18 and 1.18 kg, respectively; s.e. = 0.046) and area bone mineral density (0.93, 0.95, 0.96 and 0.93 g/cm^2, respectively; s.e. = 0.016).

Effects of feeding GM maize on organ and intestinal weights and histology

The weight of the heart, kidneys, spleen and liver were not affected by feeding GM maize (Table 5.2). Likewise, treatment did not significantly affect empty weight of the stomach, small intestine, carcass weight or dressing out percentage (Table 5.2).

Histological examination of the heart, kidney, spleen, liver, *semitendinosus* muscle and mesenteric lymph nodes did not indicate an effect of feeding GM maize (data not shown). None of the tissues presented any signs of haemorrhage or alteration of blood vessel integrity, oedema or fibrosis. Tissue hyperplasia and cell pigmentation were not present in any of the tissues examined. Subtle inflammation was observed in two of the samples (one heart from the GM treatment and one lymph node from GM/non-GM treatment) but was not present in any other samples examined. At the cellular level, no signs of nuclear or cytoplasmic fragmentation or shrinkage were seen. No pathological cytoplasmic inclusions or any signs of extensive cell apoptosis, degeneration or necrosis were observed in any of the tissues examined.

There was no effect of feeding GM maize to pigs on villus height or width, crypt depth, goblet cell number/villus and per µm of villus or villus height/crypt depth ratio measured in the duodenum, jejunum or ileum on day 110 (Table 5.3).

Effects of feeding GM maize on serum and urine biochemistry

There was no effect of feeding GM maize to pigs on serum concentrations of the enzymes ALT, ALP and GGT measured throughout the study (Table 5.4). There was an effect of time on serum ALT which increased up to day 60, decreased up to day 100 and

increased again up to day 110 ($P < 0.05$). A time effect was also observed for serum ALP which decreased up to day 100 and increased to day 110 ($P < 0.01$). Serum GGT increased up to day 60 and decreased thereafter ($P < 0.001$).

There was a treatment × time interaction for serum AST (Figure 5.1; $P < 0.05$). On day 110, pigs fed the GM treatment tended ($P = 0.06$) to have a lower serum AST concentration than pigs fed the non-GM treatment but were not different to all other treatments. Likewise, a time effect was observed for serum AST which increased up to day 60, decreased up to day 100 and increased thereafter ($P < 0.05$).

There was also a tendency for a treatment × time interaction for serum creatinine (Figure 5.2; $P = 0.10$). On day 110, pigs fed the non-GM/GM and GM/non-GM treatments had higher serum creatinine concentration than the pigs fed the non-GM and GM treatments ($P < 0.05$). A time effect was also observed for creatinine which increased throughout the study ($P < 0.05$).

There was a treatment effect for serum TP concentration (Figure 5.3; $P < 0.05$). Pigs fed the GM/non-GM treatment had an overall lower serum TP concentration compared to pigs fed the non-GM/GM treatment, however, the only time when serum TP was different between treatments was on day 110. On day 110 serum TP was lower in the serum of pigs fed the GM/non-GM diets compared with all other treatments. Serum TP also increased over time ($P < 0.05$).

Furthermore, there was a treatment × time interaction for serum urea (Figure 5.4; $P < 0.05$). On day 30, pigs fed the non-GM/GM treatment had lower serum urea than pigs on all other treatments.

On day 110, the non-GM, GM, non-GM/GM and GM/non-GM treatments showed similar urinary concentrations of protein (5.78, 6.23, 4.79 and 5.24 g/L respectively; s.e. = 1.125), creatinine (1.97, 2.14, 2.09 and 2.08 g/L respectively; s.e. = 0.352) and protein/creatinine (3.47, 3.61, 2.75 and 3.52 respectively; s.e. = 0.810).

Effects of feeding GM maize on urine dipstick analysis

Leukocytes, nitrite, urobilinogen, bilirubin or glucose were at or below the limit of detection of the urine dipstick analysis test for all pigs. There was no treatment effect on urinary protein dipstick analysis. The mode from each treatment fell within the range 0.31-1 g/L with a 95% confidence interval (CI) of 0-3 g/L. Likewise, no treatment effect was observed for urinary pH for which modes for all treatments fell within 6 and 6.5 with a 95%

CI of 6.5-8. Presence of red blood cells in urine did not differ between treatments, with modes for all treatments at the extremes of the test range (5-10 and 80-200 cells/µL), with a 95% CI of 5-200. Specific gravity was not different between treatments either, with modes for the non-GM, GM and GM/non-GM treatment being between 1.020 and 1.025 and for the non-GM/GM treatment being between 1.026 and 1.030, with a 95% CI of 1.010 to 1.030. There was no effect of treatment on urinary ketones. Ketones (5 mg/dL) were detected in the urine of a total of five pigs sampled (two from the non-GM treatment, two from the GM treatment and one from the non-GM/GM treatment). All remaining pigs sampled tested negative for urinary ketones.

5.5. Discussion

To date, several studies have investigated changes in the growth rate and carcass quality of GM maize-fed pigs (Reuter *et al*., 2002; Hyun *et al*., 2005; Custodio *et al*., 2006). This is the first pig study to date conducted to investigate the effects of feeding GM MON810 maize on growth performance and health over the entire growing and finishing period. This not only provides data for animal production but also for human health by using the pig as a model for human exposure. This is also the first study that investigates the growth and health implications of alternating between non-GM and GM maize varieties in pigs to investigate a potential age-specific GM maize effect.

Results from the current study demonstrated that long-term (110 days) feeding of GM maize to pigs had no adverse effects on growth performance and carcass characteristics. In agreement with these findings, previous work by our group also found that short-term (31 day) feeding of GM maize to weanling pigs had no adverse effects on growth (Chapter 2). Numerous studies in pigs have documented inconsistent effects on growth rates and feed intake in response to feeding GM maize (Reuter *et al*., 2002; Hyun *et al*., 2005; Custodio *et al*., 2006). We found a transient increase in ADG between day 31 and 60 in pigs fed the non-GM/GM treatment compared to all other treatments, however, this difference was only a tendency and it did not persist. The GM maize used in this study was previously reported to have a lower enzyme resistant starch content than the non-GM maize (Chapter 2). The enzyme resistant starch content of food is known to influence satiety (Willis *et al*., 2009). Changing from the non-GM maize to the GM maize diet on day 30 in the current study may have resulted in a lack of satiety in pigs fed the non-GM/GM treatment. Therefore, the lower resistant starch content of the GM maize may

account for the transient numerical increase in ADFI and the consequent tendency towards increased ADG observed in the non-GM/GM treatment compared to all other treatments. This higher concentration in enzyme resistant starch in the isogenic diet may have primed the animals to consume more of the GM feed which was lower in enzyme resistant starch. Feeding GM maize to pigs has been shown to increase feed intake in pigs from 17 to 120 kg (Custodio *et al*., 2006) and to increase daily gain in weanling pigs (Piva *et al*., 2001). Piva *et al.* (2001), however, concluded that differences were due to a higher mycotoxin concentration in the non-GM maize and Custodio *et al.* (2006) did not investigate mycotoxin contamination of the maize varieties used. It has been shown in Chapter 2 that mycotoxin concentrations for both maize lines used in the present study were below maximum allowable limits outlined in European legislation (2006/576/EC; EU/165/2010/). Similar to the findings of Reuter *et al.* (2002), we found that feeding GM maize to pigs had no effect on carcass weight and dressing out percentage. We also found that fat content of the body was unaffected by feeding GM maize to pigs. Similarly, Reuter *et al.* (2002) found no difference in the amount of leaf fat surrounding the kidneys of pigs fed GM maize compared to controls. Bone health of pigs in the current study was unaffected by feeding GM maize and to our knowledge this has not been investigated previously.

In agreement with previous findings in weanling pigs (Chapter 2), histology of the small intestine was not affected by feeding GM maize to pigs. Furthermore, there was no change in the weight of the stomach or small intestine as also shown by others in rats (Kilic and Akay, 2008) and salmon (Sanden *et al*., 2005) fed GM maize. Fares and El-Sayed (1998) described evidence of histological alterations in the ileum of mice fed potatoes treated with purified *Bacillus thuringiensis* δ-endotoxin, however, feeding GM potatoes expressing the same δ-endotoxin failed to elicit a similar response. As the pig and human digestive tract share numerous similarities (Moughan *et al*., 1992; Lewis and Southern, 2001), a lack of an adverse effect should be expected in humans following GM maize consumption.

No organ dysfunction was identified in the present study in response to feeding GM maize. Organ weights were similar between treatments and no abnormalities were observed following histopathological examination. Previous work from our group reported a tendency for higher kidney weights in weanling pigs following 30 days of GM maize feeding (Chapter 2), difference which was not associated with histopathological or blood biochemical changes. A three generation (Kilic and Akay, 2008) and a 90-day (Hammond

et al., 2006) rat study as well as an eight month study in salmon (Sanden *et al*., 2005) documented no differences in organ weights following GM maize consumption. Likewise, pigs fed GM maize during a 91 day growing period also showed no alterations in organ weight (Reuter *et al*., 2002).

We found that the liver enzyme AST tended to be lower in pigs fed the GM treatment compared to the non-GM treatment following 110 days of exposure. Previously, we reported no change in serum liver enzymes in pigs fed GM maize for 31 days (Chapter 2). A study by Kilic and Akay (2008) found no changes in serum enzymes in rats fed GM maize but focal infiltration, congestion and degeneration of the liver was reported. Elevated serum GGT and changes in nuclei structure were observed in the liver of lambs fed GM maize (Trabalza-Marinucci *et al*., 2008). Unlike our study, these studies did not fully investigate the potential for contamination of the GM maize, as it has been shown that contaminants such as mycotoxins are known to affect liver histology and liver health indicator enzymes (Casteel *et al*., 1993). In any case, further work is needed to investigate why these effects were observed in these previous studies. In the current study, we found no changes in serum GGT, ALT and ALP. The change in serum AST found in GM maize-fed pigs was not correlated with a change in liver weight or evidence of histopathology. Liver toxicity is characterized by a 10-70 fold increase in AST and a 5-10 fold concomitant increase of ALP and GGT (Kaneko, 1980; Casteel *et al*., 1993), none of which were evident in this study. In addition, the observed differences in AST were minor, were not correlated with histopathology and the values were all within the normal reference intervals for pigs of this age (Kaneko, 1980). Although in the present study significant differences between treatments did not reach values indicating toxicity, further investigation is necessary.

Serum urea concentrations were lower in pigs fed the non-GM/GM treatment on day 30. Up to this point of the study, these pigs had only been fed non-GM maize similar to pigs fed the non-GM treatment. Urea is a nitrogen source that is readily used by gastrointestinal microbiota to sustain periods of prolific growth when supplies of fermentable carbohydrates are abundant (Younes *et al*., 1995a). This increased microbial demand for urea has been shown to be satisfied through urea diffusion from the blood into the caecum and colon (Younes *et al*., 1995a, 1995b), thereby lowering serum urea concentrations. The non-GM maize used in this study was higher in enzyme resistant starch than its GM counterpart (Chapter 2). Therefore, we hypothesise that there was more

hindgut microbial fermentation in the non-GM maize-fed pigs and more urea therefore diffused from the blood into the hindgut, lowering serum urea concentrations. High dietary fibre content has previously been shown to decrease serum urea in rats (Younes *et al.,* 1995a, 1995b) and pigs (Mosenthin *et al*., 1992a, 1992b; van der Meulen *et al*., 1997). In contrast to the current findings, previous work from our group found no effect of feeding GM maize on weanling pig serum urea (Chapter 2). Pigs fed the non-GM treatment in the current study had similar serum urea concentrations to the GM and GM/non-GM treatments, although numerically lower. The change in serum urea documented in this study was transient and isolated to the day 30 sampling time point which questions the biological relevance of this change.

Feeding pigs the GM/non-GM treatment resulted in lower serum TP while serum creatinine increased in pigs fed the non-GM/GM and GM/non-GM treatments following 110 days of feeding. Kidney dysfunction is characterised by elevated serum urea and creatinine, low serum TP (Baum *et al*., 1975; Kaneko, 1980; Stonard, 1990) and increased urinary protein to creatinine ratio (P/C) (Baum *et al*., 1975). On day 110 of the present study, serum urea, urinary P/C and urine dipstick analysis revealed no differences between treatments. Although urinary dipstick analysis can only provide a gross estimate of kidney function, it is a helpful tool which may indicate differences that can be further investigated by more accurate biochemical methods. The absence of kidney histopathology and the similar kidney weights observed in the current study further support the lack of an adverse effect on pig kidney function arising from GM maize exposure. Elevated serum creatinine may also be indicative of extensive muscle catabolism (Kaneko, 1980) which was not observed by histopathological examination in the current study and no treatment effect was observed for carcass weight or body composition. Numerous studies have reported a lack of an effect of GM maize on blood biochemistry in weanling pigs (Chapter 2), calves (Shimada *et al*., 2006) and rats (Hammond *et al*., 2006) or on serum TP in sheep (Trabalza-Marinucci *et al*., 2008). A decrease in serum TP was described in rats fed GM maize for three generations (Kilic and Akay, 2008) and in rats fed GM rice (Schrøder *et al*., 2007). Killic and Akay (2008) found that serum TP of rats fed GM maize was lower only compared to rats fed non-GM maize but was similar to that of rats fed a commercial control diet. In these studies, however, mycotoxin contamination was not investigated and it has been shown that mycotoxins could lead to liver damage and thus reduced serum TP (Casteel *et al*., 1993). Similar to findings from the present study, the lower serum TP

observed by Schrøder *et al.* (2007) in rats did not occur in conjunction with histological or organ weight changes, thus questioning the biological relevance of this alteration.

Due to the complexity of organ dysfunction, a change in a single biochemical health indicator cannot be used alone to characterize pathology. Only the development of a specific pattern of biochemical changes should be used to identify the manifestation of organ dysfunction (Boone *et al*., 2005). Therefore, any conclusion regarding the development of an adverse health effect should be reached following the correlation of serum biochemistry with organ weight and/or histological evidence (Stonard, 1990; EFSA, 2008).

In conclusion, feeding GM maize to pigs from 12 days post-weaning to slaughter did not adversely affect growth, carcass characteristics, bone health or body composition. Although some changes in serum biochemistry were observed, values were all within the normal reference intervals for pigs (Kaneko, 1980; Radostits *et al*., 2007), did not conform to a pattern indicative of organ dysfunction and were not correlated with differences in organ weight or histopathology. Histological examination indicated the absence of an adverse effect of GM maize at the main site of nutrient digestion and absorption, the small intestine. This study indicates that GM Bt MON810 maize is safe as an ingredient in swine diets. Finally, there is little evidence to suggest that adverse health effects should be expected in humans following GM maize consumption. Further work is needed to assess the effects of feeding GM maize to pigs over multiple generations.

Table 5.1. Composition of experimental diets (fresh weight basis, %).

	Starter	Link		Weaner		Finisher 1		Finisher 2	
	(d -12 – 0)	(d 0 – 30)		(d 31 – 60)		(d 61 -100)		(d 101 – 110)	
Ingredient	non-GM[1]	Non-GM[1]	GM[2]	non-GM[1]	GM[2]	non-GM[1]	GM[2]	non-GM[1]	GM[2]
non-GM maize[1]	27.33	38.88	-	65.31	-	73.38	-	79.10	-
GM maize[2]	-	-	38.88	-	65.31	-	73.38	-	79.10
Soybean meal (non-GM)	24.00	25.00	25.00	28.64	28.64	22.76	22.76	17.35	17.35
Lactofeed 70[3]	25.00	20.00	20.00	-	-	-	-	-	-
Immunopro 35[4]	12.50	9.00	9.00	-	-	-	-	-	-
Soybean oil	8.00	4.00	4.00	2.37	2.37	0.06	0.06	-	-
L-Lysine HCl	0.30	0.30	0.30	0.36	0.36	0.43	0.43	0.49	0.49
DL-Methionine	0.25	0.20	0.20	0.14	0.14	0.14	0.14	0.14	0.14
L-Threonine	0.12	0.12	0.12	0.15	0.15	0.17	0.17	0.19	0.19
L-Tryptophan	0.10	0.10	0.10	0.05	0.05	0.07	0.07	0.08	0.08
Vitamin and mineral premix	0.30[5]	0.30[5]	0.30[5]	0.10[6]	0.10[6]	0.10[6]	0.10[6]	0.10[6]	0.10[6]
Formaxol[7]	0.20	0.20	0.20	-	-	-	-	-	-
Mycosorb[8]	-	-	-	0.20	0.20	0.20	0.20	0.20	0.20
Salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Dicalcium phosphate	0.50	0.50	0.50	1.19	1.19	1.13	1.13	0.90	0.90
Limestone flour	1.10	1.10	1.10	1.19	1.19	1.26	1.26	1.15	1.15

	Starter	Link		Weaner		Finisher 1		Finisher 2	
	(d -12 – 0)	(d 0 – 30)		(d 31 – 60)		(d 61 -100)		(d 101 – 110)	
Ingredient	**non-GM[1]**	**Non-GM[1]**	**GM[2]**	**non-GM[1]**	**GM[2]**	**non-GM[1]**	**GM[2]**	**non-GM[1]**	**GM[2]**
Analysed Chemical Composition (%)									
Dry matter	91.30	90.40	90.50	88.60	88.80	89.30	89.50	89.20	88.80
Crude protein	20.90	21.00	20.70	17.90	17.80	17.40	17.40	16.00	16.10
Oil (Acid hydrolysis)	9.60	6.20	6.30	5.20	5.40	3.20	3.10	3.20	3.10
Crude fibre	1.70	1.80	1.60	2.10	2.20	3.00	2.40	2.60	2.60
Ash	6.30	5.60	5.80	4.90	4.80	4.80	4.60	4.00	4.10
Lysine	1.55[i]	1.50	1.56	1.29	1.31	1.36	1.37	1.15	1.16
Ca[9]	8.30	7.80	7.80	8.00	8.00	8.00	8.00	7.00	7.00
P[9]	4.08	3.63	3.63	3.20	3.20	3.00	3.00	2.50	2.50
DE MJ/kg[9]	16.33	15.38	15.38	14.50	14.50	14.00	14.00	13.99	13.99
Pellet durability (g)	-[10]	96.4	95.8	33.0	35.0	56.1	56.8	74.8	75.1
Pellet diameter (mm)	-[10]	5.06	5.05	5.15	5.19	5.18	5.14	5.11	5.15
Pellet hardness (kg)	-[10]	4.32	4.83	1.75	1.75	2.53	2.43	3.80	3.38

GM =genetically modified; DE = digestible energy.

[1]Non-GM; isogenic parent line maize.

[2]GM; Bt MON810 maize.

[3]Lactofeed 70 contains 70% lactose, 11.5% protein, 0.5% oil, 7.5% ash and 0.5% fibre (Volac, Cambridge, UK).

[4]Immunopro 35 contains whey protein powder - protein 35% (Volac, Cambridge, UK).

[5]Premix provided per kg of complete diet: Cu, 155 mg; Fe, 90 mg; Mn, 47 mg; Zn, 120 mg, I, 0.6 mg; Se, 0.3 mg; vitamin A, 6000 IU; vitamin D_3, 1000 IU; vitamin E, 100 IU; vitamin K, 4 mg; vitamin B_{12}, 15 µg; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; choline chloride, 250 mg; vitamin B_1, 2 mg; vitamin B_6, 3 mg.

[6]Premix provided per kg of complete diet: Cu, 15 mg; Fe, 24 mg; Mn, 31 mg; Zn, 80 mg, I, 0.3 mg; Se, 0.2 mg; vitamin A, 2000 IU; vitamin D_3, 500 IU; vitamin E, 40 IU; vitamin K, 4 mg; vitamin B_{12}, 15 µg; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; vitamin B_1, 2 mg; vitamin B_6, 3 mg.

[7]Formaxol is a blend of encapsulated formic and citric acids and essential oils (Soda Feed Ingredients, Monte Carlo, Monaco).

[8]Mycosorb® is an organic mycotoxin adsorbent (Alltech, Dunboyne, Co. Meath, Ireland).

[9]Calculated values.

[10]The starter maize diet was formulated as meal.

Table 5.2. Effect of feeding GM maize to pigs for 110 days on growth and on organ and carcass weights at day 110[1].

	Non-GM[2]	GM[3]	Non-GM/GM[4]	GM/non-GM[5]	SEM	*P*-value
Day 0 BW (kg)	10.1	10.8	10.9	10.7	0.30	0.20
Day 0 to 30						
ADG (g/d)	729	695	698	737	22.5	0.37
ADFI (g/d)	1007	974	1001	1026	30.6	0.55
FCE[6]	1.38	1.41	1.44	1.40	0.017	0.14
d 30 BW (kg)	31.9	31.2	31.3	32.5	0.69	0.40
Day 31 to 60						
ADG (g/d)	952[B]	957[B]	1019[A]	959[B]	20.1	0.06
ADFI (g/d)	1712	1683	1819	1708	47.3	0.14
FCE[6]	1.81	1.77	1.79	1.78	0.032	0.83
d 60 BW (kg)	58.2	58.0	59.7	59.4	1.04	0.50
Day 61 to 110						
ADG (g/d)	1061	1024	1090	1059	34.0	0.60
ADFI (g/d)	2401	2320	2497	2412	74.8	0.40
FCE[6]	2.28	2.27	2.30	2.29	0.043	0.96
d 110 BW (kg)	108.0	106.1	110.8	109.2	2.08	0.40
Overall						
ADG (g/d)	914	892	936	918	18.0	0.31
ADFI (g/d)	1706	1659	1772	1715	56.3	0.20

	Non-GM[2]	GM[3]	Non-GM/GM[4]	GM/non-GM[5]	SEM	*P*-value
FCE[6]	1.82	1.81	1.84	1.82	0.021	0.80
Organ and carcass weights[7]						
Kidneys (g)	416.8	379.8	395.4	391.2	18.56	0.51
Spleen (g)	204.2	214.4	191.2	191.9	16.43	0.62
Heart (g)	419.3	365.3	376.1	378.1	17.02	0.13
Liver (g)	1742.1	1691.0	1595.8	1828.9	73.95	0.12
Stomach (g)	419.7	414.8	425.8	418.0	28.45	0.99
Small intestine (g)	1311.5	1264.5	1139.9	1307.9	88.34	0.45
Carcass weight (kg)	82.4	82.7	87.5	84.3	1.72	0.15
Dressing out[8] (%)	76.2	77.1	77.1	76.3	0.52	0.47

GM = genetically modified; BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; FCE = feed conversion efficiency; SEM = standard error of the mean.

[1]Data presented as treatment means.

[2]Non-GM; isogenic parent line maize diet fed from day 0 to 110.

[3]GM; Bt MON810 maize diet fed from day 0 to 110.

[4]Non-GM/GM; non-GM maize diet fed from day 0 to 30 followed by GM maize diet fed to day 110.

[5]GM/non-GM; GM maize diet fed from day 0 to 30 followed by non-GM maize diet fed to day 110.

[6]Feed conversion efficiency (FCE) was calculated as ADFI divided by the ADG.

[7]Organ weights were calculated with the final body weight as a covariate in the statistical model.

[8]Dressing out % was calculated as carcass weight divided by the day 110 body weight.

[A,B]Values within a row with different superscripts show a tendency towards statistical significance ($0.05 < P < 0.10$). Means separation was performed using Tukey-Kramer adjustment for multiple comparisons.

Table 5.3. Effect of feeding GM maize to pigs for 110 days on intestinal histology[1].

	Non-GM[2]	**GM**[3]	**Non-GM/GM**[4]	**GM/non-GM**[5]	**SEM**	***P*-value**
Duodenum						
Villus height (µm)	798.5	598.9	655.4	665.8	75.55	0.35
Crypt depth (µm)	685.1	547.7	510.5	603.4	68.34	0.30
Villus width (µm)	298.9	212.7	192.5	250.5	35.58	0.17
Villus height/crypt depth	1.32	1.33	1.44	1.29	0.130	0.83
Goblet cells/villus	19.4	27.3	23.8	20.1	2.80	0.16
Goblet cells/µm villus	0.025	0.053	0.044	0.035	0.0079	0.11
Jejunum						
Villus height (µm)	562.7	553.5	552.4	633.2	77.43	0.85
Crypt depth (µm)	422.0	424.9	372.8	358.1	43.61	0.57
Villus width (µm)	221.5	216.5	190.5	238.1	26.62	0.66
Villus height/crypt depth	1.42	1.58	1.59	1.99	0.191	0.24
Goblet cells/villus	14.4	14.8	15.9	15.4	2.31	0.96
Goblet cells/µm villus	0.027	0.029	0.034	0.027	0.0051	0.70
Ileum						
Villus height (µm)	534.9	461.3	482.3	558.9	44.20	0.34
Crypt depth (µm)	420.2	329.3	424.1	408.6	57.66	0.59
Villus width (µm)	290.9	212.9	203.6	301.4	41.69	0.20

	Non-GM[2]	**GM**[3]	**Non-GM/GM**[4]	**GM/non-GM**[5]	**SEM**	***P*-value**
Villus height/crypt depth	1.30	1.90	1.27	1.68	0.305	0.44
Goblet cells/villus	22.7	24.2	23.1	19.8	2.50	0.60
Goblet cells/µm villus	0.041	0.046	0.049	0.034	0.0050	0.18

GM = genetically modified; SEM = standard error of the mean.

[1]Data presented as treatment means.

[2]Non-GM; isogenic parent line maize diet fed from day 0 to 110.

[3]GM; Bt MON810 maize diet fed from day 0 to 110.

[4]Non-GM/GM; non-GM maize diet fed from day 0 to 30 followed by GM maize diet fed to day 110.

[5]GM/non-GM; GM maize diet fed from day 0 to 30 followed by non-GM maize diet fed to day 110.

Table 5.4. Effect of feeding GM maize to pigs for 110 days on serum biochemical parameters indicative of liver function[1].

Day	Non-GM[2]	GM[3]	Non-GM/GM[4]	GM/non-GM[5]	SEM	*P*-value		
						Treatment	Time	Treatment × time
Alanine aminotransferase (ALT, units/L)								
30	34.7	35.7	35.0	41.7	1.57	0.51		
60	40.2	41.9	35.8	42.7	1.68	0.64		
100	34.4	33.5	33.1	37.7	1.66	0.81		
110	38.6	38.3	39.0	41.6	1.66	0.90		
Overall	37.2	37.4	35.7	40.9	3.21	0.71	0.02	0.93
Gamma glutamyl transferase (GGT, units/L)								
30	44.7	43.8	46.8	44.6	3.22	0.99		
60	58.0	63.2	61.1	49.6	3.40	0.52		
100	49.3	53.0	54.3	52.9	3.36	0.97		
110	51.0	58.6	53.1	45.6	3.36	0.56		
Overall	50.7	54.7	53.8	48.2	6.05	0.83	0.003	0.82
Alkaline phosphatase (ALP, units/L)								
30	217.9	241.9	218.5	250.9	6.25	0.30		
60	148.3	164.3	143.0	156.4	6.55	0.75		
100	85.5	114.5	96.1	112.2	6.49	0.52		
110	113.8	131.1	107.6	126.5	6.49	0.67		
Overall	141.3	162.9	141.3	161.5	11.61	0.52	< 0.0001	0.91

[1]Data presented as treatment means.

[2]Non-GM; isogenic parent line maize diet fed from day 0 to 110.

[3]GM; Bt MON810 maize diet fed from day 0 to 110.

[4]Non-GM/GM; non-GM maize diet fed from day 0 to 30 followed by GM maize diet fed to day 110.

[5]GM/non-GM; GM maize diet fed from day 0 to 30 followed by non-GM maize diet fed to day 110.

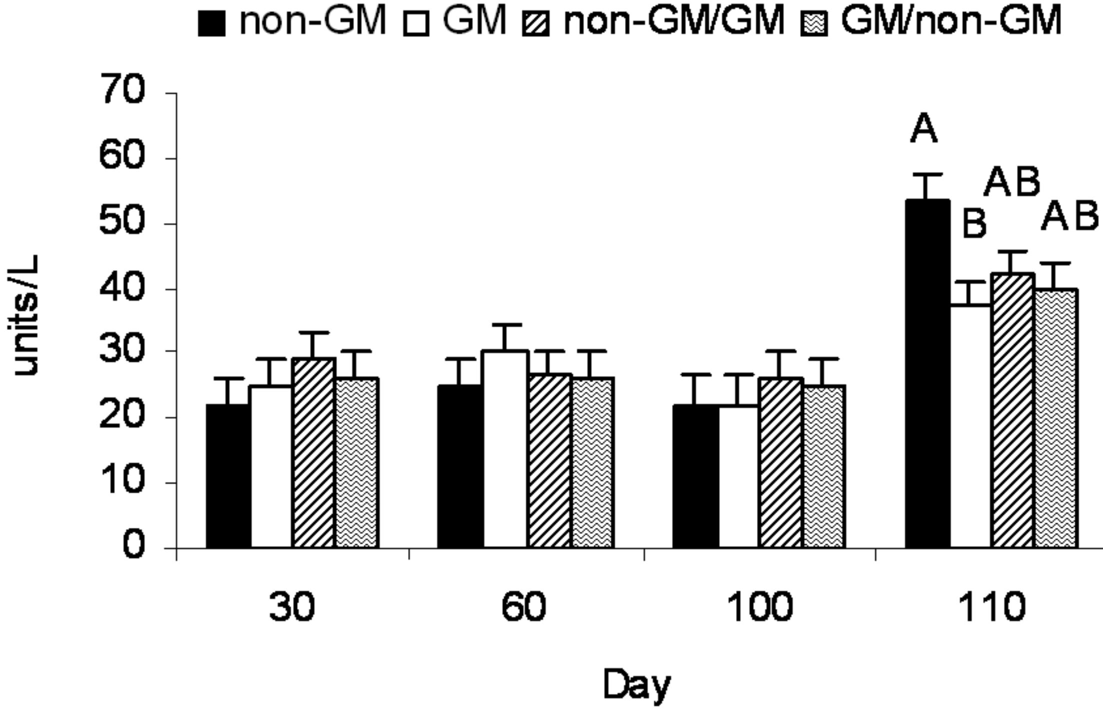

Figure 5.1. Effect of feeding GM maize to pigs on serum AST concentration (U/L). Pigs were fed one of four treatments as follows; 1) Non-GM; isogenic parent line maize diet fed from day 0 to 110, 2) GM; Bt MON810 maize diet fed from day 0 to 110, 3) Non-GM/GM; non-GM maize diet fed from day 0 to 30 followed by a GM maize diet fed to day 110 and 4) GM/non-GM; GM maize diet fed from day 0 to 30 followed by a non-GM maize diet fed to day 110. There was a treatment × time interaction for serum AST ($P < 0.05$). On day 110, pigs fed the GM treatment tended ($P = 0.06$) to have lower serum AST concentration compared to the pigs fed the non-GM treatment but were not different to all other treatments. Bars represent treatment means (n=10) with their respective standard errors. ^{AB}Means without a common superscript differ by P = 0.05-0.10. Means separation was performed using Tukey-Kramer adjustment for multiple comparisons.

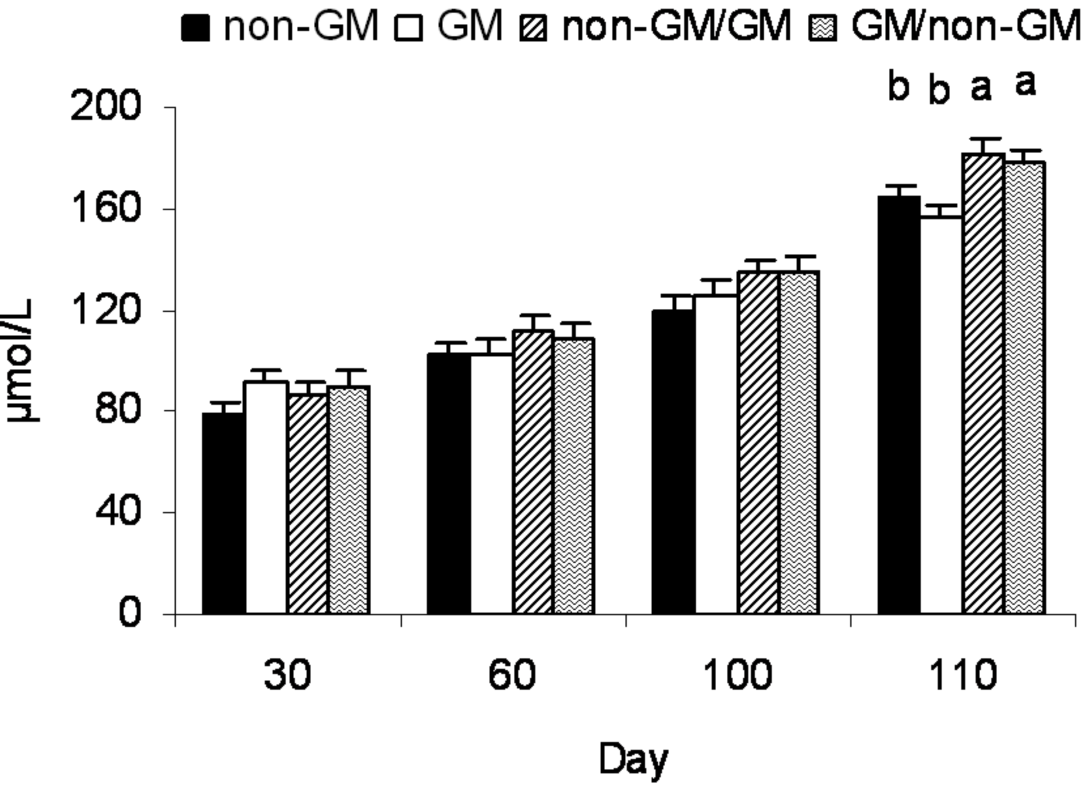

Figure 5.2. Effect of feeding GM maize to pigs for 110 days on serum creatinine concentration (µmol/L). Pigs were fed one of four treatments as follows; 1) Non-GM; isogenic parent line maize diet fed from day 0 to 110, 2) GM; Bt MON810 maize diet fed from day 0 to 110, 3) Non-GM/GM; non-GM maize diet fed from day 0 to 30 followed by a GM maize diet fed to day 110 and 4) GM/non-GM; GM maize diet fed from day 0 to 30 followed by a non-GM maize diet fed to day 110. There was a tendency for a treatment × time interaction for serum creatinine (P = 0.10). On day 110, pigs fed the non-GM/GM and GM/non-GM treatments had higher serum creatinine concentration than the pigs fed the non-GM and GM treatments ($P < 0.05$). Bars represent treatment means (n=10) with their respective standard errors. [ab]Means without a common superscript differ by $P < 0.05$. Means separation was performed using Tukey-Kramer adjustment for multiple comparisons.

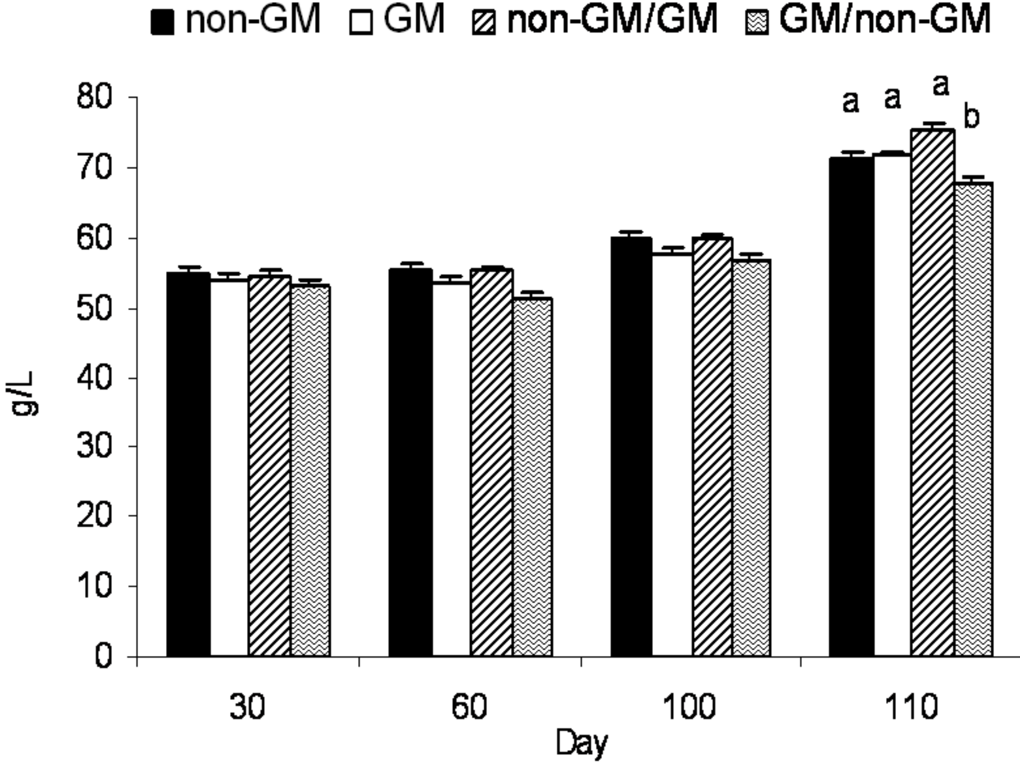

Figure 5.3. Effect of feeding GM maize to pigs for 110 days on serum total protein concentration (g/L). Pigs were fed one of four treatments as follows; 1) Non-GM; isogenic parent line maize diet fed from day 0 to 110, 2) GM; Bt MON810 maize diet fed from day 0 to 110, 3) Non-GM/GM; non-GM maize diet fed from day 0 to 30 followed by a GM maize diet fed to day 110 and 4) GM/non-GM; GM maize diet fed from day 0 to 30 followed by a non-GM maize diet fed to day 110. There was a significant treatment effect for serum total protein concentration ($P < 0.05$). This overall treatment effect was a result of significantly lower TP on day 110 in the serum of pigs fed the GM/non-GM diets compared to all other. Pigs fed the GM/non-GM treatment had lower serum total protein concentration on day 110 compared to all other treatments. Bars represent treatment means (n=10) with their respective standard errors. [ab]Means without a common superscript differ by $P < 0.05$. Means separation was performed using Tukey-Kramer adjustment for multiple comparisons.

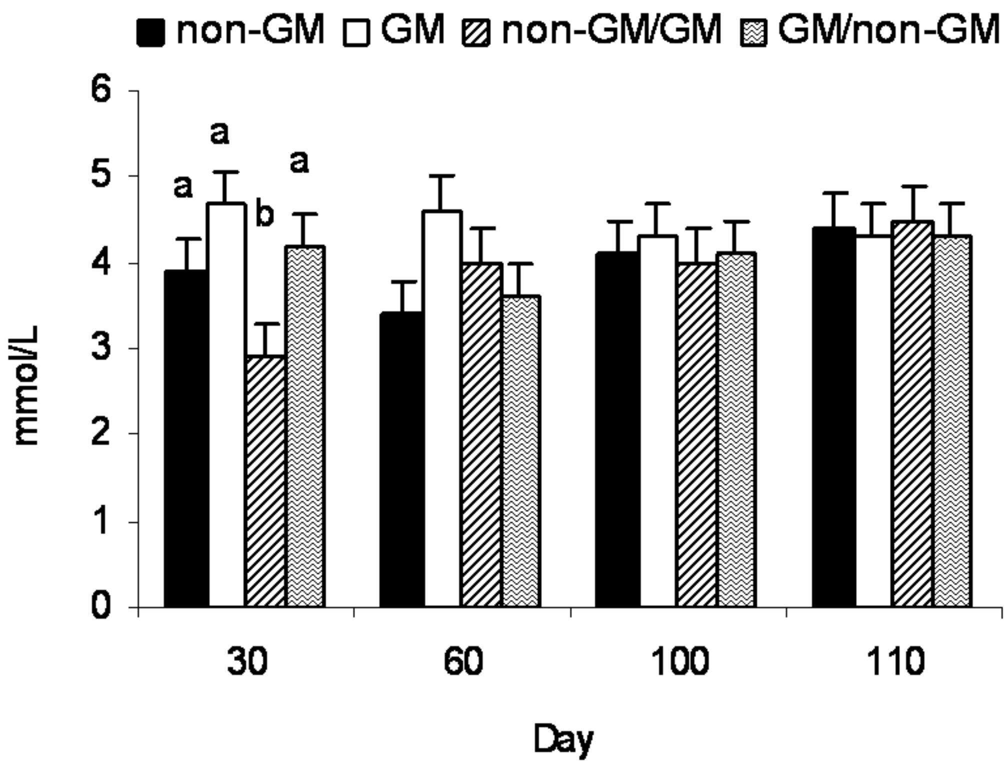

Figure 5.4. Effect of feeding GM maize to pigs for 110 days on serum urea concentration (mmol/L).

Pigs were fed one of four treatments as follows; 1) Non-GM; isogenic parent line maize diet fed from day 0 to 110, 2) GM; Bt MON810 maize diet fed from day 0 to 110, 3) Non-GM/GM; non-GM maize diet fed from day 0 to 30 followed by a GM maize diet fed to day 110 and 4) GM/non-GM; GM maize diet fed from day 0 to 30 followed by a non-GM maize diet fed to day 110. There was a treatment × time interaction for serum urea ($P < 0.05$). On d 30, pigs fed the non-GM/GM treatment had lower serum urea than pigs on all other treatments. Bars represent treatment means (n=10) with their respective standard errors. [ab]Means without a common superscript differ by $P < 0.05$. Means separation was performed using Tukey-Kramer adjustment for multiple comparisons.

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6. Effects of feeding Bt MON810 maize to pigs for 110 days on peripheral immune response and digestive fate of the *cry1Ab* gene and truncated Bt toxin

Walsh, M. C., Buzoianu, S. G., Rea, M. C., O'Donovan, O., Gelencsér, E., Ujhelyi, G., Ross, R. P., Gardiner, G. E. and Lawlor, P. G. (2012). Effects of feeding genetically modified Bt MON810 maize to growing pigs for 110 days on peripheral immune response and digestive fate of the cry1Ab gene and truncated Bt toxin. PLoS ONE 7: e36141.

6.1. Abstract

The objective of this study was to evaluate the effects of long-term (110 days) feeding of genetically modified Bt maize on peripheral immune response in pigs and to determine the digestive fate of the *cry1Ab* gene and truncated Bt toxin. Pigs (n=40) were weaned at ~28 days and fed a non-GM starter diet during a 12 day basal period prior to blocking by body weight (BW) and ancestry and random assignment to one of the following treatments (n=10/treatment); 1) non-GM isogenic parent line maize-based diet (Pioneer PR34N43) fed to day 110 (isogenic); 2) Bt maize-based diet (Pioneer PR34N44 event MON810) fed to day 110 (Bt); 3) Non-GM isogenic parent line maize-based diet (Pioneer PR34N43) fed for 30 days followed by Bt maize fed to day 110 (isogenic/Bt); and 4) Bt maize-based diet (Pioneer PR34N44 event MON810) fed for 30 days followed by the non-GM isogenic parent line maize-based diet fed to day 110 (Bt/isogenic). Blood samples were collected on d 0, 30, 60 and 100 for haematological analysis, measurement of cytokine production and Cry1Ab-specific antibody production and immune cell phenotyping. Blood samples collected on day 110 were analysed for Cry1Ab-specific antibody production and the *cry1Ab* gene and the truncated Bt toxin detection. Pigs were harvested on d 110 and digesta samples were taken from the stomach, ileum, caecum and colon and samples were taken from the liver, kidney and spleen for detection of the *cry1Ab* gene and the truncated Bt toxin. Feeding Bt maize did not affect the production of IL-6, IL-4, IL-8 or TNFα from resting or mitogen-stimulated peripheral blood mononuclear cells (PBMC) at any time during the study. Populations of $CD4^+$ and $CD8^+$ T cells did not differ between treatments on d 30, 60 or 100. However, on d 30 pigs fed isogenic/Bt tended to have a greater proportion of $CD4^+CD8^+$ T cells than pigs fed the isogenic treatment ($P = 0.08$). On d 100, leukocyte counts tended to be higher ($P = 0.06$) and lymphocyte counts were higher ($P < 0.05$) in pigs fed Bt/isogenic than pigs fed Bt or isogenic. Monocyte counts tended to be higher in pigs fed isogenic/Bt and Bt/isogenic than pigs fed Bt ($P = 0.09$). Erythrocyte counts on d 100 were lower in pigs fed Bt or isogenic/Bt than pigs fed Bt/isogenic ($P < 0.05$). Cry1Ab-specific IgG or IgA were not detected in the serum at any time during the study. Neither the truncated Bt toxin nor the *cry1Ab* gene were detected in the liver, kidneys, spleen or blood of pigs fed Bt maize. The *cry1Ab* gene was detected in stomach digesta but not in the distal gastrointestinal tract, while the stomach, caecum and colon digesta of pigs fed Bt maize were positive for Cry1Ab protein fragments. In conclusion,

there was no evidence of *cry1Ab* gene or Bt toxin translocation to organs or blood following long-term feeding. Perturbations in peripheral immune response were thought not to be age-specific and were not indicative of Th2 type allergenic or Th1 type inflammatory responses. Lymphocyte counts were sensitive to Bt maize exposure early in life and this effect was maintained following long-term feeding.

6.2. Introduction

The introduction of genetically modified (GM) technology to crop production almost 16 years ago offered the potential for a solution to the global food crisis brought about by a world population explosion. GM technology is the fastest adopted crop technology to date as it offers the possibility of higher agronomic productivity of more nutritious food without the use of pesticides (James, 2010). The global area under cultivation by GM crops has increased 87-fold since 1996, reaching 148 million hectares in 2010 (James, 2010) and new GM crops are continuously being developed. Transgenic maize is the second most important GM crop after soybean, occupying 41.3 million hectares worldwide and accounting for 31% of the global area under cultivation by GM crops (James, 2010). Bt maize is one of the most widely grown transgenic maize varieties. It is genetically engineered to express the truncated Cry1Ab toxin from *Bacillus thuringiensis* which confers resistance to the European corn borer.

The safety of GM food and feed in Europe is assessed by the European Food Safety Authority (EFSA) which recommends that 90-days studies in rodents are conducted for the detection of potential unintended effects arising from GM feed consumption (EFSA, 2008). However, some 90 day rodent studies may be insufficient to reveal late effects and longer term studies of greater than 90 days duration may be necessary to detect unintended effects due to GM ingredient consumption (Chelsea *et al*., 2011).

Abnormalities in immune response have been documented in mice fed α-amylase inhibitor peas (Prescott *et al*., 2005). Age-specific peripheral immune responses to Bt MON810 maize have previously been reported in mice (Finamore *et al*., 2008) and our group has previously documented minor changes in both the peripheral and intestinal immune response in pigs following short-term feeding of Bt maize (Chapter 3). Since the release of GM crops onto the market, concerns have been raised as to the fate of the recombinant DNA once ingested. While some animal studies have been unable to detect transgenic DNA outside the gastrointestinal tract (GIT) (Deaville and Maddison, 2005; Alexander *et al*., 2006; Yonemochi *et al*., 2010; Chapter 3), low concentrations have been documented in the organs of pigs (Mazza *et al*., 2005; Sharma *et al*., 2006).

The objectives of this study were to determine if long-term feeding and age were important factors in the peripheral immune response in pigs fed Bt maize. Another objective was to evaluate any residual effects on peripheral immune response that may

emerge in older pigs having received Bt maize in early life. The study was also designed to investigate the digestive fate of transgenic DNA and protein following long-term Bt maize consumption in an animal model that closely resembles humans.

6.3. Materials and methods

The pig study complied with European Union Council Directives 91/630/EEC (outlines minimum standards for the protection of pigs) and 98/58/EC (concerns the protection of animals kept for farming purposes) and was approved by, and a license obtained from the Irish Department of Health and Children (licence number B100/4147). Ethical approval was obtained from the Teagasc and Waterford Institute of Technology ethics committees.

Animals and experimental design

Forty crossbred (Large White × Landrace) entire male pigs were weaned at ~ 28 days of age and were fed *ad libitum* a non-GM starter diet during a 12 day basal period (day -12 to 0). The mean BW of pigs on day 0 of the study was ~10.7 kg. On day 0, pigs were blocked by weight and ancestry and within block randomly assigned to one of four treatments (n=10 pigs/treatment); 1) non-GM isogenic parent line maize-based diet (Pioneer PR34N43) fed to day 110 (isogenic); 2) GM maize-based diet (Pioneer PR34N44 event MON810) fed to day 110 (Bt); 3) Non-GM isogenic parent line maize-based diet fed for 30 days followed by GM maize fed to day 110 (isogenic/Bt); and 4) GM maize-based diet fed for 30 days followed by the non-GM isogenic parent line maize-based diet fed to day 110 (Bt/isogenic). The duration of the study was 122 days.

Housing and management

From weaning to day 60 of the study, pigs were penned individually in one of three similar rooms, each containing 24 pens. The pens were fully slatted (1.2 m × 0.9 m) with plastic slats (Faroex, Manitoba, Canada) and plastic dividers between pens. Water was available *ad libitum* from one nipple-in-bowl drinker (BALP, Charleville-Mezieres, Cedex, France) per pen. Feed was available *ad libitum* from a single stainless steel 30 cm wide feeder per pen (O'Donovan Engineering, Coachford, Co. Cork). Temperature was controlled by a hot air heating system and an exhaust fan drawing air from under slat level,

both connected to a Stienen Pcs 8400 controller (Stienen BV, Nederweert, The Netherlands). The temperature was maintained at 28 to 30ºC in the first week and reduced by 2ºC per week to 22ºC. Pigs were transferred to one of four identical finisher rooms containing 18 individual pens per room on day 60 of the study and remained there until day 110 of the study. Pens (1.81 m × 1.18 m) were fully slatted with plastic panelled partitions. Ventilation was by exhaust fans and air inlets connected to a Steinen PCS 8200 controller. Temperature was maintained at 20 to 22ºC. Feed was available *ad libitum* as dry pellets from stainless steel dry feed hoppers 30 cm in length (O'Donovan Engineering, Coachford, Co. Cork). Water was available *ad libitum* from one BALP drinking bowl. For the duration of the study, dietary treatments were equally represented in each room to avoid additional variation due to environmental conditions. Pigs showing signs of ill health were treated as appropriate and all veterinary treatments were recorded.

Maize and diets

Seeds derived from GM Bt MON810 and non-GM parent line control maize (PR34N44 and PR34N43 varieties, respectively; Pioneer Hi-Bred, Sevilla, Spain) were grown simultaneously side by side in 2007 in Valtierra, Navarra, Spain by independent tillage farmers. The Bt and isogenic control maize were purchased by the authors from the tillage farmers for use in this animal study. Samples from the Bt and isogenic maize were tested for the presence of the *cry1Ab* gene, pesticide contaminants, mycotoxins and carbohydrate composition as previously described in Chapter 2 .

All diets were manufactured and analysed for proximate analysis and amino acid concentration (Table 6.1) as previously described in Chapter 2 . All diets were formulated to meet or exceed the NRC (NRC, 1998) requirements for pigs of given weights. The non-GM starter diet was fed to all pigs from weaning (day -12) until the beginning of the study (day 0). Both isogenic and Bt maize link diets were fed from day 0 to 30, weaner diets were fed from day 31 to 60, finisher 1 diets were fed from day 61 to 100 and finisher 2 diets were fed from day 101 to day 110. Pellet hardness and durability was determined as described by Lawlor *et al.* (2003).

Blood sampling and analysis

Blood samples were collected on days 0, 30, 60 and 100 for measurement of immune parameters and on day 110 for the detection of the *cry1Ab* gene. Blood samples were

collected from the anterior vena cava of pigs of up to 30 kg BW, from the external jugular vein for heavier pigs and during exsanguination at slaughter on day 110. Whole blood samples were collected in K_2EDTA blood collection tubes (Vacuette, Greiner Bio One Ltd, Gloucestershire, UK) and stored at room temperature prior to haematological analysis which was performed within 6 h of collection. Additional whole blood samples were collected in heparinised blood collection tubes (BD Vacutainer Systems, Franklin Lakes, NJ) and stored at room temperature for PBMC isolation. Blood samples were also collected in serum collection tubes (BD Vacutainer Systems, Franklin Lakes, NJ) and centrifuged at 2500 × g for 20 min within 3 h to obtain serum. Serum was stored at -20ºC for subsequent analysis of Cry1Ab-specific antibodies. Blood samples taken at slaughter (day 110) were collected in K_2EDTA blood collection tubes and immediately placed on ice for transport to the laboratory. Within 3 h of collection blood samples were centrifuged at 2500 × g for 20 min, after which the buffy coat of white blood cells was removed and stored at -20ºC for subsequent tracking of the *cry1Ab* gene. Serum samples were also taken at slaughter as described above for Cry1Ab-specific antibody analysis.

Digesta and organ sampling

On day 110, all pigs were sacrified by electrical stunning followed by exsanguination. The last meal was administered 3 h prior to slaughter. During sampling, the following precautions were taken to prevent any cross contamination between the Bt and isogenic maize-fed pigs; all isogenic maize-fed pigs were slaughtered first followed by the Bt maize-fed pigs; all surgical instruments were cleaned with 70% ethanol between each animal and all assistants wore single-use gloves that were replaced after each sample was taken; the liver, spleen, and kidneys were removed first, to prevent contamination with digesta contents, followed by the entire GIT. Once removed, the liver, kidneys and spleen were trimmed of any superficial fat or blood clots. The outermost layer of each tissue was removed to enable sampling of the interior in order to prevent contamination by feed residue. Samples were taken from the liver (distal end, centre of central lobe), kidney (middle of cortex and medulla), and spleen (anterior end), snap frozen in liquid N and stored at -20ºC for subsequent analysis of the Bt toxin and *cry1Ab* gene. Digesta was then sampled from the stomach (cardiac region), ileum (15 cm distal to the ileo-caecal junction), caecum (tip of the blind end) and colon (60 cm from the rectum) and stored at -20ºC for subsequent analysis of the truncated *cry1Ab* gene and Bt toxin.

Isolation and stimulation of PBMC and cytokine measurement

Isolation and stimulation of PBMC from whole blood was conducted as described by Walsh *et al.* (2008) and in Chapter 3. Following PBMC stimulation, the cell culture supernatant was collected and stored at -80ºC. Concentrations of IL-4, IL-6, IL-8, and TNFα were subsequently determined in the cell supernatants using multiplex porcine-specific cytokine ELISA kits (Meso Scale Discovery, Gaithersburg, Maryland) in accordance with the manufacturer's instructions.

Immune cell phenotyping and Cry1Ab-specific antibody response

Following stimulation, PBMC were resuspended at ~2 x 10^6 cells/mL in phosphate buffered saline (PBS) containing 2% fetal bovine serum (PBS-FBS). Primary and secondary antibodies were added at concentrations determined by titration and incubated in the dark at room temperature for 15 min. Cells were washed and re-suspended in PBS-FBS and acquired using a BD FACSCanto II™ flow cytometer. Antibodies used included anti-porcine CD3 PE/Cy5 (Abcam, Cambridge, UK), anti-porcine CD4 fluorescein isothiocyanate (FITC), anti-porcine CD8 phycoerythrin (PE), and anti-mouse CD32 (all antibodies were obtained from BD Biosciences, Devon, UK unless otherwise stated). Antibodies were used according to manufacturer's recommendations. The percentages of $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ T lymphocytes were calculated on the $CD3^+$ gate. At least 50,000 events were acquired and analysed. Data were analysed using FACSDIVA software (BD Biosciences).

The detection of Cry1Ab-specific IgA and IgG in pig serum was conducted as previously described in in Chapter 3 on samples taken on days 0, 30, 60, 100 and 110.

Haematological analysis

Whole blood samples were analysed using a Beckman Coulter Ac T Diff haematology analyser (Beckman Coulter Ltd., High Wycombe, UK). The following parameters were determined; counts of white blood cells (WBC), lymphocytes (LY), monocytes (MO), granulocytes (GR), and red blood cells (RBC), haemoglobin concentration (Hgb), haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count (Plt) and mean platelet volume (MPV).

Samples were analysed according to the manufacturer's instructions and general haematology recommendations (Feldman *et al*., 2000).

Tracking of the truncated Bt toxin and *cry1Ab* gene in feed and porcine digesta, organs and blood

Cry1Ab protein quantification

Organ samples (liver, spleen and kidney) were homogenised in 0.8% saline (0.5 g/mL) and serum was diluted in 0.8% saline (0.5 mL/mL). Ten μL of 10 mM phenylmethylsulfonyl fluoride (PMSF) was added per mL of solution and samples were centrifuged for 20 min at 9390 × g. Digesta samples were centrifuged for 15 min at 540 × g and 10 μL of 10 mM PMSF was added per mL of supernatant and samples were centrifuged for 20 min at 9390 × g. The concentration of the Cry1Ab protein in both the organ and digesta samples was determined as previously outlined in in Chapter 3.

Detection of the cry1Ab gene

DNA extraction from feed, digesta, animal tissue and white blood cells was conducted as previously outlined in in Chapter 3. A preliminary cross-dilution assay was performed to determine the detection limit of the *cry1Ab*-specific PCR and the possible inhibitory effect of porcine DNA. Five primer pairs targeting two endogenous maize genes [rubisco and shrunken 2 (sh2)], two *cry1Ab* gene fragments (*cry1Ab*-1 and *cry1Ab*-2) and a porcine growth hormone gene (*sw*), respectively were obtained from Invitrogen (Paisley, UK). The primer sequences and PCR conditions used for the detection of *sh2*, *cry1Ab*-1 and sw have previously been described in in Chapter 3. The primers and conditions used for the detection of *rubisco* and *cry1Ab*-2 are outlined in Table 6.2. Two microlitres of extracted DNA was used in all PCR reactions, which were performed in a final volume of 50 μL. Each PCR reaction contained 25 μL of either REDTaq ReadyMix PCR reaction mix containing $MgCl_2$ (Sigma-Aldrich) (for white blood cells) or DreamTaq Green PCR master mix (Fermentas, Ontario, Canada) (for tissue samples and digesta), as well as 0.6 or 0.1 μM of the *cry1Ab*-2 or rubisco primers, respectively. PCR reactions were performed in a GeneAmp 2400 or 2700 thermal cycler (Applied Biosystems, Foster City, CA). Each set of PCR reactions included a positive control for the *cry1Ab* gene (DNA from Bt maize), a negative control for the *cry1Ab* and endogenous maize genes (DNA from isogenic maize), contamination controls without template DNA, and a positive control for the endogenous

porcine gene (DNA from normal pig meat). PCR products were analysed on 10% polyacrylamide gels run at 200 V for 50 min and visualized by SYBR Green-staining.

Statistical analysis

For all response criteria, the pig was the experimental unit. Immune cell phenotype and haematology data were analysed as repeated measures using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) with Tukey-Kramer adjustment for multiple comparisons using day 0 values as a covariate in the model. Cytokine data found not to be normally distributed following log transformation were analysed using the non-parametric Kruskal-Wallis test within the NPAR1WAY procedure in SAS. Cytokine data are presented as treatment median values with the 25-75th percentiles. The level of significance for all tests was $P < 0.05$ and trends were reported for $0.05 < P < 0.10$.

6.4. Results

Effect of feeding Bt and isogenic maize on immune response in growing pigs

Cytokine production

In the absence of exogenous stimuli, the spontaneous production of IL-6, IL-4 and IL-8 by resting PBMC from pigs on all four dietary treatments was comparable on days 0, 30 and 100 (Table 6.3). On day 0, (prior to commencement of dietary treatments), PBMC from pigs subsequently fed Bt maize for 110 days produced lower concentrations of TNFα than those from pigs on all other treatments ($P < 0.01$). However, TNFα production by resting PBMC was not different between treatments on day 30 or 100 of the study. The concentration of IL-6, IL-4, IL-8 and TNFα produced by mitogen-stimulated PBMC on day 0, 30 and 100 did not differ between treatments (Table 6.4).

Immune cell populations

Treatment × time interactions for all immune cell populations examined were non-significant (Table 6.5). There was no effect of feeding Bt maize to pigs on populations of $CD3^{+}$, $CD3^{+}CD4^{+}$ or $CD3^{+}CD8^{+}$ T cells isolated from PBMC at any time during the study. The proportion of $CD3^{+}$ T cells increased in pigs on day 60 of the study; however, by day 100, it was reduced to below day 30 values ($P < 0.05$). The number of both $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ T cells decreased on day 60 of the study but increased again by day 100 (*P*

< 0.001). On day 30, pigs fed isogenic maize for 30 days followed by Bt maize for 80 days tended to have a greater proportion of $CD4^+CD8^+$ T cells than pigs fed isogenic maize (P = 0.08) but cell proportions in these pigs did not differ from the other two treatments. Similar to findings for both $CD4^+$ and $CD8^+$ T cells, the proportion of $CD4^+CD8^+$ T cells decreased over time until day 60 and increased thereafter ($P < 0.001$).

Cry1Ab-specific immunoglobulin production

Cry1Ab-specific IgA and IgG were not detected in the serum of pigs fed either isogenic maize or Bt maize at any time point during the study (day 0, 30, 60, 100) or at slaughter on day 110 or in non-maize feed (control) even at the lowest dilution (data not shown).

Haematological parameters

Overall, the total count of leukocytes was not altered by dietary treatment. However, on day 100, pigs fed Bt/isogenic maize diets tended to have higher leukocytes counts than pigs fed isogenic or Bt maize diets for 110 days (Table 6.6; P = 0.06). Furthermore, leukocyte counts increased significantly as the pigs aged ($P < 0.001$). Overall, there was a tendency for higher lymphocyte counts in pigs fed Bt/isogenic maize diets compared with pigs fed isogenic or Bt maize diets for the entire 110 day period (P = 0.09). This was brought about by similar significant differences on day 100 ($P < 0.05$). Monocyte counts tended to be higher in pigs fed Bt/isogenic maize diets or isogenic/Bt maize diets compared with pigs fed Bt maize for 110 days (P = 0.09). Overall, there were no dietary treatment effects on monocyte counts; however, counts increased over time ($P < 0.01$). Granulocyte counts were not altered by dietary treatment; however, an initial decrease in granulocyte count was followed by an overall increase by day 100 of the study ($P < 0.01$).

On day 100, pigs fed Bt/isogenic maize diets had higher erythrocyte counts compared with pigs fed isogenic/Bt maize diets or Bt maize for the entire 110 days (Table 6.7; P < 0.05). Overall, however, there were no dietary treatment effects on erythrocyte counts. In addition, erythrocyte counts were found to increase up to day 30 of the study and to decrease thereafter ($P < 0.01$). Haemogloblin concentration, hematocrit or the erythrocyte parameters MCV, MCH, MCHC and RDW were not altered by dietary treatment. Likewise, platelet counts and MPV were not affected but were lower in older pigs (P < 0.001). The erythrocyte indices MCH ($P < 0.01$), and MCHC ($P < 0.001$) increased over

time, while MCV was lower in older pigs (*P* < 0.001). The erythrocyte index RDW also tended to increase over time (*P* = 0.07). Both haemogloblin concentration and hematocrit initially increased as the pigs aged but decreased thereafter (*P* < 0.01).

Fate of ingested *cry1Ab* gene and truncated Bt toxin

Detection of transgenic and endogenous plant genes in white bloods cells, tissue and digesta

Neither transgenic *cry1Ab* plant gene fragments of 211 or 149 bp were detected in liver, kidneys or spleen samples or in white blood cells of any pigs, regardless of dietary treatment (Table 6.8). Likewise, fragments of a single copy endogenous plant gene (*sh2*) were not detected in any tissue or white blood cells examined. However, a multiple copy endogenous plant gene (*rubisco*) was detected in 40-60% of liver samples, 30-50% of kidney samples, 20-50% of spleen samples and 0-20% of white blood cells examined, depending on treatment. All tissue and white blood cell samples were positive for the endogenous porcine gene (*sw*). The single copy endogenous plant gene, *sh2* was detected in the gastric digesta of 80-90% of pigs depending on treatment but was undetectable in the ileal, caecal and colon digesta (Table 6.9). Likewise, the multiple copy *rubisco* gene was found in the gastric digesta 90-100% depending on treatment. However, unlike the *sh2* gene, *rubisco* was detected in 80-100% of ileal, 30-60% of caecal and 10-40% of colon digesta samples depending on treatment. Both transgenic plant gene fragments (*cry1Ab*-1 and *cry1Ab*-2) were found in the gastric digesta of 90% of the pigs fed either Bt maize for 110 days or Bt maize for the final 80 days of the study. Faint signal bands for both *cry1Ab*-1 and *cry1Ab*-2 were detected in gastric digesta of 40% of the pigs fed either isogenic or Bt/isogenic maize diets, respectively (data not shown). This thought to have occurred due to sample contamination post-sampling. *Cry1Ab* gene contamination was not found in other digesta samples from these pigs and prior to feeding no *cry1Ab* gene fragments were detected in the isogenic maize (Chapter 3). The *cry1Ab*-1 gene fragment was detected in the ileal digesta of 20 and 10% of the pigs fed Bt maize for the entire 110 day period and the final 80 days of the study, respectively. However, the *cry1Ab*-2 gene fragment was not detected in the ileal digesta of any pigs fed Bt maize at any time. Likewise, no transgenic plant gene fragments were detected in the caecal or colon digesta of pigs fed Bt maize for 110 or 80 days. All digesta samples from pigs fed the isogenic maize diet for 110 days or Bt/isogenic maize diets did not contain either transgenic plant gene fragment.

Detection of the transgenic Cry1Ab protein in serum, tissue and digesta

The Cry1Ab protein was not detected in the kidneys, liver, spleen or in the sera of any of the pigs fed any of the four dietary treatments at any time point during the study (data not shown). The Cry1Ab protein was not detected in the stomach, caecal or colon digesta of pigs fed the isogenic maize diet or pigs fed the Bt/isogenic maize diet. It was only detected in the digesta of pigs fed Bt maize for 110 days or the isogenic/Bt maize diet (Table 6.10). In these pigs, it was detected in 90 and 80% of the gastric samples, 80 and 50% of caecal samples and 100% of colon samples, respectively 3 h after the last meal was administered. The mean concentration of Cry1Ab protein was lower in the digesta of pigs fed the isogenic/Bt maize diet compared with those fed Bt maize for the entire study period, except in the caecum where the opposite was true. In both Bt maize-fed groups, the mean concentration of Cry1Ab protein in the caecal digesta was lower than in the gastric or colon digesta (Table 6.10). In fact, the Cry1Ab protein was most concentrated in the colon digesta.

6.5. Discussion

To our knowledge, this study is the first to evaluate the effects of long-term feeding (80 or 110 days) of Bt maize on peripheral immune response of pigs. It is also the first to investigate if age at feeding impacts the response in pigs. By using a cross-over study, we were able to evaluate any residual effects on peripheral immune response that may emerge in older pigs having received Bt maize for a relatively short time period in early life (post-weaning) as well as the effects of feeding Bt maize for a longer period later in life. Changes in peripheral immune response were evaluated through measurement of cytokine production from PBMC, investigation of Cry1Ab-specific antibody production in serum, immunophenotyping and haematological analysis. In pigs fed Bt maize for the entire 110 day study period and in pigs that were older when first fed Bt maize, there was no change in the production of IL-6, IL-4, IL-8 and TNFα from mitogen-stimulated or resting PBMC between treatments at any time point. TNFα production from resting PBMC isolated from the Bt maize group was less on day 0 than all other treatments. This difference however, cannot be attributed to Bt maize, as these samples were taken prior to feeding of treatment

diets. A study examining the effects of glufosinate-ammonium tolerant triticale on the immune system of mice found increased IL-2 and decreased IL-6 in serum but no significant change in IL-4, IL-10, IL-12 or IFNγ concentrations in the fifth generation (Krzyzowska *et al*., 2010). Finamore *et al.* (2008) reported an age-specific serum cytokine response to feeding MON810 maize in mice where IL-6, IL-13, IL-12p70 and MIP-1ß were elevated in weaning mice fed MON810 maize for 30 days; however, MIP-1ß was the only cytokine elevated in weaning or old mice after 90 days of feeding. Our group previously found that feeding Bt maize to weanling pigs for a shorter time period i.e.31 days had no effect on the production of IL-10, IL-6, IL-4 or TNFα from mitogen-stimulated or resting PBMC; however a reduction in both IL-12 and IFNγ was observed (Chapter 3).

In our study, there was no effect of feeding Bt maize for 80 or 110 days on $CD3^+$, $CD4^+$ and $CD8^+$ T cells. On day 30, there tended to be a difference in $CD4^+CD8^+$ T cells between the isogenic and isogenic/Bt groups. However, at this stage both groups were receiving the same diet i.e. non-GM; therefore, this difference is not related to Bt maize. In a previous study feeding weaning mice MON810 maize for 90 days resulted in increased B cells in blood; however, when older mice were fed the same maize for 90 days, B cell and $CD8^+$ T cells populations were decreased while $CD4^+$ T cells were increased (Finamore *et al*., 2008). Krzyzowska *et al.* (2010) reported a decrease in B cells in blood of the fifth generation of mice fed GM triticale. While changes in B cell populations in response to feeding Bt maize were not evaluated in our study, we did not find the changes in $CD4^+$ and $CD8^+$ T cells observed by others in mice (Finamore *et al*., 2008).

Our group has previously found no Cry1Ab-specific antibody response in weanling pigs following 31 days of feeding Bt maize (Chapter 3) which is in agreement with the findings from this study where 80 or 110 days of feeding Bt maize also did not elicit an antigen-specific antibody response. An antigen-specific IgG1 response has been reported in mice fed diets containing rice expressing the Cry1Ab toxin and spiked with the purified Bt toxin for 28 days and a weak specific IgG2a response was evident after feeding rice expressing Bt toxin for 90 days (Kroghsbo *et al*., 2008). However, similar to our findings, Adel-Patient *et al.* (2011) found no specific-anti-Cry1Ab antibody response in serum from mice fed MON810 maize following intragastric or intraperitoneal sensitization.

On day 100, pigs fed the Bt/isogenic maize diet tended to have a higher leukocyte count than those fed either Bt or isogenic maize for 110 days. This increase was primarily a reflection of the increase in lymphocyte count in these pigs. Both leukocyte and

lymphocyte counts for all pigs fed Bt maize at some point during the study were above the normal reference range for pigs (Thorn, 2000). The immunophenotyping data indicated that T cell populations were not influenced by feeding Bt maize; however, B cells were not evaluated. While lymphocyte counts were elevated significantly in some pigs fed Bt maize, there was no indication of a Th2-mediated allergic inflammatory response to the Cry1Ab toxin in the form of antigen-specific Ig production. The spleen weight of these pigs reported previously in Chapter 5 did not differ between treatments and no histopathological indicators of organ damage were evident in the spleen or other organs. Likewise, the caecal microbial community structure was similar across treatments (Chapter 8) and as a result alterations in immune response as a consequence of changes in gut microbiota were not anticipated (Festi *et al*., 2011). A study using rats as an animal model for the safety evaluation of Bt rice found that leukocyte count and MCH were decreased in male rats; however, all haematological parameters analysed were within the reference range for rats of the age and breed used (Schroder *et al*., 2007). Krzyzowska *et al.* (2010) also found that leukocyte counts were increased when mice were fed GM triticale, but again, these values were within the normal reference range for mice. Erythrocyte counts in pigs fed Bt maize for 80 days or longer were lower than in pigs fed the Bt/isogenic maize diet. We have previously reported a decrease in erythrocyte counts in sows fed Bt maize (Walsh *et al*., 2012). However, in that study haemogloblin concentration and hematocrit were also decreased and the changes observed were not attributed to Bt maize.

In an earlier study with weanling pigs (~65 days old), we detected a small (211 bp) fragment of the *cry1Ab* gene in the gastric digesta of all pigs fed Bt maize; however, detection in the ileal and caecal digesta was limited to two and one pigs, respectively while the gene fragment was undetectable in the colon (Chapter 3). However, in the present study, in older pigs (~150 days old) *cry1Ab* gene fragments (149 and 211 bp) were detectable in the gastric digesta and the 211 bp gene fragment only was detected at low frequency in ileal digesta. The *cry1Ab* gene, regardless of amplicon size, was not detected in the caecum or colon. Nucleic acids are known to endure extensive enzymatic degradation in the GIT (Phipps and Beevers, 2000). Potentially the transgenic DNA was degraded by microbial DNAse enzymes which are most likely present at higher concentrations in the caecum and colon as a result of larger microbial populations. These findings agree with results from a wild boar study where *cry1Ab* fragments of up to 420 bp were detected in gastric contents but no fragments greater than 211 bp were found further

down the GIT (Wiedemann *et al*., 2009). In the same study, a small (173 bp) rubisco gene fragment was found throughout the GIT and a small (~100 bp) fragment of *cry1Ab* was found at very low frequency in the jejunal contents (Wiedemann *et al*., 2009). The smallest fragment we chose to detect in our study was 149 bp; therefore, smaller *cry1Ab* fragments may have been present in digesta distal to the stomach but remain undetected. Similar to Wiedemann *et al.* (2009), and Chowdhury *et al.* (2003), we detected a small (173 bp) *rubisco* fragment in small intestinal, caecal and colon digesta. Chowdhury *et al.* (2003) also detected a small *cry1Ab* fragment (110 bp) in 40 kg pigs fed Bt maize for 28 days.

The majority of studies both in monogastric and ruminant species have failed to detect transgenic DNA beyond the gastrointestinal barrier (Flachowsky *et al*., 2005; Guertler *et al*., 2008). Furthermore, our group were previously unable to detect a 211 bp fragment of the transgenic *cry1Ab* gene in the organs or blood of pigs fed Bt (MON810) maize for 31 days (Chapter 3). In agreement with these findings, a longer feeding period of 110 days did not influence the ability of *cry1Ab* to translocate across the intestinal barrier of pigs, as neither the 211 or 149 bp *cry1Ab* fragments were detectable in the blood, liver, spleen, or kidneys in the present study. Mazza *et al.* (2005), however, detected a 519 bp *cry1Ab* fragment in the plasma, liver, kidney and spleen of piglets fed Bt (MON810) maize for 35 days although the gene's smallest function unit (1800 bp) was never detected. Likewise, a 278 bp fragment of the *cp4epsps* transgene from Round-up Ready canola was found in the liver and kidneys of pigs, however, the prevalence was extremely low (Sharma *et al*., 2006). The transfer of endogenous plant DNA from the GIT into blood and organs appears to occur spontaneously in nature (Reuter and Aulrich, 2003; Nemeth *et al*., 2004; Mazza *et al*., 2005). Our findings are similar to those reported in calves (Chowdhury *et al*., 2004) and fallow deer (Guertler *et al*., 2008) where small fragments of the multiple copy endogenous chloroplast *rubisco* gene (173 or 226 bp) were detected in liver, kidney and spleen. Guertler *et al.* (2008) and Mazza *et al.* (2005) also detected fragments of the multiple copy endogenous chloroplast *zein* gene in organs of deer and pigs fed Bt maize, respectively. We were previously unable to detect fragments of the single copy endogenous chloroplast *sh2* gene in the organs or blood of pigs fed Bt maize for 31 days (Chapter 3) and similar results were found when pigs were fed Bt maize for longer in the present study. These findings suggest that copy number is the rate limiting step in the traceability of transgenic DNA. Also, the low detection frequency of the *cry1Ab* gene in

the ileum and the absence of *cry1Ab* gene detection distal to the ileum may account for the lack of detection of the single copy *cry1Ab* gene in animal tissues and blood.

Similar to our findings in weanling pigs (Chapter 3), the Cry1Ab protein was detected in the stomach, caecum and colon digesta but not in the organs or plasma of pigs fed Bt maize for 110 days. Similarly, in other studies with pigs, Yonemochi *et al.* (2010) did not detect the Cry9C protein in blood, liver or muscle samples and Wiedemann *et al.* (2009) found the Cry1Ab protein in stomach, colon and rectum samples from wild boar fed Bt176 maize but not in the organs or blood. In the present study, the Cry1Ab protein was recovered from 85% of stomach, 65% of caecal and 100% of colon samples from pigs fed Bt maize even though the *cry1Ab* gene (either 211 and 149 bp fragments) were only detected in the stomach and at low frequency in the ileum. Einspanier *et al.* (2004) reported that the use of PCR primers for shorter amplicons increased the chance of detecting plant DNA in ruminal contents. Therefore, similar to our previous study (Chapter 3), the discrepancies observed in detection frequency between the *cry1Ab* gene and protein fragments along the GIT may be due to the failure to amplify gene fragments of less than 149 bp in length. In addition, in our study, the concentration of Cry1Ab protein found in the stomach and colon digesta was twice as high as that found in the caecum. This may be due to the fact that the colon digesta is more concentrated as a result of water absorption. Furthermore, the concentration of Cry1Ab protein detected in the digesta from any site within the GIT of finisher pigs in this study is higher than that previously found by our group in weanling pigs (2.41-2.74 ng/mL) (Chapter 3). This is most likely due to the higher inclusion of Bt maize in finisher diets compared to those used for weanling pigs. The concentration of Cry1Ab protein in the digesta of pigs fed Bt maize during the entire 110 day study period was higher at all gastrointestinal sites, with the exception of the caecum than in pigs fed the isogenic/Bt maize diet. The longer duration of feeding may account for this difference. Also, the Cry1Ab protein was no longer present in the digesta of pigs fed the Bt/isogenic diet. This is not surprising as these pigs had not been fed Bt maize for 80 days. This demonstrates that once Bt maize feeding is ceased, the Cry1Ab protein is no longer detectable in the GIT.

In conclusion, long-term feeding of Bt maize to pigs did not elicit an allergic or inflammatory-type peripheral immune response. This was evidenced by the lack of antigen-specific antibody production and the absence of alterations in T cell populations and inflammatory cytokine production. Peripheral immune response to Bt maize did not

appear to be age-related, as there were no differences in cytokine, antigen-specific Ig production or T cell populations between pigs fed Bt maize from 40 or 70 days of age. However, a residual effect on lymphocyte count was apparent in older pigs administered Bt maize in early life only, an effect which was not present following long-term Bt or isogenic maize consumption. Intestinal degradation of transgenic DNA was enhanced in older pigs, as the *cry1Ab* gene was detected in gastric digesta and at low frequency in the ileum but not in the distal GIT, unlike transgenic protein fragments which were found in the colon. Furthermore, long-term feeding of Bt maize did not result in the translocation of transgenic DNA or protein from the GIT to organs or blood. Findings from this study can offer assurance to regulators and consumers as to the safety of long-term consumption of Bt maize.

Table 6.1. Composition of experimental diets (fresh weight basis, %).

	Starter (d -12 – 0)	Link (d 0 – 30)		Weaner (d 31 – 60)		Finisher 1 (d 61 -100)		Finisher 2 (d 101 – 110)	
Ingredient	**non-GM[1]**	**Isogenic[1]**	**Bt[2]**	**Isogenic[1]**	**Bt[2]**	**Isogenic[1]**	**Bt[2]**	**Isogenic[1]**	**Bt[2]**
non-GM maize[1]	27.33	38.88	-	65.31	-	73.38	-	79.10	-
GM maize[2]	-	-	38.88	-	65.31	-	73.38	-	79.10
Soybean meal (non-GM)	24.00	25.00	25.00	28.64	28.64	22.76	22.76	17.35	17.35
Lactofeed 70[3]	25.00	20.00	20.00	-	-	-	-	-	-
Immunopro 35[4]	12.50	9.00	9.00	-	-	-	-	-	-
Soybean oil	8.00	4.00	4.00	2.37	2.37	0.06	0.06	-	-
L-Lysine HCl	0.30	0.30	0.30	0.36	0.36	0.43	0.43	0.49	0.49
DL-Methionine	0.25	0.20	0.20	0.14	0.14	0.14	0.14	0.14	0.14
L-Threonine	0.12	0.12	0.12	0.15	0.15	0.17	0.17	0.19	0.19
L-Tryptophan	0.10	0.10	0.10	0.05	0.05	0.07	0.07	0.08	0.08
Vitamin and mineral premix	0.30[5]	0.30[5]	0.30[5]	0.10[6]	0.10[6]	0.10[6]	0.10[6]	0.10[6]	0.10[6]
Formaxol[7]	0.20	0.20	0.20	-	-	-	-	-	-
Mycosorb[8]	-	-	-	0.20	0.20	0.20	0.20	0.20	0.20
Salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Dicalcium phosphate	0.50	0.50	0.50	1.19	1.19	1.13	1.13	0.90	0.90
Limestone flour	1.10	1.10	1.10	1.19	1.19	1.26	1.26	1.15	1.15

	Starter	**Link**		**Weaner**		**Finisher 1**		**Finisher 2**	
	(d -12 – 0)	**(d 0 – 30)**		**(d 31 – 60)**		**(d 61 -100)**		**(d 101 – 110)**	
Ingredient	**non-GM[1]**	**Isogenic[1]**	**Bt[2]**	**Isogenic[1]**	**Bt[2]**	**Isogenic[1]**	**Bt[2]**	**Isogenic[1]**	**Bt[2]**
Analysed Chemical Composition (%)									
Dry matter	91.30	90.40	90.50	88.60	88.80	89.30	89.50	89.20	88.80
Crude protein	20.90	21.00	20.70	17.90	17.80	17.40	17.40	16.00	16.10
Oil (Acid hydrolysis)	9.60	6.20	6.30	5.20	5.40	3.20	3.10	3.20	3.10
Crude fibre	1.70	1.80	1.60	2.10	2.20	3.00	2.40	2.60	2.60
Ash	6.30	5.60	5.80	4.90	4.80	4.80	4.60	4.00	4.10
Lysine	1.55[i]	1.50	1.56	1.29	1.31	1.36	1.37	1.15	1.16
Ca[9]	8.30	7.80	7.80	8.00	8.00	8.00	8.00	7.00	7.00
P[9]	4.08	3.63	3.63	3.20	3.20	3.00	3.00	2.50	2.50
DE MJ/kg[9]	16.33	15.38	15.38	14.50	14.50	14.00	14.00	13.99	13.99
Pellet durability (g)	-[10]	96.4	95.8	33.0	35.0	56.1	56.8	74.8	75.1
Pellet diameter (mm)	-[10]	5.06	5.05	5.15	5.19	5.18	5.14	5.11	5.15
Pellet hardness (kg)	-[10]	4.32	4.83	1.75	1.75	2.53	2.43	3.80	3.38

GM =genetically modified; DE = digestible energy.

[1]Non-GM; isogenic parent line maize.

[2]GM; Bt MON810 maize.

[3]Lactofeed 70 contains 70% lactose, 11.5% protein, 0.5% oil, 7.5% ash and 0.5% fibre (Volac, Cambridge, UK).

[4]Immunopro 35 contains whey protein powder - protein 35% (Volac, Cambridge, UK).

[5]Premix provided per kg of complete diet: Cu, 155 mg; Fe, 90 mg; Mn, 47 mg; Zn, 120 mg, I, 0.6 mg; Se, 0.3 mg; vitamin A, 6000 IU; vitamin D_3, 1000 IU; vitamin E, 100 IU; vitamin K, 4 mg; vitamin B_{12}, 15 µg; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; choline chloride, 250 mg; vitamin B_1, 2 mg; vitamin B_6, 3 mg.

[6]Premix provided per kg of complete diet: Cu, 15 mg; Fe, 24 mg; Mn, 31 mg; Zn, 80 mg, I, 0.3 mg; Se, 0.2 mg; vitamin A, 2000 IU; vitamin D_3, 500 IU; vitamin E, 40 IU; vitamin K, 4 mg; vitamin B_{12}, 15 µg; riboflavin, 2 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; vitamin B_1, 2 mg; vitamin B_6, 3 mg.

[7]Formaxol is a blend of encapsulated formic and citric acids and essential oils (Soda Feed Ingredients, Monte Carlo, Monaco).

[8]Mycosorb® is an organic mycotoxin adsorbent (Alltech, Dunboyne, Co. Meath, Ireland).

[9]Calculated values.

[10]The starter maize diet was formulated as meal.

Table 6.2. Primers and PCR conditions used for the detection of target genes in porcine organ, white blood cell and digesta samples.

Primer name	Sequence (5'-3')	Specificity	Target gene	Amplicon size (bp)	PCR conditions	Reference
rubisco - F	AGC TAA TCG TGT GGC TTT AGA AGC C	Plant (endogenous)	Ribulose bisphosphate carboxylase	173	94°C × 3 min 94°C × 30 s, 62°C × 30 s, 72°C × 30 s } × 35 72°C × 7 min	Guertler *et al.,* 2009
rubisco - R	TGG TAT CCA TCG CTT TGA AAC CA					
cry1Ab-2 - F	ACT ATC CTT CGC AAG ACC CTT CCT C	Plant (transgenic)	*cry1Ab*	149	95°C × 3 min 95°C × 25 s, 62°C × 30 s, 72°C × 45 s } × 30 72°C × 7 min	Nemeth *et al.*, 2004
cry1Ab-2 - R	GCA TTC AGA GAA ACG TGG CAG TAA C					

Table 6.3. Cytokine production by resting PBMC from pigs fed diets containing Bt and isogenic maize (pg/mL)[1].

Cytokine	Isogenic	Bt	Isogenic/Bt	Bt/isogenic	*P*-value
Day 0					
IL-6	4.5	1.2	6.5	4.0	0.16
	(0.1-8.4)	(0.2-15.1)	(1.4-61.1)	(2.5-8.8)	
IL-4	11.8	3.5	8.9	9.4	0.42
	(1.8-57.2)	(0.6-21.3)	(0.2-14.0)	(4.6-22.7)	
IL-8	44.7	69.6	121.0	109.4	0.44
	(8.59-435.42)	(2.1-129.8)	(25.3-5277.9)	(5.5-1497.9)	
TNFα	2.6	0.6	2.4	2.9	0.01
	(0.40-15.84)	(0.2-1.3)	(0.5-20.0)	(1.3-4.9)	
Day 30					
IL-6	2.7	2.2	6.8	4.4	0.14
	(0.3-15.8)	(0.1-6.9)	(2.9-82.0)	(0.3-8.8)	
IL-4	7.2	7.9	9.7	6.1	0.45
	(1.8-24.6)	(5.1-18.2)	(4.5-34.9)	(2.4-14.2)	
IL-8	150.9	381.0	703.7	448.6	0.35
	(95.8-2428.5)	(72.3-2349.1)	(175.1-5415.5)	(33.9-1177.5)	
TNFα	4.1	6.9	7.8	6.7	0.65
	(0.7-22.1)	(0.4-17.6)	(3.6-79.0)	(1.7-18.6)	
Day 100					

Cytokine	Isogenic	Bt	Isogenic/Bt	Bt/isogenic	*P*-value
IL-6	0.4	1.2	4.5	1.5	0.28
	(0.04-1.7)	0.03-9.7	0.5-8.4	(0.5-2.0)	
IL-4	3.5	6.7	3.0	2.6	0.12
	(0.31-7.18)	(2.8-35.9)	(1.3-5.8)	(0.5-14.3)	
IL-8	553.8	150.1	787.4	354.9	0.14
	(142.1-2109.8)	(11.6-6428.9)	(270.3-2807.1)	(57.1-2397.7)	
TNFα	1.3	2.1	1.4	1.0	0.94
	(0.4-5.9)	(0.1-13.0)	(0.3-10.4)	(0.2-4.8)	

[1]Values are given as the median with 25th to 75th percentiles in parentheses.

Table 6.4. Cytokine production by mitogen stimulated PBMC from pigs fed diets containing Bt and isogenic maize (pg/mL)[1].

Cytokine	Isogenic	Bt	Isogenic/Bt	Bt/isogenic	*P*-value
Day 0					
IL-6	18.8	34.9	23.2	112.0	0.84
	(10.3-550.3)	(1.0-416.1)	(3.19-268.6)	(4.1-454.9)	
IL-4	24.0	38.8	34.3	65.1	0.96
	(6.8-148.9)	(13.9-82.5)	(1.8-205.9)	(4.33-125.8)	
IL-8	5438.2	10696.7	9516.9	11739.5	0.89
	(1435.5-22783.7)	(565.9-21144.7)	(469.4-21224.0)	(317.4-27328.6)	
TNFα	43.7	66.9	58.7	125.2	0.85
	(24.2-543.1)	(6.9-304.9)	(7.1-367.1)	(1.3-480.5)	
Day 30					
IL-6	23.3	30.6	19.6	65.9	0.50
	(8.7-91.6)	(11.1-49.7)	(11.3-66.9)	(4.7-134.6)	
IL-4	23.4	33.1	18.9	30.9	0.87
	(5.8-149.1)	(12.1-80.8)	(8.9-108.8)	(10.5-141.1)	
IL-8	4080.5	4009.9	2576.3	5985.2	0.46
	(789.3-13561.9)	(748.9-12187.9)	(373.3-9668.0)	(923.1-12288.2)	
TNFα	30.5	38.3	26.7	55.9	0.72
	(13.8-120.4)	(15.7-80.2)	(12.3-115.6)	(14.9-169.1)	
Day 100					

Cytokine	Isogenic	Bt	Isogenic/Bt	Bt/isogenic	*P*-value
IL-6	2.9	2.2	6.5	4.8	0.44
	(0.3-7.7)	(0.4-8.7)	(0.3-19.1)	(1.4-15.1)	
IL-4	3.2	9.9	7.6	2.7	0.17
	(1.3-8.7)	(5.1-33.8)	(0.03-38.8)	(1.0-54.2)	
IL-8	2266.3	1838.2	2447.0	3440.3	0.59
	(812.7-6732.5)	(345.6-9734.9)	(1383.6-10625.4)	(398.0-23765.5)	
TNFα	8.6	10.7	17.1	9.0	0.52
	(4.4-18.2)	(9.2-21.0)	(3.9-24.9)	(1.9-34.3)	

[1]Values are given as the median with 25th to 75th percentiles in parentheses.

Table 6.5. Effects of feeding Bt and isogenic maize on immune cell phenotypes of peripheral blood mononuclear cells. The $CD4^+$, $CD8^+$, $CD4^+CD8^+$ immune cell populations are given as proportions of $CD3^+$ T cells (%). All values are shown ± SE.

Day	Treatment					*P*-value		
	Isogenic	Bt	Isogenic/Bt	Bt/isogenic	Mean	Treatment	Time	Treatment × time
	$CD3^+$ T cells							
30	55.5 ± 6.63	53.5 ± 5.95	52.1 ± 6.76	49.8 ± 6.51	52.7 ± 3.11	0.94		
60	68.4 ± 6.92	60.4 ± 6.39	62.9 ± 6.71	56.9 ± 7.08	62.2 ± 3.36	0.76		
100	53.5 ± 6.63	43.1 ± 6.31	50.9 ± 7.30	54.9 ± 6.51	50.6 ± 3.35	0.51		
Mean	59.0 ± 4.75	52.3 ± 4.04	55.3 ± 4.56	53.9 ± 4.63		0.82	0.02	0.84
	$CD3^+CD4^+$ T cells							
30	16.1 ± 2.65	18.3 ± 2.36	19.7 ± 2.64	20.5 ± 2.48	18.7 ± 1.20	0.68		
60	5.2 ± 2.61	7.6 ± 2.44	8.6 ± 2.63	5.7 ± 2.59	6.7 ± 1.27	0.77		
100	10.8 ± 2.65	11.4 ± 2.45	11.0 ± 2.82	10.3 ± 2.48	10.9 ± 1.27	0.99		
Mean	10.7 ± 2.14	12.4 ± 1.89	13.1 ± 2.14	12.2 ± 2.02		0.90	0.001	0.82
	$CD3^+CD8^+$ T cells							
30	26.5 ± 2.37	26.7 ± 2.22	27.6 ± 2.46	31.3 ± 2.45	28.0 ± 1.15	0.45		
60	15.1 ± 2.36	12.8 ± 2.34	11.2 ± 2.46	12.2 ± 2.56	12.9 ± 1.24	0.70		
100	24.0 ± 2.37	20.2 ± 2.34	25.3 ± 2.66	22.1 ± 2.45	22.9 ± 1.24	0.52		
Mean	21.9 ± 1.62	19.9 ± 1.55	21.4 ± 1.68	21.9 ± 1.76		0.78	0.001	0.4

	$CD4^+CD8^+$ T cells							
30	$7.3^{b} \pm 1.21$	$8.8^{ab} \pm 1.14$	$11.8^{a} \pm 1.26$	$9.6^{ab} \pm 1.19$	9.4 ± 0.59	0.08		
60	4.0 ± 1.20	5.2 ± 1.21	3.5 ± 1.26	2.6 ± 1.26	3.8 ± 0.63	0.48		
100	3.9 ± 1.21	4.2 ± 1.20	4.7 ± 1.36	4.0 ± 1.19	4.2 ± 0.63	0.97		
Mean	5.1 ± 0.81	6.1 ± 0.79	6.7 ± 0.85	5.4 ± 0.81		0.51	0.001	0.29

[ab] Within a row means without a common superscript differ by $P < 0.10$ by means separation using Tukey-Kramer adjustment for multiple comparisons.

Table 6.6. Effects of feeding of Bt and isogenic maize on immune cell counts (×1000/μL) ± SE in growing pigs.

Day	Treatment					*P*-value		
	Isogenic	Bt	Isogenic/Bt	Bt/isogenic	Mean	Treatment	Time	Treatment × Time
	Leukocytes[1]							
30	18.0 ± 2.96	22.0 ± 2.44	25.1 ± 2.54	26.4 ± 2.71	22.9 ± 1.20	0.28		
60	19.5 ± 2.80	23.6 ± 2.72	25.8 ± 2.80	28.4 ± 2.71	24.3 ± 1.34	0.25		
100	22.7^{b} ± 2.70	28.6^{b} ± 2.56	29.5^{ab} ± 2.80	35.2^{a} ± 2.71	29.0 ± 1.26	0.06		
Mean	20.0 ± 2.26	24.7 ± 1.86	26.8 ± 2.01	30.0 ± 2.12		0.13	0.001	0.95
	Lymphocytes[2]							
30	12.9 ± 1.86	14.7 ± 1.73	15.4 ± 1.74	17.1 ± 1.80	15.0 ± 0.85	0.49		
60	13.5 ± 1.80	16.1 ± 1.93	18.5 ± 1.87	18.9 ± 1.180	16.7 ± 0.94	0.19		
100	13.8^{y} ± 1.71	15.4^{y} ± 1.81	17.9^{xy} ± 1.87	21.4^{x} ± 1.80	17.1 ± 0.87	0.04		
Mean	13.4^{c} ± 1.30	15.4^{bc} ± 1.28	17.2^{ab} ± 1.25	19.1^{a} ± 1.30		0.09	0.15	0.87
	Monocytes[3]							
30	1.7 ± 0.23	1.4 ± 0.22	1.7 ± 0.22	1.6 ± 0.22	1.61 ± 0.10	0.78		
60	1.6 ± 0.23	1.7 ± 0.25	1.9 ± 0.23	1.9 ± 0.22	1.79 ± 0.12	0.76		
100	2.2^{ab} ± 0.21	1.7^{b} ± 0.23	2.3^{a} ± 0.23	2.5^{a} ± 0.22	2.17 ± 0.11	0.09		
Mean	1.84 ± 0.14	1.60 ± 0.14	1.98 ± 0.13	2.00 ± 0.13		0.21	0.002	0.77
	Granulocytes							
30	2.6 ± 2.5	6.8 ± 1.84	8.5 ± 1.97	8.1 ± 2.17	6.5 ± 0.90	0.45		
60	3.5 ± 2.45	6.7 ± 2.02	5.6 ± 2.15	8.1 ± 2.17	6.0 ± 0.10	0.64		

Day	Treatment				Mean	*P*-value		
	Isogenic	Bt	Isogenic/Bt	Bt/isogenic		Treatment	Time	Treatment × Time
100	5.9 ± 2.39	12.4 ± 1.91	8.8 ± 2.15	11.9 ± 2.17	9.7 ± 0.94	0.24		
Mean	3.7 ± 2.14	8.7 ± 1.45	7.6 ± 1.64	9.3 ± 1.81		0.45	0.002	0.68

[1] Normal reference range in pigs = 11,000-22,000/µL (Thorn, 2000).

[2] Normal reference range in pigs = 4,300-13,600/µL (Thorn, 2000).

[3] Normal reference range in pigs = 200-2,200/µL (Thorn, 2000).

[xy] Within a row means without a common superscript differ by $P < 0.05$ by means separation using Tukey-Kramer adjustment for multiple comparisons.

[abc] Within a row means without a common superscript differ by $P < 0.10$ by means separation using Tukey-Kramer adjustment for multiple comparisons.

Table 6.7. Effects of feeding of Bt and isogenic maize on haematological parameters in growing pigs. All values are shown ± SE.

Day	Treatment				Mean	*P* -value		
	Isogenic	Bt	Isogenic/Bt	Bt/isogenic		Treatment	Time	Treatment × Time
	Erythrocyte, 1,000,000/µL							
30	6.5 ± 0.21	6.8 ± 0.18	6.5 ± 0.20	7.1 ± 0.23	6.7 ± 0.09	0.17		
60	7.3 ± 0.22	7.2 ± 0.20	7.2 ± 0.21	7.7 ± 0.23	7.4 ± 0.10	0.42		
100	7.2^{xy} ± 0.20	6.8^{y} ± 0.19	6.8^{y} ± 0.21	7.7^{x} ± 0.23	7.1 ± 0.09	0.02		
Mean	7.0 ± 0.16	6.9 ± 0.14	6.8 ± 0.16	7.5 ± 0.20		0.11	0.0001	0.43
	Haemogloblin, g/dL							
30	11.2 ± 0.55	11.8 ± 0.47	11.5 ± 0.49	12.0 ± 0.64	11.6 ± 0.22	0.79		
60	13.1 ± 0.54	12.8 ± 0.50	12.8 ± 0.52	13.4 ± 0.64	13.0 ± 0.24	0.85		
100	12.9 ± 0.53	12.4 ± 0.48	12.0 ± 0.52	13.5 ± 0.64	12.7 ± 0.23	0.28		
Mean	12.4 ± 0.47	12.3 ± 0.40	12.1 ± 0.43	13.0 ± 0.59		0.64	< 0.0001	0.48
	Hematocrit, L/L							
30	0.36 ± 0.02	0.39 ± 0.01	0.40 ± 0.02	0.40 ± 0.02	0.38 ± 0.01	0.40		
60	0.40 ± 0.02	0.41 ± 0.02	0.38 ± 0.02	0.42 ± 0.02	0.40 ± 0.01	0.95		
100	0.38 ± 0.02	0.38 ± 0.01	0.40 ± 0.02	0.41 ± 0.02	0.39 ± 0.01	0.61		
Mean	0.38 ± 0.01	0.39 ± 0.01	0.38 ± 0.01	0.4± 0.02		0.65	0.005	0.75
	MCV, fL							
30	54.5 ± 1.48	57.1 ± 1.27	55.8 ± 1.47	56.4 ± 1.81	56.0 ± 0.67	0.71		
60	53.9 ± 1.46	55.9 ± 1.31	56.3 ± 1.52	55.6 ± 1.77	55.4 ± 0.69	0.76		

Day	Treatment				Mean	*P*-value		
	Isogenic	Bt	Isogenic/Bt	Bt/isogenic	Mean	Treatment	Time	Treatment × Time
100	52.5 ± 1.45	54.8 ± 1.28	55.1 ± 1.54	54.2 ± 1.77	54.2 ± 0.68	0.68		
Mean	53.7 ± 1.39	55.9 ± 1.19	55.7 ± 1.41	55.4 ± 1.71		0.73	0.001	0.87
	MCH, g/dL							
30	16.9 ± 0.51	17.3 ± 0.44	17.6 ± 0.47	17.6 ± 0.54	17.3 ± 0.21	0.83		
60	17.4 ± 0.51	17.5 ± 0.46	17.7 ± 0.49	18.1 ± 0.54	17.7 ± 0.23	0.84		
100	17.7 ± 0.50	17.9 ± 0.45	17.7 ± 0.49	18.1 ± 0.54	17.8 ± 0.22	0.93		
Mean	17.3 ± 0.47	17.6 ± 0.41	17.6 ± 0.44	17.9 ± 0.50		0.91	0.01	0.81
	MCHC, %							
30	31.2 ± 0.51	30.4 ± 0.44	31.9 ± 0.51	31.2 ± 0.61	31.2 ± 0.24	0.23		
60	32.7 ± 0.52	31.4 ± 0.47	31.7 ± 0.52	32.1 ± 0.58	32.0 ± 0.25	0.38		
100	33.9 ± 0.49	32.8 ± 0.46	32.4 ± 0.56	33.0 ± 0.58	33.0 ± 0.24	0.38		
Mean	32.6 ± 0.44	31.6 ± 0.39	32.0 ± 0.46	32.1 ± 0.52		0.49	0.0001	0.49
	RDW, %							
30	20.4 ± 1.35	17.1 ± 1.11	19.8 ± 1.18	20.3 ± 1.59	19.4 ± 0.53	0.24		
60	21.2 ± 1.34	17.6 ± 1.15	19.7 ± 1.22	19.5 ± 1.59	19.5 ± 0.56	0.23		
100	21.6 ± 1.33	17.9 ± 1.13	21.7 ± 1.22	19.8 ± 1.59	20.2 ± 0.54	0.13		
Mean	21.1 ± 1.27	17.5 ± 1.04	20.4 ± 1.11	19.9 ± 1.53		0.21	0.07	0.36

Day	Treatment				Mean	*P* -value		
	Isogenic	Bt	Isogenic/Bt	Bt/isogenic		Treatment	Time	Treatment × Time
	Platelets, 1000/µL							
30	511.4 ± 67.82	572.1 ± 58.82	637.0 ± 70.29	664.9 ± 84.06	596.3 ± 28.76	0.49		
60	454.1 ± 65.60	547.1 ± 63.61	518.4 ± 77.37	545.5 ± 84.06	516.3 ± 31.54	0.80		
100	467.1 ± 63.77	465.4 ± 60.82	435.6 ± 77.37	476.5 ± 84.06	461.2 ± 30.05	0.98		
Mean	477.5 ± 55.91	528.2 ± 49.19	530.3 ± 65.08	562.3 ± 75.04		0.86	<0.0001	0.54
	MPV, fL							
30	8.6 ± 0.70	9.4 ± 0.60	9.9 ± 0.63	10.0 ± 0.89	9.5 ± 0.30	0.62		
60	9.0 ± 0.69	10.0 ± 0.63	9.5 ± 0.65	9.5 ± 0.89	9.5 ± 0.32	0.81		
100	7.9 ± 0.68	8.7 ± 0.61	8.2 ± 0.65	8.9 ± 0.89	8.4 ± 0.31	0.80		
Mean	8.5 ± 0.64	9.4 ± 0.55	9.2 ± 0.58	9.5 ± 0.85		0.82	< 0.0001	0.46

MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; RDW = red cell distribution width; MPV = mean platelet volume.

[xy] Within a row means without a common superscript differ by $P < 0.05$ by means separation using Tukey-Kramer adjustment for multiple comparisons.

[abc] Within a row means without a common superscript differ by $P < 0.10$ by means separation using Tukey-Kramer adjustment for multiple comparisons.

Table 6.8. Detection of endogenous maize and porcine genes and transgenic *cry1Ab* gene in the organs and blood of pigs fed Bt and isogenic maize[1].

Fragment amplified	Organ/Tissue															
	Liver				**Kidney**				**Spleen**				**White blood cells**			
Dietary treatment[2]	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Endogenous																
sh2 (maize)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
rubisco (maize)	6	5	4	5	4	3	3	5	3	2	5	4	2	2	0	1
sw (porcine)	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Transgenic																
cry1Ab-1 (211 bp; maize)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
cry1Ab-2 (149 bp; maize)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[1]Number of samples that tested positive for the gene of interest out of 10 samples analysed. One sample was tested per pig (*n* =10 pigs per treatment).

[2]Dietary treatments; 1) isogenic maize diet for 110 days, 2) Bt maize diet for 110 days, 3) isogenic maize diet for 30 days followed by Bt maize diet for 80 days and 4) Bt maize diet for 30 days followed by isogenic maize diet for 80 days.

Table 6.9. Detection of endogenous maize and porcine genes and transgenic *cry1Ab* gene in stomach, ileal, caecal and colon digesta of pigs fed Bt and isogenic maize diets [1].

Fragment amplified	Stomach				Ileum				Caecum				Colon			
Dietary treatment[2]	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Endogenous																
sh2 (maize)	9	9	9	8	0	0	0	0	0	0	0	0	0	0	0	0
rubisco (maize)	10	9	9	8	10	10	8	9	4	5	6	3	1	1	1	4
Transgenic																
cry1Ab-1 (211 bp)	0	9	9	0	0	2	1	0	0	0	0	0	0	0	0	0
cry1Ab-2 (149 bp)	0	9	9	0	0	0	0	0	0	0	0	0	0	0	0	0

[1]Number of samples that tested positive for the gene of interest out of 10 samples analysed. One sample was tested per pig (*n* =10 pigs per treatment).

[2]Dietary treatments; 1) isogenic maize diet for 110 days, 2) Bt maize diet for 110 days, 3) isogenic maize diet for 30 days followed by Bt maize diet for 80 days and 4) Bt maize diet for 30 days followed by isogenic maize diet for 80 days.

Table 6.10. Mean Cry1Ab protein concentrations (ng/mL) in gastrointestinal digesta of pigs fed Bt and isogenic maize[1].

Treatment	Isogenic	Bt	Isogenic/Bt	Bt/isogenic
Stomach	BLD[2] (0)	28.26 (90)	12.97 (80)	BLD (0)
Caecum	BLD (0)	7.92 (80)	11.45 (50)	BLD (0)
Colon	BLD (0)	32.74 (100)	18.05 (100)	BLD (0)

BLD = below the limit of detection (stomach; 0.82 ng/mL, caecum; 2.16 ng/mL, colon; 1.48 ng/mL).

[1]Values in parentheses correspond to the percentage of pigs within each treatment that tested positive for the Cry1Ab protein.

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7. The effect of feeding Bt MON810 maize to pigs for 110 days on intestinal microbiota

Buzoianu, S. G., Walsh, M. C., Rea, M. C., O'Sullivan, O., Crispie, F., Cotter, P. D., Ross, R. P., Gardiner, G. E. and Lawlor, P. G. (2012). The effect of feeding Bt MON810 maize to pigs for 110 days on intestinal microbiota. PLoS ONE 7: e33668.

7.1. Abstract

Objective: To assess the effects of feeding Bt MON810 maize to pigs for 110 days on the intestinal microbiota.

Methodology/Principal findings: Forty male pigs (~40 days old) were blocked by weight and litter ancestry and assigned to one of four treatments; 1) Isogenic maize-based diet for 110 days (Isogenic); 2) Bt maize-based diet (MON810) for 110 days (Bt); 3) Isogenic maize-based diet for 30 days followed by a Bt maize-based diet for 80 days (Isogenic/Bt); 4) Bt maize-based diet for 30 days followed by an isogenic maize-based diet for 80 days (Bt/Isogenic). Using culture-based methods, *Enterobacteriaceae*, *Lactobacillus* and total anaerobes were enumerated in the faeces on days 0, 30, 60 and 100 of the study and in ileal and caecal digesta on day 110. No differences were found between treatments for any of these counts at any time point. The relative abundance of caecal bacteria was also determined using high-throughput 16S rRNA gene sequencing. No differences were observed in any bacterial taxa between treatments, with the exception of the genus *Holdemania* which was more abundant in the caecum of pigs fed the isogenic/Bt treatment compared to pigs fed the Bt treatment (0.012 vs 0.003%; $P \leq 0.05$).

Conclusions/Significance: Feeding pigs a Bt maize-based diet for 110 days did not affect counts of any of the culturable bacteria enumerated in the faeces, ileum or caecum. Neither did it influence the composition of the caecal microbiota with the exception of a minor increase in the genus *Holdemania*. As the role of *Holdemania* in the intestine is still under investigation and no health abnormalities were observed, this change is not likely to be of clinical significance. These results indicate that feeding of Bt maize to pigs in the context of its influence on the porcine intestinal microbiota is safe.

7.2. Introduction

As the relationship between dietary habits and intestinal microbiota is becoming clearer with respect to a variety of illnesses, such as diabetes, obesity and inflammatory bowel disease (Cani and Delzenne, 2009; Sekirov *et al*., 2010; Vrieze *et al*., 2010; Buddington and Sangild, 2011), consumers are becoming increasingly aware of the impact of certain foodstuffs on the intestinal microbiota (Saarela *et al*., 2002). This comes at a time when the controversy surrounding genetically modified (GM) food and animal feed is far from being resolved (Gaskell, 2005; Martinez-Poveda *et al*., 2009; Rollin *et al*., 2011), thus fuelling potential consumer concerns about safety in relation to the intestinal microbiota.

Although Bt maize expressing the insecticidal Cry1Ab protein has been thoroughly tested during pre-market risk assessment and has been approved for inclusion in food and feed (EFSA, 2009), studies investigating its effects on animal health and production have not provided a definitive answer as to the safety of Bt maize (Finamore *et al*., 2008; Domingo and Giné Bordonaba, 2011).

As studies investigating the antimicrobial properties of the Cry1Ab protein *in vitro* have yielded contradictory results (Koskella and Stotzky, 2002; Yudina *et al*., 2007), *in vivo* studies are required to clarify this issue. Several groups, including ours, have investigated the effect of Bt maize on the intestinal microbiota in short-term pig-feeding studies (Chapter 4), cattle studies (Einspanier *et al*., 2004; Wiedemann *et al*., 2007) and long-term sheep studies (Trabalza-Marinucci *et al*., 2008). However, short-term studies may fail to adequately address consumer concerns, which are mainly related to the safety of Bt maize following long-term consumption. Also, pigs are a more suitable model for humans than ruminants, both physiologically and anatomically, as well as in terms of composition of the intestinal microbiota (Moughan *et al*., 1992). Therefore, feeding studies in pigs are more likely to provide an accurate insight into the potential impact of Bt maize in humans. In addition, no data are available with respect to the potential carry-over effect of Bt maize following its exclusion from the diet.

As culture-based microbiological analysis is becoming increasingly outdated, novel, more powerful methods, such as high-throughput 16S rRNA gene sequencing are becoming increasingly popular for analysis of the intestinal microbiota. Such technologies offer the

potential to generate large amounts of data (Rothberg and Leamon, 2008; Tringe and Hugenholtz, 2008) and may prove to be a useful tool for testing the safety of Bt maize.

Therefore, the objective of this study was to investigate the effect of long-term (110 days) feeding of Bt maize to pigs on the intestinal microbiota using both culture-dependent and independent methods. In addition, we investigated the effect of changing diets following 30 days of feeding to assess the possibility of a carry-over effect as a result of exposure to Bt maize early in life.

7.3. Materials and methods

Ethical approval

The pig study complied with European legislation concerning minimum standards for pig protection (European Union Council Directive 91/630/EEC) and the protection of animals kept for farming purposes (European Union Council Directive 98/58/EC). Ethical approval was obtained from the ethics committees of Teagasc and Waterford Institute of Technology. An experimental license (number B100/4147) was obtained from the Irish Department of Health and Children.

Animals and experimental design

Forty crossbred (Large White × Landrace) entire male pigs were weaned at ~28 days of age and allowed a 12 day adaptation period. During this adaptation period, pigs were provided with *ad libitum* access to a non-GM starter diet. Pigs were then blocked by weight and ancestry and, within block, randomly assigned to one of four treatments at ~40 days of age (n=10 pigs/treatment); 1) isogenic maize-based diet (isogenic parent line; Pioneer PR34N43) for 110 days (isogenic); 2) Bt maize-based diet (Bt; Pioneer PR34N44; event MON810) for 110 days (Bt); 3) Isogenic maize-based diet for 30 days followed by Bt maize-based diet for 80 days (isogenic/Bt); and 4) Bt maize-based diet for 30 days followed by isogenic maize-based diet for 80 days (Bt/isogenic). The duration of the study was 110 days. Pigs were individually housed in identical pens in similar climatically controlled rooms and were allowed *ad libitum* access to water and feed. For the duration of the study, all dietary treatments were equally represented in each room to remove any variation due to

environmental conditions. Pigs showing signs of ill health were treated as appropriate and all veterinary treatments were recorded.

Maize and diets

In accordance with established guidelines (OECD, 2002; Hartnell *et al.*, 2007; EFSA, 2008), the maize lines used in the present study were MON810 maize and its closest comparator, the isogenic maize from which it was derived. Furthermore, to ensure similar growing conditions, Bt MON810 maize and its isogenic counterpart (PR34N44 and PR34N43, respectively; Pioneer Hi-Bred, Seville, Spain) were grown in neighbouring plots in Valtierra, Navarra, Spain by independent farmers. The Bt and isogenic control maize were purchased by the authors from the tillage farmers for use in this animal study. Samples from the isogenic and Bt maize were tested for the presence of the *cry1Ab* transgene and for the presence of pesticide contaminants and mycotoxins as described in Chapter 2. Proximate composition and amino acid content of the maize and diets, as well as carbohydrate composition of the maize, were also determined as described in Chapter 2.

All diets were formulated to meet or exceed the National Research Council requirements for pigs (NRC, 1998) and were manufactured as outlined in Chapter 2. The isogenic and Bt diets were formulated with identical maize inclusion rates. As a precautionary measure a mycotoxin binder was included in all diets used in the study (Mycosorb™, Alltech, Dunboyne, Co. Meath, Ireland). A succession of diets was fed to pigs according to their age group as follows: link diets from day 0 to 30, weaner diets from day 31 to 60, finisher 1 diets from day 61 to 100 and finisher 2 diets from day 101 to 110. The composition of the diets has previously been reported in Chapter 5.

Faecal and caecal and ileal digesta sampling and microbiological analysis

Faecal samples were collected in sterile containers by rectal stimulation from 10 pigs/treatment on days 0, 30, 60 and 100. Digesta samples from the caecum (terminal tip of the caecum) and ileum (15 cm before the ileo-caecal junction) were obtained following euthanasia on day 110 when pigs were ~150 days of age. The last meal was provided 3 hours before euthanasia. Digesta samples were removed aseptically, placed in sterile plastic containers and stored at 4°C in sealed anaerobic jars containing Anaerocult™ A gas packs (Merck, Darmstadt, Germany) until analysis (within 12 hours). Enumeration of

Lactobacillus and *Enterobacteriaceae* from individual faecal samples and ileal and caecal digesta was performed as described by Gardiner *et al.* (2004). To inhibit growth of yeasts and moulds, nystatin (Sigma Aldrich Ireland Ltd., Wicklow, Ireland) was added to the *Lactobacillus* selective medium at a concentration of 50 units/mL. Total anaerobic bacteria from individual faecal, ileal and caecal samples were enumerated as previously described by Rea *et al.* (2007).

DNA extraction and PCR

The QIAamp DNA Stool kit (Qiagen, Crawley, West Sussex, UK) was used to extract total DNA from individual caecal digesta samples, according to the manufacturer's instructions with some modifications. To increase DNA yield an additional bead beating step was included (Rantakokko-Jalava and Jalava, 2002) and the initial lysis temperature was increased from 70 to 90°C. For PCR, forward and reverse primers targeting the V4 region of the bacterial 16S rRNA gene were used, as previously described by Murphy *et al.* (2010). These primers are predicted to bind to 96.4% of all 16S rRNA genes (Murphy *et al*., 2010). To allow detection of individual amplicons from samples that were pooled at the sequencing stage, unique molecular identifiers were incorporated into the forward primer (Table 7.6) (Murphy *et al*., 2010).

Each PCR reaction contained 2 µL of template DNA, 200 nM of forward primer, and 50 nM of each of the four reverse primers, 25 µL of Biomix Red (Bioline, London, UK) and 21 µL of sterile double distilled water. Each set of PCR reactions contained a negative control in which template DNA was replaced with sterile double distilled water and a positive control containing previously amplified caecal bacterial DNA. The PCR cycle started with denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation (94°C for 1 minute), annealing (52°C for 1 minute) and elongation (72°C for 1 minute). A final elongation step was performed at 72°C for 2 minutes. Amplicons were detected by UV visualization following electrophoresis in 1.5% agarose gels containing 0.3 ng/µL ethidium bromide. PCR products were purified using the High Pure PCR product purification kit (Roche Applied Science, Mannheim, Germany). DNA was quantified using a NanoDrop 3300 spectrophotometer (Fisher Scientific, DE, USA) following staining using the Quant-it Pico Green dsDNA kit (Invitrogen Ltd. Paisley, UK).

16S rRNA gene sequencing

Sequencing was performed on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd., Burgess Hill, West Sussex), according to manufacturer's instructions. De-noising, sequence length cut-off, quality score cut-off, checking of sequencing reads and assignment to NCBI taxonomies were performed as previously described by Murphy *et al.* (2010). Principal coordinate analysis was performed using the QIIME software tool (Caporaso *et al*., 2010). Population indices such as Chao1 richness estimation, Shannon diversity and Good's coverage were computed at the genus level using MOTHUR software (Schloss *et al*., 2009).

The number of reads assigned to each caecal bacterial taxonomical rank was divided by the total number of reads assigned to the highest rank (phylum) to obtain the relative abundance values. Therefore, relative abundance is presented as a ratio, with values ranging from 0 (0%) to 1 (100%).

Statistical analysis

To ensure normality, bacterial counts and relative abundance values were log transformed to the base 10. Faecal bacterial counts were analysed with sampling day as a repeated measure using the MIXED procedure in SAS version 9.1.3 with day 0 values as a covariate in the model. Fixed effects included treatment and sampling day, while block was included as a random effect in the model. The *slice* option was used to determine significance for simple main effects. Ileal and caecal bacterial counts, as well as the relative abundance data, which were normally distributed were analysed as a complete randomized block design using the GLM procedure in SAS. Data which were not normally distributed following log transformation were analysed using the non-parametric Kruskal-Wallis test within the NPAR1WAY procedure in SAS. In this case, data were presented as treatment median values and the 5-95th percentiles. Statistical significance was considered for $P \leq 0.05$ and tendencies were reported up to $P = 0.10$. Means separation was performed using the Tukey-Kramer post-hoc adjustment for normally distributed data. For data that were analysed using the Kruskal-Wallis non-parametric test, significance between treatments was determined by using the GLM procedure in SAS on the Wilcoxon signed ranks test and means separation was performed using the Tukey-Kramer post-hoc adjustment. For all analyses the individual pig was the experimental unit.

7.4. Results

At approximately day 70 of the study, two pigs from the isogenic treatment and one from the Bt treatment displayed symptoms of diarrhoea and were treated with injectable Enrofloxacin (2.5 mg/kg body weight) for three consecutive days. As a result, data from these pigs were not included in the analysis.

Maize and diets

Both the proximate composition and the amino acid content of the isogenic and the Bt diets were similar (Chapter 5). The Bt maize used in this study was previously found to have a higher starch and sucrose content and a lower content of enzyme resistant starch than the isogenic maize (Chapter 2); however, amino acid profile and proximate composition were within the normal range for maize (ILSI, 2012; OECD, 2002; Chapter 2). The concentrations of mycotoxins and pesticides in the isogenic and Bt maize were previously reported to be below the maximum allowable limits for animal feedstuffs (Chapter 2).

Culture-based investigation of the effects of feeding Bt maize on the intestinal microbiota of pigs

No significant differences were found between the four dietary treatments for faecal bacterial counts of *Enterobacteriaceae*, *Lactobacillus* or total anaerobes on day 30, 60 or 100 (Table 7.1). *Enterobacteriaceae* counts increased from day 30 to 100 ($P < 0.05$) and total anaerobe counts decreased over time for all treatments ($P < 0.05$). Similar to faecal counts, ileal and caecal counts of *Enterobacteriaceae*, *Lactobacillus* and total anaerobes did not differ between treatments (Table 7.2).

Effects of feeding Bt maize on the relative abundance of the caecal microbiota

Raw sequencing data has been uploaded to http://www.ebi.ac.uk. A total of 151,608 16S rRNA reads (239 bp long) were generated from high-throughput sequencing corresponding to an average of 4097 reads per caecal sample (ranging from 2251 to 10296 reads per sample). From this, 138,854 (91.6%) were assigned at the phylum level, 79,368

(52.4%) were assigned at the family level and 58,914 (38.9%) were assigned at the genus level. Rarefaction curves were similar between treatments (Figures 7.4, 7.5, 7.6 and 7.7). The Shannon diversity index [an unweighted measure of the number of species present in a community (Krause *et al*., 1995)] was similar across all four treatments (Table 7.3). Likewise, Good's coverage and Chao 1 richness estimator were similar between treatments (Table 7.3). No clustering corresponding to a specific treatment group was observed following beta diversity analysis (Figure 7.8).

A total of 15 phyla were detected in the caecum of the 150 day old pigs with *Firmicutes* being the most abundant (61.5%) followed by *Bacteroidetes* (19.6%) and *Proteobacteria* (8.3%; Figure 7.1). Together, these three major phyla accounted for 89% of the porcine caecal bacteria, while the other 12 phyla made up the remaining 11% (Table 7.5). No difference between treatments was observed with respect to the relative abundance of bacterial phyla in the caecum.

The 16S rRNA reads from the caecum of 150 day old pigs were assigned to a total of 36 families. The porcine caecal microbiota was dominated by *Clostridiaceae* (9.6%), *Prevotellaceae* (9.1%), *Veillonellaceae* (6.2%), *Ruminococcaceae* (5.2%) and *Bacteroidaceae* (3.8%; Figure 7.2). No significant differences in relative abundance were detected between treatments for any of the bacterial families. However, although not reaching statistical significance, *Veillonellaceae* tended to be less abundant in the caecum of pigs fed the isogenic/Bt treatment compared to pigs fed the Bt/isogenic treatment (3.7 vs. 9%; P = 0.08; Figure 7.2) but was not different from the other two treatments. Also, *Succinivibrionaceae* tended to be lower in the caecum of pigs fed the isogenic/Bt treatment compared to pigs fed the isogenic, Bt and the isogenic/Bt treatments (P = 0.08; Table 7.4) but this difference was not statistically significant. In contrast, *Erysipelotrichaceae*, although not significantly different, tended to be more abundant in the caecum of pigs fed the isogenic/Bt treatment compared to pigs fed the Bt treatment (P = 0.07; Table 7.4) but was not different from the other two treatments. There was no effect of feeding Bt maize to pigs on any of the remaining families identified (Table 7.5).

Sequencing analysis identified 49 genera within the caecum of 150 day old pigs. Almost one quarter of the sequenced bacteria in the pig caecum (23.9%) were comprised of the genera *Clostridium* (9.1%), *Prevotella* (6.2%), *Oscillospira* (3.8%), *Acidaminococcus* (3.7%) and *Peptococcus* (1.1%; Figure 7.3). No significant differences were observed between treatments at the genus level, with the exception of *Holdemania* which was more

abundant in the caecum of pigs fed the isogenic/Bt treatment than in the caecum of pigs fed the Bt treatment ($P \leq 0.05$; Table 7.4) but was not different to pigs fed the isogenic or the Bt/isogenic treatments. Although statistical significance was not reached, there was a tendency for lower relative abundance of *Succinivibrio* in the caecum of pigs fed the isogenic/Bt treatment compared to all other treatments ($P = 0.07$; Table 7.4). *Acidaminococcus* also tended to be less abundant in the caecum of pigs fed the isogenic/Bt treatment compared to pigs fed the Bt/isogenic treatment ($P = 0.09$; Figure 7.3) but was not different from the isogenic or Bt treatments. *Eubacterium* were only detected in pigs fed the isogenic/Bt and Bt/isogenic treatments and not in those fed the Bt and isogenic treatments. Consequently, although not statistically significant, *Eubacterium* tended to have greater abundance in the caecum of pigs fed the Bt/isogenic treatment compared to pigs fed the isogenic and the Bt treatments ($P = 0.09$; Table 7.4) but was not different from pigs fed the isogenic/Bt treatment.

7.5. Discussion

When considering the impact of GM food and feed on the intestinal microbiota, horizontal transfer of the transgene to the microbiota is one of the major safety concerns (EFSA, 2008). However, gene transfer was not the focus of the present study and in fact, we have investigated it in a previous study (Buzoianu *et al*., unpublished). The purpose of the present study was to investigate effects of the Bt maize on microbial community structure within the porcine intestinal tract. To our knowledge, this is the first pig feeding study to evaluate the effects of long-term exposure of Bt maize on intestinal microbial communities as assessed by high-throughput 16S rRNA gene sequencing. A limited number of studies have investigated the effect of short-term feeding of Bt maize on porcine (Chapter 4) or bovine or intestinal microbiota using molecular techniques, such as 16S rRNA gene sequencing (Einspanier *et al*., 2004) or real-time PCR (Wiedemann *et al*., 2007). However, these studies were conducted in weanling pigs fed Bt maize for only 31 days (Chapter 4) or in mature cattle fed Bt maize for four weeks (Einspanier *et al*., 2004) or 11 days (Wiedemann *et al*., 2007). Such studies provide valuable insight into the effect of Bt maize on both an unstable microbial community in young animals or an established climax community in mature animals (Einspanier *et al*., 2004; Wiedemann *et al*., 2007). However, evidence suggesting that six weeks of exposure are required for the intestinal

microbiota to adapt to feed structure (Castillo *et al*., 2007) highlights the limitations of short-term feeding studies. Other potential limitations relate to fears expressed by some consumers that health effects arising from exposure to GM crops may only become evident following long-term exposure (Dona and Arvanitoyannis, 2009). In addition, differences in digestive physiology between humans and ruminants (Cummings and Macfarlane, 1991) make the latter less suitable as a human model. By conducting a long-term feeding study throughout the pig's entire productive life we have increased the potential to detect discrete changes in intestinal microbiota that may not be obvious following short-term exposure.

The absence of an effect of Bt maize on faecal, ileal and caecal counts of *Enterobacteriaceae*, *Lactobacillus* and total anaerobes is in agreement with previous findings by our group following 31 days of Bt maize exposure in weanling pigs (Chapter 4). Similarly, Bt rice, expressing the Cry1Ab protein was found to have no impact on faecal coliforms, *Lactobacillus* or total anaerobes in rats following 90 days of consumption (Schrøder *et al*., 2007). However, in contrast to our findings, the latter study found that coliform counts increased in the ileum and counts of bifidobacteria decreased in the duodenum of rats fed Bt rice.

Faecal *Lactobacillus* counts were found to be stable over time in the present study. This is in agreement with published literature which indicates that lactobacilli reach a stable community after the first week of life (Naito *et al*., 1995; Castillo *et al*., 2007; Walter, 2008). Likewise, the rise in *Enterobacteriaceae* counts over time reported in the present study is in agreement with previous studies which found lower counts during the first 30 days post-weaning (Gardiner *et al*., 2004) and higher counts as pigs mature (Poletto *et al*., 2009). Similar to published findings for pigs (Jensen and Jorgensen, 1994; Franklin *et al*., 2002; Canibe *et al*., 2005), faecal counts of total anaerobes remained high throughout the study.

Our findings also agree with previous work from our group (Chapter 4) and others (Poroyko *et al*., 2010; Vahjen *et al*., 2010; Kim *et al.* 2011) in that the porcine caecum was dominated by the phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria*, although in previous studies the proportions differed depending on age and diet. Similarly, in humans, *Firmicutes* and *Bacteroidetes* comprise the "core" bacteria of the large intestine (Eckburg *et al*., 2005; Flint *et al*., 2007) and have been used as biomarkers for the metabolic status of the host (Armougom *et al*., 2009; Larsen *et al*., 2010). In accordance with our previous research in 60 day-old pigs (Chapter 4) and with results from Kim *et al.* (2011) in 22 week-

old pigs, *Clostridiaceae* and *Prevotellaceae* were the dominant families in the pig caecum in the present study. Similarly, Leser *et al.* (2002) also found that *Clostridiaceae*, *Prevotellaceae* and *Bacteroides* dominated the digestive tract of pigs of different ages fed different diets. *Clostridia* and *Prevotellaceae* have also been recovered in high numbers from human intestinal and faecal samples (Chassard *et al*., 2008; Larsen *et al*., 2010; Agans *et al*., 2011; Gosalbes *et al*., 2011). These similarities underline the value of the pig as a model for predicting the influence of Bt maize on the major taxa of the human intestinal microbiota.

The composition of the caecal microbiota was similar for the isogenic maize control treatment and the Bt maize treatment in the present study. Similarly, real-time PCR analysis revealed no effects of feeding Bt176 maize silage on any of six ruminal bacterial species in cows (Wiedemann *et al*., 2007). Another study also demonstrated that feeding Bt176 maize silage to cows for four weeks did not influence the composition of ruminal microbiota as assessed by 16S rRNA gene sequencing (Einspanier *et al*., 2004). Likewise, total ruminal amylolytic and cellulolytic bacterial populations, as well as protozoal numbers and composition and microbial metabolites did not differ between sheep fed Bt176 or non-GM maize for three years (Trabalza-Marinucci *et al*., 2008).

The only statistically significant difference that was observed within the caecal microbiota was that the genus *Holdemania* was more abundant in pigs fed the isogenic/Bt treatment compared to pigs fed the Bt treatment. Although the presence of *Holdemania* at low relative abundance has been established in the porcine intestine (Leser *et al*., 2002; Kim *et al*.), the role of this genus in the intestine is not fully understood. In the present study, the difference in caecal *Holdemania* abundance was not associated with any effects on small intestinal weight and morphology (Chapter 5). Furthermore, none of the minor differences in blood biochemistry observed between treatments in these pigs (Chapter 5) could be related to changes in the intestinal microbiota. Therefore, although statistically significant, the difference in caecal *Holdemania* abundance observed in the present study is not believed to be of biological significance or to have a major impact on pig health. The increased abundance in caecal *Holdemania* is believed to be related to the time at which maize source was changed rather than a response to feeding Bt maize *per se*.

In conclusion, no changes were observed within the caecal microbial community of healthy pigs following long-term exposure to Bt maize or following a cross-over between isogenic and Bt maize after 30 days of feeding, with the exception of *Holdemania*. The fact

that no difference between the Bt and isogenic treatments was observed provides evidence that the intestinal microbiota are tolerant to Bt maize and substantiates our previous findings that Bt maize is safe for long-term consumption. Changing maize source following 30 days of feeding did not affect the intestinal microbiota with the exception of *Holdemania* which indicates the absence of a residual effect following Bt maize exposure early in life. Also, neither Bt maize nor changing maize source affected counts of faecal *Enterobacteriaceae*, *Lactobacillus* or total anaerobes at any time during the study. These findings indicate that Bt maize is well tolerated by the 'normal' intestinal microbiota of healthy pigs and fails to alter its composition, at least in the caecum, even after long-term exposure. However, as stress is known to affect the response to stimuli, future studies are needed to investigate potential effects of Bt maize in physiologically stressed animals.

Table 7.1. Effect of feeding isogenic or Bt maize-based diets to pigs from 12 days post-weaning for 110 days on faecal bacterial counts[1].

	Treatments						*P*-value[6]		
Day	**Isogenic[2]**	**Bt[3]**	**Isogenic/Bt[4]**	**Bt/isogenic[5]**	**Mean**	**SEM**	**Treatment**	**Time**	**Treatment × Time**
			Enterobacteriaceae						
30	5.47	6.08	5.45	5.40	5.60	0.192	0.57		
60	6.36	6.74	6.40	6.60	6.53	0.205	0.94		
100	7.50	7.14	7.29	7.15	7.27	0.202	0.95		
Mean	6.44	6.65	6.38	6.39		0.417	0.92	<0.0001	0.61
			Lactobacillus						
30	8.47	8.71	8.75	8.61	8.63	0.153	0.94		
60	7.97	8.63	8.52	8.28	8.35	0.163	0.58		
100	8.25	8.45	8.54	8.50	8.43	0.163	0.95		
Mean	8.23	8.60	8.60	8.47		0.314	0.79	0.27	0.97
			Total anaerobes						
30	9.79	9.86	9.96	9.86	9.87	0.097	0.95		
60	9.15	9.66	9.73	9.50	9.51	0.106	0.33		
100	9.18	9.49	9.27	9.47	9.35	0.103	0.77		
Mean	9.37	9.67	9.65	9.61		0.191	0.68	0.0002	0.71

[1]Bacterial counts are presented as means of $\log_{10}$ CFU g^{-1} wet weight.

[2]Isogenic - isogenic parent line maize-based diet for 110 days (n = 8 pigs/treatment).

[3]Bt - Bt maize-based diet for 110 days (n = 9 pigs/treatment).

[4]Isogenic/Bt - isogenic maize-based diet for 30 days followed by a Bt maize-based diet for 80 days (n = 10 pigs/treatment).

[5]Bt/isogenic - Bt maize-based diet for 30 days followed by a isogenic maize-based diet for 80 days (n = 10 pigs/treatment).

[6]Significance values computed using the *mixed* procedure in SAS.

Table 7.2. Effect of feeding isogenic or Bt maize-based diets to pigs from 12 days post-weaning for 110 days on caecal and ileal bacterial counts[1].

	Treatments					
	Isogenic[2]	**Bt[3]**	**Isogenic/Bt[4]**	**Bt/isogenic[5]**	**SEM**	***P*-value[6]**
			Enterobacteriaceae			
Ileal	8.34	7.29	7.63	6.64	0.423	0.24
Caecal	7.96	7.21	7.34	7.04	0.292	0.22
			Lactobacillus			
Ileal	6.11	6.08	6.96	5.91	0.500	0.21
Caecal	7.94	7.09	7.14	7.28	0.452	0.88
			Total anaerobes			
Ileal	8.59	8.03	8.14	7.83	0.322	0.55
Caecal	9.13	9.22	8.91	9.02	0.143	0.33

[1] Bacterial counts are presented as means of log_{10} CFU g^{-1} wet weight.

[2]Isogenic - isogenic parent line maize-based diet for 110 days (n = 8 pigs/treatment).

[3]Bt - Bt maize-based diet for 110 days (n = 9 pigs/treatment).

[4]Isogenic/Bt - isogenic maize-based diet for 30 days followed by a Bt maize-based diet for 80 days (n = 10 pigs/treatment).

[5]Bt/isogenic - Bt maize-based diet for 30 days followed by a isogenic maize-based diet for 80 days (n = 10 pigs/treatment).

[6]Significance values computed using a one-way ANOVA in SAS.

Table 7.3. Estimation of bacterial diversity at 97% similarity in the caecum of pigs fed isogenic or Bt maize-based diets[1,2].

	Treatments			
	Isogenic[3]	**Bt[4]**	**Isogenic/Bt[5]**	**Bt/isogenic[6]**
Chao 1 richness estimation	1238	1390	1451	1388
Shannon diversity index	5.56	5.90	5.77	5.77
Good's coverage	0.91	0.91	0.91	0.91

[1]Estimates of diversity were computed using MOTHUR software.

[2]Data presented as treatment means.

[3]Isogenic - isogenic parent line maize-based diet was fed for 110 days (n = 8 pigs/treatment).

[4]Bt - Bt MON810 maize-based diet was fed for 110 days (n = 9 pigs/treatment).

[5]Isogenic/Bt - isogenic maize-based diet was fed for 30 days followed by a Bt MON810 maize-based diet for 80 days (n = 10 pigs/treatment).

[6]Bt/isogenic - Bt MON810 maize-based diet was fed for 30 days followed by a isogenic maize-based diet for 80 days (n = 10 pigs/treatment).

Table 7.4. Effect of feeding isogenic or Bt maize-based diet to pigs from 12 days post weaning for 110 days on the relative abundance of caecal bacterial taxa in pigs[1].

	Treatments					
	Isogenic[2]	**Bt[3]**	**Isogenic/Bt[4]**	**Bt/isogenic[5]**	**5-95th percentile**	***P*-value[6]**
Family						
Succinivibrionaceae	0.012^{x}	0.019^{x}	0.003^{y}	0.012^{x}	0-0.14	0.08†
Erysipelotrichaceae	0.020^{xy}	0.010^{y}	0.022^{x}	0.018^{xy}	0.003-0.037	0.07*
Genus						
Succinivibrio	0.012^{x}	0.019^{x}	0.003^{y}	0.012^{x}	0-0.10	0.07†
Eubacterium	0^{y}	0^{y}	0.0017^{xy}	0.0022^{x}	0-0.008	0.09†
Holdemania	0.005^{ab}	0.003^{b}	0.012^{a}	0.007^{ab}	0-0.03	0.05†

[1]Data reported as median values.

[2]Isogenic - isogenic parent line maize-based diet for 110 days (n = 8 pigs/treatment).

[3]Bt - Bt maize-based diet for 110 days (n = 9 pigs/treatment).

[4]Isogenic/Bt - isogenic maize-based diet for 30 days followed by a Bt maize-based diet for 80 days (n = 10 pigs/treatment).

[5]Bt/isogenic - Bt maize-based diet for 30 days followed by a isogenic maize-based diet for 80 days (n = 10 pigs/treatment).

[6]Significance values computed using a one-way ANOVA (*) or the Kruskal-Wallis non parametric test (†) in SAS.

Within a row, medians with different superscripts are different at $^{a,b}P \leq 0.05$ or $^{x,y}P < 0.10$.

Table 7.5. Bacterial taxa detected in the caecum of ~150 day-old pigs[1].

	Isogenic[2]	Bt[3]	Isogenic/ Bt[4]	Bt/isogenic[5]
Phylum				
Firmicutes	0.636	0.624	0.549	0.618
Bacteroidetes	0.169	0.207	0.206	0.234
Proteobacteria	0.080	0.066	0.084	0.086
Spirochaetes	0.011	0.008	0.006	0.012
Verrucomicrobia	0.000	0.007	0.017	0.006
Planctomycetes	0.000	0.002	0.001	0.003
Lentisphaerae	0.000	0.001	0.000	0.002
Fusobacteria	0.000	0.000	0.000	0.001
Cyanobacteria	0.000	0.000	0.000	0.000
Deferribacteres	0.000	0.000	0.000	0.000
Fibrobacteres	0.000	0.000	0.000	0.000
Actinobacteria	0.000	0.000	0.000	0.000
Tenericutes	0.000	0.000	0.000	0.000
Chlamydiae	0.000	0.000	0.000	0.000
Elusimicrobia	0.000	0.000	0.000	0.000
Family				
Clostridiaceae	0.097	0.096	0.093	0.088
Prevotellaceae	0.060	0.084	0.104	0.111
Veillonellaceae	0.070	0.057	0.037	0.090
Ruminococcaceae	0.054	0.056	0.043	0.064
Bacteroidaceae	0.030	0.033	0.048	0.036
Erysipelotrichaceae	0.021	0.010	0.022	0.018
Desulfovibrionaceae	0.004	0.012	0.022	0.013
Peptococcaceae	0.011	0.007	0.010	0.010
Succinivibrionaceae	0.012	0.019	0.003	0.012
Spirochaetaceae	0.011	0.008	0.004	0.012
Lachnospiraceae	0.003	0.007	0.005	0.005
Enterobacteriaceae	0.009	0.006	0.004	0.005
Alcaligenaceae	0.005	0.004	0.004	0.005

	Isogenic[2]	Bt[3]	Isogenic/ Bt[4]	Bt/isogenic[5]
Eubacteriaceae	0.003	0.004	0.004	0.005
Streptococcaceae	0.004	0.004	0.005	0.004
Lactobacillaceae	0.000	0.003	0.005	0.003
Verrucomicrobia subdivision 5	0.000	0.007	0.001	0.006
Rhodospirillaceae	0.000	0.000	0.000	0.000
Acetobacteraceae	0.000	0.000	0.000	0.000
Rickettsiaceae	0.000	0.000	0.000	0.000
Pasteurellaceae	0.000	0.000	0.001	0.000
Helicobacteraceae	0.000	0.001	0.000	0.001
Campylobacteraceae	0.000	0.000	0.000	0.000
Oxalobacteraceae	0.000	0.000	0.000	0.000
Rhodocyclaceae	0.000	0.000	0.000	0.000
Porphyromonadaceae	0.000	0.000	0.000	0.000
Clostridiales Family XIII Incertae Sedis	0.000	0.000	0.000	0.000
Fusobacteriaceae	0.000	0.000	0.000	0.000
Fibrobacteraceae	0.000	0.000	0.000	0.000
Bifidobacteriaceae	0.000	0.000	0.000	0.000
Coriobacteriaceae	0.000	0.000	0.000	0.000
Verrucomicrobia	0.000	0.000	0.000	0.000
Anaeroplasmataceae	0.000	0.000	0.000	0.000
Mycoplasmataceae	0.000	0.000	0.000	0.000
Chlamydiaceae	0.000	0.000	0.000	0.000
Victivallaceae	0.000	0.000	0.000	0.000
Genus				
Clostridium	0.091	0.094	0.086	0.084
Prevotella	0.054	0.056	0.060	0.066
Oscillospira	0.030	0.045	0.035	0.034
Acidaminococcus	0.046	0.041	0.026	0.056
Peptococcus	0.011	0.011	0.010	0.010
Desulfovibrio	0.004	0.010	0.015	0.011

	Isogenic[2]	Bt[3]	Isogenic/ Bt[4]	Bt/isogenic[5]
Succinivibrio	0.012	0.019	0.003	0.012
Holdemania	0.005	0.003	0.012	0.007
Treponema	0.010	0.008	0.004	0.006
Faecalibacterium	0.007	0.007	0.004	0.006
Sutterella	0.005	0.004	0.004	0.005
Turicibacter	0.004	0.003	0.003	0.006
Streptococcus	0.003	0.004	0.003	0.004
Ruminococcus	0.002	0.005	0.001	0.004
Lactobacillus	0.000	0.003	0.005	0.003
Eubacterium	0.000	0.000	0.002	0.002
Bacteroides	0.001	0.002	0.008	0.001
Rickettsia	0.000	0.000	0.000	0.000
Anaerobiospirillum	0.000	0.000	0.000	0.000
Actinobacillus	0.000	0.000	0.000	0.000
Helicobacter	0.000	0.001	0.000	0.001
Campylobacter	0.000	0.000	0.000	0.000
Spirochaeta	0.000	0.000	0.000	0.000
Odoribacter	0.000	0.000	0.000	0.000
Lactococcus	0.000	0.000	0.000	0.000
Acetitomaculum	0.000	0.000	0.000	0.000
Butyrivibrio	0.000	0.000	0.000	0.000
Lachnospira	0.000	0.000	0.000	0.000
Dorea	0.000	0.000	0.000	0.000
Coprococcus	0.000	0.000	0.000	0.002
Megasphaera	0.000	0.003	0.000	0.001
Sarcina	0.000	0.000	0.000	0.000
Veillonella	0.000	0.000	0.000	0.000
Mitsuokella	0.000	0.000	0.000	0.000
Blautia	0.000	0.000	0.000	0.000
Mogibacterium	0.000	0.000	0.000	0.000
Anaerofilum	0.000	0.000	0.000	0.000

	Isogenic[2]	**Bt[3]**	**Isogenic/ Bt[4]**	**Bt/isogenic[5]**
Catenibacterium	0.000	0.000	0.000	0.002
Fibrobacter	0.000	0.000	0.000	0.000
Bifidobacterium	0.000	0.000	0.000	0.000
Spirulina	0.000	0.000	0.000	0.000
Olsenella	0.000	0.000	0.000	0.000
Anaeroplasma	0.000	0.000	0.000	0.000
Mycoplasma	0.000	0.000	0.000	0.000
Fusobacterium	0.000	0.000	0.000	0.000
Denitrobacterium	0.000	0.000	0.000	0.000
Chlamydia	0.000	0.000	0.000	0.000
Synechococcus	0.000	0.000	0.000	0.000
Victivallis	0.000	0.000	0.000	0.000

[1]Relative abundance presented as median values. Zero values correspond to taxa which were detected in a low number of samples per treatment with an abundance of < 0.001.

[2]Isogenic - isogenic parent line maize-based diet for 110 days (n = 8 pigs/treatment).

[3]Bt - Bt maize-based diet for 110 days (n = 9 pigs/treatment).

[4]Isogenic/Bt - isogenic maize-based diet for 30 days followed by a Bt maize-based diet for 80 days (n = 10 pigs/treatment).

[5]Bt/isogenic - Bt maize-based diet for 30 days followed by a isogenic maize-based diet for 80 days (n = 10 pigs/treatment).

Table 7.6. Individual molecular identifiers used for PCR amplification of the 16S rRNA gene fragments from porcine caecal samples.

Pig	Treatment	Molecular identifier
52[1]	isogenic[2]	AGAGAGAG
64	isogenic	AGAGCAGC
68	isogenic	AGATCATC
17	isogenic	AGATGAGC
5	isogenic	AGATGCTC
83	isogenic	ATCAGCTG
85	isogenic	ATCTCATC
1[1]	isogenic	ATCTCTGC
61	isogenic	ATCTGATG
72	isogenic	ATCTGCTC
47[1]	Bt[3]	AGCAGAGC
8	Bt	AGCAGCAG
74	Bt	AGCATCTG
16	Bt	AGCTCATG
9	Bt	AGCTGCTG
39	Bt	ATGAGAGC
87	Bt	ATGAGCAG
31	Bt	ATGCATGC
88	Bt	ATGCTCAG
70	Bt	ATGCTCTC
48	isogenic/Bt[4]	AGCAGATG
24	isogenic/Bt	AGCAGCTC
2	isogenic/Bt	AGCATGAG
44	isogenic/Bt	AGCTCAGC
95	isogenic/Bt	AGCTGATC
73	isogenic/Bt	ATGAGATG
27	isogenic/Bt	ATGAGCTC
20	isogenic/Bt	ATGATCTG
57	isogenic/Bt	ATGATGAG

Pig	Treatment	Molecular identifier
32	isogenic/Bt	ATGCAGAG
29	Bt/isogenic[5]	AGAGATGC
30	Bt/isogenic	AGAGCATG
81	Bt/isogenic	AGATCTGC
86	Bt/isogenic	AGATGATG
21	Bt/isogenic	AGATGCAG
78	Bt/isogenic	ATCAGATC
93	Bt/isogenic	ATCATCAG
14	Bt/isogenic	ATCATCTC
41	Bt/isogenic	ATCTGAGC
71	Bt/isogenic	ATCTGCAG

[1]Removed from the analysis following antibiotic treatment.

[2]Isogenic - isogenic parent line maize-based diet for 110 days.

[3]Bt - Bt maize-based diet for 110 days.

[4]Isogenic/Bt - isogenic maize-based diet for 30 days followed by a Bt maize-based diet for 80 days.

[5]Bt/isogenic - Bt maize-based diet for 30 days followed by an isogenic maize-based diet for 80 days.

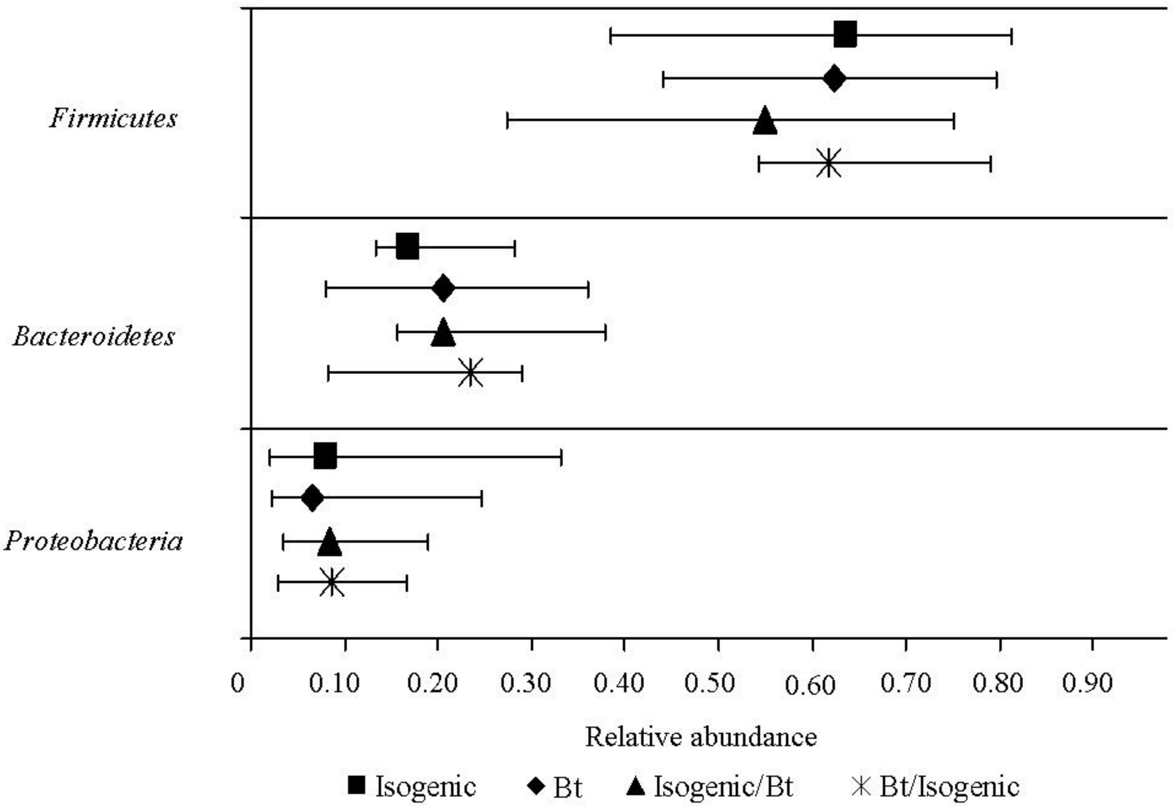

Figure 7.1. Effect of feeding Bt maize to pigs on relative abundance of major caecal bacterial phyla.

Data presented as medians ± 5th-95th percentiles.

Isogenic - isogenic parent line maize-based diet was fed for 110 days (n = 8 pigs/treatment).

Bt - Bt maize-based diet was fed for 110 days (n = 9 pigs/treatment).

Isogenic/Bt - isogenic maize-based diet was fed for 30 days followed by Bt maize-based diet for 80 days (n = 10 pigs/treatment).

Bt/isogenic - Bt maize-based diet was fed for 30 days followed by isogenic maize-based diet for 80 days (n = 10 pigs/treatment).

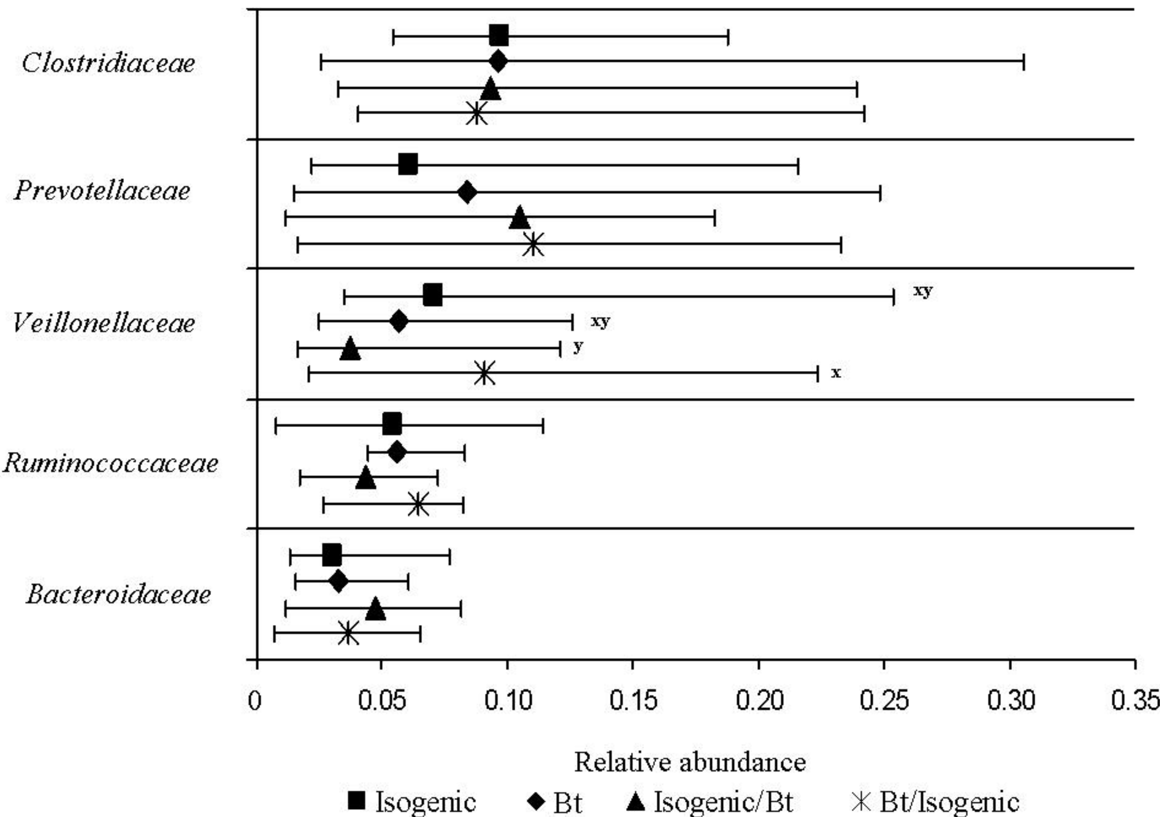

Figure 7.2. Effect of feeding Bt maize to pigs on relative abundance of major caecal bacterial families.

Data presented as medians ± 5th-95th percentiles.

Isogenic - isogenic parent line maize-based diet was fed for 110 days (n = 8 pigs/treatment).

Bt - Bt maize-based diet was fed for 110 days (n = 9 pigs/treatment).

Isogenic/Bt - isogenic maize-based diet was fed for 30 days followed by Bt maize-based diet for 80 days (n = 10 pigs/treatment).

Bt/isogenic - Bt maize-based diet was fed for 30 days followed by isogenic maize-based diet for 80 days (n = 10 pigs/treatment).

^{x,y}Medians with different superscripts indicate a tendency towards statistical significance (0.05 < *P* < 0.10).

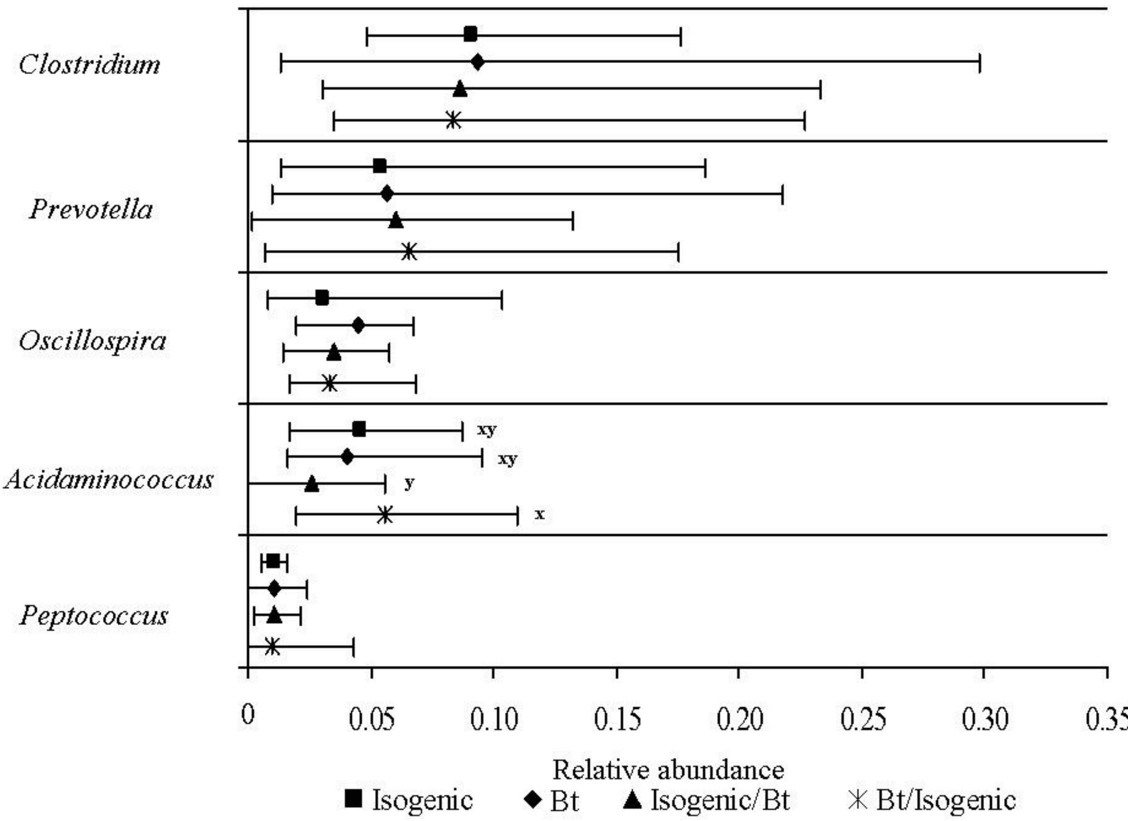

Figure 7.3. Effect of feeding Bt maize to pigs on relative abundance of major caecal bacterial genera.

Data presented as medians ± 5th-95th percentiles.

Isogenic - isogenic parent line maize-based diet was fed for 110 days (n = 8 pigs/treatment).

Bt - Bt maize-based diet was fed for 110 days (n = 9 pigs/treatment).

Isogenic/Bt - isogenic maize-based diet was fed for 30 days followed by Bt maize-based diet for 80 days (n = 10 pigs/treatment).

Bt/isogenic - Bt maize-based diet was fed for 30 days followed by isogenic maize-based diet for 80 days (n = 10 pigs/treatment).

^{x,y}Medians with different superscripts indicate a tendency towards statistical significance ($0.05 < P < 0.10$).

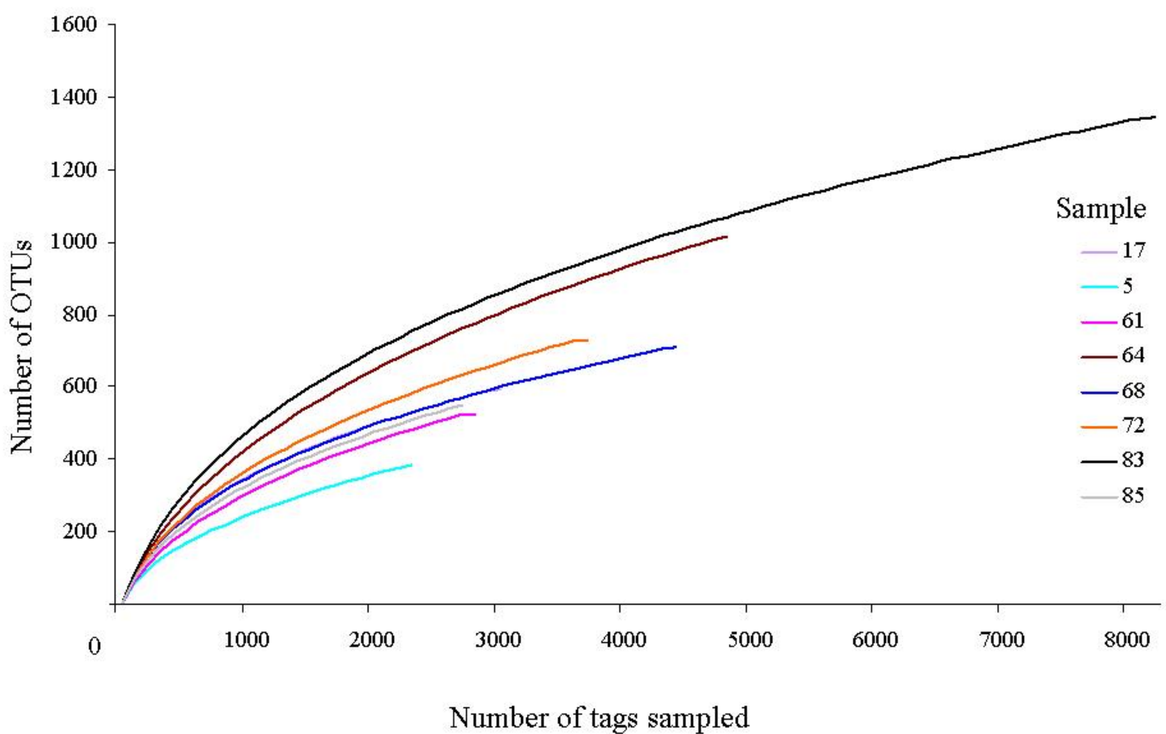

Figure 7.4. Bacterial alpha diversity at 97% similarity in the caecum of ~150 day-old pigs. Pigs were fed an isogenic maize-based diet for 110 days.

OTU = operational taxonomical unit.

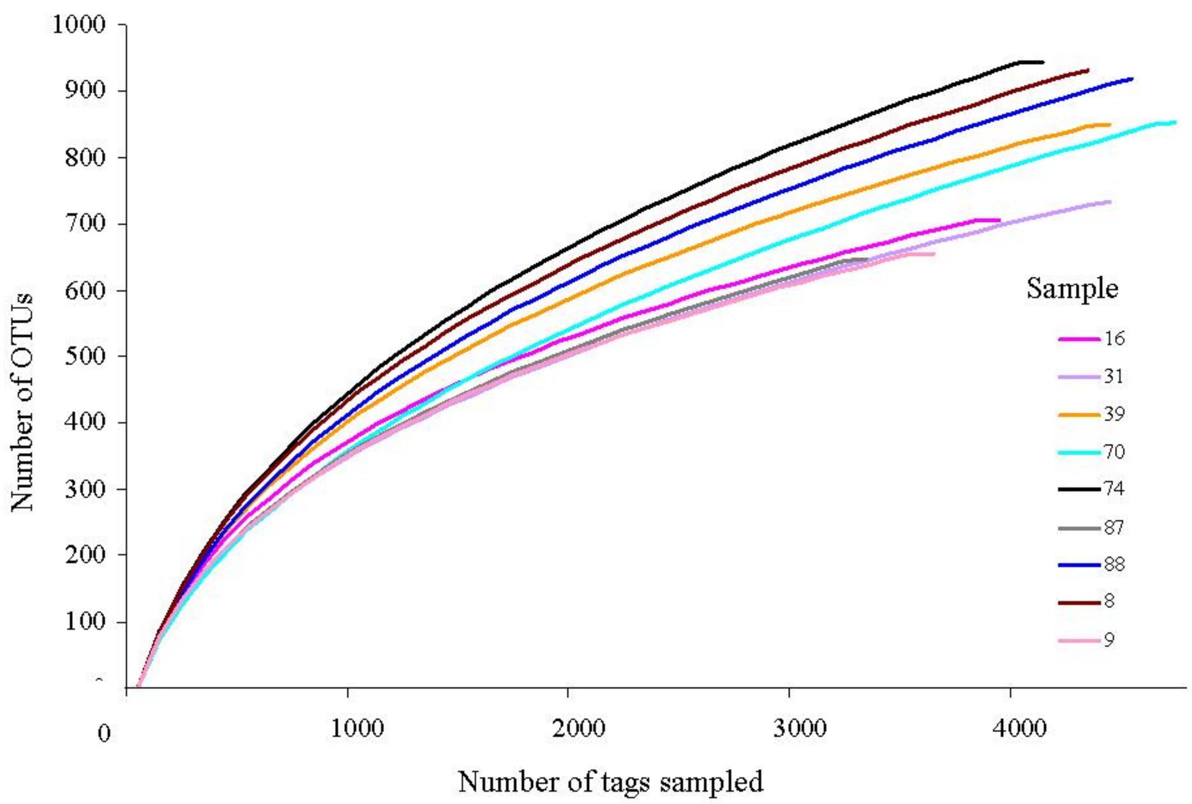

Figure 7.5. Bacterial alpha diversity at 97% similarity in the caecum of ~150 day-old pigs. Pigs were fed a Bt maize-based diet for 110 days.

OTU = operational taxonomical unit.

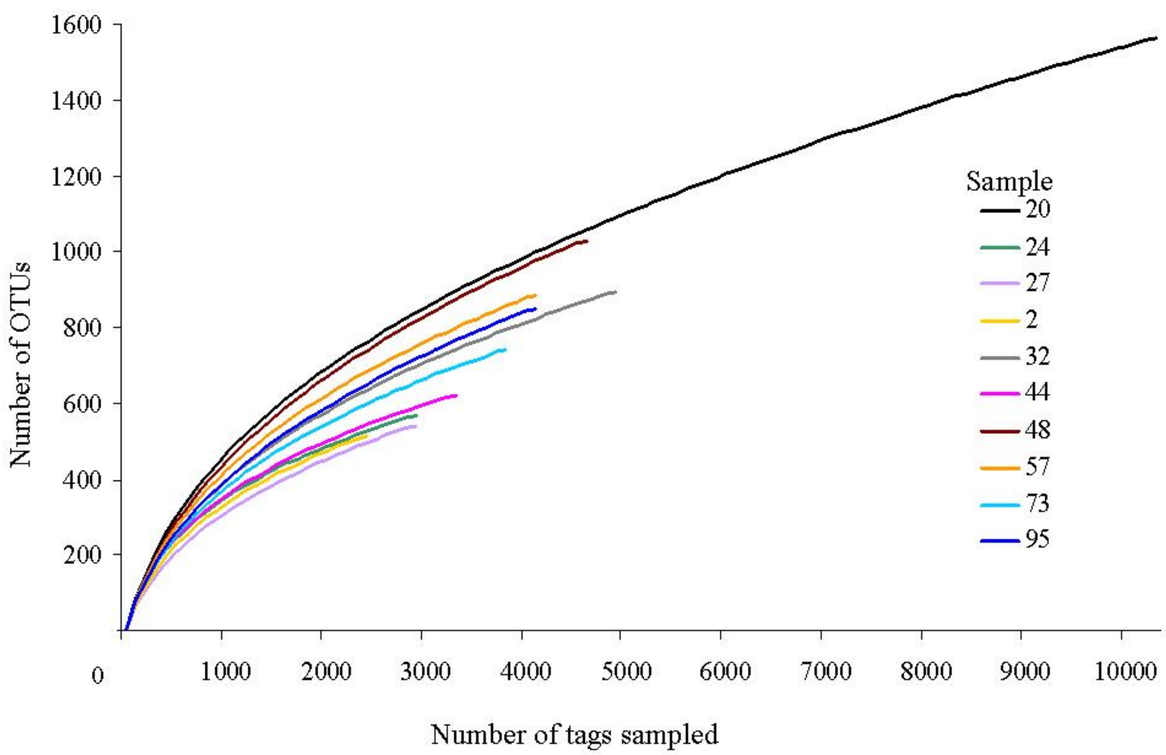

Figure 7.6. Bacterial alpha diversity at 97% similarity in the caecum of ~150 day-old pigs. Pigs were fed an isogenic maize-based diet for 30 days followed by a Bt maize-based diet for 80 days.

OTU = operational taxonomical unit.

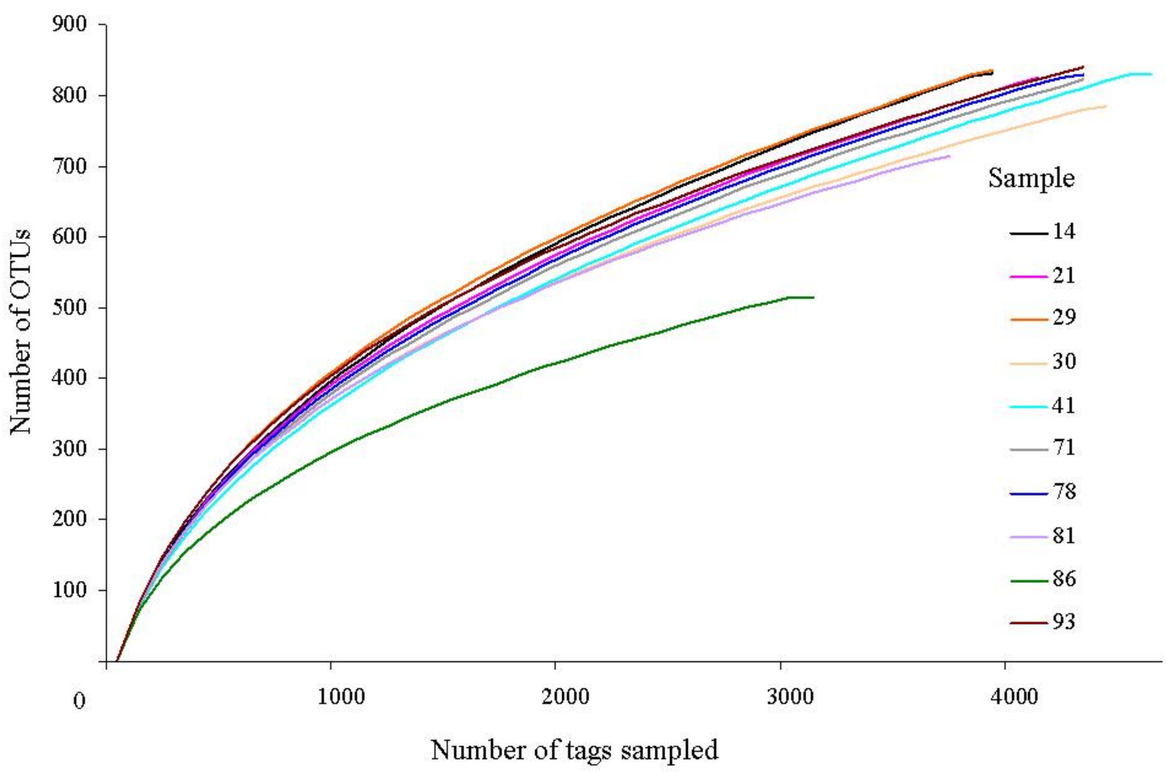

Figure 7.7. Bacterial alpha diversity at 97% similarity in the caecum of ~150 day-old pigs. Pigs were fed a Bt maize-based diet for 30 days followed by an isogenic maize-based diet for 80 days.

OTU = operational taxonomical unit.

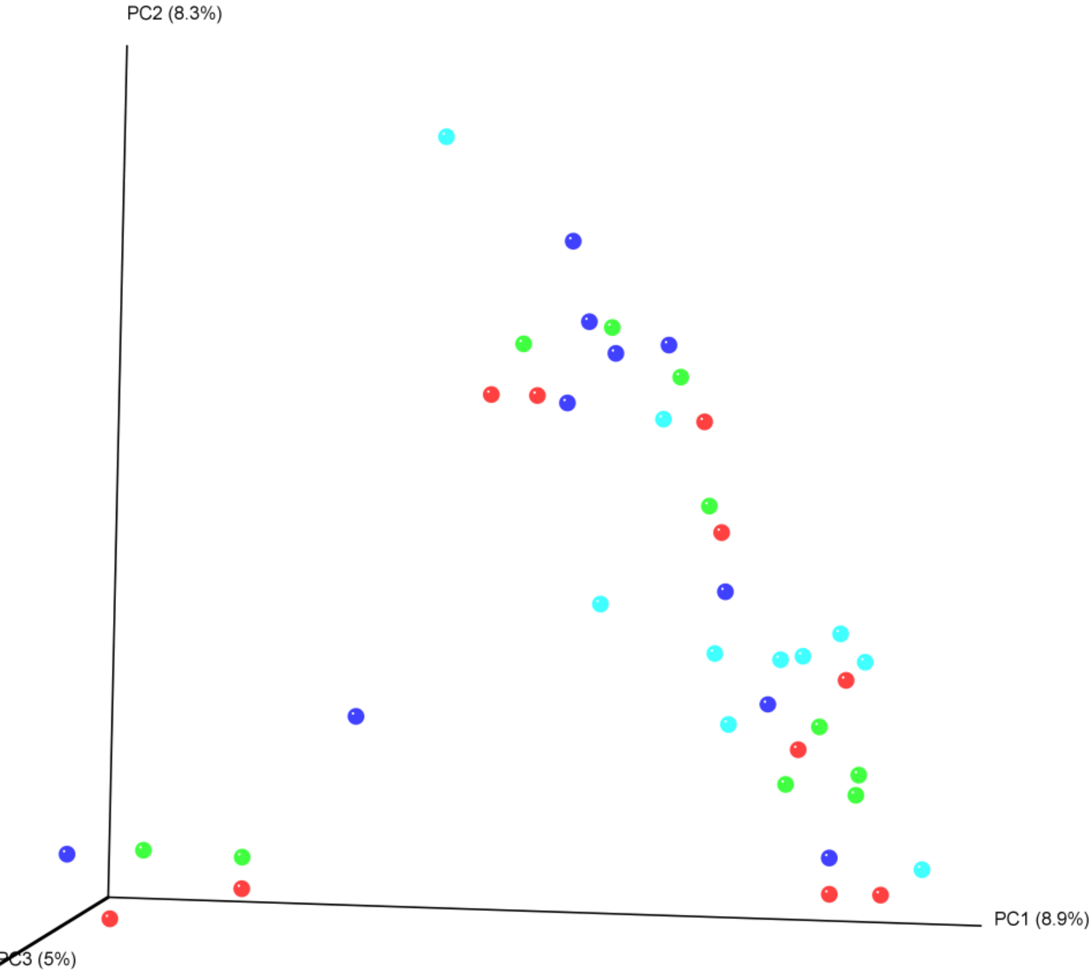

Figure 7.8. Unweighted bacterial beta diversity in the caecum of ~150 day-old pigs. Unweighted beta diversity was computed using QIIME software.

Blue - isogenic maize-based diet was fed for 110 days.

Green - Bt maize-based diet was fed for 110 days

Red - isogenic maize-based diet was fed for 30 days followed by a Bt maize-based diet for 80 days.

Light blue - Bt maize-based diet was fed for 30 days followed by a isogenic maize-based diet for 80 days.

7.6. References

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Overall conclusions

1. Although both the transgenic protein and the cry1Ab transgene were detected along the porcine GIT following short- and long-term feeding, the transgene was largely degraded as it was found only in the proximal GIT.

2. The cry1Ab transgene and protein did not translocate across the intestinal barrier into blood or organs.

3. Porcine growth performance and slaughter characteristics were largely unaffected by short- or long-term feeding of Bt maize or by changing maize sources from isogenic to Bt or vice-versa following 30 days of feeding.

4. The minor differences observed in serum biochemistry and haematology were mainly within the normal range for pigs, were not associated with any organ histopathology or differences in organ weights.

5. Differences in health and growth parameters between Bt and isogenic maize-fed pigs were not associated with signs of intestinal or organ pathology and were not consistently observed across the two studies.

6. Short- and long-term feeding of Bt maize to pigs did not result in localised or systemic allergic or inflammatory responses.

7. Bt maize is well tolerated by the porcine intestinal microbiota following short- and long-term feeding.

8. Overall discussion

The objectives of this work were to investigate any potential effects on pig growth and health arising from both short- and long-term consumption of GM Bt maize and to assess the degradability of the *cry1Ab* transgene and transgenic protein along the GIT and potential transfer to animal tissues.

The few studies that have investigated the effects of feeding Bt maize to pigs to date, have mainly focused on animal production performance. As pigs are considered an excellent model for humans and pig meat is widely consumed, studies in pigs are needed to reveal any potential health effects that may occur following Bt maize consumption. By investigating a range of health indicators in addition to growth performance, this study increased the chances of detecting more subtle changes. This holistic approach also enabled us to examine any changes detected in the context of their overall biological significance. By changing diets from isogenic to Bt maize and *vice-versa* following 30 days of feeding, the potential for an age-specific effect was also investigated. This also mirrors an on-farm situation where feed batches may contain maize from different sources.

The data obtained from these studies are important not only for pig producers and the feed industry but also for consumers, as pigs are known to be similar to humans in numerous aspects of their physiology and metabolism (Moughan *et al*., 1992; Kararli, 1995). Furthermore, as pig meat and organs are among the most consumed foods worldwide (FAO, 2012), the potential introduction of transgenic products into the human food chain through pig meat and organs could be perceived as a risk by consumers.

In the present study, growth performance of pigs was not adversely affected following consumption of Bt maize for either 31 or 110 days. Pigs fed Bt maize from 6 days post-weaning for 31 days had higher feed intake and were less efficient in converting feed to body mass between days 14 and 30 of the trial. However, this was not observed at any time point or overall in the long-term study or in the 35-day immune response study. The absence of adverse effects on growth is in accordance with most of the published literature which has shown either no effect (Reuter *et al*., 2002; Aeschbacher *et al*., 2005; Shimada *et al*., 2006; Flachowsky *et al*., 2007; Trabalza-Marinucci *et al*., 2008) or higher feed intake and poorer feed conversion efficiency (Custodio *et al*., 2006) in pigs, cows and sheep fed Bt maize. Similar to the growth performance results, no differences in carcass characteristics, organ weights and fat percentage were found as a result of feeding Bt maize. This is in accordance with other groups who have investigated the effect of feeding

Bt maize to pigs (Custodio *et al*., 2006), cattle (Flachowsky *et al*., 2007) or poultry (Brake *et al*., 2003). Although higher yields in breast muscles were observed in poultry (Brake and Vlachos, 1998; Taylor *et al*., 2003), this does not suggest a detrimental effect. The lack of an adverse effect on growth was expected, as no major nutrient differences were observed between the maize lines or the diets used in these studies. However, although growth performance and slaughter characteristics are valuable parameters for overall safety assessment, they are less powerful when used individually and therefore, need to be correlated with other health indicators to provide a more accurate safety assessment.

As the intestine is the route of entry of dietary constituents into the body, a more subtle effect of feeding Bt maize to pigs may have a higher chance of being observed at this site. Weaning is one of the most stressful events in the life of mammals (Pluske *et al*., 1997; Weary *et al*., 2008); therefore, pigs are most sensitive to external stimuli immediately post-weaning. Chronic toxicity, however, would not be evident until later in life, following long-term exposure. However, no changes in small intestinal architecture were observed in response to feeding Bt maize to pigs for 31 or 110 days, indicating the absence of intestinal toxicity. Likewise, no differences in the empty weight of the stomach and small intestine were observed following long-term Bt maize consumption. The absence of intestinal pathology is in agreement with previous findings in sheep (Trabalza-Marinucci *et al*., 2008) and rats (Kilic and Akay, 2008; Hammond *et al*., 2006), indicating the absence of a direct effect of Bt maize on intestinal architecture. However, not only direct toxic effects of Bt maize on the intestine are of concern, but also indirect effects.

A potential antimicrobial effect of the Bt maize could alter the composition of the intestinal microbiota in a manner that is detrimental to the host. An *in vitro* antimicrobial effect of the Cry1Ab protein has been observed against two *Clostridium* species (*Clostridium butyricum* and *Clostridium acetobutylicum*) and an Archaea (*Metanosarcina barkeri)* by Yudina *et al.* (2007). However, the Cry1Ab protein lacked antibacterial activity when tested against a wider range of bacterial species (*Proteus spp., Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Agrobacterium radiobacter*, *Arthrobacter globiformis, Bacillus subtilis and Bacillus cereus*) (Koskella and Stotzky, 2002). The approach which was adopted in the present study to evaluate effects on the highly dynamic intestinal microbiota is of greater relevance to the overall safety assessment of Bt maize. No effects were observed on counts of culturable *Enterobacteriaceae* (indicator of pathogenic bacteria), *Lactobacillus* (indicator

of beneficial bacteria) or total anaerobes in the short-term study or at any time point during the long-term study. The higher caecal abundance of *Enterococcaceae* observed following short-term Bt maize consumption is not believed to be of major biological significance as they were present at a relatively low abundance. Furthermore, some members of the family are considered beneficial, as they produce bacteriocins and others are used as probiotics (Fisher and Phillips, 2009). On the other hand, enterococci are able to translocate across the intestinal epithelium, leading to bacteremia or localized infections (Fisher and Phillips, 2009). However, in the present study, histological examination of intestinal tissue did not reveal any signs of intestinal damage or inflammation. The lower abundance of *Erysipelotrichaceae* as a result of short-term feeding of Bt maize may be a result of the higher feed intake in these pigs, as an increase in *Erysipelotrichaceae* has recently been associated with increased dietary fat intake, higher body weight and fat deposition in mice (Fleissner *et al*., 2010). However, the higher feed intake observed is believed to have occurred as a result of the lower enzyme resistant starch content of the Bt maize. However, this difference in enzyme resistant starch is not necessarily linked to the Cry1Ab transgenic protein *per se* and is most likely a result of natural variation, as similar compositional variation exists between non-GM maize varieties (OECD, 2002; Gajda *et al*., 2005; Hernot *et al*., 2008; García-Rosas *et al*., 2009; ILSI, 2010; Zilic *et al*., 2011; FAO, 2012). Additionally, variation in chemical composition due to seasonality has been shown to be higher than variation due to genetic modification (Barros *et al*., 2010). The lower abundance of *Blautia* encountered in the caecum of Bt maize-fed pigs may also be a result of lower enzyme resistant starch in the Bt maize, as *Blautia* are known to utilise hydrogen resulting from fermentation of dietary fibre (Flint *et al*., 2008; Rey *et al*., 2010). Therefore, a potentially lower level of fermentation in the intestine of Bt maize-fed pigs may have resulted in lower hydrogen concentrations in the intestine, thereby creating a less suitable environment for *Blautia* to thrive. However, Trabalza-Marinucci *et al.* (2008) did not find any difference in volatile fatty acid production in the rumen of ewes fed Bt maize for 3 years, potentially indicating no difference in microbial fermentation. The increase in *Bifidobacteriaceae* and *Bifidobacterium* following short-term feeding of Bt maize to pigs is also not believed to be of major biological significance. While bifidobacteria are associated with beneficial health effects in humans (Sekirov *et al*., 2010), their role in the intestine of pigs is not yet established as they only comprise a minor proportion of the microbiota. Effects on the caecal microbiota observed during the short-term study were not mirrored in

the long-term study, in which the only effect seen was an increase in *Holdemania*. However, relative abundance of this genus only differed between the pigs that switched diets on day 30 from isogenic to Bt maize compared to the Bt group and was not different between the Bt and isogenic groups. Moreover, this finding is not believed to be of clinical significance, as the role of *Holdemania* in the porcine intestine is not yet established. Furthermore, the fact that differences observed at day 31 in the short-term study were not mirrored at day 110 in the long-term study, may mean that these differences are age-specific. Overall, the changes observed in the relative abundance of caecal microbial populations in response to feeding Bt maize to pigs were not associated with any changes in intestinal architecture, stomach or intestinal weights or with any signs of inflammation in the mesenteric lymph nodes. Although no studies are available in pigs for comparison, the finding that feeding Bt maize had no adverse effects on the intestinal microbiota are in agreement with short- (Einspanier *et al*., 2004; Wiedemann *et al*., 2007) and long-term studies (Trabalza-Marinucci *et al*., 2008) in ruminants. These studies are valuable for overall safety assessment; however, due to differences in microbiology of the rumen, pigs are considered a more suitable model for humans (Cummings and Macfarlane, 1991).

While evaluation of effects on intestinal morphology and microbiota are essential in the safety assessment of dietary components, a potentially harmful compound absorbed from the intestine could be responsible for pathology in the main detoxifying and excretion organs of the body, the liver and kidney. Due to the physiological complexity of these organs, pathology should be assessed by a combination of multiple measurements in order to obtain a more definitive conclusion (Stonard, 1990; Boone *et al*., 2005; EFSA, 2008). No effect of either short- or long-term Bt maize consumption on liver weight was observed in pigs. Furthermore, serum indicators of hepato-cellular damage (ALT) and hepato-biliary dysfunction (ALP and GGT) did not differ between the Bt and isogenic treatments following either short- or long-term exposure. While no differences were observed in the short-term study, serum AST tended to be lower for pigs fed Bt maize for 110 days compared with pigs fed isogenic maize. No changes in liver structure were observed following histopathological examination. Liver dysfunction is characterised by increased serum AST accompanied by an increase in other serum enzymes; therefore, the observed changes in serum biochemistry observed in this study are unlikely to be of clinical significance.

Kidney function was not impaired by feeding Bt maize to pigs in the short- or long-term. While a tendency towards increased kidney weight was observed following short-term feeding, no evidence of kidney dysfunction was observed following histopathological examination. Neither serum creatinine nor serum urea were affected by 31 days of feeding Bt maize to pigs. However, an increase in serum urea was observed in pigs fed Bt maize following 30 days of feeding in the long-term study. This change was age-specific, as it did not persist to the end of the study and no differences in kidney weight, urinary kidney function indicators or signs of histopathology were observed at the end of the long-term study. The hypothesis was that the tendency towards higher kidney weights observed in the short-term study and the higher serum urea observed following 30 days of feeding in the long-term study are a result of lower fibre fermentation in the intestine of pigs fed Bt maize due to the lower enzyme resistant starch content of the maize. There is evidence that blood urea can be used as a substrate by intestinal fibre-fermenting bacteria (Younes *et al*., 1995; Younes *et al*., 1995; Mosenthin *et al*., 1992; Mosenthin *et al*., 1992). Therefore, a lower urea demand in the intestine of pigs fed Bt maize diets may have lead to an increased pool of serum urea which was excreted by the kidney leading to a tendency towards increased kidney weights. However, as no signs of kidney histopathology were evident in any of the studies and serum biochemistry parameters remained within or close to the normal range for pigs, the changes observed are believed to be part of an adaptive response rather than a sign of pathology. No differences in serum creatinine or TP were observed in the short-term study. Lower serum TP on day 110 in the Bt/isogenic treatment group compared with all other treatments and higher serum creatinine in the in the isogenic/Bt and Bt/isogenic groups compared with the isogenic and Bt groups were observed during the long-term study. As these differences were not associated with any signs of kidney dysfunction and values remained within the normal range for pigs, their clinical significance is considered to be minor. Our findings demonstrating a lack of adverse effects of Bt maize on serum health indicators in pigs is supported by published data from calves (Shimada *et al*., 2006) and rats (Hammond *et al*., 2006). Trabalza-Marinucci *et al*., (2008), however, reported a decrease in serum GGT in ewes fed Bt maize silage for 3 years and nuclear changes in hepatocytes and pancreatic cells in lambs of ewes fed Bt maize. (Kilic and Akay, 2008) also observed a decrease in serum TP and subtle hepatic and renal pathology in response to feeding Bt maize to rats for three generations at a 20% dietary inclusion rate. However, these authors did not specify whether the non-GM maize was the isogenic parent line and

did not investigate potential mycotoxin or pesticide contamination of the maize used, making it difficult to interpret the observed changes. To minimise interference from contaminants in this study, mycotoxin binders were included in the diets, the isogenic parent line was used as a comparator and maize samples were thoroughly analysed in accordance with international guidelines (Hartnell *et al*., 2007; EFSA, 2008). In addition, to maximize the chances of detecting any potential adverse effects, the maize dietary inclusion rate was 48-90 times higher than the maximum European maize intake for humans and 24-39 times higher than the maximum estimated maize intake for humans worldwide (FAO, 2012).

While no signs of toxicity were evident from the biochemical parameters measured, a potential immune response could be of major concern to consumers, especially in the context of the recent rise in occurrence of allergies (Branum and Lukacs, 2009). Following short-term Bt maize feeding we observed an increase in $CD4^+$ T cells in the ileum, associated with increased production of IL-4 and IL-8 which could potentially indicate an allergic response; however, these changes were not accompanied by an increase in either macrophage or B cell proportion or the B cell activator, IL-6 in the ileum. Furthermore, no Bt maize-specific antibodies were detected in plasma and no differences in intestinal architecture indicative of inflammation were observed. A B cell and macrophage inhibitor role was proposed for IL-4 in pigs by Murtaugh *et al.* (2009) which may explain the changes in intestinal immune cells observed. While splenocyte production of IL-4 and IL-6 was increased in pigs in response to short-term feeding of Bt maize, it was not accompanied by changes in B cell populations or Bt maize-specific antibody production and is thus unlikely to indicate an allergic response. A decrease in IFN-γ production from PBMC in response to short-term feeding of Bt maize was also observed, which holds little biological significance, as only an increase would be correlated with an inflammatory response. These differences are not believed to be of clinical significance, as the increase observed for these cytokines was not sufficient to trigger recruitment of macrophages or differentiation of B cells in the intestine. The basal concentration of both IL-12 and IFNγ from resting (unstimulated) PBMC isolated from isogenic maize-fed pigs was greater than in Bt maize-fed pigs. By nature of the transgene insertion, Bt maize is protected from insect damage. As insect damage has been correlated with mould and bacterial contamination of maize, the insect-resistant Bt maize could have less bacterial growth and as a result contain less bacterial endotoxin than its isogenic counterpart. The potentially

greater exposure to endotoxins from feeding isogenic maize may account for the elevated Th1 cytokine profile evident in both resting and stimulated PBMC isolated from pigs fed isogenic maize. The differences in immune cells distribution and cytokine production observed following short-term feeding of Bt maize to pigs were not mirrored in pigs fed Bt maize for 110 days. A lack of antibody response to intra-peritoneal or intra-gastric administration of Bt maize was also observed in mice (Adel-Patient *et al*., 2011). Similar to our short-term study, a lower ileal population of B cells and a lower splenic $CD4^+$ T cell population were observed by Finamore *et al.* (2008) in weaning mice fed Bt maize for 30 days. However, contrary to our study, the lower B cell proportion was accompanied by a decrease in ileal $CD4^+$ T cells. In contrast to our findings, Finamore *et al.* (2008) also observed an increase in γδ T cells in mice fed Bt maize diets. Such an increase has been linked to allergy and production of allergy-indicative cytokines in the serum. However, similar to our findings, most of the changes were no longer detected following long-term (90 day) exposure of mice.

An increase in lymphocyte counts was observed on day 100 in the long-term study in pigs fed the Bt/isogenic treatment compared with pigs fed the isogenic and Bt treatments and, as a result a tendency towards a similar pattern was observed for white blood cells. However, these changes are not believed to have occurred as a result of Bt maize consumption, as no differences were observed between pigs fed Bt maize for 110 days and the control group and counts remained close to the normal range for pigs (Feldman *et al*., 2006). The changes in lymphocytes observed in the long-term study could potentially indicate a sensitization early in life which persisted to day 100. However, an allergic response to Bt maize would be accompanied by Bt maize-specific antibody production which was not observed at any point during the study. Furthermore, no pathology was present in the intestine, no signs of inflammation were observed in the mesenteric lymph nodes and spleen and no changes in intestinal bacterial populations were observed. The higher red blood cell (RBC) counts observed for the Bt/isogenic group compared with Bt and isogenic/Bt group were also within the normal range for ~4 month-old pigs (Feldman *et al*., 2006). Such an increase in red blood cells could be a sign of abnormal proliferation of blood cells. However, proliferation of RBC would be accompanied by spleen pathology and increased spleen weight (Feldman *et al*., 2006) which were not observed, indicating that the observed differences are unlikely to be of clinical significance. Similar to our

study, no adverse effects on haematological parameters were observed in rats fed Bt maize (Hammond *et al*., 2006) and sheep (Trabalza-Marinucci *et al*., 2008).

While both the 211 bp and 149 bp *cry1Ab* gene fragments were detected in the stomach of all pigs fed Bt maize diets for short and long periods, the transgene was degraded along the GIT. It was detected in only two of 10 ileal samples from the short-term study and three of 20 ileal samples from the long-term study. In the short-term study, the 211 bp transgene fragment was detected further down the GIT but only in one caecal sample and was not detected in the colon. In the long-term study, neither the 211 bp transgene fragment nor a smaller 149 bp fragment were detected in the caecum or colon. The maize-native *sh2* gene fragment showed similar behaviour to that of the *cry1Ab* gene in both studies. This is not surprising as the *sh2* gene, while native to maize, is a single-copy gene, similar to c*ry1Ab*. This indicates that transgenic DNA is degraded in a similar manner to that of native plant DNA. The multi-copy *rubisco* maize-native gene fragment used in the long-term study was detected in the stomach of 36 out of 40 pigs and in the colon of only 7 of 40 pigs, confirming a high level of degradation along the porcine GIT. The multicopy *rubisco* plant gene was detected in all intestinal segments, but with lower frequency towards the colon. Although none of the single copy genes was detected in the blood and organs of pigs, the multicopy *rubisco* was detected in the liver, kidneys, spleen and white blood cells of pigs fed Bt maize in the long-term study. While this indicates that DNA fragments from ingested native plant multi-copy genes can translocate into porcine blood and organs, such an effect was not observed for the transgene. This is in contrast to other studies in the literature which report the presence of *cry1Ab* in the blood and organs of weanling pigs (Mazza *et al*., 2005) and in the digesta of growing pigs, as far down the GIT as the rectum (Chowdhury *et al*., 2003; Reuter and Aulrich, 2003). Similar to our results, *cry1Ab* transgene fragments were only detected in the proximal digestive tract of wild boars (Wiedemann *et al*., 2009) and poultry (Rossi *et al*., 2005). They have also been detected in the faeces of ruminants (Guertler *et al*., 2010). Although small fragments are observed along the GIT, the intact transgene (3470 bp) or its minimum functional unit (1800 bp) were not detected. Identification of such large DNA fragments has been difficult even from feed (Mazza *et al*., 2005) and stomach digesta (Wiedemann *et al*., 2009). While the presence of both transgenic and plant DNA has been confirmed in blood and various organs, there is no evidence to suggest that transgenic DNA is degraded differently from native plant DNA (Jonas *et al*., 2001). Furthermore, stable incorporation of plant DNA into

mammalian cells has not yet been proven, although DNA has always been a dietary component (Jonas *et al*., 2001). It is known that DNA degradation begins with chewing, and is accelerated in the low pH environment of the stomach (Jonas *et al*., 2001). However, the food matrix may protect some DNA from degradation, providing an explanation for the persistence of DNA in the small intestine. DNA degradation continues in the small intestine due to the action of porcine pancreatic DNAses (Lewis and Southern, 2001) and the remaining free DNA is likely to be further degraded by bacterial DNAses in the large intestine. Therefore, the results are not surprising, confirming a similarly high DNAse activity along the GIT of ~60 and ~150 day-old pigs. The differences between the current studies and others in the literature may have occurred due to differences in timing of the last meal, gastrointestinal pH, quantity of digesta present in the GIT and feed formulation.

Similar to its coding gene, the Cry1Ab protein was largely degraded in the GIT in the both the short- and long-term studies, with concentrations up to 90 times lower in the GIT than in raw feed. Furthermore, the Cry1Ab protein was not detected in the blood or any of the organs examined. These results are in agreement with published literature demonstrating Cry1Ab protein degradation along the GIT (Chowdhury *et al*., 2003) and absence from blood and organs (Chowdhury *et al*., 2003). This is expected, as proteolytic activity is high along the porcine GIT (Lewis and Southern, 2001). The differences in frequency of detection observed between the short- and long-term studies may be a result of different maize inclusion rates or different gastrointestinal motility and volume between young and adult animals. In the short-term study, the Cry1Ab protein was detected in 3 out of 10 pigs in the stomach and caecum, and in 8 out of 10 pigs in the ileum and colon. This pattern may have occurred due to physiological dilution or concentration at different sites within the GIT. For example, in the short-term study, dilution with saliva and gastric juices in the stomach probably lead to the low frequency of detection (i.e. in only 30% of the pigs). On the other hand, water absorption in the small intestine would have concentrated the ileal digesta, leading to a greater number (80%) of pigs being positive for the protein. Dilution would have occurred at the terminal ileum, lowering caecal Cry1Ab concentrations and finally the digesta was concentrated due to extensive water absorption in the colon, leading to a higher quantity of Cry1Ab per gram of digesta. This phenomenon may also be responsible for the similarity between Cry1Ab concentrations found in the stomach and large intestine digesta. It is known that younger pigs have a faster gut transit

time compared to adult pigs (Kararli, 1995) and this may explain the lower frequency of the Cry1Ab protein detection in the stomach of pigs from the short-term study.

Power analysis was performed to calculate the minimum effect size (% difference) that is likely to be detected with the sample size used for the exepriments in this thesis. Calculations based on an 80% power [(1-β) × 100] with an α of 5% were performed for both the short- and long-term studies. These calculations revealed that the sample size (n=12-15/treatment) used in growth performance studies was sufficient to detect differences of between 6.0 and 12.6%. Evaluation of slaughter characteristics and organ weights in this thesis used a sample size of n=10/treatment and power calculations found that this sample size could determine an effect of greater than 2.6 to 26.0% (depending on parameter). Likewise, 3.8-28.3% differences between treatments could be determined in the present studies for microbial culturing where sample size was n=9/treatment. For haematology and blood biochemistry differences greater than 2.5-59.7%, depending on parameter, could be determined at the sample size of 10/treatment used for this analysis.

8.1. References

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Future research

Although the changes observed in our studies did not indicate pathological effects due to Bt maize consumption in pigs, all animals used in our studies were of a high health status. Considering that the response of an immuno-compromised or challenged individual may differ significantly from that of a healthy individual, future research should perhaps focus on investigating the effects of Bt maize on immunocompromised animals or those with underlying pathology or who are under physiological stress such as pregnancy.

Likewise, as horizontal gene transfer is most likely to occur in tissues with a high rate of proliferation, such as those of the foetus, studies investigating gene transfer from mother to foetus are warranted. Studies investigating horizontal transgene transfer to the intestinal microbiota should also be performed as part of a comprehensive risk assessment.

While changes in bacterial populations may be indicative of an effect of feeding Bt maize, the role of many bacteria in the intestine is still under investigation. Therefore, functional metagenomic studies could shed light on bacterial enzymatic activity in the intestine in response to Bt maize feeding. Likewise, measurement of microbial metabolites, such as short chain fatty acids in the intestine will help clarify the effects of Bt maize on functionality of the intestinal microbiota.

As we have seen that the quantity of Cry1Ab protein was lower in the GIT compared to the feed, future studies could investigate the size of protein fragments along the GIT and correlate this with the quantitative data.

Implications

Results from this study provide valuable data for the overall safety assessment of Bt maize. Their dissemination will assist consumers in making an informed decision on whether to accept the use of Bt maize as a feed or food ingredient. The findings also provide information to pig producers and the feed industry on the safety of Bt maize, one of the main ingredients of pig diets. As production of Bt maize is cheaper than conventional non-insect resistant varieties, its use may have financial benefits for the pig industry. Therefore, findings from this study should provide a guarantee to pig farmers that, if used, Bt maize will not be detrimental to pig growth performance or slaughter characteristics. Furthermore, farmers should be reassured that Bt maize does not pose a health risk when fed to pigs from shortly after weaning to slaughter. Findings from the experiments conducted in this study should also help assure consumers of the safety of Bt maize, as a similar lack of adverse effects should be expected in humans, for which pigs are considered an excellent model.

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