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# High-Throughput Sequence-Based Analysis of the Intestinal Microbiota of Weanling Pigs Fed Genetically Modified MON810 Maize Expressing *Bacillus thuringiensis* Cry1Ab (Bt Maize) for 31 Days

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**The objective of this study was to investigate if feeding genetically modified (GM) MON810 maize expressing the *Bacillus thuringiensis* insecticidal protein (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) maize 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. Fecal, cecal, 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 compositions of the cecal microbiotas. The only differences were that pigs fed the Bt maize diet had higher cecal abundance of *Enterococcaceae* (0.06 versus 0%; $P < 0.05$), *Erysipelotrichaceae* (1.28 versus 1.17%; $P < 0.05$), and *Bifidobacterium* (0.04 versus 0%; $P < 0.05$) and lower abundance of *Blautia* (0.23 versus 0.40%; $P < 0.05$) than 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 cecal microbiotas. 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.**

Maize is one of the main nutrient sources for humans and animals worldwide (13). 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 (25). Most of the GM maize cultivated worldwide is resistant to insect damage via expression of the Cry1Ab transgenic protein originally identified in *Bacillus thuringiensis* and is referred to as Bt maize.

Although many benefits are associated with the presence of the Cry1Ab protein in maize (5), its presence in human and animal diets has raised concerns, mainly related to potential health risks. Alternatively, effects may be mediated by potential changes in intestinal bacterial populations following GM plant consumption. Research on bacterium-host interactions has revealed the enormous influence of the intestinal bacteria on host physiology (49). Therefore, any effect a GM plant may have on intestinal bacteria could potentially affect the host, especially immunocompromised individuals (43).

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 (10). 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 (37, 45).

An *in vitro* study has demonstrated antimicrobial activity of the Cry1Ab protein in both intact (130-kDa) and fragmented (~60-kDa) forms against *Clostridium butyricum*, *Clostridium acetobutylicum*, and *Methanosarcina barkeri* (57). However, conflicting data were obtained by Koskella and Stotzky (27), 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. (50) also found that Bt maize had no effect on *Fibrobacter succinogenes* and *Streptococcus bovis* populations *in vitro* following a 12- or 24-h 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 (8, 11, 55). 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. (56) employed

**Received** 1 February 2012 **Accepted** 26 March 2012
**Published ahead of print** 30 March 2012
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Supplemental material for this article may be found at http://aem.asm.org/.

doi:10.1128/AEM.00307-12

real-time PCR analysis and found no effects of Bt maize on six ruminal bacterial strains following 11 days of feeding. Einspanier et al. (11) 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 (6). Likewise, Trabalza-Marinucci et al. (51) 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 (48). 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 (33). 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 intestinal microbial populations.

The Organisation 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 (10, 20, 36). Furthermore, the recommendations state that the plants compared should be grown under similar conditions (20). 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 (23, 48, 56), others have not (51). 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 (3).

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 isogenic parent line maize grown under environmental conditions similar to those for 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.

## 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 had 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; body weight, 7.5 ± 1.5 kg) were weaned at ~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: (i) non-GM isogenic parent line maize-based diet (Pioneer PR34N43) and (ii) GM maize-based diet (Bt maize; Pioneer PR34N44 event MON810). Both treatments were fed for 31 days. Pigs were penned individually in a total of four rooms, with 4 or 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 Bt maize 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 (10, 20). The isogenic maize 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 by Walsh et al. (54). 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 (35). For both diets, either isogenic or Bt maize was included at identical levels. Diets were manufactured and analyzed as previously described by Walsh et al. (54). As a precaution, an organic mycotoxin absorbent (Mycosorb; Alltech, Dunboyne, Co. Meath, Ireland) was included in all diets.

**Sample collection.** Individual fecal 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 ileocecal junction) and cecal (from the terminal tip of the cecum) digesta were collected from all pigs. All fecal and digesta samples were stored in sterile containers at 4°C in anaerobic jars containing Anaerocult A gas packs (Merck, Darmstadt, Germany) until analysis (within 12 h).

**Microbiological analysis.** *Lactobacillus* and *Enterobacteriaceae* counts were determined in individual fecal, ileal, and cecal samples as indicators of beneficial and pathogenic bacteria, respectively (7). This was performed as previously described by Gardiner et al. (18), 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 molds. Total anaerobic bacterial counts were performed in individual fecal, ileal, and cecal samples as described by Rea et al. (41). To maintain anaerobiosis, all manipulations with these samples were performed in a Whitley A85 anaerobic workstation (DW Scientific, Shipley, United Kingdom), and the plates were also incubated anaerobically within the workstation. Cecal digesta samples were frozen at −20°C for subsequent 16S rRNA gene sequencing.

**DNA extraction, PCR, and 16S rRNA gene sequencing.** Total DNA was extracted from individual cecal digesta samples using the QIAamp DNA stool minikit (Qiagen, Crawley, West Sussex, United Kingdom) 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 rRNA primers predicted to bind to 94.6% of all 16S rRNA genes, as previously outlined by Murphy et al. (34). 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′ CTACDSRGGTMTCT AATC 3′), and R4 (5′ TACNVGGGTATCTAATC 3′) (34). Each PCR contained 2 μl of template DNA, 200 nM forward primer, 50 nM each of the four reverse primers, and 25 μl Biomix Red (Bioline, London, United Kingdom) in a total volume of 50 μl. A negative control sample in which template DNA was replaced with double-distilled water and a positive control sample containing previously amplified cecal bacterial DNA were included. The PCR cycle began with initial denaturation at 94°C for 2 min,

followed by 35 cycles of 1 min of denaturation at 94°C, annealing at 52°C for 1 min, followed by 1 min of elongation at 72°C. A final elongation step was performed at 72°C for 2 min. All PCR amplifications were performed in a G-Storm GS1 thermal cycler (G-Storm, Somerton, Somerset, United Kingdom). 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 double-stranded DNA (dsDNA) kit (Invitrogen Ltd., Paisley, United Kingdom) according to the manufacturer's instructions and then quantified using a NanoDrop 3300 spectrophotometer (Fisher Scientific, DE). Sequencing was performed on a 454 genome sequencer FLX platform (Roche Diagnostics Ltd., Burgess Hill, West Sussex, United Kingdom) according to 454 protocols. Sequencing reads were checked and assigned to NCBI taxonomies as previously described by Murphy et al. (34). Denoising 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 (46). Trimmed FASTA sequences were then BLASTed (1) against a previously published 16S-specific database (52) using default parameters. The resulting BLAST output was parsed using MEGAN (22). 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 cutoff, BLAST bitscore 86; relative cutoff, 10% of the top hit) (34, 52). The relative abundance of each bacterial taxonomic rank in the pig cecum 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 base 10 in an attempt to ensure normal distribution. Only data which were normally distributed and with equal variances (47) were analyzed as a one-factor analysis of variance (ANOVA) using the GLM procedure of SAS (44) (SAS Inst. Inc., Cary, NC). Data which were nonnormally distributed following log transformation or which had unequal variances were subjected to nonparametric analysis using the Kruskal-Wallis test within the NPAR1WAY procedure of SAS. For analysis of day 30 fecal 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 a $P$ value of ≤0.05. Tendencies were reported up to a $P$ value of ≤0.10. Bacterial counts are presented as means ± standard error (SE) of the log-transformed values, while relative abundances are presented as medians with 5th and 95th percentiles (47).

## RESULTS

**Maize and diets.** The compositions of maize lines and diets used in the present study are presented in Tables 1 and 2, respectively. No major differences were observed between the Bt maize and the isogenic maize, and values were mostly within the normal range of variation for maize varieties (Table 1). The Bt maize was found to have a lower enzyme-resistant starch content and a higher overall starch content than the isogenic maize, but the values remained within the natural variability for maize varieties cited in the literature. Amino acid contents were similar for the two maize lines. Both the Bt and isogenic maize diets had similar proximate compositions and amino acid contents (Table 2).

**TABLE 1** Chemical composition of maize lines included in pig diets

| Composition | Value (%) for indicated maize line | | Normal value (%)[a] (reference) |
|---|---|---|---|
| | Isogenic | Bt | |
| DM[b] (% of fresh wt) | 88.1 | 87.4 | 85.6–90.6 (36) |
| % of DM | | | |
| Crude protein | 8.40 | 8.81 | 6.00–12.70 (36) |
| Fat | 4.31 | 3.78 | 1.65–6.02 (24) |
| | | | 3.10–5.80 (36) |
| Crude fiber | 2.95 | 2.29 | 0.35–3.24 (24) |
| Ash | 1.36 | 1.83 | 0.62–6.02 (24) |
| | | | 1.27–1.52 (36) |
| Starch | 70.26 | 73.34 | 25.6–75.4 (24) |
| | | | 54.6–69.9 (58) |
| Sugar (sucrose) | 1.36 | 2.47 | 1.81[c] (12) |
| | | | 2.39–4.25 (58) |
| ADF[d] | 4.57 | 3.98 | 3.63–4.76 (58) |
| | | | 3.00–4.30 (36) |
| NDF[e] | 13.05 | 12.81 | 11.02–14.72 (58) |
| | | | 8.30–11.90 (36) |
| ADL[f] | 1.15 | 1.16 | 0.29–0.80 (58) |
| Enzyme-resistant starch | 6.73 | 4.10 | 3.59–3.90[c] (17) |
| | | | 16.3[g] (21) |
| | | | 25.2[g] (4) |
| | | | 34.9 (16) |
| Water-soluble carbohydrate | 2.42 | 3.62 | NA[h] |
| Lysine | 0.36 | 0.35 | 0.20–0.38 (36) |
| Methionine | 0.18 | 0.17 | 0.10–0.28 (36) |
| Cystine | 0.25 | 0.25 | 0.12–0.27 (36) |
| Threonine | 0.39 | 0.37 | 0.27–0.49 (36) |
| Tryptophan | 0.11 | 0.11 | 0.05–0.12 (36) |

[a] 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.
[b] DM, dry matter.
[c] Calculated at a DM content of 88%.
[d] ADF, acid detergent fiber.
[e] NDF, neutral detergent fiber.
[f] ADL, acid detergent lignin.
[g] No information about maize line is provided.
[h] NA, not available.

**Culture-based investigation of the effect of feeding Bt maize on intestinal microbiota of weanling pigs.** Fecal counts of *Lactobacillus*, *Enterobacteriaceae*, and total anaerobes did not differ between the isogenic and Bt maize groups at day −1, i.e., prior to the administration of experimental diets ($P > 0.05$) (Table 3). A tendency toward decreased *Lactobacillus* was, however, observed on day −1 in the feces 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. Fecal counts of *Lactobacillus*, *Enterobacteriaceae*, and total anaerobes were not affected by 30 days of Bt maize exposure ($P > 0.05$) (Table 3). Likewise, there were no differences in the counts of *Lactobacillus*, *Enterobacteriaceae*, or total anaerobes in the ilea or ceca of pigs fed isogenic or Bt maize-based diets for 31 days ($P > 0.05$).

**High-throughput 16S rRNA gene-sequencing analysis of porcine cecal 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

**TABLE 2** Composition of experimental diets[g]

| Composition | Value (%) for: | | |
|---|---|---|---|
| | Baseline (day −6 to −1) (isogenic maize) | Experimental (day 0 to 31) | |
| | | Isogenic maize | Bt maize |
| Ingredient (%) | | | |
| Maize (isogenic) | 27.33 | 38.88 | |
| Maize (Bt MON810) | | | 38.88 |
| Soybean meal (non-GM) | 24.00 | 25.00 | 25.00 |
| Lactofeed 70[a] | 25.00 | 20.00 | 20.00 |
| Immunopro 35[b] | 12.50 | 9.00 | 9.00 |
| Soybean oil | 8.00 | 4.00 | 4.00 |
| L-Lysine HCl (78.8) | 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[c] | 0.30 | 0.30 | 0.30 |
| Mycosorb[d] | 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 |
| Analyzed 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 fiber | 1.7 | 2.1 | 1.9 |
| Ash | 6.3 | 5.5 | 5.6 |
| Lysine | 1.55[e] | 1.42 | 1.42 |
| Ca[e] | 0.83 | 0.78 | 0.78 |
| P[e] | 0.61 | 0.59 | 0.59 |
| DE[f] (MJ of DE/kg)[e] | 16.33 | 15.38 | 15.38 |

[a] Lactofeed 70 contains 70% lactose, 11.5% protein, 0.5% oil, 7.5% ash, and 0.5% fiber (Volac, Cambridge, United Kingdom).
[b] Immunopro 35 is a whey protein powder product containing 35% protein (Volac, Cambridge, United Kingdom).
[c] 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, 6,000 IU; vitamin $D_3$, 1,000 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.
[d] Mycosorb is an organic mycotoxin adsorbent (Alltech, Dunboyne, Co. Meath, Ireland).
[e] Calculated values.
[f] DE, digestible energy.
[g] Analysis expressed on a fresh weight basis.

**TABLE 3** Effect of feeding a Bt maize-based diet for 31 days on fecal and intestinal microbial counts in weanling pigs[a]

| Sample and bacterial group | Count ($log_{10}$ CFU/g) for indicated maize-based diet | | Pooled SE[d] | *P* value[e] |
|---|---|---|---|---|
| | Isogenic[b] | Bt[c] | | |
| Feces, day −1[f] | | | | |
| *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 |
| Feces, 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 |
| Cecal 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 |

[a] Mean of $n = 9$ pigs/treatment.
[b] Isogenic maize-based diet fed for 31 days.
[c] Bt maize-based diet fed for 31 days.
[d] Pooled standard error of the mean.
[e] *P* value from the one-way ANOVA test.
[f] Variability present at day −1 has been accounted for by including these day −1 values as covariates in the statistical model.

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, and mean values were then obtained for each dietary treatment (Table 4). At the 97% similarity level, the Shannon index, a metric for community diversity, revealed similar levels of overall biodiversity for both treatments, with values ranging from 5.1 to 6.8. Good's coverage at the 97% similarity level ranged between 92 and 96%. Rarefaction curves showed similar levels of cecal bacterial diversity between treatments (see Fig. S1a and S1b in the supplemental material). Beta diversity analysis using the unweighted option did not reveal a split between treatments (see Fig. S2).

A full outline of the relative abundances of all bacterial taxa in the porcine cecum is available in Table S1 in the supplemental material. Major bacterial taxa are presented in Fig. 1, while minor taxa which were statistically significant or showed tendencies toward significance are presented in Table 5. A total of 15 different bacterial phyla were detected in the porcine cecum. 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 (Fig. 1a; also see Table S1). No significant differences were observed with respect to the relative abundances of bacterial phyla in the ceca of pigs fed the Bt maize versus the isogenic maize. A low prevalence was observed for *Fusobacte-*

**TABLE 4** Estimations of bacterial diversity at 97% similarity within the ceca of weanling pigs fed isogenic and Bt maize-based diets[a]

| Parameter | Value for indicated maize-based diet | |
|---|---|---|
| | Isogenic[b] | Bt[c] |
| Chao 1 richness estimation | 3,904 | 3,149 |
| Shannon's index for diversity | 6.2 | 6.1 |
| Good's coverage | 95% | 96% |

[a] Mean of $n = 9$ pigs/treatment.
[b] Isogenic maize-based diet fed for 31 days.
[c] Bt maize-based diet fed for 31 days.

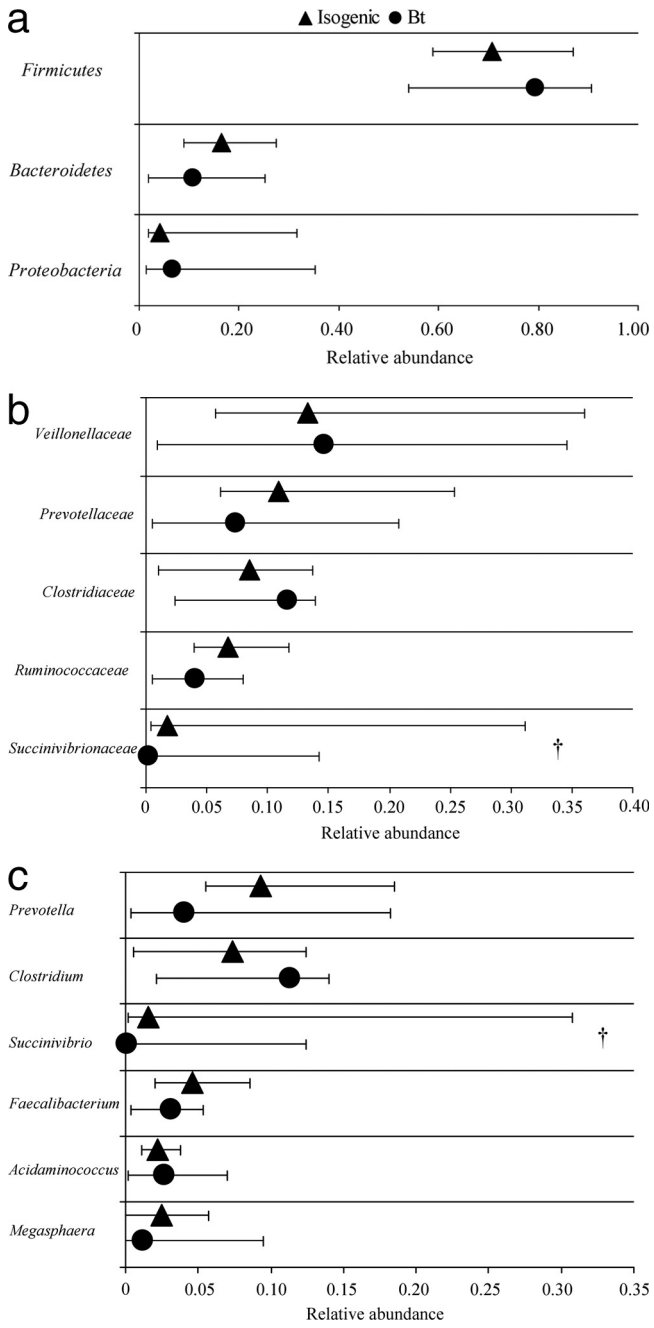


**FIG 1** Effect of feeding a Bt or isogenic maize-based diet for 31 days on the mean relative abundances of the major phyla (a), families (b), and genera (c) in the ceca of weanling pigs. A full outline of the relative abundance of all bacterial taxa in the porcine cecum is available in Table S1 in the supplemental material, and minor taxa which were statistically significant or showed tendencies toward significance are presented in Table 5. 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 larger 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 nonparametric test.

*ria*, which were detected in the cecum of only one of nine pigs fed the isogenic maize-based diet and in the ceca of 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 5). Similarly, a lower 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 5).

A total of 39 bacterial families were identified in the weanling pig cecum (see Table S1 in the supplemental material). 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%) (Fig. 1b). There were no significant differences between treatments in the relative abundance of any of these major families (Fig. 1b). A greater abundance of *Enterococcaceae* and *Erysipelotrichaceae* ($P \leq 0.05$) was observed in the ceca of pigs fed the Bt maize-based diet than for pigs fed the isogenic maize-based diet (Table 5). No other family differed with respect to cecal abundance between pigs fed the isogenic and the Bt maize-based diets (Table 5; also see Table S1). However, the cecal abundance of *Succinivibrionaceae* tended to be lower for pigs fed the Bt treatment than for pigs fed the isogenic treatment (0.17% versus 1.80%; $P = 0.08$) (Fig. 1b). A low prevalence was observed for the *Bifidobacteriaceae* family, which was detected in the ceca of only two pigs from the isogenic treatment and five pigs from the Bt treatment. This resulted in a tendency toward higher *Bifidobacteriaceae* abundance in the ceca of pigs fed Bt maize diets than in the ceca of pigs fed isogenic maize diets ($P = 0.06$) (Table 5).

A total of 54 genera were identified in the ceca of pigs in the present study (see Table S1 in the supplemental material). Figure 1c summarizes the six most abundant genera identified in the weanling pig cecum, 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 ($P > 0.05$) (Fig. 1c). However, the cecal abundance of *Blautia* ($P < 0.05$) (Table 5) was lower for pigs fed the Bt maize diet compared to pigs fed the isogenic maize diet. Similar to that of its corresponding family (*Bifidobacteriaceae*), *Bifidobacterium* prevalence in the ceca 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 5). No other genera differed significantly in relative cecal abundance between pigs fed the Bt or isogenic maize-based diets (see Table S1). The genus *Succinivibrio* tended to be less abundant in the ceca of pigs fed the Bt treatment than in the ceca of pigs fed the isogenic treatment ($P = 0.10$) (Fig. 1c), similar to findings for the corresponding family, *Succinivibrionaceae*. Cecal *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 5).

## DISCUSSION

To date, research investigating the effect of feeding Bt maize on gastrointestinal bacterial communities has been limited to studies in ruminants (11, 51, 56). To our knowledge, the present study is the first to characterize porcine intestinal microbiota composition following Bt maize consumption. A culture-independent high-

**TABLE 5** Effect of feeding a Bt maize-based diet for 31 days on the relative abundance of cecal bacterial taxa in weanling pigs[a]

| Taxon | Value for indicated maize-based diet | | | | *P* value[e] | *n*[f] |
|---|---|---|---|---|---|---|
| | Isogenic[b] | | Bt[c] | | | |
| | Relative abundance | 5th–95th percentiles[d] | Relative abundance | 5th–95th percentiles | | |
| Phyla | | | | | | |
| *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 |
| Families | | | | | | |
| *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 |
| Genera | | | | | | |
| *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 |

[a] 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 cecum is available in Table S1 in the supplemental material.
[b] Isogenic maize-based diet fed for 31 days.
[c] Bt maize based-diet fed for 31 days.
[d] The 5th percentile is larger than 5% of the values and the 95th percentile is larger than 95% of the values.
[e] *P* value from the one-way ANOVA test (*) or the Kruskal-Wallis nonparametric test (†).
[f] *n*, number of animals in which the bacterial taxon was present (isogenic versus Bt).

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 cecum and uses the largest number of independent samples to date (18 pigs).

Culturable fecal, cecal, 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., who reported that Bt rice administration for 90 days had no effects on fecal or small intestinal counts of *Lactobacillus* in rats (48). 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 (9), physiology, feed intake and diet (39), 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 (19, 33).

Sequence-based compositional analysis of the cecal 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 (28, 29, 31, 38). Similarly, Poroyko et al. detected 11 phyla in the ceca of 21-day-old pigs but found that *Bacteroidetes* dominated, followed by *Firmicutes* and *Proteobacteria* (40). 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 (53).

The presence of *Enterococcaceae* at low abundance as well as at low prevalence in the porcine intestine has previously been reported (29, 53). 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 (14). On the other hand, enterococci are able to translocate across the intestinal epithelium, leading to bacteremia or localized infections (14). In the present study, *Enterococcaceae* were more abundant in the ceca of Bt maize-fed pigs; however, histological examination of intestinal tissue and mesenteric lymph nodes from these pigs did not reveal any signs of intestinal damage or inflammation (54).

The increase in *Erysipelotrichaceae* in pigs fed Bt maize may have occurred as a result of the higher feed intake in these pigs (54). 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 (54). An increase in colonic *Erysipelotrichaceae* has recently been associated with increased dietary fat intake, body weight and fat deposition, and decreased fecal short-chain fatty acid (SCFA) concentrations in mice (15). 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 rumens of sheep fed Bt maize for 3 years (51). However, in the present study, no differences were observed in relative abundance of cecal microbiota with known fiber-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.

Cecal *Bifidobacterium* and the family to which it belongs (*Bifidobacteriaceae*) was 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 (49). 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 (26, 32).

Members of the genus *Blautia* are known to utilize hydrogen and to produce acetate in the intestine (42). The lower abundance of *Blautia* in the ceca 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 fiber-fermenting capacity of the intestinal microbiota. This is because lower fiber 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 the prevalence of these bacterial populations is low. In any case, the differences in relative abundance of certain cecal 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 (54).

Previous studies in cows failed to demonstrate any effects of Bt maize silage on ruminal bacteria (11, 56). However, Wiedemann et al. (56) used only four cows and studied only six ruminal species, making assessment of a community-wide effect difficult. Trabalza-Marinucci et al. also found no effect of Bt maize on culturable amylolytic and cellulolytic bacteria in the rumens of ewes fed Bt maize silage for 36 months (51). Furthermore, ruminal microbiotas differ considerably from those found in monogastrics, such as pigs and humans (30).

Although the Cry1Ab protein has previously been shown to have antibacterial activity against *Clostridium* spp. *in vitro* (57), the present study did not reveal any anticlostridial effects within the porcine cecum 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 (27). However, the concentration of Cry1Ab protein detected in the cecal digesta of pigs in the present study was 2.41 ng/ml, which was 90 times lower than that in the feed (55) and ~4,000 times lower than the concentrations used by Koskella and Stotzky (27) and Yudina et al. (57) 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 cecal bacteria in pigs fed the Bt maize 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 (2, 51). Although nutrient differences between the Bt maize 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 (12, 16, 17, 21, 24, 36, 58). Furthermore, it has been concluded in a study by Barros et al. (3) that environmental factors can affect maize composition more than the genetic modification itself. In any case, the composition of the isogenic maize and Bt maize used in the present study falls, for the most part, within the normal variation reported in the literature (12, 16, 17, 21, 24, 36, 58).

In conclusion, 31 days of Bt maize consumption had only minimal impact on microbial community structure in the ceca of pigs, resulting in statistically significant differences in abundance of only 2 of 39 bacterial families and 2 of 54 genera detected. However, the low abundance and frequency of detection of some taxa, as well as the lack of information on their role within the intestine, make 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, we enumerated a limited number of culturable bacterial groups and investigated only taxonomic distribution. Therefore, additional analyses, using, for example, quantitative PCR and functional metagenomics, are needed to fully elucidate the effects of Bt maize consumption on the functional capacity of intestinal bacterial populations and consequently host health.

## ACKNOWLEDGMENTS

The research leading to these results received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement number 211820 and the Teagasc Walsh Fellowship Programme. None of the authors had a financial or personal conflict of interest regarding the present study, and the work was conducted independently of any commercial input, financial or otherwise.

We thank Fiona Crispie and Gemma McCarthy for technical assistance.

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