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High-throughput Sequencing-based Analysis of the Intestinal Microbiota of Broiler Chickens Fed Genetically Modified Rice Expressing Cry1Ac/Cry1Ab Chimeric *Bacillus thuringiensis* Protein

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Many types of *Bacillus thuringiensis* (Bt)-crops are being grown worldwide, triggering concerns about their potential impact on humans and livestock. To ensure better yield and food safety in China, an attempt has been made to develop Bt-rice targeting a broad range of insects. We aimed to investigate whether feeding genetically modified rice expressing the Bt chimeric Cry1Ac/Cry1Ab protein has any effects on the intestinal microbiota of broilers. Broilers were fed either Bt-rice or its unmodified isogenic parent line for 42 days, and total DNA was isolated from cecum contents for high-throughput sequencing of the 16S rRNA gene. In total, 1,241,005 reads, assigned to 12 phyla, 31 families, and 48 genera were generated. No significant differences were observed in the relative abundance of organisms identified among the major phyla, families, and genera, except for two less abundant families, *Thermoanaerobacteraceae* and *Peptostreptococcaceae*, and two less abundant genera, *Anaerotruncus* and *Gelria*. The results were in agreement with those from culture-based analysis and Biolog EcoPlates. These results illustrate that feeding Bt-rice has no adverse effects on the broiler intestinal microbiota and provide sufficient support for the food safety of Bt-rice.

Key words: broilers, Bt-rice, high-throughput sequencing-based analysis, intestinal microbiota

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Introduction

Rice (*Oryza sativa)* is a key food crop, in 2013 it was grown on over 160. 96 million hectares worldwide with production reaching 476.06 million metric tons. Thirty percent of the rice is produced in China on 18.8% of the world's rice land (USDA, 2014). Insect pests of rice cause tremendous economic and environmental losses. A single species of rice stem borers can cause annual losses of 11.5 billion Yuan (Sheng *et al*., 2003). To ensure better yield and food security in China, attempts have been made to develop *Bacillus thuringiensis* (Bt)-rice targeting a broad range of insects (Chen *et al*., 2011). Although several genetically modified (GM) rice varieties have been approved for commercialization globally, only one variety, TT51 which harbors *cry1Ac/cry1Ab*, was granted a safety certificate in China in 2009. It has been reported that variety TT51 showed high resistance to *Scirpophaga incertulas* and *Pyralidae* in field tests, which reduced the use of pesticides and increased rice production (Tu *et al*., 2000).

The accumulated hectarage of GM crops planted globally in 2013 exceeded 1.75 billion hectares (James, 2014). Many types of Bt-crops have been commercialized worldwide, triggering concerns about their potential impact on the health of humans and livestock. A large number of feeding studies have shown that the Cry proteins expressed by Bt-crops are non-toxic to humans and mammals (Kılıç *et al*., 2008; Buzoianu *et al*., 2013; Zeljenková *et al*., 2014). Diets containing Bt-corn had no deleterious effects on weight gain and carcass yields of broilers (Brake *et al*., 2003; Taylor *et al*., 2003; McNaughton *et al*., 2007). As for Bt-rice, only two studies have been conducted to investigate the effects of

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feeding KMD1 rice expressing the Cry1Ab protein (Schrøder *et al*., 2007) or T2A-1 rice expressing the Cry2A protein (Yuan *et al*., 2013) on the health of rats; no toxic effects were found. There were no significant biological differences in the reproductive system of rats fed TT51 Bt-rice for one or two generations (Wang *et al*., 2013, 2014a).

Moreover, an increasing number of studies have analyzed the effects of Bt-crops on the intestinal microbiota of mammals (Buzoianu *et al*., 2012a, b, 2013) as well as on other non-target organisms (Geng *et al*., 2013; Jiang *et al*., 2013; Wang *et al*., 2014b). A balanced intestinal microbiota plays an important role in metabolic reactions and nutritional absorption in the intestinal tract (Harris *et al*., 2012; Davila *et al*., 2013). Historically, several methods such as culture-based approaches, denaturing-gradient gel electrophoresis (DGGE), and real-time polymerase chain reaction (PCR) have been used widely to analyze the composition of intestinal microflora. However, culture-based methods could identify only 10−40% of bacteria (Tannock *et al*., 2001, 2002), co-migration of phylogenetically heterogeneous bands reduced the sensitivity of DGGE (Yang *et al*., 2000; Sekiguchi *et al*., 2001), and real-time PCR could only be used to monitor selected bacterial taxa (Matsuki *et al*., 2004; Yuan *et al*., 2011). The advent of high-throughput sequencing has offered a new way of investigating the microbial community. Syrups supplemented with high concentrations of Cry1Ie toxin (20 μg/mL) did not significantly affect the diversity of the midgut bacteria in honey bees (Jia *et al*., 2016). GM MON810 maize had no adverse effects on the intestinal microbiota of pigs or their offspring (Buzoianu *et al*., 2012a, b, 2013). However, GM rice expressing lectin or PAT protein has been reported to cause significant changes to the intestinal microflora of rats (Poulsen *et al*., 2007a, b). Yet, when selected intestinal bacteria (Yuan *et al*., 2011) and microflora composition (Yuan *et al*., 2013) of rats fed T2A-1 rice were analyzed by real-time PCR or DGGE, no significant effects were observed.

Therefore, in this study, we investigated the effects of feeding Bt-rice for 42 days on the microbiota of broiler chickens.

Materials and Methods

Rice Materials

The rice used in this study was the GM insect-resistant rice TT51, which harbors a synthetic *cry1Ac*/*cry1Ab* gene (patent no. ZL 95119563.8) that was introduced into the elite indica rice restorer line Minghui 63, provided by Huazhong Agricultural University, Wuhan, China.

Sample Collection and Cultivation

All experimental procedures involving animals were approved by Animal Care and Use Committee, Institute of Animal Science, CAAS. In total, 140 one-day-old female Arbor Acres broilers (Beijing Huadu Broiler Corp., Beijing, China) were evenly divided and randomly allocated to two groups; one group was fed transgenic rice TT51 and the other group was fed the parent line Minghui 63. Seven broilers were selected randomly from each group and samples of the content of the cecum were collected in sterile containers after 42 days of isogenic or Bt-rice consumption. Broilers were euthanized using CO_2 asphyxiation, followed by exsanguination. The last meal was administered 10 h prior to euthanasia. Immediately after the euthanasia, the ceca were collected from the broilers and stored in liquid nitrogen until analysis.

For conventional culture, 0.2 g fecal sample was suspended in 19.8 mL of 0.85% NaCl and shaken for 30 min. Total aerobes and *Enterobacteriaceae* were cultured at 37℃ for 24 h and 72 h, respectively. Total anaerobic bacteria and *Lactobacillus* were maintained in a Whitley VA500 anaerobic workstation (DW Scientific, Shipley, United Kingdom).

Bacterial Functional Diversity

Bacterial functional diversity was studied using Biolog EcoPlates^{TM}. Samples of conventional cultures were diluted 1,000 times for inoculation. Plates were incubated in the Whitley VA500 anaerobic workstation at 25℃, and absorbance at 595 nm was measured daily for 7 d. The average well-color development (AWCD) value was calculated for each well separately.

DNA Extraction and PCR

Total DNA was isolated from the contents of cecum using a QIAamp DNA stool mini kit (Qiagen, Hilden, Germany). The precipitated DNA was washed and purified using a QIAamp spin column (Qiagen) according to the manufacturer's instructions and finally eluted in 200 μL of purified water.

The microbial composition of these samples was determined by sequencing the 16S rRNA V6, V7, V8, and partial V9 variable regions (434 bp). The V6, V7, V8, and partial V9 regions of the 16S rRNA gene were amplified using the universal primers, 5′-AACGCGAAGAACCTTAC-3′ and 5′-CGGTGTGTACAAGACCC-3′.

16S rRNA Gene Sequencing and Bioinformatics Analysis

Next-generation sequencing library preparation and Illumina MiSeq sequencing were conducted at GENEWIZ, Inc. (Beijing, China). DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), and DNA quality was assessed by electrophoresis on a 0.8% agarose gel.

A sequencing library was constructed using a MetaVx^{TM} Library Preparation kit (GENEWIZ, Inc., South Plainfield, NJ, USA). Indexed adapters were added to the ends of the 16S rRNA amplicons by limited-cycle PCR. DNA libraries were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and quantified by Qubit analysis and real-time PCR (Applied Biosystems, Carlsbad, CA, USA). DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument according to manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using a 2×250 paired-end configuration; image analysis and base calling were conducted using the MiSeq Control Software (MCS) on the MiSeq instrument. Initial taxonomic analysis was carried out on the Illumina BaseSpace cloud computing platform.

Sequence reads were clustered into operational taxonomi-

cal units (OTUs) using the QIIME suite of software tools (http://wwwqiime.org). Subsequently, beta diversities of the samples were calculated. Principal coordinate analysis (PCoA) and hierarchical clustering of samples were implemented.

Statistical Analysis

The number of reads was log-transformed to base 10 to ensure a normal distribution of the data. The relative abundance values were calculated by dividing the number of reads assigned to each taxonomic rank by the number of reads assigned to the phylum. Data were analyzed with SPSS version 19.0 (SPSS Inc., Chicago, IL, USA), and the Shapiro–Wilk test was used to check for normality. Data that were normally distributed were analyzed using a paired *t*-test, and non-normally distributed data were analyzed using a non-parametric test (Kruskal–Wallis).

Results

Culture-based Analysis of the Effect of Feeding Bt-rice on Broiler Cecal Microbiota

The direct bacterial counts of fecal samples from the two treatments were from 5.22 to 6.00 $\log_{10}$ CFU/g (Table 1). There were no differences in the counts of *Lactobacillus*, *Enterobacteriaceae*, total aerobes or total anaerobes in the ceca of broilers fed isogenic or Bt rice-based diets for 42 days ($p>0.05$).

Effect of Bt-rice on Functional Diversity of Cecal Microbial Communities of Broilers Fed a Bt-rice-based Diet

The potential effects of Bt-rice on the functional diversity of cecal microbial communities were analyzed using Biolog EcoPlates. In this study, no significant differences between Bt- and isogenic-rice-fed broilers were observed in AWCD curves ($p>0.05$, Fig. 1). Additionally, no obvious differences were found among the Shannon, Simpson, and McIntosh indices for Bt and isogenic rice ($p>0.05$, Table 2).

High-throughput 16S rRNA Gene-sequencing Analysis of Broiler Cecal Microbiota

The microbial composition of the cecal samples was determined by sequencing of the 16S rRNA gene to determine the effects of feeding the broilers a Bt-rice diet. In total, 1,241,005 reads from the V6, V7, V8, and partial V9 variable regions of the 16S rRNA gene were generated by high-throughput sequencing of the DNA obtained from cecal content samples of the broilers. Of these sequences, 1,239,269 reads (99.9%) were assigned to the phylum level; 1,154,190 (93.0%) to the family level; and 1,094,310 (88.2%) to the genus level.

The relative abundances of all cecal bacterial taxa from broilers fed an isogenic- or Bt-rice-based diet are shown in Table S1. In total, 12 bacterial phyla were detected in the broiler intestine (Fig. 2a). However, 91.0% of the sequence reads assigned at the phylum level were derived from three phyla, *Firmicutes* (51.0%), *Bacteroidetes* (27.8%), and *Proteobacteria* (12.2%), with the remaining 10 assigned phyla

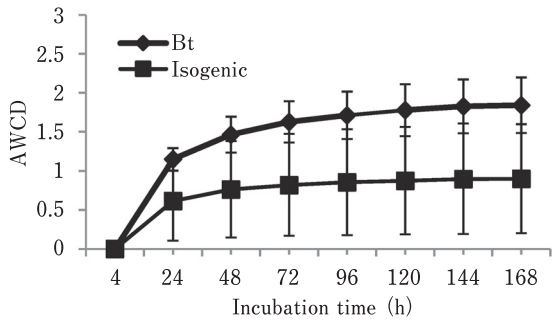

Fig. 1. **The AWCD variations of gut microbial community during incubation.**

Table 1. **Effect of different varieties of rice on cecum microbial community of broilers fed for 42 days (lg_{10} CFU g^{-1})**

Bacterial group	Isogenic rice based diet	Bt-rice based diet	P value
Enterobacteriaceae	5.22±0.689	5.31±0.380	0.783
Lactobacillus	5.88±0.461	6.00±0.217	0.538
Total aerobes	5.39±0.617	5.65±0.168	0.311
Total anaerobes	5.85±0.400	5.97±0.285	0.549

Table 2. **Functional diversity of microbe community in broiler cecum fed Bt rice or isogenic rice**

	Isogenic rice based diet	Bt-rice based diet	P value
Shannon	4.17±0.327	4.17±0.171	0.986
McIntosh	9.26±5.741	9.84±1.991	0.802
Simpson	118.46±239.756	242.38±32.385	0.200

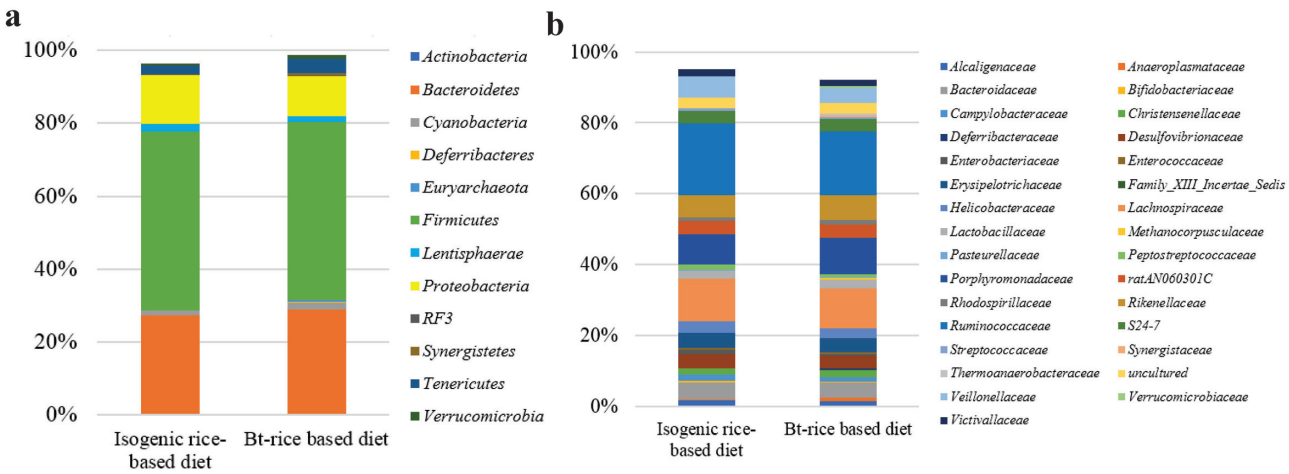

Fig. 2. **The mean relative abundances of the phyla (a) and families (b) in the intestine of broilers fed a Bt or isogenic rice-based diet.**

accounting for 9.0% of the sequence reads (Fig. 3a and Table S1). No significant differences were found in the relative abundance of bacterial phyla in the cecum of broilers fed on Bt-rice and those fed isogenic rice.

In total, 31 bacterial families were identified from the DNA present in the broiler cecal contents (Fig. 2b and Table S1). The most abundant were *Ruminococcaceae* (overall average of 19.2%), *Lachnospiraceae* (11.6%), and *Porphyromonadaceae* (9.3%; Fig. 3b). No significant differences were observed with respect to the relative abundance of any of these major families in the cecal contents of broilers fed isogenic *vs*. Bt-rice (Fig. 3b). However, as for the less abundant families, *Thermoanaerobacteraceae* were present at higher levels in broilers fed Bt-rice ($p=0.03$, Table 3) while *Peptostreptococcaceae* were present at higher levels in broilers fed isogenic rice ($p=0.02$, Table 3).

In total, 48 genera were identified from the DNA obtained from the broiler cecal contents. The five most abundant genera identified here were *Bacteroides* (overall average of 4.6%), *Barnesiella* (4.0%), and *Alistipes* (4.0%; Fig. 3c). There were no differences in the relative abundance of any of these major genera between the broilers fed on the different types of rice (Fig. 3c). However, the cecal abundance of *Anaerotruncus* was higher for broilers fed the isogenic rice diet than for broilers fed the Bt-rice diet ($p=0.02$, Table 3), and *Gelria* was present at higher levels in broilers fed Bt-rice ($p=0.03$, Table 3).

Discussion

Several studies have focused on the effects of GM crops on the cecal microbiota of mammals (Buzoianu *et al*., 2012a, b, 2013) and on other non-target organisms (Geng *et al*., 2013; Jiang *et al*., 2013). To our knowledge, the present study is the first to characterize the composition of the intestinal microbiota of broiler chickens following Bt-rice consumption employing high-throughput sequencing. By using deep sequencing of the 16S rRNA gene, we determined the microbial community structure in broiler cecum comprehensively, and we obtained 1,241,005 reads, which were assigned to 12 phyla, 31 families, and 48 genera. Only minimal impact on the broiler cecal microbiome, reflected as statistically significant differences in the relative abundances of two families and genera, were observed upon Bt-rice consumption. By using culture-based analysis and Biolog EcoPlates, we observed no differences in the quantity and functional diversity of cecal microbial communities between broilers fed an isogenic- or Bt-rice-based diet for 42 days. Furthermore, no deleterious effects on body weight and immune function of broilers fed a Bt rice-based diet were found in our experiment (Liu *et al*., 2016).

Sequence-based analysis of the cecal microbiota community revealed no significant differences in the relative abundances of bacterial phyla between the Bt- and isogenic rice-fed broilers, indicating that the Bt-rice is well tolerated by the host and cecal microbiota at the phylum level. Our deep 16S rRNA gene-sequencing approach detected 12 bacterial phyla in broilers, which was less than that previously detected in pigs (Buzoianu *et al*., 2012b, 2013). The main phyla identified were *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, and this was in agreement with previously reported data on the cecal microbiota of broilers using 16S rRNA gene sequencing (Qu *et al*., 2008). Another study previously identified 11 phyla in the ceca of 49-day-old broilers, but found a predominance of *Proteobacteria*, followed by *Firmicutes* and *Bacteroidetes* (Singh *et al*., 2012). However, Cressman *et al*. (2010) detected only four phyla in the cecum of 42-day-old broilers, but, consistent with our results, found that *Firmicutes* dominated.

Among the 31 bacterial families identified, the main families were *Ruminococcaceae*, *Lachnospiraceae*, and *Porphyromonadaceae*. Ceca of 49-day-old and 42-day-old broilers have been reported to be dominated by *Ruminococcaceae*

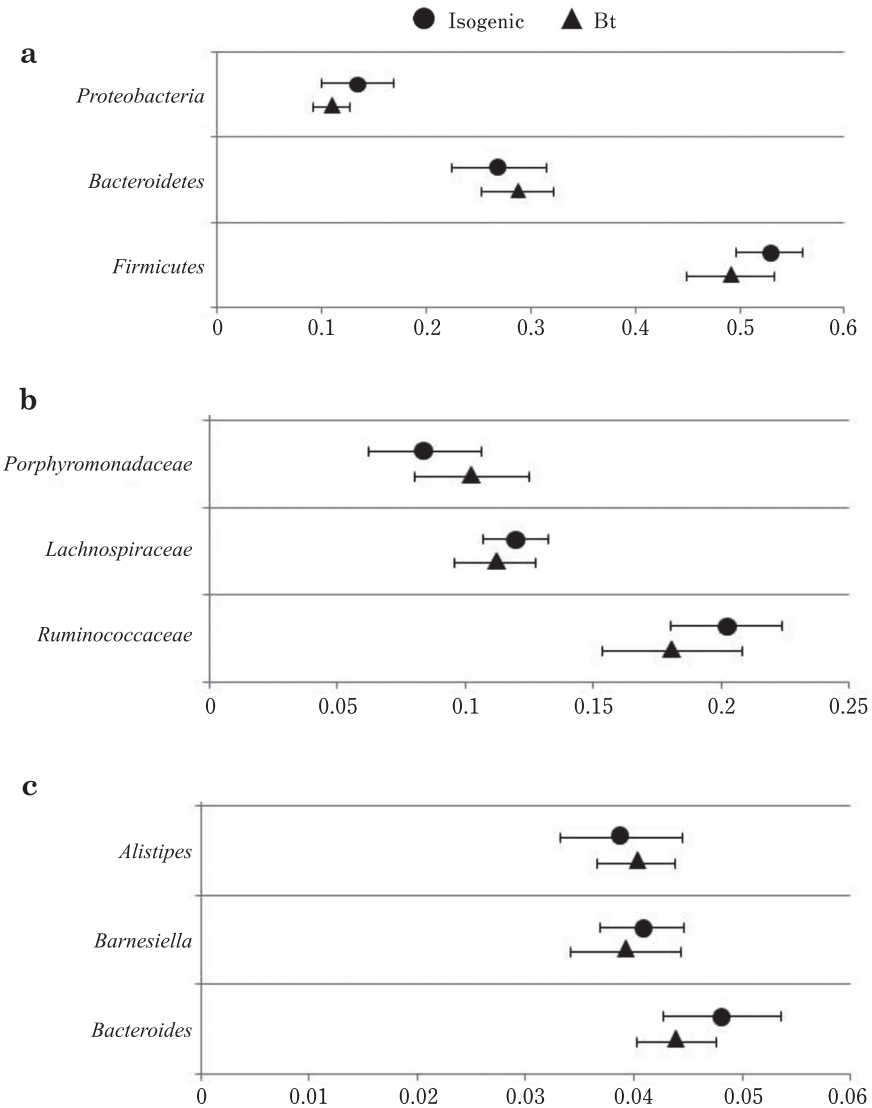

Fig. 3. **Effect of feeding a Bt or isogenic rice-based diet on the mean relative abundances of the major phyla (a), families (b), and genera (c) in the intestine of broilers.** Data are presented as the mean from 8 samples per treatment. Whiskers on each bar represents 95% confidence intervals.

and *Lactobacillaceae*, respectively (Cressman *et al*., 2010). No marked differences were observed in the major families. However, the less abundant family, *Thermoanaerobacteraceae*, which is non-enteropathogenic (Moon *et al*., 2013), was present at higher levels in broilers fed Bt-rice ($p=0.03$, Table 3). *Peptostreptococcaceae,* a pathogen associated with enteric and atopic diseases in infants (Penders *et al*., 2007), was present at lower levels in broilers fed Bt-rice than in those fed isogenic rice ($p=0.02$, Table 3).

Two out of the 48 genera identified here showed a difference in relative abundance in broilers fed isogenic rice and those fed Bt-rice. *Anaerotruncus* was less abundant in broilers fed Bt-rice than in those fed isogenic rice; *Anaerotruncus colihominis* has been reported to cause bacteraemia (Lau *et al*., 2006). *Gelria*, which is a glutamate-degrading anaerobe that may play a role in cellulose methanization (Plugge *et al*., 2002; Lü *et al*., 2014), was present at a higher level in broilers fed Bt-rice. These minimal differences observed at the genus level are not believed to be of major biological importance, and these were not associated with any adverse health effects. A previous high-throughput sequencing-based study of pigs fed Bt-maize for 31 days also demonstrated a minimal impact on the cecal microbial community structure, with only two of 39 bacterial

Table 3. **Effect of feeding a Bt rice based diet on the relative abundance of intestinal bacterial taxa of broilers**

	Value for different rice based diet				P value
	isogenic		Bt		
	Relative abundance	95% confidence intervals	Relative abundance	95% confidence intervals	
Family					
Peptostreptococcaceae	0.0145	0.0108−0.0182	0.0088	0.0049−0.0127	0.02
*Thermoanaerobacteraceae**	0	0	0.0031	−0.0003−0.0119	0.03
Genus					
Anaerotruncus	0.0280	0.0244−0.0316	0.0228	0.0197−0.0258	0.02
*Gelria**	0	0	0.0031	0−0.0063	0.03

P value from the paired *t* test. * *P* value from the Kruskal–Wallis nonparametric test.

families and two of 54 genera showing statistically significantly different relative abundances (Buzoianu *et al*., 2012b).

Conclusion

No significant differences were found in the relative abundances of bacterial phyla, families, and genera between Bt- and isogenic-rice-fed broilers, except for two families and two genera that were less abundant (0−2.8%). *Peptostreptococcaceae* and *Anaerotruncus*, which are reportedly associated with enteric and atopic diseases and bacteraemia, respectively, were lower in Bt-rice-fed broilers.

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