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Effects of Cry1Ab Bt maize straw return on bacterial community of earthworm *Eisenia fetida*

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HIGHLIGHTS

- *E. fetida* accelerated Cry1Ab protein degradation in Bt maize straw and soil.
- Bt maize straw return has significant effect on soil nutrients (e.g. N levels).
- Bt straw return affected soil bacterial community on the 75th and 90th d.
- Bt straw return affected the bacterial community of earthworm casts.
- Changes in cast bacterial community were related to Cry1Ab and soil N levels.

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ABSTRACT

The eco-toxicological effects of *Bacillus thuringiensis* (Bt) maize on earthworm life-history traits were widely studied and the results were controversial, while their effects on earthworm bacterial community have been rarely studied. Here, effects of two hybrids of Bt maize [5422Bt1 (event Bt11) and 5422CBCL (MON810)] straw return on *Eisenia fetida* bacterial community were investigated by the terminal restriction fragment length polymorphism (T-RFLP) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) combing with DNA sequencing, compared to near-isogenic non-Bt maize (5422). Bt maize straw return had significant effects on soil nutrients, especially for available nitrogen (N). The significant differences were shown in soil bacterial community between Bt and non-Bt maize treatments on the 75th and 90th d, which was closely correlated with soil available N, P and K rather than Cry1Ab protein. There was no statistically significant difference in the bacterial community of earthworm gut contents between Bt and non-Bt maize treatments. The significant differences in the bacterial community of earthworm casts were found among three maize varieties treatments, which were closely correlated with Cry1Ab protein and N levels. The differentiated bacterial species in earthworm casts mainly belonged to Proteobacteria, including *Brevundimonas*, *Caulobacter*, *Pseudomonas*, *Stenotrophomonas*, *Methylobacterium*, *Asticcacaulis* and *Achromobacter* etc., which were associated with the mineralization, metabolic process and degradation of plants residues. Therefore, Bt maize straw return caused changes in the bacterial community of *E. fetida* casts, which was possibly caused by the direct (Cry1Ab protein) and non-expected effects (N levels) of Bt maize straw.

1. Introduction

Genetically modified (GM) crops expressing insecticidal crystalline (Cry) proteins derived from *Bacillus thuringiensis* (Bt) are being cultivated with increasing frequency worldwide. Bt maize

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(*Zea mays*) carries genes encoding Cry proteins that are toxic to maize borers (e.g., *Ostrinia nubilalis*) and maize rootworms (*Diabrotica* spp.) and has become one of the most rapidly commercialized anti-insect crops in the world (James, 2015). However, a major concern with the cultivation and return of Bt maize is their potential effects on soil ecosystems (soil microorganisms, microbe-mediated processes and functions, and soil-dwelling invertebrates) due to the presence of insecticidal proteins which may result from root exudates, pollen dispersal and plant residues remained in the field for extended periods (Losey et al., 1999; Saxena et al., 2004; Clark et al., 2005; Icoz et al., 2008; Icoz and Stotzky, 2008; Miethling-Graff et al., 2010; Zurbrügg et al., 2010; Fließbach et al., 2012). As a toxin, Cry protein in Bt maize straw or released to soil from Bt straw return has been shown to degrade slowly and to accumulate in soil with insecticidal activity (Zurbrügg et al., 2010; Feng et al., 2011), which presents potential risk for non-target organisms in soil environment, such as earthworms (e.g., Saxena et al., 1999; Saxena and Stotzky, 2000; Zwahlen et al., 2003; Icoz et al., 2008; Zhang et al., 2012).

Bt maize residues have been shown to exhibit large quantitative differences in plant components such as carbohydrates, cellulose, lignin, carbon and nitrogen with conventional maize (Saxena and Stotzky, 2001; Rossi et al., 2003; Zwahlen et al., 2003; Poerschmann et al., 2005; Flores et al., 2005; Icoz and Stotzky, 2008). The changes in above plant components could affect nutrition parameters of plant material (Clark and Coats, 2006) and the decomposability of plant residues in soil (Flores et al., 2005; Zwahlen et al., 2007; Hönemann and Nentwig, 2009), which consequently cause the unintended effects on soil non-target organisms, including earthworms (Escher et al., 2000; Icoz et al., 2008; Icoz and Stotzky, 2008; Zhang et al., 2012; Shu et al., 2015).

In arable soils, earthworms represent crucial non-target organisms (Icoz and Stotzky, 2008), which are a large and common component of soil ecosystem and are often considered as the keystone group within soil food webs (Wall et al., 2010). We reviewed the potential impact of Bt crops on the earthworms and found that in the majority of studies Bt crops expressing insecticidal proteins have no detrimental effects on earthworms (Zhang et al., 2012). Additionally, some studies have identified minor adverse effects relative to the earthworms receiving Bt maize treatments (Vercesi et al., 2006; Hönemann and Nentwig, 2009; van der Merwe et al., 2012). However, previous studies have focused on the potential eco-toxicological of Bt crops occurring to the life-history traits or the population of earthworms (Hönemann et al., 2008; Zeilinger et al., 2010; Shu et al., 2011, 2015), while the effects of Bt crops on earthworm bacterial community have been rarely studied.

Earthworms perform many critical ecosystem functions (nutrient cycling and residue decomposition) depending on the interaction between them and bacterial community in living and internal environment, which are also controlled by the quality and quantity of plant litter (Dijkstra et al., 2006; Thakuria et al., 2010). Thakuria et al. (2010) found that food resource type can cause shifts in the gut wall-associated bacterial community, which in turn strongly affected decomposition through gut associated processes, i.e. via the effects of ingestion, digestion and assimilation of the organic matter and microorganisms, and then released in earthworm casts (Aira et al., 2009; Monroy et al., 2011). Thus, the direct (Cry toxin) and unintended effects [e.g., nutrient resource and living environment (soil)] of Bt maize straw on the bacterial community (gut contents and casts) of earthworms should be necessary for GM crops risk assessment.

In a previous study, multilevel assessment of Cry1Ab Bt maize (5422Bt1, 5422CBCL) straws return affecting the epigeic earthworm *Eisenia fetida* were investigated using 90-day microcosm (Shu et al., 2015). 5422Bt1 straw return had no deleterious effects, while 5422CBCL presented negative effects on adult earthworms with the corresponding response in enzymes [Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD)] activity and genes [heat shock protein 70 (Hsp70), translationally controlled tumour protein (TCTP), SOD and annetocin (ANN)] expression. We did not find that effects of Bt maize straw return on *E. fetida* were related to Cry1Ab protein in the straw and soil. Compared to 5422Bt1 and control, changes in decomposition and nutrients in 5422CBCL likely resulted in negative effects on the growth and reproduction of earthworm. However, the controversial results in adult earthworms from the 2nd and 3rd generation under 5422CBCL treatments did not support above speculation. The more complicated reason needs further studies, for example, the changes in living environment (soil nutrients and bacterial community) and bacterial community in earthworms themself. Hence, evaluating the potential effects of Bt maize straw return on bacterial community in earthworms and soil might further reveal the mechanism of their impact on fitness parameters (growth and reproduction) of *E. fetida*.

In the present study, we continue to carry out a 90-day microcosm study with the epigeic earthworm species *E. fetida* (Lumbricidae) bred in soil surface-applied with two hybrids of Bt maize (5422Bt1, 5422CBCL) expressing Cry1Ab protein and near isogenic, non-Bt maize (5422). The bacterial community in soil, gut contents and casts of earthworms were determined by the Terminal restriction fragment length polymorphism (T-RFLP) and Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) combing with DNA sequencing; soil nutrients [organic matter (OM), total nitrogen (N), total phosphor (P), total potassium (K), available N, available P and available (K) and the Cry1Ab protein concentrations in straw, soil and earthworm gut contents were also measured. The aim of the present study was therefore to investigate whether Bt maize straw return affects the bacterial community of earthworms. We also determined whether these effects are related to the direct (Cry1Ab protein) and unintended effects (soil nutrient status and bacterial community) of Bt maize straw return.

2. Materials and methods

2.1. Soil, maize plants, and earthworms

Soil was collected from the top layer (5–25 cm) of the conventional maize field at the Agriculture Experiment Station (23°08′N, 113°15′E) of South China Agriculture University, Guangzhou, China. No GM maize had been grown previously at or around this site. The processing of soil was described as Shu et al. (2015). The soil was a red clay loam with a pH of 5.7, containing 17.57 g kg^{-1} OM, 1.00 g kg^{-1} total N, 1.19 g kg^{-1} total P, 24.04 g kg^{-1} total K, 116.05 mg kg^{-1} available N, 99.78 g kg^{-1} available P, and 144.9 g kg^{-1} available K.

Two Bt maize hybrids from Beck's Superior Hybrids were cultivated in a greenhouse, 5422Bt1 (Event Bt11) and 5422CBCL (MON810), both expressing Cry1Ab protein. Their conventional (non-Bt) parent line 5422 served as a control in this study. The cultivation of maize plants was described as Shu et al. (2015). Three weeks after pollen was shed, the straw, including the leaves and stalks of the maize, were cut into approximately 2- to 4-cm-length pieces, freeze-dried, ground, and sieved through a 1 mm mesh. The plant material was stored at −20 °C until used in the experiments. The basic straw characteristics of the three maize varieties were listed in Table 1 in Shu et al. (2015).

The test species earthworm, *E. fetida* Daping No. 2, was bred in our lab and was originally derived from a culture purchased from Hollen Ecological Agricultural Company, Guangzhou, China. Prior to the experiment, the earthworms were kept in a climate-controlled

Table 1
The contents of nutrients in soil from different treatments.

		Background	Day 0–15	Day 0–30	Day 0–45	Day 0–60	Day 0–75	Day 0–90
Organic matter (g kg^{-1})	5422	17.57 ± 1.30	22.11 ± 1.26	23.43 ± 1.32	23.41 ± 1.31	23.99 ± 1.27	**21.77 ± 0.75b**	26.29 ± 2.41
	5422Bt1		20.28 ± 0.36	22.32 ± 0.43	24.15 ± 1.06	25.82 ± 1.33	**26.39 ± 1.57a**	25.31 ± 1.11
	5422CBCL		21.94 ± 1.36	23.63 ± 1.49	22.87 ± 1.11	23.99 ± 1.43	**24.15 ± 0.73ab**	24.15 ± 1.18
Total nitrogen (g kg^{-1})	5422	0.99 ± 0.09	1.19 ± 0.09	1.30 ± 0.11	1.31 ± 0.08	**1.34 ± 0.03b**	**1.31 ± 0.06b**	**1.55 ± 0.19a**
	5422Bt1		1.22 ± 0.05	1.31 ± 0.08	1.39 ± 0.07	**1.61 ± 0.1a**	**1.61 ± 0.09a**	**1.60 ± 0.08a**
	5422CBCL		1.13 ± 0.01	1.20 ± 0.02	1.30 ± 0.08	**1.38 ± 0.05b**	**1.47 ± 0.05a**	**1.26 ± 0.08b**
Total phosphorus (g kg^{-1})	5422	1.19 ± 0.08	1.21 ± 0.01	1.29 ± 0.03	1.36 ± 0.08	1.32 ± 0.01	1.34 ± 0.04	1.40 ± 0.11
	5422Bt1		1.25 ± 0.04	1.29 ± 0.02	1.45 ± 0.08	1.41 ± 0.05	1.38 ± 0.05	1.36 ± 0.02
	5422CBCL		1.25 ± 0.03	1.28 ± 0.02	1.31 ± 0.01	1.31 ± 0.01	1.39 ± 0.02	1.37 ± 0.04
Total potassium (g kg^{-1})	5422	24.04 ± 0.39	24.04 ± 0.34	24.53 ± 0.87	**25.96 ± 0.66ab**	25.40 ± 1.43	25.67 ± 0.10	25.92 ± 1.35
	5422Bt1		24.08 ± 0.83	24.74 ± 0.48	**26.36 ± 0.54a**	25.82 ± 0.37	22.87 ± 2.26	24.7 ± 2.14
	5422CBCL		24.80 ± 1.02	25.80 ± 1.41	**25.09 ± 0.19b**	26.02 ± 0.04	25.22 ± 0.11	25.09 ± 1.87
Available nitrogen (g kg^{-1})	5422	116.05 ± 3.14	**204.74 ± 11.32a**	**175.12 ± 40.51a**	**187.46 ± 25.85a**	**232.67 ± 34.20b**	**195.25 ± 12.17b**	**256.41 ± 43.43b**
	5422Bt1		**190.69 ± 11.04a**	**176.21 ± 12.57a**	**184.47 ± 15.16a**	**314.15 ± 7.73a**	**331.24 ± 55.38a**	**373.22 ± 32.09a**
	5422CBCL		**154.99 ± 20.62b**	**167.67 ± 24.49b**	**148.15 ± 59.63b**	**220.68 ± 8.55b**	**165.01 ± 19.93c**	**155.80 ± 23.44c**
Available phosphorus (mg kg^{-1})	5422	99.78 ± 19.20	127.37 ± 5.41	118.05 ± 8.97	**129.55 ± 5.82ab**	**133.13 ± 1.96b**	**131.85 ± 3.22ab**	139.01 ± 5.85
	5422Bt1		118.17 ± 5.83	112.56 ± 3.39	**133.77 ± 3.85a**	**141.05 ± 3.24a**	**143.35 ± 4.09a**	140.93 ± 5.53
	5422CBCL		124.69 ± 4.81	119.07 ± 4.99	**122.13 ± 5.06b**	**123.03 ± 0.22c**	**129.36 ± 8.15b**	128.47 ± 7.17
Available potassium (mg kg^{-1})	5422	144.97 ± 6.22	834.26 ± 48.77	752.98 ± 97.48	823.61 ± 64.36	**967.35 ± 29.46a**	**891.17 ± 16.67a**	**912.32 ± 71.05b**
	5422Bt1		750.23 ± 33.21	697.61 ± 30.12	851.92 ± 22.22	**954.10 ± 77.73a**	**943.44 ± 56.09a**	**1036.94 ± 49.91a**
	5422CBCL		779.17 ± 67.85	763.71 ± 54.92	888.44 ± 29.62	**846.53 ± 27.68b**	**707.71 ± 37.76b**	**655.72 ± 47.64c**
ANOVA								
Organic matter			0.181	0.495	0.188	0.253	**0.01**	0.668
Total nitrogen			0.067	0.327	0.259	**0.003**	**0.006**	**0.008**
Total phosphorus			0.35	0.69	0.134	0.017	0.364	0.929
Total potassium			0.618	0.393	**0.03**	0.682	0.218	0.906
Available nitrogen			**0.002**	**0.006**	**0.009**	**<0.0001**	**0.004**	**<0.0001**
Available phosphorus			0.12	0.12	**0.026**	**<0.0001**	**0.038**	0.089
Available potassium			0.398	0.279	0.479	**0.028**	**0.001**	**<0.0001**

Values of the content of nutrients in soil from the different treatments, four replicates for each maize variety treatment per day. The difference of the content of nutrients in soil among three maize varieties treatments on the same testing day was analyzed by one way-ANOVA. Bold values followed by the different little letter within a line were significantly different.

chamber (25 °C, 65% relative humidity, 24 h darkness) in the same soil used for the experiments and fed powdered straw from conventional, field-grown maize. Individual adult *E. fetida* that were approximately 2 months old with a clitellum and an average weight of approximately 200 mg (180–220 mg) were chosen for experiment. Before the experiments, they were placed onto clean moist filter paper in the dark for 24 h to void gut contents, and then were washed and dried before use. They were then placed on the surfaces of the test substances in preparation for the subsequent experiments.

2.2. Experimental design

In a 90-day microcosm study, we tested the effects of Bt maize hybrids (5422Bt1 and 5422CBCL) on bacterial community as well as soil nutrients in comparison with 5422. Twenty-four replicates per maize variety treatment were conducted, and four replicates from each maize variety treatment were sampled every 15 days.

Every microcosm in a plastic container (11 cm width × 16 cm length × 10 cm depth) was received 500 g of substances, consisting of air-dried soil and 25 g (5% of the total weight) of powdered maize straw. The straw was evenly distributed onto the surface of the soil. The water content within the plastic container was maintained at 50% of the water holding capacity with distilled water. The healthy, selected 9 individuals of *E. fetida* were added to the microcosm, and the container was closed with gauze to ensure that earthworms could easily breathe over the course of the experiments. Additionally, the containers without *E. fetida* were also carried out as the same as above microcosm to investigate the effect of earthworm presence on the Cry1Ab protein degradation in Bt straw and soil. The study was performed in a climate-controlled chamber (25 °C, 65% relative humidity, 24 h darkness).

2.3. Enzyme-linked immunosorbent assay (ELISA) of Cry1Ab

2.0 g of test maize straw, soil from each container with or without earthworms during the corresponding sampling time was collected, flash frozen, weighed, lyophilized and weighed again. Additionally, earthworms were picked from every treatment and washed in distilled water until nothing remained on their surface. After dissection in phosphate-buffered saline (PBS; 137 mM NaCl; 2.7 mM KCl; 8 mM Na_2HPO_4; 1.5 mM KH_2PO_4; pH 7.5), gut contents of *E. fetida* were collected. The samples were stored at −80 °C until used for Cry1Ab protein detection. The Cry1Ab protein concentrations in above samples were measured using a Cry1Ab/Ac enzyme-linked immunosorbent assay (ELISA) kits, following the manufacturer's protocol (Catalogue number: PSP 06200; Agdia, Elkhart, Indiana, USA). Absorbance was measured at 650 nm with a microplate reader (Molecular Devices, California, USA). The Cry1Ab protein concentration was calculated using a six-point standard curve developed with purified Cry1Ab (supplied with the kit). Test results were validated with both positive and negative controls.

2.4. Determination of soil nutrients

10.0 g of soil from each container during the corresponding sampling time was sampled, flash frozen, weighed, lyophilized and weighed again. The samples were stored at −80 °C until used for soil nutrients extraction. The determination of OM, total N, total P, total K, available N, available P and available K in soil referred to Bao (2000).

2.5. DNA extraction

Containers with *E. fetida* were described in Section 2.2, eighteen replicates per maize variety. Every 15 days, three replicates from

each maize variety were sampled. The surviving adult earthworms were isolated from each replicate and washed in distilled water until nothing remained on their surface. After dissection, the gut contents were collected. In addition, 2.0 g of soils and earthworm casts from each container during the corresponding sampling time were also collected. The samples were frozen immediately in liquid nitrogen and stored at −80 °C until used for DNA extraction.

Total DNA was extracted from samples using the cetyltrimethylammonium bromide method or a FastDNA Spin Kit (BIO 101 Systems, California, USA), respectively, according to the protocols provided by the manufacturers. DNA quality was examined by 1.5% agarose gel electrophoresis in 1 × TAE (Tris-acetate-EDTA) buffer, and the DNA concentration was quantified using an ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, USA). The resulting DNA samples were stored at −80 °C prior to PCR amplification.

2.6. The terminal restriction fragment length polymorphism (T-RFLP) analysis

Bacterial community in soil, earthworm gut contents and casts were investigated through T-RFLP analysis. PCR mixture contained 1 μl total DNA (approximately 100 ng), 2 μl of 2.5 mM dNTPs, 0.5 μl Taq DNA polymerase (5 U m L^{-1}), 0.8 μl of universal primers (10 mM; 8F-FAM, 5′-AGA GTT TGA TCC TGG CTC AG-3'; 926R, 5′-CCG TCA ATT CCT TTR AGT TT-3′), and 2 μl of 10-fold PCR buffer (Takara, Japan) in a final volume of 20 μl. The cycling conditions started with a 4-min initial denaturation at 94 °C, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, followed by 10 min at 72 °C. PCR products were purified following the E.Z.N.A. Gel Extraction Spin Protocol (Omega, Georgia, USA) and were digested with *Msp*I or *Hae*III restriction enzyme (New England BioLabs, Beijing, China). The resulting samples were loaded onto an ABI capillary sequencer (Applied Biosystems, California, USA) with LIZ-500 as the size standard. All steps of the T-RFLP were performed with a negative control.

The quality of T-RFLP data was first visually inspected by Gene Scanner Software v1.0 (Applied Biosystems, California, USA) and then transferred to T-Rex (Culman et al., 2009) with a clustering threshold of 1.5 and exclusion of T-RFs less than 45 bp in length. True peaks were identified for both labels as those for which the area exceeded 1% of peak area computed over all peaks and divided by two. Nonmetric multidimensional scaling (NMDS) with Brays-Curtis distance measure and 10,000 permutations were used to assess the similarity of bacterial communities in the samples. We also estimated bacterial richness by dividing total peaks (forward and reverse) by two to approximate the bacterial richness in each sample. Bacterial Shannon, Simpson and evenness's index were evaluated using the program PAST 3.0 (University of Oslo, Norway).

2.7. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE)

Based on the results of T-RFLP, partial 16S rRNA genes were amplified from the extracted genomic DNA by PCR using a PTC 100 thermal cycler (MJ Research, Watertown, MA). Each PCR mixture (a total volume of 50 μl) contained 50 ng genomic DNA, 200 μM each deoxynucleotide triphosphate, 50 pmol universal primers, 2.0 mM $MgCl_2$, 5 μl of 10 × PCR buffer, and 5 units Taq DNA polymerase. The 16S rRNA genes of the bacteria were amplified with the 40-nucleotide guaninecytosine (GC)-clamp primer PRBA338F-GC [5′-(CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) AC TCC TAC GGG AGG CAG CAG-3′] and the PRUN518R (5′-ATT ACC GCG GCT GCT GG-3′; Nakatsu et al., 2000) using a thermo cycling program consisting of a 5-min initial denaturation at 94 °C, 30 cycles of 92 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by 15 min at 72 °C. All PCR products were incubated at 4 °C until processed further.

DGGE was performed on a D-Gene apparatus (Bio-Rad, Hercules, CA). Samples containing equal amounts of PCR amplicons were loaded onto 8% (w/v) polyacrylamide gels (37.5: 1, acrylamide: bisacrylamide) in 0.53 TAE with a denaturing gradient ranging from 40% to 60% denaturant for bacterial 16S rRNA genes [100% denaturant contains 7 M urea and 40% (v/v) formamide in 0.53 TAE]. Electrophoresis was performed at a constant 80 V for 14 h at 65 °C. After electrophoresis, the gels were rinsed and stained for 20 min in an ethidium bromide solution (0.5 mg L^{-1}), followed by 1 min of distaining in water repeated three times. The DGGE profile images were digitally captured and recorded (Gel DocTM XR170-8170 Molecular Imager System, Bio-Rad).

The most prominent bands were excised and transferred to a micro-centrifuge tube containing 20 μl of sterile distilled water, and incubated overnight at 4 °C. The gel-extracted product was used as a template for an additional PCR reaction with the same conditions as above in order to test the existence of double bands and contamination. DGGE was carried out using previous PCR products as molecular weight markers. Only those extracted products whose reamplicons presented a single band with the same migration distance as the marker were chosen for a further nested PCR. The nested primers PRBA338F (without a GC-clamp) and PRUN518R were applied using the same protocol as described above.

PCR products (amplified with PRBA338F/PRUN518R) were purified with a PCR purification kit (Takara, Dalian, China) and ligated into the cloning vector pGEM-T (Promega, Madison, USA) following the manufacturer's instructions. Ligated DNA was transformed into competent *Escherichia coli* DH5α cells. Plasmid inserts were extracted by the alkaline lysis method (Sambrook et al., 1989). Clones from each DGGE band were selected randomly for sequencing with an automated ABI 3100 sequencer using a T7 primer. Nucleotide sequence data obtained in this study were compared with those from the GenBank (http://www.ncbi.nlm.nih.gov/) using the BlastN program.

2.8. Statistical analysis

The statistical analyses were conducted using the software package SPSS (version 22; SPSS, Inc., Chicago, USA). For all tests, a significant level of 5% was applied. The statistical significance of Cry1Ab concentrations in maize straw and soil between earthworm and non-earthworm treatments was determined by an independent sample *T* test. The generalized linear models (GLM) distinguished the effects of sampling time, maize variety or enzyme on the contents of soil nutrients, the indexes of bacterial community in soil, earthworm gut contents and casts. Descriptive statistics followed by explore were used to test Cry1Ab concentrations and soil nutrients data for normality. One-way analyses of variance (ANOVA) followed by Tukey HSD test was performed to test for significant differences in soil nutrients among the three maize varieties during the corresponding sampling time. Nonparametric tests followed by Kruskal-Wallis test was used to test for significant differences in Shannon, Simpson, and evenness's index of bacterial community in soil, earthworm gut contents and casts among the three maize varieties treatments during the corresponding sampling time. The measures were log transformed when necessary to verify variance homogeneity.

Canonical correspondence analysis (CCA) was used to the correlations between bacterial community of soil, earthworm gut contents, casts and the soil chemical properties, Cry1Ab protein concentrations in straw, soil, etc. measured for each sample. Arrows indicated the direction and magnitude of measurable variables

associated with bacterial community structures. CCA were also performed by the program PAST 3.0 (University of Oslo, Norway).

3. Results

3.1. Cry1Ab protein concentrations in different samples

3.1.1. Cry1Ab protein concentrations in Bt straw

Cry1Ab protein concentrations in 5422Bt1 and 5422CBCL straw without earthworms decreased over time (Fig. 1A and B). Cry1Ab protein concentration in 5422Bt1 straw decreased during the first 15 d, being 4587.6 ± 492.1 ng g^{-1}, about 30.19% of initial Cry1Ab concentration (15,194 ± 1850 ng g^{-1}) in straw. For the remaining sampling time, Cry1Ab protein concentrations in 5422Bt1 straw were 3392.6 ± 677.7, 2169.7 ± 332.0, 1855.7 ± 279.8, 1724.7 ± 130.2 and 1305.8 ± 41.2 ng g^{-1}, being 22.32%, 14.28%, 12.21%, 11.35% and 8.59% of initial Cry1Ab protein concentration, respectively. Cry1Ab protein concentrations in 5422CBCL straw decreased sharply during the first 15 d, and then decreased gradually. From 15 to 90 d, Cry1Ab concentrations in straw were 4992.1 ± 1471.8 to 1804.5 ± 504.9 ng g^{-1}, about 31.17%, 30.02%, 25.99%, 12.31%, 11.90% and 11.27% of initial Cry1Ab protein concentration in 5422CBCL straw (16,012 ± 1100 ng g^{-1}), respectively.

When two types of trials (earthworm and non-earthworm treatments) were compared, Cry1Ab protein concentrations in 5422Bt1 straw from earthworm treatments were significantly lower than those from non-earthworm treatments on the 15th, 30th, 45th, 60th, 75th, and 90th d (Fig. 1A). On the 30th, 45th, 60th, 75th and 90th d, the Cry1Ab protein concentrations in 5422CBCL straw from earthworm treatments were significantly lower than those from non-earthworm treatments (Fig. 1B).

3.1.2. Cry1Ab protein concentrations in soil

The concentration curves of the Cry1Ab protein in soil without earthworms from 5422Bt1 and 5422CBCL treatments were similar over time (Fig. 1C and D), where there was a sharp decline from 15 to 30 d and a slow decrease from 30 to 90 d. On the 15th, 60th, 75th and 90th d, Cry1Ab protein concentrations in 5422Bt1 treated soil were significantly higher than those in 5422CBCL treated soil.

When two types of trials (earthworm and non-earthworm treatments) were compared, Cry1Ab protein concentrations in 5422Bt1 treated soil with earthworms were significantly lower than those in non-earthworm treatments on the 15th and 30th d, and their concentrations in soil from non-earthworm treatments were almost 3 to 26 times higher than those in earthworm treatments (Fig. 1C). Concentration curves of the Cry1Ab protein in soil with and without earthworms from 5422CBCL treatments were significantly different during 45 d. Cry1Ab protein concentrations

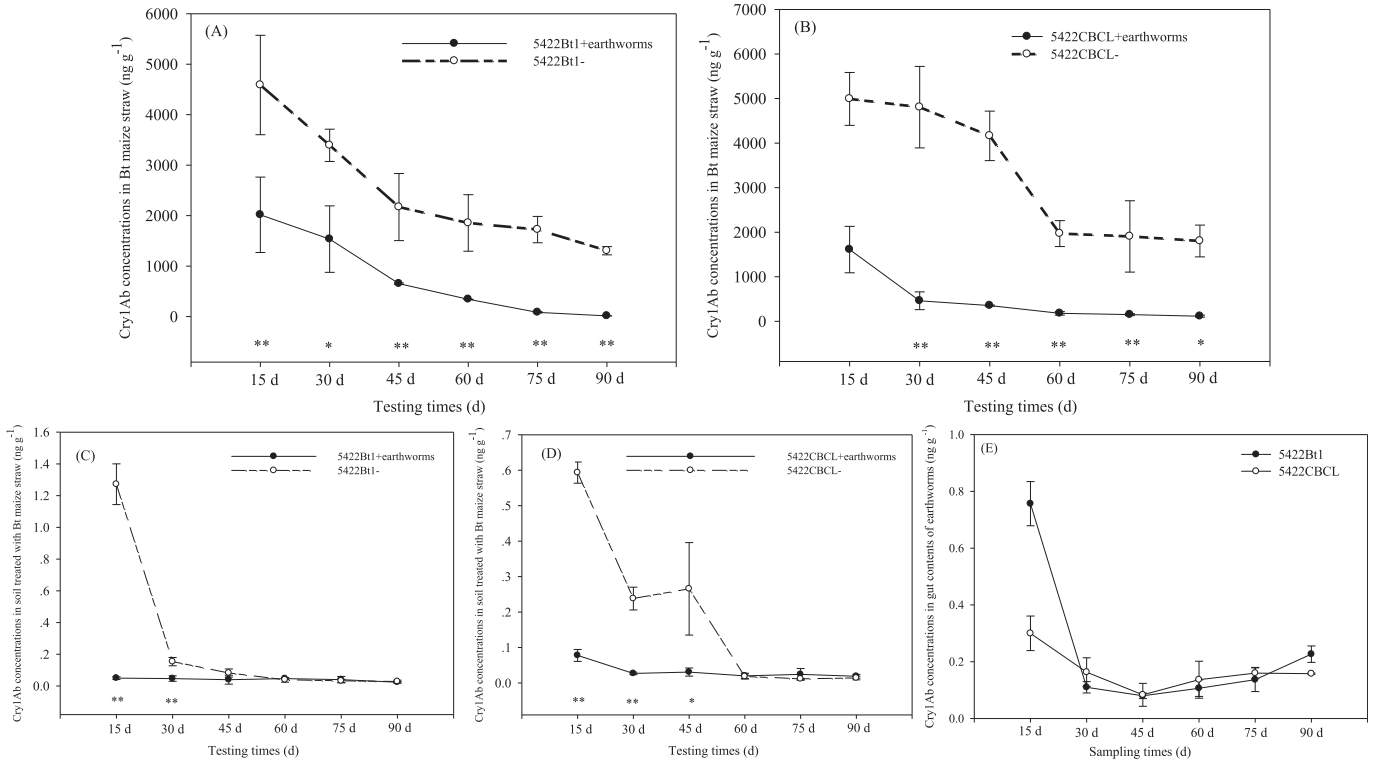

Fig. 1. Cry1Ab protein concentrations in Bt maize straw, soil and gut contents from different treatment groups. The bars represent the average (±SD) of Cry1Ab protein concentrations in 5422Bt1 (A) and 5422CBCL (B) straw on the different testing day, four replicates for each maize variety treatment. The difference of Cry1Ab protein concentrations in Bt maize of between treatments with and without earthworms was analyzed by *T*-test [5422Bt1 treatment: $F = 0.790$, $Pr = 0.004$ (the 15th d); $F = 1.175$, $Pr = 0.044$ (the 30th d); $F = 5.745$, $Pr = 0.005$ (the 45th d); $F = 7.245$, $Pr = 0.002$ (the 60th d); $F = 7.233$, $Pr = 0.0001$ (the 75th d); $F = 2.912$, $P = 0.0001$ (the 90th d); 5422CBCL treatment: $F = 3.364$, $P = 0.071$ (the 15th d); $F = 10.222$, $Pr = 0.0001$ (the 30th d); $F = 3.592$, $Pr = 0.0001$ (the 45th d); $F = 3.717$, $Pr = 0.001$ (the 60th d); $F = 4.022$, $Pr = 0.005$ (the 75th d); $F = 5.191$, $P = 0.016$ (the 90th d)]. The bars represent the average (±SD) of Cry1Ab concentrations in soil treated with 5422Bt1 (C) and 5422CBCL (D) straw on the different testing day, four replicates for each maize variety treatment. The difference of Cry1Ab concentrations in Bt maize of between treatments with and without earthworms was analyzed by *T*-test [5422Bt1 treatment: $F = 6.693$, $Pr = 0.0001$ (the 15th d); $F = 0.655$, $Pr = 0.004$ (the 30th d); $F = 0.019$, $Pr = 0.127$ (the 45th d); $F = 2.622$, $Pr = 0.445$ (the 60th d); $F = 2.443$, $Pr = 0.578$ (the 75th d); $F = 0.880$, $P = 0.434$ (the 90th d); 5422CBCL treatment: $F = 1.311$, $P = 0.0001$ (the 15th d); $F = 12.165$, $Pr = 0.0001$ (the 30th d); $F = 8.240$, $Pr = 0.036$ (the 45th d); $F = 0.033$, $Pr = 0.776$ (the 60th d); $F = 13.267$, $Pr = 0.260$ (the 75th d); $F = 0.000$, $P = 0.313$ (the 90th d)]. The bars represent the average (±SD) of Cry1Ab concentrations in gut contents (E) of earthworm from 5422Bt1 and 5422CBCL treatments on the different sampling day, four replicates for each maize variety treatment. The difference of Cry1Ab concentrations in gut contents of between 5422Bt1 and 5422CBCL treatments was analyzed by *T*-test [$F = 8.542$, $Pr = 0.013$ (the 15th d); $F = 5.621$, $Pr = 0.030$ (the 30th d); $F = 3.571$, $Pr = 0.070$ (the 45th d); $F = 3.638$, $Pr = 0.068$ (the 30th d); $F = 13.856$, $Pr = 0.005$ (the 75th d); $F = 141.765$, $Pr = 0.0001$ (the 90th d)]. "*" and "**" indicated $Pr < 0.05$ and $Pr < 0.01$, respectively.

in 5422CBCL treated soil with earthworms were significantly lower than those in the earthworm treatments during 45 d (Fig. 1D). On other sampling time, no significant differences were found between earthworm and non-earthworm treatments.

3.1.3. Cry1Ab protein concentrations in gut contents of earthworms

The concentration curves of the Cry1Ab protein in gut contents of earthworms from 5422Bt1 and 5422CBCL treatments were similar over time (Fig. 1E), where there was a sharp decline from 15 to 45 d and a slow increase from 45 to 90 d. On the 15th and 90th d, Cry1Ab protein concentrations in gut contents from 5422Bt1 treatments were significantly higher than those from 5422CBCL treatments, whereas the contrast case was found between 5422Bt1 and 5422CBCL treatments on the 30th d.

3.2. Effects of Bt maize straw return on soil nutrients

The maize straw return increased soil nutrients (OM, total N, P, K and available N, P, K) compared to soil background values (Table 1). The soil OM from 5422Bt1 treatments on the 75th d was significantly higher than that from 5422 treatment, while no significant differences were found among three maize varieties treatments during other sampling time. The contents of total N in 5422Bt1 treated soil were significantly higher than those from 5422 treated soil on the 60th and 75th d. On the 90th d, the contents of total N in 5422CBCL treated soil were significantly lower than those from 5422 to 5422Bt1 treated soil. No significant differences among three maize varieties were present in the contents of total P, irrespective of sampling time. Except the treatment on the 45th d, no significant differences among three maize varieties treatments were found in the contents of total K during the corresponding sampling time. Over the whole experiment period, the significant differences among three maize varieties treatments were found in the content of available N, where the content of available N in 5422 treated soil was significantly higher than those from the corresponding 5422CBCL treatments. During the later sampling time (on the 60th, 75th, and 90th d), the content of available N in 5422Bt1 treated soil was significantly higher than those from the corresponding 5422 treatments. On the 45th, 60th and 75th d, the significant differences among three maize varieties treatments were found in the content of available P during the corresponding treatments, where the highest and lowest contents of available P in soil were found in 5422Bt1 and 5422CBCL treatment, respectively. During the later sampling time (on the 60th, 75th and 90th d), the contents of available K in 5422CBCL treated soil were significantly lower than those from the corresponding 5422Bt1 and 5422 treatments.

3.3. Bacterial community diversity in soil, the gut contents and casts of E. fetida

T-RFLP revealed 30, 28 and 26 unique T-RF signals in soil, gut contents and casts samples digested by *Hae*III, respectively; additionally, 87, 51 and 60 unique T-RF signals were revealed in soil, gut contents and casts samples digested by *Msp*I, respectively. These signals contributed to more than 1% to the total signal area across all treatments.

GLM results showed that maize variety individually affected the Simpson and Evenness's index of bacterial community in soil (Table 2). Sampling time and enzyme (*Msp*I and *Hae*III) were found to have a strong significant effect on the Simpson and Shannon's index of bacterial community. The interactions between maize variety and sampling time also significantly affected the Shannon, Simpson, and Evenness's index of the bacterial community (Table 2). However, the mutual interaction among maize variety, sampling time and enzyme was not present in the bacterial community in soil samples. When individual maize variety and sampling time points were examined, there were some significantly different data points in the Simpson, Shannon and Evenness's index of the bacterial community (see Table 1 in the Supplemental Data). With *Hae*III treatments, a significant difference was detected in Simpson and Shannon's index of bacterial community in soil collected from Bt and non-Bt treatments on the 75th and 90th d. Simpson and Shannon's index of bacterial communities in soil collected from 5422CBCL and 5422 treatments digested by *Msp*I were also significantly different on the 15th, 75th and 90th d.

GLM results revealed that maize variety had no individual effect on the Simpson and Shannon's index of the bacterial community in earthworm gut contents, while the interaction between maize variety and sampling time significantly affected the bacterial community in gut contents (Table 2). Sampling time and enzyme (*Msp*I and *Hae*III) were found to have a strong significant effect on the bacterial community. When individual maize variety and sampling time points were examined, there were only three significantly different data points in Evenness's index of the bacterial community in earthworm gut contents digested by *Hae*III on the 15th, 45th and 60th d (see Table 2 in the Supplemental Data). With *Msp*I treatments, Evenness's index of the bacterial community in earthworm gut contents collected from 5422Bt1 and non-Bt maize straw treatments were significantly different on the 30th d. On the 75thd, Shannon and Simpson's index in 5422CBCL treatments were significantly higher than those in 5422 and 5422Bt1 treatments.

Maize variety and sampling time individually and mutually affected the richness of the bacterial community in earthworm casts (Table 2). When individual straw variety and sampling time points were examined, there were two significantly different data points in richness indices of the bacterial community in earthworm casts digested by *Msp*I and *Hae*III, on the 45th and 60th d. On the 15th d, three indices of the bacterial community were significantly different in earthworm casts digested by *Msp*I (see Table 3 in the Supplemental Data).

To explore whether the bacterial community composition in soil, earthworm gut contents and casts was associated with Bt maize straw, an NMDS plot was used to compare the parameter of Bt treatments with non-Bt treatment (Fig. 2). Data points that were close together represented samples that were highly similar in community composition. With *Msp*I treatments, there were statistically significant differences in the bacterial community in soil associated with 5422Bt1 and 5422 straw on the 15th, 75th and 90th d (Fig. 2A-1). With *Hae*III treatments, there were statistically significant differences in the bacterial community composition associated with 5422Bt1 and 5422 straw at three sampling time (on the 15th, 45th and 75th d) (Fig. 2A-2), while there were four significantly different data points found associated with 5422CBCL and 5422 straw (on the 15th, 30th, 75th and 90th d).

Although NMDS plots indicated that there was no statistically significant difference in the bacterial community composition of earthworm gut contents associated with Bt and non-Bt maize straw over the whole experiment period, the magnitude and direction of priority effects on the bacterial community structure were clearly variable between samples treated with Bt or non-Bt maize straw. Additionally, with each enzyme treatment, the structure of the bacterial community was overlapped and exhibited a relatively homogeneous distribution between Bt and non-Bt maize straw treatments (Fig. 2B-1 and B-2).

NMDS plots indicated that there were statistically significant differences in the bacterial community in earthworm casts associated with 5422Bt1 and 5422 or 5422CBCL straw at four sampling time (on the 30th, 45th, 60th, and 75th d) digested by *Msp*I (Fig. 2C-1), while there was only one significantly different data point found

Table 2
GLM analysis of the bacterial Simpson, Shannon, and Evenness indices based on terminal restriction fragments (TRFs) in the soil, earthworm gut contents and casts treated with Bt and non-Bt maize straw.

Source		*df*	Simpson		Shannon		Evenness	
			F	P	F	P	F	P
Soil	Enzymes	1	300.541	**0.0001**	223.868	**0.0001**	21.533	**0.0001**
	Maize varieties	2	4.151	**0.020**	2.708	0.073	3.583	**0.033**
	Sampling time	5	4.462	**0.001**	4.620	**0.001**	1.233	0.303
	Maize varieties × Enzymes	2	3.137	**0.049**	0.947	0.393	0.484	0.618
	Sampling time × Enzymes	5	4.241	**0.002**	4.799	**0.001**	0.592	0.706
	Maize varieties × Sampling time	10	2.452	**0.014**	2.238	**0.025**	2.074	**0.038**
	Maize varieties × Sampling time × Enzymes	10	1.116	0.362	1.125	0.356	0.874	0.561
Earthworm gut contents	Enzymes	1	14.876	**0.0001**	23.138	**0.0001**	5.359	**0.023**
	Maize varieties	2	0.854	0.430	0.249	0.780	7.665	**0.001**
	Sampling time	5	4.229	**0.002**	3.674	**0.005**	8.236	**0.0001**
	Maize varieties × Enzymes	2	1.200	0.307	1.622	**0.205**	1.564	0.216
	Sampling time × Enzymes	5	0.634	0.675	1.083	0.377	3.096	**0.002**
	Maize varieties × Sampling time	10	2.339	**0.019**	2.230	**0.025**	3.096	**0.002**
	Maize varieties × Sampling time × Enzymes	10	0.695	0.726	0.597	0.811	2.032	**0.042**
Earthworm casts	Enzymes	1	2.703	0.105	12.175	**0.001**	4.720	**0.033**
	Maize varieties	2	2.473	**0.041**	3.963	**0.023**	16.086	**0.0001**
	Sampling time	5	3.297	**0.010**	3.252	**0.011**	5.038	**0.001**
	Maize varieties × Enzymes	2	0.287	0.751	0.955	0.390	1.255	0.291
	Sampling time × Enzymes	5	1.135	0.350	1.705	0.145	4.111	**0.002**
	Maize varieties × Sampling time	10	3.036	**0.003**	4.793	**0.0001**	3.644	**0.001**
	Maize varieties × Sampling time × Enzymes	10	2.073	**0.038**	1.857	0.066	2.453	**0.014**

Significant *P*-values were indicated in bold type.
Maize varieties: 5422Bt1, 5422CBCL, and 5422.
Sampling times: 15th, 30th, 45th, 60th, 75th, and 90th d.
Enzymes: *MSP*I and *Hae*III.

associated with 5422CBCL and 5422 straw (on the 15th d). With *Hae*III treatments, there were statistically significant differences in the bacterial community composition associated with 5422Bt1 and 5422 straw at three sampling time (30th, 45th, 75th d) (Fig. 2C-2), while no statistically significant difference was found in the bacterial community composition associated with 5422CBCL and 5422 straw during the whole test.

3.4. DGGE analysis of bacterial communities in soil, earthworm casts

13 DGGE bands derived from the 16S rDNA gene were observed in Bt or its non-Bt isoline samples from the earthworm casts collected on the 15th, 30th and 60th d; A total of 52 operational taxonomic units (OTUs) were detected. Using the program BLAST, sequences with the most similarity to reference strains were found in the GenBank database (Table 3). On the 15th d, B1, B3, B5 and B6 were observed in 5422Bt1 treatment. DNA sequencing revealed that the differentiated bacteria possibly contained *Brevundimonas* and *Caulobacter* (B1), uncultured *Rhizobiales* bacterium, *Stenotrophomonas* and *Pseudomonas* (B3), *Streptomyces* and *Arthrobacter* (B5), *Cellulosimicrobium*, *Rhizobium*, *Agrobacterium* and *Devosia* (B6), respectively. Likewise, B2 (*Rhizobium*, *Agrobacterium tumefaciens* and *Devosia*), and B4 (*Pedobacter* and *Algoriphagus*) were only found in 5422CBCL treatment. On the 30th d, the differentiated bacteria in the presence of B7 (*Asticcacaulis*, *Microbacterium* and *Pantoea*) and B8 (*Microbacterium*, *Achromobacter*, *Bacterium*, etc.) was observed between 5422Bt1 and 5422 or 5422CBCL treatments. In addition, B9 (*Bacillus* etc.), B11 (*Blastococcus* etc.) and B13 were only found in 5422CBCL treatment on the 60th d. B10 (*Algoriphagus*, *Streptomyces*, *Isoptericola*, *Bacillus aryabhattai* and *Bacillus*) and B12 (*Flexibacteraceae bacterium* P3, *Frigoribacterium*, *Cryobacterium*, *Salinibacterium*, *Leifsonia*, uncultured Actinobacterium and uncultured Bacteroidetes) were differentially present in earthworm casts collected from 5422 treatments on the 60th d. Furthermore, one DGGE band was observed in soil collected from 5422CBCL treatment on the 75th d, and 2 operational taxonomic units (OTUs) were detected. BLAST similarity results showed that they were *Bacillus*, etc.

3.5. Correlations of Cry1Ab protein or nutrients data and bacterial communities

To investigate correlations between the bacterial community in soil, earthworm gut contents, casts and measured Cry1Ab protein or nutrients variables, canonical correspondence analysis (CCA) were used to analyze different bacterial phyla classes (Fig. 3). The influence of the above data on the CCA bioplot is indicated by arrows in which lengths are proportional to their importance (Liu et al., 2009). Available nutrients (available N, P, K) showed the longest arrows, indicating that they were the most important in influencing the bacterial community, regardless of enzyme treatment (Fig. 3A-1 and A-2). Regardless of enzyme treatment, correlations of Cry1Ab protein in Bt straw, soil and soil bacterial community were similar, having shorter arrows, which also presented the opposite effect when compared with those of nutrients data (Fig. 3A-1 and A-2).

N, P (total and available levels), as well as OM, showed the longer arrows, indicating that they were more important in influencing the bacterial community of gut contents than other nutrients data and Cry1Ab protein, regardless of enzyme treatment (Fig. 3B-1 and B-2). Total K presented the opposite effect on the bacterial community of gut contents between *Hae*III and *MSP*I (Fig. 3B-1 and B-2). Cry1Ab protein in soil, maize straw and earthworm gut contents (Fig. 1E), having shorter arrows, presented similar influence on the bacterial community in gut contents, which were opposite when compared with those of nutrients data (Fig. 3B-1 and B-2).

Cry1Ab protein in soil, maize straw and earthworm casts [Fig. 6 in Shu et al. (2015)] had the longer arrows and similar directions, suggesting that they were more important in influencing the bacterial community of earthworm casts than other data, regardless of

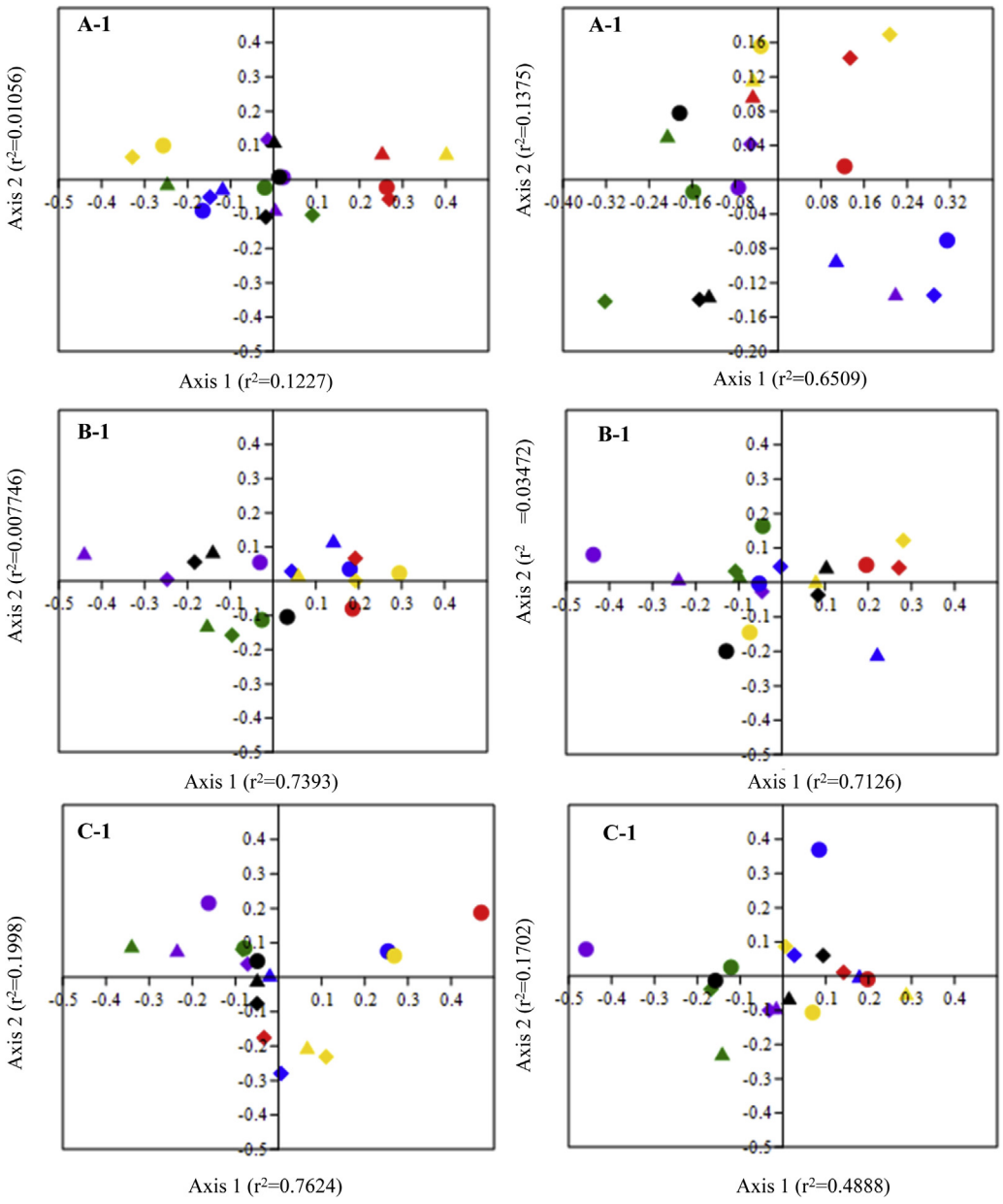

Fig. 2. Nonmetric multidimensional scaling (NMDS) ordination plots of the bacterial communities in soil, earthworm gut contents and casts based on TRFs for each enzyme. A, Soil; B, Earthworm gut contents; C, Earthworm casts; 1 = *Msp*I; 2 = *Hae*III. Different shapes (full diamond, dot and full triangle) indicated different maize varieties (5422, 5422Bt1 and 5422CBCL), respectively. Different colors (green, purple, black, blue, yellow and red) in figures indicated samples collected from different testing days (on the 15th, 30th, 45th, 60th, 75th and 90th d), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enzyme treatment (Fig. 3C-1 and C-2). Total N had longer arrow compared with other nutrients data, suggesting that it was the most important in influencing the bacterial community of earthworm casts. Moreover, available N and P also had longer arrows, indicating they were more important factors affecting the bacterial community of earthworm casts.

4. Discussion

Earthworm *E. fetida* could accelerate the degradation of Cry1Ab protein in Bt maize straw, which was consistent with Schrader et al. (2008) and Emmerling et al. (2011), who revealed degradation of Bt protein from litter material was accelerated by earthworm activity. This result might be attributed to that *E. fetida*, as epigeic earthworm species, can enhance the incorporation of litter material into soils and stimulate their decomposition (Bossuyt et al., 2005; Pérès et al., 2010; Butt and Briones, 2011). Additionally, this result might be attributed in turn to an increase in microbial activity when earthworms are present (Edwards and Fletcher, 1988; Ernst et al., 2008), since earthworm secreted mucus that promotes microbial activity and thus leads to an increase in microbial decay of proteins (Brown, 1995).

Bt protein may be released into soil from decaying crop residues (Zwahlen et al., 2003). In our study, ELISA showed the presence of detectable Cry1Ab protein in the soil and significantly higher concentrations of Cry1Ab protein from non-earthworm treatments in comparison with earthworm treatments. This result was consistent with the trend of Cry1Ab protein in straw. However, it was in

Table 3
Results of a BLAST analysis on the sequences of 16S rDNA sequences derived from DGGE bands.

DGGE band	Closest relatives Microorganisms	Accession number	Similarity (%)[b]
B1[a]	Uncultured bacterium	JQ337403, etc.	100
	Brevundimonas	KF501480, etc.	100
	Caulobacter	KF536028, etc.	100
B2	*Rhizobium*	KC589290, etc.	100
	Agrobacterium tumefaciens	AB826000, etc.	100
	Uncultured bacterium	KC607460, etc.	100
	Rhizobium	KC934840, etc.	100
	Devosia	KC464847, etc.	100
B3	Uncultured *Rhizobiales* bacterium	HM108433.1	100
	Stenotrophomonas	JX842795, etc.	100
	Pseudomonas	JX134078, etc.	100
B4	*Pedobacter*	FJ377316, etc.	100
	Uncultured bacterium	HF558957, etc.	100
	Algoriphagus	FJ196000, etc.	100
	Marine sediment bacterium	AY911170, etc.	100
B5	*Streptomyces*	KF454864, etc.	100
	Arthrobacter	KF177259, etc.	100
B6	*Cellulosimicrobium*	KF562805, etc.	100
	Rhizobium	KF170819, etc.	100
	Agrobacterium	AB826000, etc.	100
	Uncultured bacterium	KC607460, etc.	100
	Devosia	KC464847, etc.	100
B7	Uncultured bacterium	HG003554, etc.	100
	Asticcacaulis	FN794207, etc.	100
	Microbacterium	KF254727, etc.	100
	Pantoea	KF527217, etc.	100
B8	*Microbacterium*	KF534779, etc.	100
	Uncultured bacterium	HE965986, etc.	100
	Achromobacter	AB824289, etc.	100
	Bacterium	AB127844, etc.	100
	Uncultured beta proteobacterium	HM798729, etc.	100
	Uncultured bacterium	GU291493, etc.	100
B9	Uncultured bacterium	JF223433, etc.	100
	Bacillus	KF483225, etc.	100
B10	Uncultured bacterium	HF558957, etc.	100
	Algoriphagus	FJ196000, etc.	100
	Streptomyces	KF479190, etc.	100
	Isoptericola	KF428918, etc.	100
	Bacillus aryabhattai	KF436688, etc.	100
	Bacillus	KC585037, etc.	100
B11	*Blastococcus*	KF040437, etc.	100
	Uncultured bacterium	KF366575, etc.	100
	Uncultured beta proteobacterium	GU929365, etc.	100
B12	Uncultured bacterium clone	JX948680, etc.	100
	Flexibacteraceae bacterium P3	AY429700.1	100
	Frigoribacterium	KC256952, etc.	100
	Cryobacterium	KC986996, etc.	100
	Uncultured Actinobacterium	KC994764, etc.	100
	Salinibacterium	KC894033, etc.	100
	Leifsonia	KC160918, etc.	100
	Uncultured Bacteroidetes	EF471633, etc.	100
B13	Uncultured *bacterium*	FJ716016, etc.	99
B14	*Bacillus*	KC236698, etc.	99
	Uncultured bacterium	KC993597, etc.	99

[a] Lowercase letters indicates that more than one operational taxonomic unit (OTU) was detected from the same DGGE band.
[b] Similarity (%) = no. of identical nucleotides/total no. of nucleotides.

contrast to Ahmad et al. (2006), who found significantly higher concentrations of Cry3Bb1 protein in the soil of earthworm treatments. These differences can be explained by the differences in the experimental design between both studies. In contrast to Ahmad et al. (2006), we sampled soil and casts separately measuring Cry1Ab protein released through Bt maize straw applied to soil. The possible reasons for higher Cry1Ab protein from non-earthworm treatments than earthworm treatments were as follows: earthworms increase the contact between Cry1Ab protein and soil microorganisms, thereby accelerating their degradation in soil; earthworms can immobilize pollutants in their casts and promote the persistence of pollutants significantly against biodegradation (Dendooven et al., 2011). Our previous study indicated that Cry1Ab protein detected in soil of earthworm treatments was significantly lower than that in casts (Shu et al., 2015), which agrees with this result of Cry1Ab protein in soil.

After straw return in soil, the content of OM, N, P and K in soil could increase (Coppens et al., 2006), which was consistent with our results that soil nutrient contents increased with the decomposition process of maize straw. Maize variety significantly affected the soil nutrients (not including OM), where the total and available N in 5422Bt1 treated soil were generally higher than those in 5422 and 5422CBCL treated soil during later sampling time. Available N in 5422CBCL treated soil was generally lower than that in 5422 treated soil over the whole experiment period. This was probably attributed to chemical properties of maize straw. The contents of

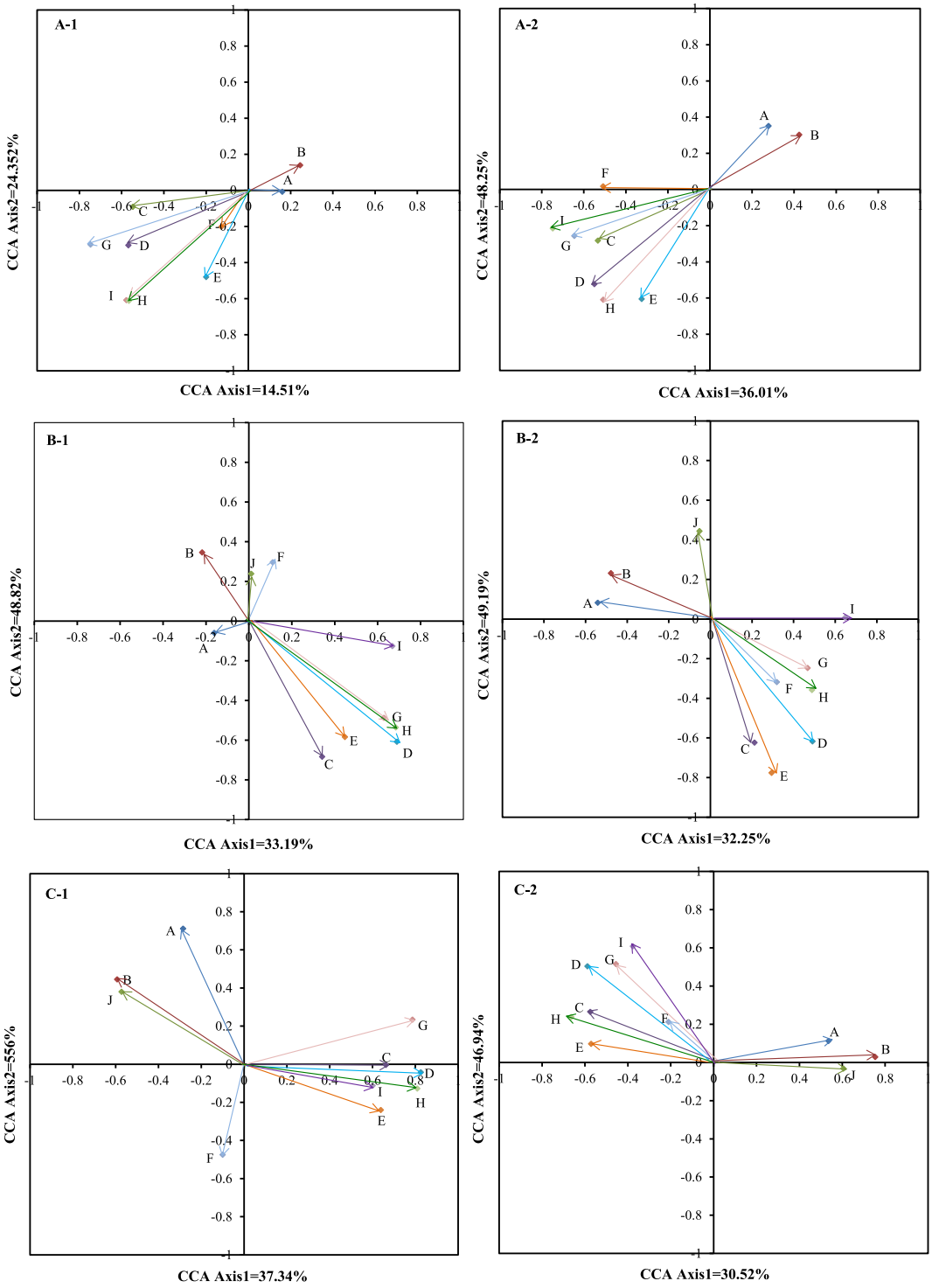

Fig. 3. Canonical correspondence analysis (CCA) for bacterial community in soil, earthworm gut contents, casts and soil nutrients, Cry1Ab protein concentrations. Arrows indicated the direction and magnitude of measurable variables associated with bacterial community structures. A, Soil; B, Earthworm gut contents; C, Earthworm casts; 1 = *Msp*I; 2 = *Hae*III. Different capital letters in the figures indicated different measurable variables (A: Cry1Ab protein concentrations in soil; B: Cry1Ab protein concentrations in straw; C: Soil organic matter; D: Soil total N; E: Soil total P; F: Soil total K; G: Soil available N; H: Soil available P; I: Soil available K; J: Cry1Ab protein concentrations in gut contents or casts, respectively).

total protein and N extracted from 5422Bt1 and 5422 straw were significantly higher than those of 5422CBCL (Shu et al., 2015). The other possible reason was the decomposition rate that was associated with C: N ratio and lignin content. Increased lignin content and C: N ratio contribute to reduce Bt maize decomposition rates (Flores et al., 2005; Zeilinger et al., 2010). Saxena and Stotzky (2001) showed that Bt (Cry1Ab protein) maize has significantly higher lignin. Flores et al. (2005) demonstrated that transgenic Bt plants decompose less in soil than non-Bt plants, which was due to higher C: N ratio and lignin detected in Bt maize than its near-isogenic

non-Bt maize. 5422CBCL straws have higher C: N ratio than 5422, since similar organic carbon and lower total N were detected in 5422CBCL than 5422 straw (Shu et al., 2015). Moreover, the lignin content of 5422CBCL was higher than 5422 at the harvesting time (unpublished results). Thus, the decomposition rate of 5422CBCL may be slower than that of 5422, which resulted in the lower soil available nutrients detected in 5422CBCL treated soil.

Overall, the straw return of two hybrids of Bt maize in soil resulted in the presence of Cry1Ab protein and changes in nutrients (especially for N levels) in soil, which may cause a potential change in soil bacterial community. T-RFLP results revealed that Bt maize straw return had significant effect on richness or diversity of soil bacterial community during later sampling time, where Simpson and Shannon's index of 5422CBCL treated soil were significantly higher than those of 5422 and 5422Bt1 treated soil. DGGE patterns further revealed altered bacterial community in the soils amended with 5422CBCL straw compared with 5422 on the 75th d. DNA sequencing revealed that the different bacterial species were *Bacillus* (Similarity 99%) and uncultured bacterium (Similarity 99%). Fang et al. (2007) demonstrated that incorporation of Bt residue significantly affected the structure of microbial community compared with the residues from its non-Bt isoline, which was associated with higher lignin and lignin/N ratio in soil rather than Bt protein released from Bt maize residues. In our study, the correlations between Cry1Ab protein or soil nutrients and bacterial community revealed that changes in bacterial community were closely correlated with soil available nutrients rather than Cry1Ab protein released in Bt maize straw or soil. Furthermore, we speculated that the richness or diversity of bacterial community in soil was negatively correlated with available nutrients, where lower available nutrients and higher Simpson and Shannon's index in 5422CBCL treated soil when compared to those of 5422 and 5422Bt1 treated soil.

Since epigeic species are surface-dwellers that feed on fresh surface litter and do not make permanent burrows (Lubbers et al., 2013), *E. fetida* take the maize straw but not soil as a food source, and they could ingest lots of maize straw that then was introduced in the earthworm gut (Lavelle and Gilot, 1994) and was more highly processed OM in earthworm casts (Edwards and Fletcher, 1988; Schönholzer et al., 1999; McLean et al., 2006). Afterwards, *E. fetida* could secrete mucus that aggregate with soil and maize straw, and thus they consume and digest above aggregation after maize straw return in soil for a period of time. Therefore, changes in microbial community of earthworm gut contents and casts were mainly caused by the ingested maize straw during the earlier stage, while the factors influencing the microbial community during the later stage are complex, including straw, soil and their interactions. Food resource type can cause shifts in the gut wall-associated bacterial community (Thakuria et al., 2010), which was not present in our study that Bt maize straw return had no consistent significant effect on bacterial community of gut contents, although there were significant differences (Cry1Ab and nutrients) between Bt and non-Bt maize straw.

However, the GLM with Kruskal-Wallis test using T-RFLP results showed that maize variety presented a significant effect on bacterial community in earthworm casts and the significant differences occurred between 5422CBCL and 5422 or 5422Bt1 treatments on the 15th, 45th and 60th d. NMDS analysis also indicated that the significant differences occurred between 5422Bt1 and 5422 or 5422CBCL treatments on the 15th, 30th, 45th, 60th and 75th d. Furthermore, DGGE patterns revealed that significant differences occurred between non-Bt and Bt maize straw treatments on the 15th, 30th and 60th d. DNA sequencing demonstrated that the majority of different bacterial species in earthworm casts between 5422Bt1 and 5422 treatments on the 15th d belonged to Proteobacteria, including *Brevundimonas* (Its genomes code for antitoxin proteins that neutralize a specific toxin, Scott and Ely, 2016), *Caulobacter*, *Cellulosimicrobium*, *Pseudomonas*, *Stenotrophomonas*, *Agrobacterium*, *Devosia* and *Methylobacterium*. It was well known that Proteobacteria, as a dominant cellulolytic bacterium group, can hydrolyse carbohydrates and cellulose, which was closely associated with the decay of litter (Scott and Ely, 2016). Additionally, two species belonged to Actinobacteria, *Streptomyces* (they are major contributors to biological buffering of soils and have roles in OM decomposition conductive to crop production, Ningthoujam et al., 2009) and *Arthrobacter* were found in earthworm casts between 5422Bt1 and 5422 treatments on the 15th d. Likewise, the majority of different bacterial species in earthworm casts between 5422CBCL and 5422 treatments on the 15th d belonged to Proteobacteria, including *Rhizobium*, *Agrobacterium tumefaciens* (Both are gram-negative soil bacteria that fix N) and *Devosia*. *Pedobacter* and *Algoriphagus*, belonging to Bacteroidetes that are related to the hydrolysis and mineralization of OM, were also found in different bacterial species. Similarly, the different bacterial species in earthworm casts between 5422Bt1 and 5422 treatments on the 30th d belonged to Proteobacteria, including *Asticcacaulis*, *Pantoea* and *Achromobacter*, etc. These results suggested that the differential species between Bt and non-Bt treatments were mainly associated with the decomposition and mineralization of OM, the metabolism of carbohydrates, organic salts and amino acids, etc., which might be caused by the differences of the decomposition rate between Bt and non-Bt straw. This was coincided with that Bt plants decompose less in soil than non-Bt plants and was coincided with that the content of soluble sugar extracted from Bt straw was significantly higher than 5422 straw (Shu et al., 2015). CCA results revealed N levels in soil also presented important factors influencing the bacterial community in earthworm casts (Fig. 3C), which in turn reflected the non-expected effects (the decomposition rate and nutrients release) of Bt maize on the bacterial community in earthworm casts.

Additionally, the different bacterial species in earthworm casts between 5422CBCL and 5422 treatments on the 60th d were *Bacillus*. Moreover, the different bacterial species, such as *Caulobacter* and *Brevundimonas*, involved in coding for antitoxin proteins and growing at very low levels of nutrients, which suggested that there was stressful living environment for earthworm. This case may attribute to Cry1Ab protein or the other harmful chemicals released from Bt straw. The CCA results revealed the closely correlations between the bacterial community in earthworm casts and Cry1Ab protein in straw, soil and casts (Fig. 3C). Shu et al. (2015) demonstrated that the immunoreactive Cry1Ab protein in casts of earthworms from Bt-maize treatments, was absolutely stronger than that of non-Bt-maize treatments on the 15th d and significantly higher Cry1Ab protein concentrations in casts found in 5422Bt1 treatments than 5422CBCL and 5422 treatments, and these results were in agreement with the differences of bacterial community in earthworm casts on the 15th and 30th d.

The different bacterial species in earthworm casts between Bt and non-Bt treatments on the 60th d mainly belonged to three phylum, Actinobacterium (*Streptomyces*, *Isoptericola*, *Leifsonia*, *Salinibacterium*, etc.), Bacteroidetes (*Algoriphagus*, *Flexibacteraceae* bacterium P3), Firmicutes (*Bacillus aryabhattai*, *Bacillus*, *Frigoribacterium Cryobacterium*). They were environmental bacteria, depending on growing conditions. This case indicated the significant difference of earthworm casts was found between Bt and non-Bt treatments, thereby in turn to reflect the difference of earthworms growth, metabolism and functions between Bt and non-Bt treatments.

Interestingly, N levels presented significant effects on bacterial community in soil, earthworm gut contents and casts. Moreover,

the differential species in earthworm casts between Bt and non-Bt maize treatments contained *Rhizobium*, *Agrobacterium tumefaciens* and *Agrobacterium* that are agriculturally important bacteria capable of inducing N fixation. Furthermore, the total and available N in 5422Bt1 treated soil was generally higher than that in 5422 and 5422CBCL treated soil during later stage. Available N in 5422CBCL straw treated soil was generally lower than that in 5422 treated soil during the whole stage. These results suggested that N released Bt maize straw during their decaying process played an important role in causing changes in the bacterial community of earthworm *E. fetida* bred in soil with Bt maize straw. Thus, the N cycles (N fixation, nitrification and denitrification) in soil with earthworm after Bt maize straw return deserved the further study, as N availability is often the limiting factor in terrestrial ecosystem productivity and abundance and community structure of microorganisms (Levy-Booth et al., 2014).

5. Conclusion

The present study demonstrated the significant differences were shown in soil bacterial community between Bt and non-Bt maize treatments on the 75th and 90th d, which was closely correlated with soil available N, P and K rather than Cry1Ab protein concentrations in straw and soil. Additionally, Bt maize return had significant effects on soil nutrients, especially for available N. These results suggested that Bt maize straw return caused changes in living environment of earthworm *E. fetida*, mainly including soil nutrient levels and bacterial community, which might ultimately affect the growth, metabolism and functions of earthworms. For example, the lower soil available nutrients and higher Simpson and Shannon's index of bacterial community were detected in 5422CBCL treated soil, compared to 5422 treatments, which were corresponded to the previous study that the effects on the growth and reproduction of adult earthworms from the 1st generation were observed in 5422CBCL treatments (Shu et al., 2015).

There was no statistically significant difference in the bacterial community of earthworm gut contents associated with Bt and non-Bt maize straw. However, a significant difference in bacterial community of earthworm casts were found among three maize varieties treatments, which was closely correlated with Cry1Ab protein during the earlier stage and the N levels over the whole stage. The differentiated bacterial species in earthworm casts mainly belonged to Proteobacteria, including *Brevundimonas*, *Caulobacter*, *Pseudomonas*, *Stenotrophomonas*, *Methylobacterium*, *Asticcacaulis* and *Achromobacter* etc., which were associated with the mineralization, metabolic process and degradation of plants residues. Therefore, Bt maize straw return caused changes in the bacterial community in *E. fetida* casts, which was possibly caused by the direct (Cry1Ab protein) and non-expected effects (N levels) of Bt maize straw.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.01.023.

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