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Impact of cadmium on the bacterial communities in the gut of *Metaphire posthuma*

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ABSTRACT

The effects of cadmium (Cd) contamination in soil onto the bacterial communities of the guts pooled from ten *Metaphire posthuma* were addressed during 14 days' incubation. We found that about 50% of Cd (5 mg/kg, dry weight soil) in the contaminated soil was bio-accumulated into the earthworms. DNA was extracted from the guts of *M. posthuma* and their dwelling soil irrespective of Cd treatment for the analysis of the bacterial communities of guts in *M. posthuma* and in soil by PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis). A distinctive cluster of bacterial communities of the guts in the earthworm with and without Cd treatment using the analysis of unweighted pair-group method using arithmetic averages (UPGMA) was observed, indicating that the bacterial community of guts could be changed by Cd. However, no differences in the bacterial communities in soil irrespective of Cd treatment were observed, which could be resulted from the bioremediation of Cd by earthworms leading to insignificant effect of Cd on bacterial communities in soil. For the sequencing of some of the dominant bands in the DGGE profile, *Bradyrhizobium japonicum*, *Stenotrophomonas* sp. D2, and *Labrys*, sp. CC-BB4, whose sequences display an identity of more than 97% using blast program against a known sequence in the GeneBank database and Ribosomal database, were identified.

Collectively, our results showed that earthworm treatment can decrease the concentrations of Cd in soil, and Cd cause a shift in the bacterial communities in the guts of *M. posthuma*. The application of *M. posthuma* for Cd bioremediation would be desired.

1. Introduction

The soil contamination with heavy metals is ubiquitous, presenting a possibly unacceptable risk to human and ecological health [1]. Earthworm as a common organism in soil is inevitably affected [2]. However, earthworms can survive in heavy metal-contaminated mine soil [3–5] with concentration of cadmium (Cd) exceeding 350 μg/g [6]. Indeed, cadmium can be accumulated in the earthworms inhabiting these metal-contaminated mine sites [7]. The ability of earthworms to live in the presence of toxic metals has promoted interest in their potential application as biomarkers for metals [7]. Therefore, many studies on the bioaccumulation of metals by earthworm have been extensively investigated mainly for the purpose of using earthworm as bioindicators of soil pollution or metal availability [8–12].

Studies of various earthworm species have generally shown that a large portion of the microbial population in soil passing through the gastrointestinal tract of the earthworm unchanged while representatives of some phyla increase in abundances [13]. The differences in microbial activity, bacterial numbers and certain bacterial species between the gut of earthworm or burrow and bulk soil suggested that the bacterial community structure of these habitats are different from those of the soil [13]. However, the structure and function of the bacterial community inhabiting the gut of earthworm is still poorly understood [14].

In this study, the exploration of bacterial community in gut of earthworms after Cd treatment was first performed using the denaturing gradient gel electrophoresis (DGGE) technique. DGGE is widely used to study the microbial composition of a variety of different habitats including the gut microflora of humans [15], pigs [16], wasps [17] or termites [18]. By DGGE, we herein presented evidences that only a shift of bacterial communities in the gut of earthworms but not in their dwelling soil with Cd treatment compared with their corresponding control groups (without Cd treatment) was observed.

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2. Materials and methods

2.1. Earthworms treated with Cd

The soil without Cd contamination was collected in a remote site in mountain area of Chang-Liou, Kaohsiung Country. Subsequently, the soil was homogenized by sieving through a 0.2-mm mesh. This type of soil was stored at 4 °C for less than 3 days before the experiments were performed. The earthworm, *Metaphire posthuma*, was obtained from a local earthworm-breeding farm. For each of the three independent experiments, earthworms were maintained in three plastic containers (500 ml) filled with soil (dry weight, 200 g; the contents of water, 40 ± 5%; the pH values, 7.0) for at least 3-d acclimation before the determination of Cd concentration in earthworm or its dwelling soil. Theses containers were kept at 25 °C and in the dark room for at least 24 h. Adult *M. posthuma* with a well-developed clitellum and without Cd contamination were used for this study.

Three experimental settings (groups A, B and C) were performed. Soil containing ten earthworms without Cd treatment was designated as group A. Group B constituted only soil with Cd treatment (5 mg/kg, dry weight basis). Group C was composed of ten earthworms and Cd-treated soil (5 mg/kg, dry weight basis). The concentration level selected in this study referred to the maximum contaminant level (MCL) of Cd of 10 mg/kg in soil as regulated by Taiwan Environmental Protection Administration. The contents of water, the pH value, and Cd concentration in each group were determined in triplicate independently. The bacterial community between soil and the gut of earthworms was compared using DGGE analysis as described below.

2.2. The determination of Cd concentration in earthworms or soil

Ten earthworms were removed from groups A and C, respectively, at sampling days 0, 3, 7, 10, and 14 during 14-d incubation, and then were pooled to determine the level of Cd in the earthworm. Additionally, the residual Cd in soil in which the ten earthworms dwelled was tested. Determination of Cd concentration in earthworms or soil from groups A and C was performed by at least triplicate analyses.

Group A was served as the negative control because it contained no Cd. For the digestion of soil and earthworm samples, 2 g of sample (wet weight) was transferred to a digestion vessel. Ten milliliters of concentrated hydrochloric acid was added to the sample. Subsequently, the sample was covered with a ribbed watch glass, and then was continued heating the digestate until the volume was reduced to approximately 5 ml. The digestate was filtered through Whatman No. 41 filter paper (or equivalent) before the collection of digestate in a 100 ml volumetric flask. Finally, this sample was analyzed by flame atomic absorption spectrometry (FLAA). Before the analysis of Cd by FLAA, the standard stock solution was prepared. For this standard solution, 1.000 g of Cd was dissolved in 20 ml of 1:1 HNO_3, and then was diluted to 1 l with reagent water. Thereafter, we prepared five concentration levels of standard solution for the determination of Cd concentration. The detection limit of Cd concentration was 0.005 mg/l.

2.3. DNA extraction and PCR amplification of target DNA

Earthworms were sedated, surface sterilized with ethanol (70%). Subsequently, the gut of the earthworms were depurated under sterile conditions. The whole gut content of each specimen was extracted with a small sterile spatula and directly transferred into sterile 1.5 ml micro-centrifugation tube. Each gut sample was separately used for DNA extraction. DNA extraction from the ten of *M. posthuma* gut and their dwelling soil was performed using Soil DNA extraction kit (GeneMark, Taiwan) according to the manufacture's protocol. Extracted DNA was amplified in a PCR thermocycle (Eppendorf, Germany) with a set of primer F968gc and R1401 as described [19]. Each PCR mixture contained 5–15 ng extracted DNA, 0.5 μM of each primer, and 25 μl Master Mix RED (Ampliqon, Denmark) containing 0.4 mM dNTP-Mix, 2.5 mM $MgCl_2$, 0.025 U/μl ampliqon taq DNA polymerase, and DNA polymerase buffer. For amplification of the bacterial 16 S rDNA primer F968gc-R1401, a touchdown-PCR program with an initial denaturation step at 94 °C for 4 min, followed by 5 thermal cycles of 1 min, 1 min at 60 °C decreased by 1 °C every cycle, 1 min at 72 °C, followed by 27 thermal cycles of 94 °C for 1 min, 1 min at 52 °C decreased by 1 °C every cycle, 1 min at 72 °C, and final extension step at 72 °C for 10 min were set.

2.4. DGGE analysis and sequencing of DGGE bands

Denaturing gradient gel electrophoresis (DGGE) was performed with D-Code™ universal mutation detection system (BioRad Lab., USA). PCR product were loaded on to 8% (w/v) polyacrylamide gels with a denaturing gradient of 40–55% (100% denaturant according to 7 M urea plus 40% formamide in 1× TAE buffer) and were run for 16 h at 80 V and at a constant temperature in 1× TAE buffer (pH 7.4). After electrophoresis, the gels were stained with SYBE Safe TM (Invitrogen, UK), and then were put in a shaker at 150 rpm for 10 min, followed by excision bands from the gel with a pipette tip under UV illumination. The excised bands were directly placed in 1.5 ml micro-centrifugation tubes containing 50 μl ultrapure DNase/RNase-free sterile water and incubated at 4 °C for overnight to elute DNA. Ten microliters of the supernatants was used as template for the re-amplification (as described above). The resulting amplicons were again electrophoresed on a DGGE gel to verify the position of the original band. Subsequently, the amplicons were purified using the Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan) and sequences (Mission Biotech. Co., Taiwan). All sequences were compared to those in the GeneBank database and Ribosomal database.

2.5. Statistical analysis

DGGE banding patterns were analyzed using Quantity One software (BioRad, USA). For cluster analysis, PCR-DGGE of one primer set was performed in duplicate. Calculation of the pair-wise

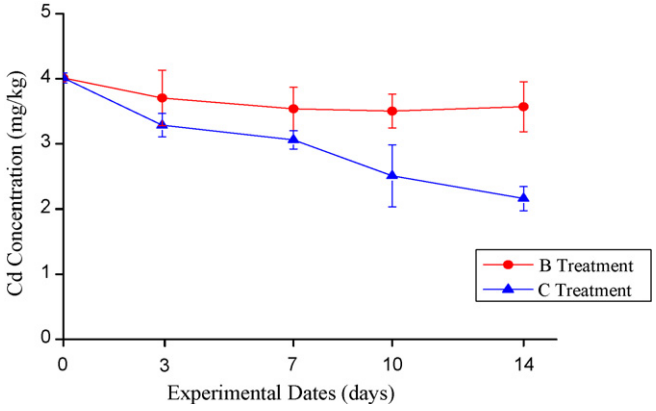

Fig. 1. The level of Cd (5 mg/kg dry weight of soil) in soil was decreased by the addition of ten earthworms into the soil (group C treatment), while group B treatment indicated that no earthworms were added into the this Cd-contaminated soil. The values of percentage were calculated from the residual Cd concentrations divided by 5 mg/kg and then multiplied by 100. Each value was derived form the average of three independent experiments.

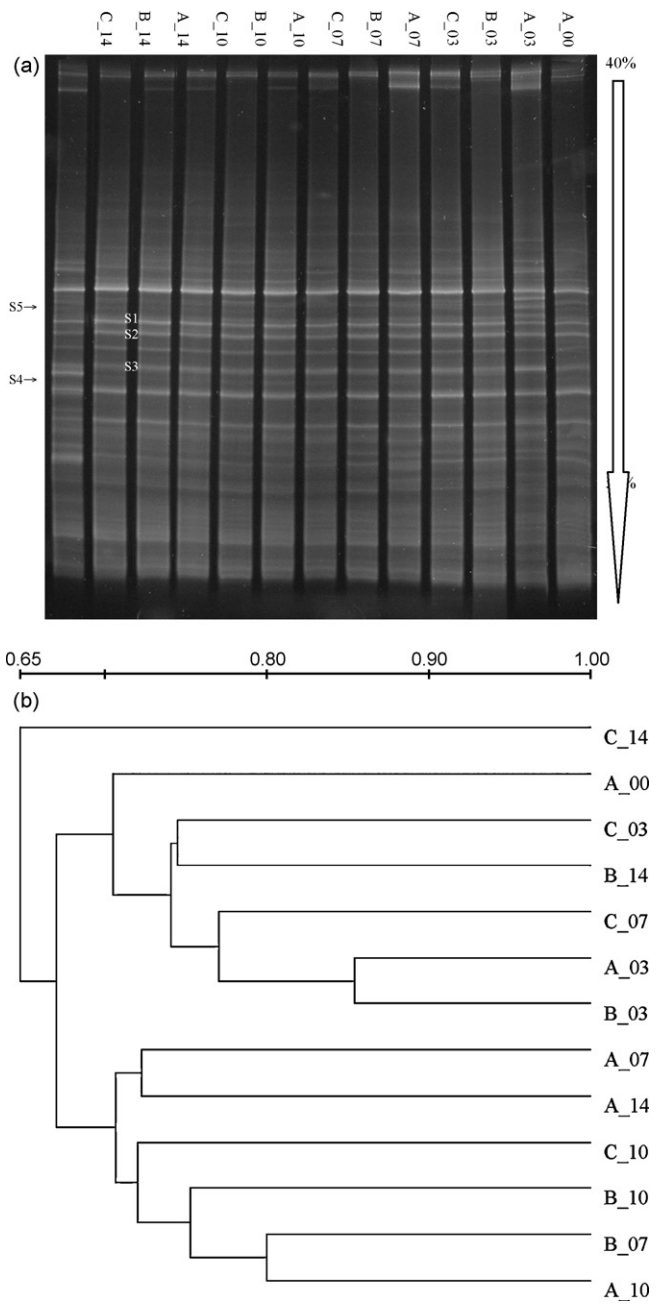

Fig. 2. (a) A bacterial fingerprints based on 16S rDNA extracted from the non-contaminated soils with earthworms (A group), Cd-contamination soils (B group), and Cd-contaminated soil with earthworms (C group). Values near the side of each group indicate for incubation time. (b) Subsequently, this DGGE fingerprint was analyzed by the UPGMA method.

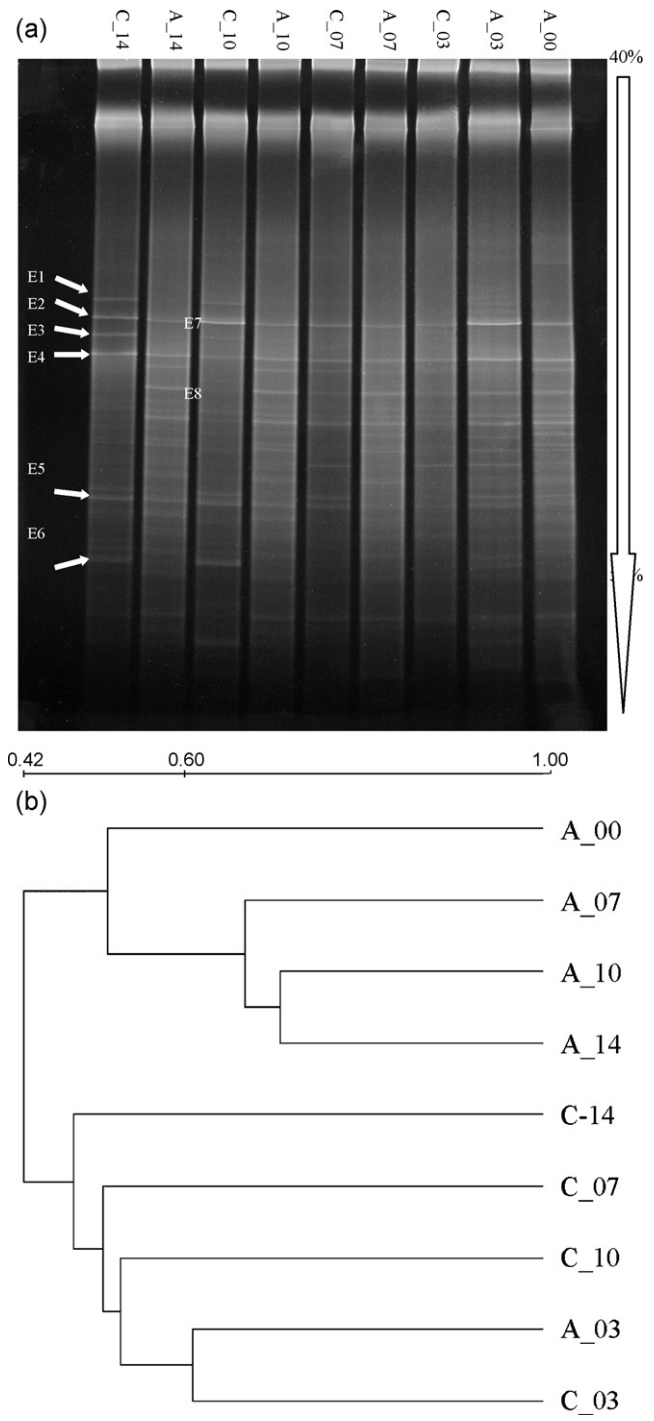

Fig. 3. (a) A bacterial fingerprints based on 16S rDNA extracted from the guts of earthworms without Cd treatment (A group) and earthworms with Cd treatment (C group). Values near the side of each group indicate for incubation time. (b) Subsequently, this DGGE fingerprint was analyzed by the UPGMA method.

similarities was based on the Dice correlation coefficient. Dendrograms were created using the algorithm of unweighted pair-group method using arithmetic averages (UPGMA) [20].

Student *t*-test was used to compare the difference of the weight of earthworm with Cd and without Cd treatment. Analysis of variance (ANOVA) and Turkey's multiple range test ($p < 0.05$) were performed to compare the difference of Cd concentration in soil and earthworm in the tested three groups using SPSS software, version 10.0.7C. All of the experiments were performed in at least three replicates.

3. Results

3.1. The effects of Cd on earthworm weight and survival

To avoid the influence of the gut bacteria by ingested food, the earthworms were not fed during the 14-d incubation with Cd. The weight of earthworms (group A) or (group C) was decreased to the same level after 14-d incubation (data not shown). After analysis of *t*-test, this result indicated that Cd did not affect earthworms' weight ($p > 0.05$). For the survival analysis, the survival rates of

earthworms derived from the ratio of the live earthworms to the death earthworms in both groups were over 97% after 14-d incubation, suggesting that Cd level was not lethal to those earthworms' survival.

3.2. Cd accumulation by earthworms

During 14-d incubation, the Cd concentration in soil remained constant (i.e., 5 mg/kg) in group B (without earthworm), while a decreased concentration of Cd was observed in soil belonging to group C (with earthworm) (Fig. 1). To evaluate the potential of Cd removal by earthworm, the concentration of Cd in the earthworm was also determined. In the group C treatment (Fig. 1), results revealed that the levels of Cd in earthworm (the contents of Cd/dry weight of earthworms) or soil were below detection limit or 5.0 ± 0.5 mg/kg, 3.45 ± 0.44 mg/kg or 3.29 ± 0.18 mg/kg, 5.07 ± 0.78 mg/kg or 3.06 ± 0.14 mg/kg, 7.35 ± 0.55 mg/kg or 2.51 ± 0.48 mg/kg, and 11.3 ± 0.60 mg/kg or 2.16 ± 0.18 mg/kg at the sampling days 0, 3, 7, 10, and 14, respectively. When the value of bioconcentration factor (BCF) derived from the ratio of Cd concentration in the earthworm/Cd concentration in soil is smaller than 1, it shows that earthworms can only absorb but not accumulate heavy metals. On the other hand, it shows that earthworms can accumulate metals if BCF > 1. Our results revealed that the values of BCF were 1.05, 1.66, 2.93 and 5.23 at sampling day, 3, 7, 10, or 14, indicating that Cd can be accumulated in the earthworms. As shown in Fig. 1, the level of Cd (5 mg/kg) remained constant in soil without earthworms (group B) but was decreased at days 10 and 14 ($p < 0.001$) after earthworms addition (group C), which could highlight the fact that Cd can be removed by earthworms.

3.3. Band identification

Although a high number of bands appeared when performing PCR-DGGE analysis with universal bacterial primers, it was only possible to identify few bands by excising them from the DGGE. Weak and close bands were difficult to cut off the gel and could not be re-amplified or only produced multiple DNA sequences. After 10-d or 14-d incubation, the prominent bands of group A, group C or differential bands in group C as compared to group A, which was indicated in Figs. 2a and 3a, were excised. Subsequently, the excised bands were sequenced and then were compared to some database such as GeneBank. Results (Table 1) showed that these sequences are separately assigned to *Alcaligenes* sp. (S1), Uncultured Xanthomonadaceae bacterium (S2), Uncultured Rhodocyclaceae bacterium (S3), *Nitrosomonas* sp. Nm58 (S4), Verrucomicrobia bacterium (S5), *Bacilllus* sp. NN106 (E1), *Stenotrophomonas* sp. D2 (E2), Actinobacterium (E3), *Bacillus marinus* (E4), *Labrys*, sp. CC-BB4 (E5), *Bradyrhizobium japonicum* (E6), and *Stenotrophomonas* sp. D2 (E7).

3.4. Comparison of bacterial community in soil and the gut of earthworm among groups

DGGE banding patterns were normalized and analyzed using Quantity One software (BioRad, USA). The similarities of these banding patterns were calculated based on the Dice correlation coefficient. Subsequently, the UPGMA method was used for cluster analysis. Cluster analysis of PCR-DGGE fingerprinting patterns of soil in groups A, B, and C during 14-d incubation was found in Fig. 2b, revealing that distinctive separations of the bacterial community in these soil cannot be detected. However, comparing the banding patterns of the bacterial community of the gut of earthworm with Cd treatment (group C) and without Cd treatment (group A), we found a clear separation of the bacterial community depending on Cd treatment although the fingerprinting patterns of group A at day 3 incubation showed less similarities than those of group A at days 0, 7, 10, and 14 (Fig. 3b), whereas group B was not included for this analysis due that this group contains only Cd-contaminated soil. This result suggested that the bacterial community of the gut was changed by Cd treatment.

4. Discussion

In this study, we analyzed the bacterial community in the gut of the *M. posthuma*, the common species found in soil of Taiwan, under the exposure of Cd by using a molecular fingerprinting method-DGGE. DGGE was an appropriate tool to simultaneously analyze samples from the soil with or without Cd treatment and the gut of *M. posthuma* on one gel and compare their banding patterns.

Our results demonstrated that Cd can be bio-accumulated by *M. posthuma* based on the value of the BCF. BCF was calculated as the ratio of the content of heavy metal in the earthworms to that in the soil. By using the BCF, we can confirm whether earthworms absorb or accumulate heavy metals [9]. Although it was reported that Cd can be accumulated by some species of earthworms such as *Dendrobaena veneta* [20], *Enchytraeus buchholzi* [21], *Lumbricus rebellus* [7], and *Elsenia fetida* [19], the effects of Cd accumulation in the bacterial community of the gut of earthworms are not known to date.

Cadmium has been reported to affect the microbial community of the contaminated soil as compared to the control set [22–25]. It is anticipated that the bacterial community in soil with anthropogenic chemical such as Cd was different from that without Cd. Furthermore, the microbial community of soil may also be

Table 1
Closest relatives of excised DGGE bands as revealed by BLAST N research in GeneBank database.

DGGE[a] band	Closest relative	Accession number	Similarity[b] %
S1	Uncultured *Alcaligenes* sp.	EF173341	94
S2	Uncultured Xanthomonadaceae bacterium	EF018313	97
S3	Uncultured Rhodocyclaceae bacterium	EF018551	96
S4	*Nitrosomonas* sp. Nm58	AY123799	92
S5	Uncultured Verrucomicrobia bacterium	AM690912	96
E1	*Bacillus* sp. NN106	AJ973278	93
E2	*Stenotrophomonas* sp. D2	DQ839619	99
E3	Uncultured Actinobacterium	EF220604	97
E4	*Bacillus marinus*	AJ237708	93
E5	*Labrys* sp. CC-BB4	DQ062742	98
E6	*Bradyrhizobium japonicum*	DQ517954	99
E7	*Stenotrophomonas* sp. D2	DQ839619	99
E8	Uncultured bacterium (unclassified)	DQ340200	93

[a] The excised DGGE bands were indicated in Figs. 2a and 3a.
[b] The similarity of all sequences was obtained from comparison to those in the GeneBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

profoundly affected by the presence of earthworm [13]. An increase in microbial numbers of in the gut of earthworms after the ingestion of soil containing a large portion of bacteria was reported [13], whereas heavy metal contamination decreased the diversity of bacterial community in the soil [9]. Although no distinctive clusters performed with UPGMA analysis for these soils were obtained, this analysis can be applied for analyzing the gut dependent on the presence of Cd (Fig. 3b). After the exposure to Cd in soil, the gut bacterial communities of the earthworms were changed as compared to that of earthworms in soil without Cd treatment based on the observations that a shift of some prominent bands and a decreased number of bands in DGGE profiles was found in the gut treated with Cd compared with that without Cd treatment (Fig. 3a). Thus, we concluded that bacteria communities in the gut were changed after the exposure of the earthworms to Cd in soil at a sub lethal concentration (5 mg/kg) which did not affect the earthworms' viability.

Although DGGE has been widely used for the exploration of the microbial community qualitatively in soil, it still has limitation that its profiles represent only the most dominant phylotypes in the investigated samples [26]. Therefore, our banding patterns reflect only the abundant organisms, whereas less abundant representatives of the microbial community or microbes strongly attached to the gut wall resulting in the difficulty in the DNA extraction of these bacteria, which were hardly analyzed by DGGE, are not likely to be included in our analysis. The efficiency of DNA extraction from some sample such as soil often presents a major obstacle for subsequent DGGE analysis. Another limitation is that it is frequently not possible to obtain a pure DNA sequence from a band, as DGGE bands are overlapping or cannot be separated efficiently from each other during excision [27]. Moreover, the UV light used to make bands visible when cutting them off the DGGE may destroy the DNA and lead to sequence variation artifacts [28]. Thus, to ensure our DGGE profile reliable and avoid the occurrence of these limitations when performing the DGGE analysis, we added some known bacterial species such as the representative gram-positive (e.x. *Bacillus* sp.) and gram-negative species (e.x. *Pseudomonas* sp.), which were common seen in the environment, to the analyzed soil or gut as the internal standards, and then detect their presence from the excised bands in the DGGE profiles. Our results revealed that DGGE profiles were highly reproducible in a triplicate-independent manner.

Based on the criteria that only sequences displaying an identity of more than 97% were considered to represent the same species [29], *Bradyrhizobium japonicum*, *Labrys* sp. CC-BB 4, and *Stenotrophomonas* sp. D2 from the gut of the earthworms in soil contaminated with and without Cd were identified in Table 1. One species of *Stenotrophomonas* sp., *Stenotrophomonas maltophilia*, has the ability to remove 2 mM Cd by increasing the level of cysteine to form CdS-cluster formation [30]. *Stenotrophomonas* sp. CD02 can remove 80% of high concentrations of cadmium ion (up to 4 mM) [31]. *Bradyrhizobium japonicum* is a soil bacterium involved in symbiotic nitrogen fixation with Glycine max, the common soybean, and its strain USDA 122 but not USDA 123 is resistant to Cd toxicity [32]. The ability of *Labrys* sp. to detoxify or resist Cd toxicity is still not known. Since some of these identified bacteria, particularly in *Stenotrophomonas* sp. D2 and *Bradyrhizobium japonicum*, were found in both the guts of the earthworms and their dwelling soil, the presence of these bacteria in the gut could be indigenous or mainly from the ingestion of soil by earthworms. Thus, whether *Stenotrophomonas* sp. D2 and *Bradyrhizobium japonicum* were related with the ability of *M. posthuma* to bio-accumulate Cd still needs to be investigated.

5. Conclusion

The results of this study demonstrated that DGGE was a useful molecular technique for analyzing the bacterial community in the gut of *M. posthuma*. Excising bands from the DGGE did not allow for the identification of all bands of interest; however, this technique made it possible to identify some of the most abundant bacteria in the earthworm gut. Additionally, the setting up of a clone library from the gut samples will be necessary to fully elucidate the bacterial community in gut, offering a more detailed overview about the gut microbiota of *M. posthuma*. Collectively, we first demonstrated that the bacterial communities in the gut but not in their surrounding soil are changed by Cd. Alterations in the composition of microbial communities have often been proposed to be a sensitive indicator anthropogenic effects on soil ecology [33].

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