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The Biochemical Properties and Microbial Profiles of Vermicomposts Affected by the Age Groups of Earthworms

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Abstract.- The aim of this work was to assess the effect of age groups of earthworms on the quality of vermicomposts. For this, *Eisenia foetida* grouped into hatchlings, juveniles and adults were used for vermicomposting of food wastes spiked with vermicomposted materials. Compared to control treatment (without earthworms), vermicomposting treatment caused the significant reductions of TOC and C/N, while increases of pH and inorganic ions after 60 days. Vermicomposts by *E. foetida* showed the lowest TOC content and microbial activity and population in hatchlings, the largest increase of nitrogen in adults and the maximum enrichment of inorganic ions in juveniles, respectively. PCR-DGGE analysis revealed vermicomposts displayed the analogous microbial community structures. Overall results indicated that age-specific *E. foetida* did significantly affect the biochemical properties of their vermicomposts, but they could not effect on the microbial community profiles of the vermicomposts.

Keywords: Earthworms, vermicomposts, food waste recycling, PCR-DGGE.

INTRODUCTION

Vermicomposting is a biochemical process for degradation of organic materials by means of the joint actions of earthworms and microorganisms. According to Domínguez *et al*. (2010), a direct function of earthworms and an indirect role of microorganisms are interdependent and their interactions regulate the humification and breakdown of organic waste during vermicomposting. Despite the microorganisms' participation in the decomposition of organic materials, earthworms act as critical driver of this process through breaking up the organic matter, increasing the area of aerobic microbial activity and triggering the enzymatic activity, thus in turn, stimulating the decomposition further (Domínguez *et al*., 2010).

Hence, features of earthworms appear to affect the properties of vermicompost and its potential for agricultural utilization. Indeed, the quality of the end product differs with the species and ecological groups of earthworms (Gajalakshmi *et al*., 2001; Tripathi and Bhardwaj, 2004). Vermicomposting with miscellaneous species or ecological categories of earthworms are deemed to be more efficient than conventional monoculture vermin-reactors for managing organic wastes (Khwairakpam and Bhargava, 2009; Suthar and Singh, 2008). The density of earthworms is a feature of intraspecific relationship that exerts some effect on the chemical feature of vermicomposts and their biomass (Ndegwa *et al*., 2000; Hait and Tare, 2011).

Age is a natural feature in biology. In general, the life cycle of earthworms is composed of cocoon, hatchling, juvenile and adult. However, little is known about earthworms age-related vermicomposting process. In terms of the physiological features of earthworms, significant differences in the intestinal enzymatic activities and the capacity of enrichment in carbon and nitrogen were revealed regarding the hatchling, juvenile and adult earthworms (Ranganathan and Vinotha, 1998; Sampedro and Domínguez, 2008; Whalen and Janzen, 2002). Consequently, it can reasonably be hypothesized that the age of earthworms would, to some extent, affect the biochemical properties of vermicomposts. However, to our best acknowledge, the selection of age group of earthworms was not uniformed in earlier vermicomposting trials (Table I). Nevertheless, taking into account of vermicomposts having the commercial potential of being used for soil conditioner, understanding the influence by the age stages of earthworms involves the commercial value of vermicomposting products.

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However, the comparison investigation of vermicompost properties processed by the different age classes of earthworms is limited.

Table I.- Summary in different age class of earthworms used in earlier vermicomposting experiments.

Age groups	Earthworms species	Reference
Non-clitellated earthworms (Hatchlings/ Juveniles)	*Eisenia andrei*	Fernández-Gómez *et al.* (2010a)
	Eisenia foetida	Li *et al*. (2011); Garg *et al*. (2012)
Clitellated earthworms (Adults)	*Eisenia foetida*	Sangwan *et al*. (2008); Suthar (2010)
	Eudrilus eugeniae	Mainoo *et al*. (2009)
Both non-clitellated and clitellated earthworms	*Eisenia foetida*	Hait and Tare (2011); Gómez-Brandón *et al.* (2011a)
	Eisenia foetida, Eudrilus eugeniae, Perionyx excavatus	Khwairakpam and Bhargava (2009)

In view of the above, the objective of this study was to seek the evidence whether and to what extent the age of earthworms affects the properties of vermicompost. For this, *E. foetida* that is one of the most widely used species in vermicomposting systems, was chosen for this experiment. Moreover, the physicochemical, biochemical properties and microbial profiles were comprehensively determined to have an insight into the difference of vermicomposts resulted by the age-specific earthworms.

METHODS

Experimental set up

Vegetable wastes (cabbage, lettuces and potato peels) were obtained from Kanesue, a supermarket at Gifu, Japan. The waste were dried at 55°C in hot air oven and then chopped. In order to soften the waste and create a comfortable condition for earthworms' growth, short composting using vegetable waste combined with old vermicompost produced from cow dung at the rate of 1:2 (vegetable waste: vermicompost, dry weight basis) was carried out for 2 weeks.

Table II.- Chemical properties of vegetable wastes, bulking materials and initial substrate.

	Vegetable wastes	Bulking materials	Initial substrate
pH	5.5 ± 0.02	7.6 ± 0.01	7.1 ± 0.02
TOC (g/kg)	391.2 ± 4.41	279.9 ± 3.17	337.9 ± 5.36
Total N (g/kg)	13.8 ± 0.32	13.0 ± 0.28	13.4 ± 0.05
C/N ratio	28.5 ± 0.42	21.6 ± 0.23	25.2 ± 0.32
Inorganic N (g/kg)	1.8 ± 0.04	0.5 ± 0.03	0.8 ± 0.06
PO_4^{3-} (g/kg)	3.5 ± 0.12	4.0 ± 0.36	3.8 ± 0.29
K^+ (g/kg)	0.5 ± 0.02	1.5 ± 0.08	1.4 ± 0.01
Mg^{2+} (g/kg)	1.1 ± 0.06	1.0 ± 0.06	1.0 ± 0.03
Ca^{2+} (g/kg)	0.6 ± 0.01	0.5 ± 0.03	0.6 ± 0.02

The chemical properties of vegetables, bulking materials and initial substrate (after short composting) are shown in Table II. Stock earthworms were reared by fresh vegetable wastes for one year under laboratory condition. *E. foetida* were collected and then divided into three groups, namely 'hatchlings', 'juveniles' and 'adults' based on their body lengths and weights (wet basis) and stages of clitellum development. 'Hatchlings' were newly hatched earthworms with a mean wet weight of 0.1g and body length of 1.5-2cm. Juveniles were approximately 30 days hatched earthworms that had not formed clitellum with a mean wet weight of 0.25g and body length of 3-4 cm. 'Adults' were old earthworms that developed clitellum with a mean weight of 0.5g and body length of 5-6 cm. Subsequently, 6g individuals of hatchlings, juveniles and adults were allowed to settle in separate perforated plastic bins containing 100 g mixed substrate (dry weight basis). For each age group of earthworms, three reactors were run in parallel. All reactors were kept in dark at 25°C. Deionized water was regularly sprinkled to control the moisture. After 60 days, earthworms and their cocoons were picked out by hands and cleaned immediately by wetted paper. Then earthworms were counted by hands and their body weights were measured. The resulting vermicompost was homogenized and divided into two subsamples. One was dried and finely pulverized for chemical analysis and another one was stored at -20°C for enzyme activity and DNA analysis.

Physicochemical analysis and dehydrogenase activity

The microstructure of dried samples (not ground) was determined by scanning electron microscope. (Hitachi S-4300, Japan).

The pH value was measured in a solution by mixing the sample with water (dry weight of sample/weight of water = 1/10). Total organic carbon (TOC) was measured by using the method provided by Nelson and Sommers (1982). Total nitrogen was determinated by the method of oxidation with potassium persulfate after digestion with H_2SO_4-H_2O_2. Concentrations of inorganic nitrogen (nitrate plus ammonia), phosphate, potassium, magnesium and calcium were extracted by Milli-Q water and shook up 6 h then measured by ion chromatography analyzer (SHIMADAZU, Japan).

Total microbial activity was determined by measuring dehydrogenase activity with the triphenyl-tetrazolium chloride (TTC) method. 1 g of sample was incubated using TTC for 6 h and the amount of formazan formed was extracted with toluene and then measured by colorimetric method at 485 nm.

DNA extraction and PCR-DGGE protocol

250 mg of the subsample of each vermicomposting product was subjected to DNA extraction using the MoBio UltraClean Soil DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's protocol. The primers GC341F and 907R were used to amplify the fragments, according to Huang et al. (2013). PCR reactions were performed by adding 1 μl of each DNA extract to a total volume of 50 μl containing 20 μM each primer (0.5 μl), 10×Ex Taq buffer (5 μl), 2.5 mM dNTPs (4 μl), 0.1 % bovine serum albumin (BSA) (1 μl), 250 μM Ex Taq enzyme (0.25 μl) and sterilized pure water (37.75 μl). The PCR program initiated by denaturation at 95°C for 5 min, followed by 35 amplification cycles of 30 s at 95°C, 30s at 57°C and 40 s at 72°C, and finally by an extension step for 10 min at 72°C. The PCR products were tested by electrophoresis in 1.2 % agarose gels stained with 0.5 μg/ml ethidium bromide.

DGGE was conducted by loading about 100 ng of each PCR product into 7% (w/v) polyacrylamide gel with a denaturing gradient of 40-65 % and then running for 800 min at 100 V and a constant temperature of 60°C in 1×TAE buffer using an BIO-RAD Dcode™ system (Bio-Rad, USA). Subsequently, the gel was strained for 20 min in 1×TAE buffer containing a 1:10,000 dilution of SYBR® Green Nucleic Acid Gel Stain (Takara) and visualized with the Gel Doc 2000 System (Bio-Rad, USA).

Real time qPCR assay

The universal primers of 16S rDNA, com1 (5'-CAGCAGCCGCGGTAATAC-3') and com2 (5'-CCGTCAATTCCTTTGAGTTT-3'), were used for quantifying the number of 16S rDNA copies. PCR reactions were preformed in a 25 μl volume containing 2 μl of DNA extract, 12.5 μl of SYBR Ex Taq (TAKARA), 0.5 μl each primer (0.4 μM) and 9.5 μl of sterilized pure water. Three replicates for each extracted DNA were included. The real-time qPCR program consisted of initial denaturation at 95°C for 5 min followed by 35 three-step cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. All experiments included negative (without target DNA) and 10 fold serial dilutions of an *Escherichia coli* DNA standard. DNA amplification and quantification were performed with a real time PCR system (Thermal Cycler Dice, TP800, Takara).

Data analysis

The growth rates of different age groups earthworms were calculated on the mg/g worms /day using the data of initial and final biomass of worms. Significant differences in the means of parameters between the control and vermicomposting treatments were tested based on one-way analysis of variance (ANOVA) with mean separation by Tukey's significant difference (HSD) test at 95 % confidence level using the software of STATISTIC 8.0. Digitalized DGGE fingerprints were analyzed using the Quantity One image analysis software v.4.2 (Bio-Rad Laboratories, Hercules, CA, USA) to compute a similarity matrix using the Dice's coefficient. The Shannon index of the general diversity *H* was calculated from the following equations:

$$H = \sum\left\{\left(\frac{N_i}{N}\right)\ln\left(\frac{N_i}{N}\right)\right\}$$

where N_i is the height of the peak of the band *i* and *N* is the sum of all peak heights in the targeted lane of the obtained DGGE profile.

RESULTS AND DISCUSSION

Physical appearance of control and vermicomposts

Vermicomposts processed by *E. foetida* exhibited a distinct physical appearance in contrast to the substrate after gut transit process, as evidenced from more homogenous surface obtained (Fig. 1). This indicated that vermicomposting process resulted in a more compacted structure of organic materials as compared to undigested substances. The observations corresponded to the previous results that the appearance of humic acid fractions in substrate was shifted to more closed-grained and lumpy after vermicomposting (Li *et al*., 2011). The appearance structure of organic materials was probably altered by the viscous force generated from mucus of digestive tract and the constraint force produced from musculus sphincter during gut transit process. Unexpectedly, obvious divergence was not observed in vermicomposts of hatchling, juvenile and adult *E. foetida*, which suggests that the appearance feature is unconcerned with the age groups of *E. foetida*.

Chemical characteristics of control and vermicomposts

As shown in Figure 2, vermicomposting significantly altered the chemical properties of initial material. The pH increased significantly in vermireactors compared to the control treatment. The higher pH values could be ascribed to the decomposition of organic acid in vegetable wastes and release of cation from vegetable tissue during the mineralization process. Similar findings were also reported in vermicomposting and composting of other food wastes (Fernández-Gómez *et al.*, 2010a; Mainoo *et al.*, 2009; Nasreen and Qazi, 2012).

Dramatic decline occurred in TOC content in control (12.6%) and vermicomposts (26.7-31.4%), compared to initial substrate. This shows that earthworms can enhance the loss of carbon. The microbial respiration and earthworms feeding give rise to the carbon loss from the substrate. Moreover, the TOC content in vermicomposts was significantly affected by different age class of *E. foetida* with the greater reduction in hatchlings treatment. This observation can be explained by the growth of hatchlings which result in higher assimilation of carbon into their tissues than in adults that had already reached their final size (Whalen and Janzen, 2002). Leachate may be an important factor for the loss of carbon and nitrogen during vermicomposting process (Mainoo *et al.*, 2009). But, no leachate was observed in this study, suggesting that the nutrient loss was mainly due to the contribution of earthworms and microbes.

Compared to control, a significant decrease of TN content was recorded in vermicompost of hatchlings, while a significant increase was observed in vermicompost of adults. This suggests that age-specific earthworms exert significant effect on the TN content of the end products. The decrease of TN in hatchlings is mainly because of conversion of organic nitrogen of substrate into the protein of earthworm. Thus, this suggests more consumption of nitrogen source is required for hatchlings to construct their body cells than juveniles and adults. Indeed, the highest growth rate was found in hatchlings (7.2 mg/g/day) followed by juveniles (3.2 mg/g/day) and adults (1.1 mg/g/day) in this study. Hatchling of *E. foetida* hence assimilates higher content of carbon and nitrogen for their body development relative to juveniles and adults.

In contrast, vermicomposting may enrich the nitrogen content because the presence of earthworms containing mucus, enzymes and nitrogenous excretory substances has been exemplified by plenty of studies (Sangwan *et al*., 2008; Suthar, 2010; Garg *et al*., 2012). Hence, adult *E. foetida* showed higher total nitrogen content in present study is related to the TN content of their excretion. Whalen *et al.* (2000) elucidated that the nitrogen excretion rates of juvenile *Lumbricus terrestris* were significantly lower than the adults.

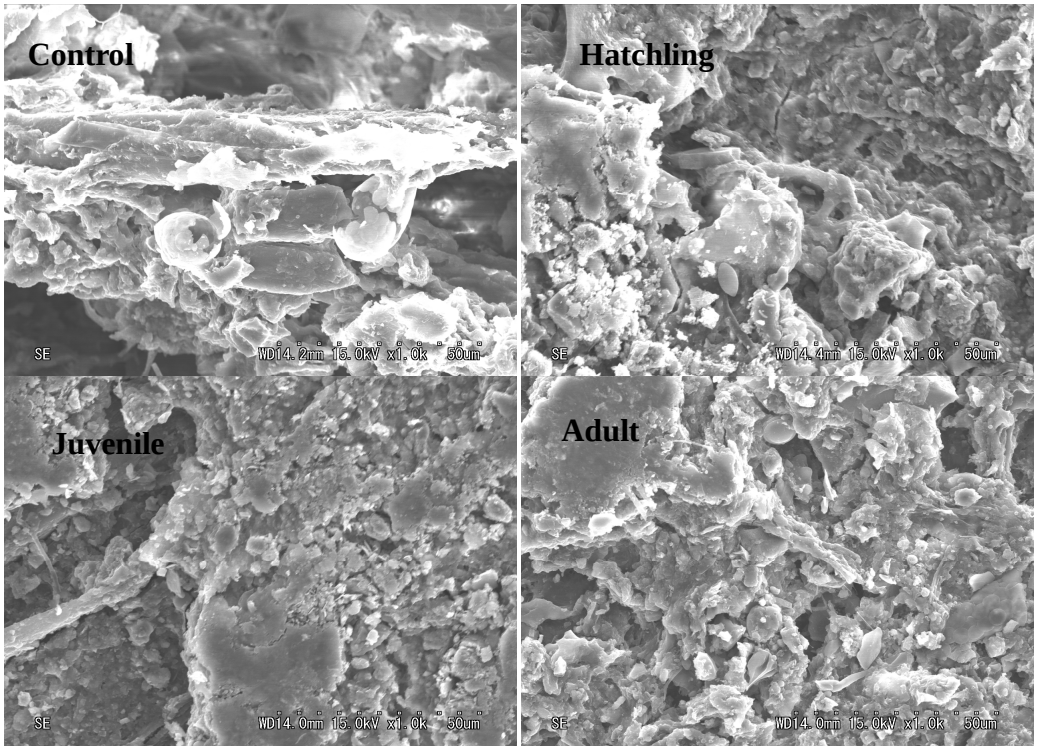

Fig. 1. The scanning electron micrographs of control and vermicomposts

As evidenced by Morais and Queda (2003), the C/N ratio below 20 is indicative of acceptable maturity. Hence, in present study, C/N ratio significantly dropped below 20 in all worms indicates that a high degree of organic material stabilization was achieved in vermicomposts. Compared to control, the C/N ratio decreased 15.5-20% in adult *E. foetida* showing the maximum decrease (Fig. 2). The loss of carbon as CO_2 by earthworms and microorganisms and adding TN excrements by inoculated earthworms lowers the C/N ratio during vermicomposting.

Inorganic N content in vermicomposted materials was relatively higher than control. The juvenile *E. foetida* displayed the largest increase. Similarly, P, K, Mg and Ca content increased in vermicomposted materials compared to controls, with the juveniles showing the greatest enhancement (Table III). The enrichment of inorganic ions in end products implies that the vermicomposts have the agronomic potential of being used as plant growth media or soil conditioner. This enrichment probably responds to the high mineralization of nutrients during vermicomposting process, which is partly performed by earthworms gut, and further release of nutrients continue to be processed by the microbial activities of deposited casts (Drake and Horn, 2007; Domínguez *et al.*, 2010). The increase in available nutrients content after vermicomposting has been reported by others also (Fernández-Gómez *et al.*, 2010b; Singh and Suthar, 2012). The greatest enhancement of all nutrients after juvenile treatment may be correlated to the organisms that allowed the strongest activity relative to other age groups. However, it should be noted that the reduction of volume and weight (organic matter) could increase the relative content of nutrients in the end products.

Microbial activity and population during vermicomposting

Dehydrogenase activity has been often used to estimate the microbial activity in the composting process, which catalyzes metabolic reactions producing ATP through the oxidation of organic matter.

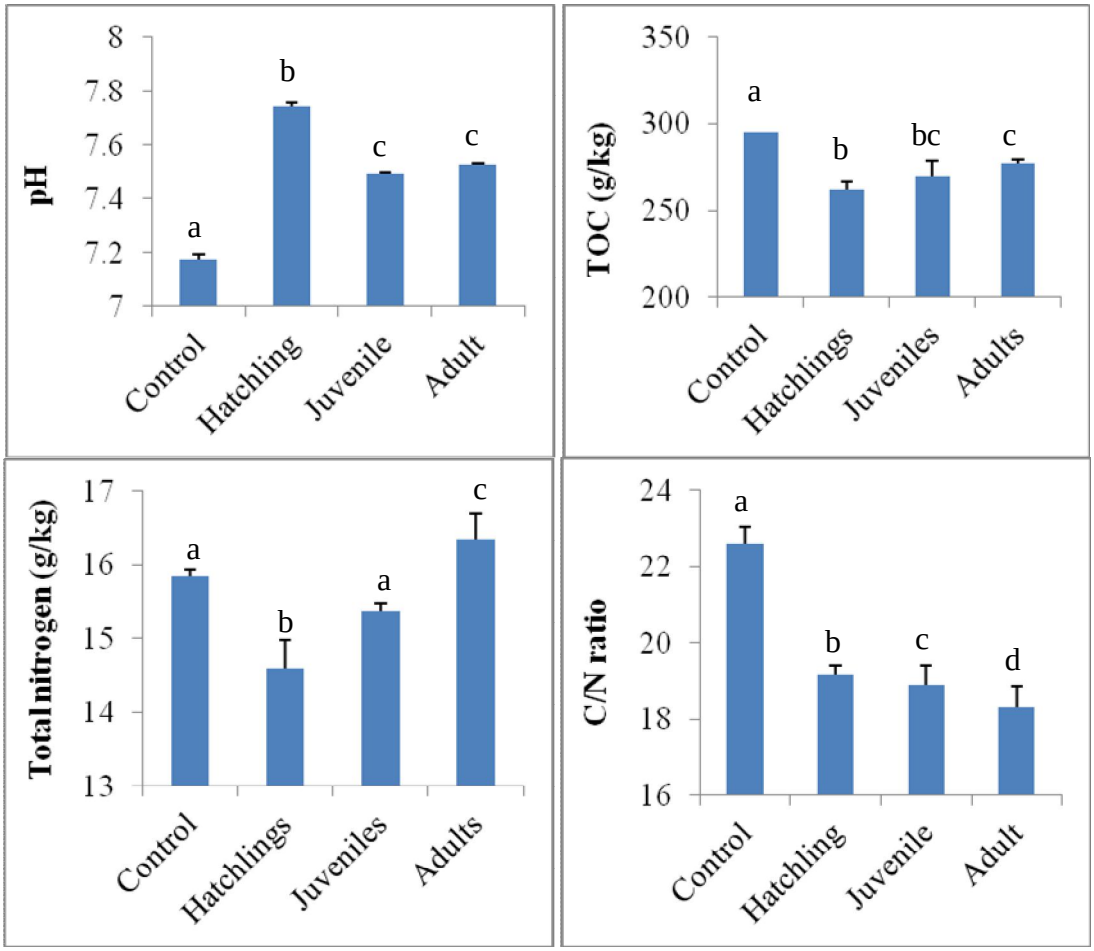

Fig. 2. Chemical properties of control and vermicomposts. Different letters in same column show the observed difference is significant (p<0.05).

As depicted in Figure 3, the dehydrogenase activity showed the maximum activity at the beginning of initial substrate, which indicates the preliminary treatment of short composting before vermicomposting leads to high biological activity. In contrast to control, dehydrogenase activity is significantly decreased in the vermicomposts, which further suggested that the matured products were built up by earthworms. Moreover, the dehydrogenase activities of vermicomposts differed significantly by the earthworms of different age classes, because the hatchling of *E. foetida,* displayed the lowest value compared with juveniles and adults. Similarly, decreased bacterial population was dependent on the age of earthworms since the lowest value was recorded in the hatchling *E. foetida* treatment (Fig. 3). These results could be related to TOC contents of end products (Fig. 2), since pronounced correlations have been established in TOC content and dehydrogenase activity and bacterial population in the present study (Data not shown). Sen and Chandra (2009) also observed a significant correlation of total heterotrophic bacterial population and dehydrogenase activity during vermicomposting and composting process, concluding that the biological activity was closely related to the microbial population. In line with this, Gómez-Brandón *et al*. (2013) found that reduction of microbial biomass and activity occurred throughout the process of vermicomposting.

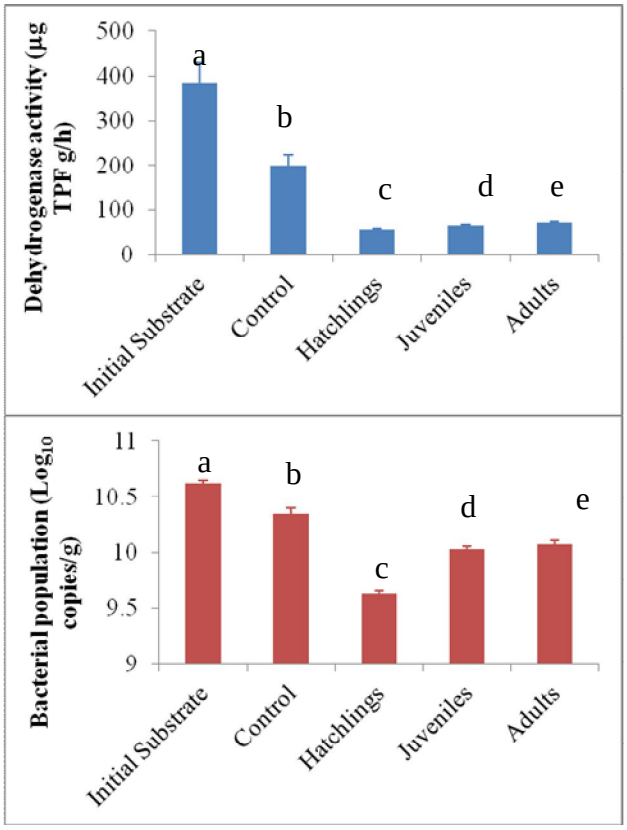

Fig. 3. Bacterial population and dehydrogenase activity of control and vermicomposts. Different letters in same column show the observed difference is significant (p<0.05).

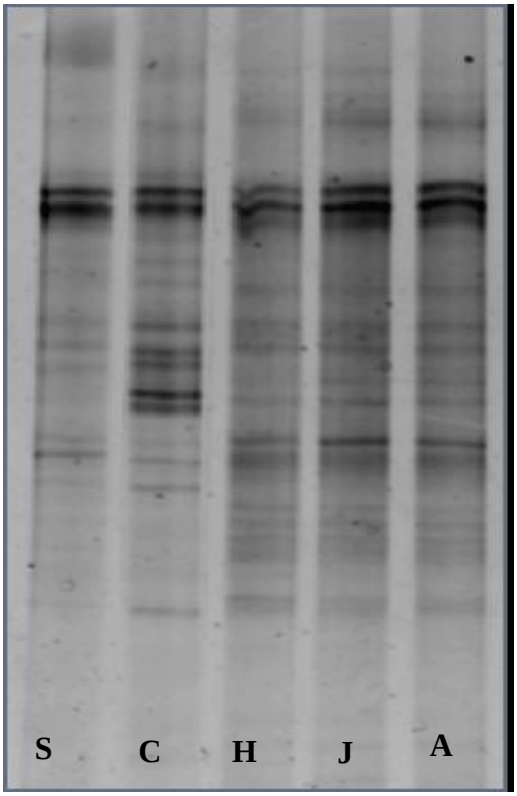

Fig. 4. DGGE Image of 16S rDNA of the PCR products from substrate, control and vermicomposts. The letters of S, C, H, J and A coded in each lane represent substrate, control, hatchling, juvenile and adult, respectively.

According to Ranganathan and Vinotha (1998), the enzymes activities of earthworm intestinal tract are correlated to the significant growth exhibited by the clitellated stage, with the clitellated earthworm showing the maximum enzymatic activities in their gut. As a consequence, it is speculated that the higher enzymatic activity in adult vermicompost in present study could be resulted by the gut digestive process.

Changes of microbial community profiles during vermicomposting

The bacterial DGGE image of the 16S rDNA genes extracted from initial substrate, control and vermicomposts is illustrated in Figure 4. It is apparent that the divergent profiles displayed in control and vermicomposts compared to initial substrate, indicating the distinguishing microbial succession arose in control and vermicomposts. Compared to control, vermicomposting treatments showed higher shannon index value (Table IV), suggesting that a greater bacterial diversity existed in the worm-worked treatments. Current finding regarding a greater bacterial diversity in worm-worked products is in accordance with other authors (Sen and Chandra, 2009; Vivas *et al.*, 2009). They also stated that the diverse microbial community structure in composts and vermicomposts in spite of the end products derived from the same materials, which implied the contribution of earthworm to microbial diversity and function. The increased microbial communities in the vermicomposts are explained by the indirect priming effect produced from mucus, undigested materials and fresh cast, and direct gut selective effect of earthworms (Bernand *et al.*, 2012). Furthermore, it is better evidenced that the food quality and earthworm

Table III.- Plant-available nutrient characteristics of control and vermicomposts.

	Inorganic N (g/kg)	PO_4^{3-} (g/kg)	K^+ (g/kg)	Mg^{2+} (g/kg)	Ca^{2+} (g/kg)
Control	1.2 ± 0.04 a	4.2 ± 0.12 a	1.5 ± 0.08 a	1.1 ± 0.04 a	0.6 ± 0.02 a
Hatchlings	1.5 ± 0.05 a	5.0 ± 0.09 b	1.8 ± 0.01 a	1.5 ± 0.02 b	0.8 ± 0.01b
Juveniles	2.6 ± 0.06 b	6.1 ± 0.11 c	2.7 ± 0.15 b	2.0 ± 0.09 c	1.0 ± 0.03 c
Adults	1.7 ± 0.02 c	5.4 ± 0.21 bc	1.8 ± 0.08 a	1.4 ± 0.07 b	0.8 ± 0.04 b

Data are mean and standard deviation of triplicates. Different letters in same column show the observed difference is significant (p<0.05).

Table IV.- Similarity matrix and Shannon index of initial substrate, control and all vermicomposts based on DGGE image.

	Substrate	Control	Hatchlings	Juveniles	Adults
Substrate	1				
Control	0.69	1			
Hatchlings	0.64	0.61	1		
Juveniles	0.68	0.58	0.87	1	
Adults	0.64	0.65	0.80	0.84	1
Shannon index	2.12	2.80	2.91	3.04	2.99

species do affect the microbial community profiles after gut associated process (Gómez-Brandón *et al*., 2011b, 2012). However, earthworms marking the contribution to microbial communities did not predominated by their age class in present study since vermicomposts processed by three different age stage of earthworms exhibited their bacterial fingerprints with more than 80 % similarity degree (Table 4). This could be partly ascribed to the same materials and earthworm species used for vermicomposting (Fernández-Gómez *et al*., 2012). As reported by Toyota and Kimura (2000), the gut of *E. foetida* hosted indigenous specific-species of microflora which could contribute to the analogous microbial community in vermicomposts. Also, Huang and Li (2012) pointed out that the microbial community structure of fresh casts excreted from hatchling, juvenile and adult of *E. foetida* was irrespective of their age class, which is consistent with this study. However, an important fact should not be overlooked that the hatchlings could grow into the juveniles or adults during the composting process of 60 days, which leads to the same age classes of earthworms in all treatments. As a result, this point may be a possible reason for the similar bacterial communities in all final products of vermicomposts.

CONCLUSIONS

Based on the observations, the age stages of *E. foetida* showed significant influence on the chemical properties and microbial densities and activities of vermicomposts, but they did not affect the physical appearance and microbial communities of vermicomposts. The vermicomposts produced by hatchlings exhibited the lowest TOC, microbial activity and population, suggesting that they could be the best candidate for stabilization of organic materials. The products vermicomposted by juveniles displaying the greatest enhancement in plant available nutrients indicated that they could be the optimal age class for mineralization of organic matter.

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