El Colegio de la Frontera Sur

Roles enzimáticos asociados a la anatomía intestinal de la lombriz de tierra *Eisenia fetida* (Savigny, 1826)

TESIS
presentada como requisito parcial para optar al grado de
Maestría en Ciencias en Recursos Naturales y Desarrollo Rural
Con orientación en Biotecnología Ambiental

Por

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Este trabajo se lo dedicó a mi madre que ha estado apoyándome en todo momento.

A mi familia

A mis compañeros de generación por la gran amistad que me brindaron

A mi hijo que es lo mas hermoso de mi vida

A ottoniel por el apoyo brindado en ciertos momentos

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Resumen

El objetivo del trabajo fue evaluar la dinámica enzimática a lo largo de la anatomía intestinal de *Eisenia fetida* (*Oligochaeta, lumbricidae*), para proponer las posibles vías catabólicas que pudieran actuar sobre los principales componentes de los residuos orgánicos durante el proceso de lombricompostaje. Para abordar el estudio se determinó la actividad de 19 enzimas hidrolíticas en tres diferentes secciones del tracto digestivo de *Eisenia fetida* (A, boca, molleja; B, intestino anterior; C, intestino intermedio y posterior*)* en periodo de ayuno, 24 horas, 30, 60 y 90 días, respectivamente; además del análisis químico proximal del sustrato en cada período. La información obtenida aporta al conocimiento de la fisiología digestiva de la lombriz adaptada a un clima tropical; tales como la correlación entre las máximas actividades de glicosilhidrolasas con la sección B (que concuerda con la disminución de hemicelulosa del sustrato) y la proteasa α-quimotripsina en la sección C. Esto puede ser de gran interés para el desarrollo de alternativas viables para problemas de contaminación (como el aprovechamiento de los residuos orgánicos); así como la bioprospección de enzimas de interés para aplicaciones biotecnológicas.

Palabras clave: Enzimas, microbiota, tracto digestivo, residuos orgánicos, lombricompostaje.

CAPÍTULO I. INTRODUCCIÓN

Las lombrices de tierra juegan un papel crucial en los ecosistemas terrestres porque se les reconoce el papel funcional que realizan en el suelo, tales como aumentar su porosidad, reorganizar la estructura del suelo, estimular la microflora del suelo y la mineralización de los nutrientes (Clause et al. 2014). Estos invertebrados tienen la capacidad de degradar la mayor parte de la materia orgánica por la interacción con los microorganismos que se alojan en su intestino (Edwards & Fletcher 1988). El sistema digestivo o canal alimentario de la lombriz es un tubo que se extiende desde la boca hasta el ano y está formado por la cavidad bucal, faringe, buche, molleja y el intestino, por el cual pasa el material orgánico ingerido (Drake & Horn 2007). Barois y Lavelle (1986) han descrito el intestino de la lombriz de tierra como un "sistema digestivo mutualista" y proponen la división del tracto intestinal en tres secciones (anterior, media y posterior). Sin embargo, el intestino ha sido subdividido de diversas maneras según el propósito de la investigación; para este trabajo esta indicado en la Fig. 1.

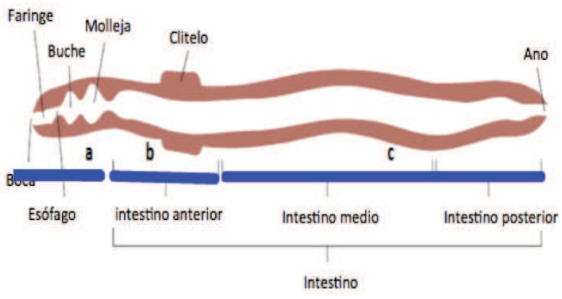


Figura 1. Representación esquemática de la anatomía del tracto digestivo de la lombriz. Señaladas en color azul: región **A** (boca, faringe, buche y molleja), región **B** (intestino anterior), y región **C** (intestino medio y posterior). Basado en Edward (1996) y Horn (2003).

Los residuos orgánicos ingeridos por la lombriz experimentan modificaciones físicas, químicas y biológicas importantes en su paso por el tracto digestivo (Barois & Lavelle 1986). En una fase preliminar, el agua y la materia orgánica fácilmente asimilable se incorporan y el pH aumenta significativamente dentro del tracto intestinal a 6.8 (Barois & Lavelle 1986). En la molleja la mezcla se intensifica y se homogeniza el medio semilíquido, donde la microbiota permanece en contacto directo con los sustratos orgánicos (ya sea materia orgánica o mucus intestinal). Aquí la actividad microbiana al principio es fuertemente estimulada por condiciones físicas más favorables y aumenta a medida que el moco intestinal se consume; esto conduce a que la microbiota aumente su capacidad de digerir la materia orgánica más compleja en beneficio de la lombriz de tierra en el intestino posterior (Barois & Lavelle 1986).

Lavelle et al. (1995) mencionan que el moco intestinal secretado por las lombrices es una mezcla de bajo peso molecular soluble en agua compuesta por aminoácidos, glucósidos y una glicoproteína, que no está presente en la parte media del intestino ya que parte de ella ha sido metabolizado por los microorganismos y otra parte probablemente reabsorbido y reciclado dentro de la lombriz. La capacidad que tienen los microorganismos de moverse en el suelo es limitada y no pueden aprovechar los recursos orgánicos a su alrededor; por lo tanto, la microbiota tiene mínima actividad metabólica pero cuando están en contacto con el mucus intestinal de la lombriz se potencia su actividad metabólica

para digerir la materia orgánica más compleja; este evento es considerado como el efecto “priming” o también denominado la “teoría de la bella durmiente”.

El lombricompostaje involucra la oxidación y estabilización de los residuos orgánicos a través de la acción conjunta de las lombrices y los microorganismos (Aira et al., 2007). Este sistema se divide en dos subprocesos: el primero se lleva a cabo por los eventos asociados al paso a través de sus intestinos (PAIs), que involucra toda la serie de procesos directos que resultan en la modificación de los residuos orgánicos durante ese trayecto, incluyendo la transformación de los nutrientes, modificación e incremento de la diversidad microbiana, el proceso de digestión, la homogeneización del sustrato, la asimilación y la excreción. El segundo está asociado a las deyecciones (PADs), que son los efectos indirectos que derivan del intestino, tales como el envejecimiento de las excretas y los componentes que no fueron modificados en su paso por el intestino (Domínguez et al., 2009). Hasta el momento, se sabe que los PADs modifican la biomasa microbiana y sus actividades enzimáticas en el sistema de lombricompostaje (Aira et al. 2007).

La información disponible sobre los procesos asociados al paso a través de sus intestinos (PAIs), se ha enfocado a las interacciones entre las lombrices y los microorganismos, desde el punto de vista ecológico de alimentación (Curry & Schmidt 2007). Por ejemplo, han contribuido a conocer el papel que desempeñan los microorganismos aerobios celulolíticos en la digestión de lignocelulosa, donde sugieren que estos microorganismos se activan en la parte anterior del intestino de

la lombriz, pero al entrar en contacto con la zona anaerobia se desactivan, mientras se activan los microorganismos anaerobios (Fujii et al. 2012). Esta información es de gran relevancia para conocer la interrelación entre las diferentes especies lombrices y los microoorganismos asociados a su tracto digestivo porque nos aporta información desde el punto de vista de su ecología de alimentación. Sin embargo, esta hipótesis está basada en evidencias indirectas obtenidas de estudios *in vitro* con bacterias aisladas del intestino de la lombriz.

La información que existe con respecto a las enzimas digestivas de las lombrices describe su relación con hábitos de alimentación y grupo ecológico al que pertenecen: epígeas, endógeas y anécicas (Zhang et al., 2000). Esta clasificación ha sido de la siguiente manera: las lombrices epígeas viven cerca de la superficie del suelo y se alimentan principalmente de residuos vegetales, las lombrices anécicas viven en madrigueras en el suelo mineral pero se alimentan de residuos en la superficie del suelo, mientras que las endógeas viven en las profundidades del suelo y se alimentan del suelo mezclado con residuos orgánicos (Curry & Schmidt 2007).

Las lombrices epígeas estimulan, deprimen o modifican las comunidades microbianas a través de los procesos asociados a su intestino (Monroy et al. 2008); por consiguiente, afectan la descomposición de los residuos orgánicos en el sistema de lombricompostaje (Huang et al. 2013). Estas lombrices poseen un conjunto de enzimas digestivas que les permite digerir bacterias, protozoarios, hongos y restos vegetales parcialmente descompuestos durante el paso del

material orgánico (Zhang et al. 2000). Sin embargo, cada especie tienen diferentes conjunto de enzimas. Un estudio comparativo sobre la actividad de enzimas digestivas de lombrices epígeas (*Eudrilus euginae* y *E. fetida)* alimentadas con residuos orgánicos municipales, mostró que *E. fetida* tiene las mayores actividades de celulasa, xilanasa, fosfatasa ácida y fosfatasa alcalina, mientras que *E. euginae* tiene las actividades mas altas de amilasa-celobiosa, endoglucanasa y nitrato reductasa (Prabha et al. 2007).

E. fetida ha sido ampliamente reconocida en el lombricompostaje debido a su fácil manejo y tolerancia a factores ambientales, y los estudios realizados con 16S rDNA han revelado la estructura de la comunidad bacteriana en su intestino y fueron divididos en 12 géneros: *Aeromonas (*6%), *Agromyces* (3%), *Bacillus* (31%), *Bosea* (1%), *Gordonia* (6%), *Klebsiella* (6%), *Microbacteium* (7%), *Nocardia* (2%), *Pseudomonas* (10%), *Rhodococcus* (19%), *Tsukamurella* (2%) y *Streptomyces* (7%); siendo *Bacillus* el grupo dominante (Kim et al. 2004).

Toyota y Kimura (2000) caracterizaron la comunidad microbiana autóctona en esta lombriz en periodo de ayuno y encontraron exclusivamente bacterias Gram-negativas que son capaces de sobrevivir al ambiente intestinal que las Gram-positivas y bacterias fermentativas que utilizan los azúcares disponibles, una de las especies identificadas fue *Aeromonas hydrophila*; por el contrario, como respuesta a la alimentación se ha observado un cambio rápido y homogéneo de la microbiota intestinal (Rudi et al. 2009).

En general, la mayoria de los estudios han aportado resultados interesantes para dar a una aproximación *in vivo* de los procesos que ocurren en el sistema digestivo de las lombrices. Esto ha sido con lombrices endógeas donde se ha estudiado las glicosilhidrolasas en diferentes secciones del tracto digestivo en tejidos *in vitro* y tejidos *in vivo*, para determinar el origen de estas enzimas (Garvín et al. 2000). Mientras en lombrices epígeas como *Eisenia andrei* se ha reportado enzimas xilanolíticas en todo el contenido intestinal (Merino-Trigo et al. 1999). No obstante, no se ha estudiado el papel de estas enzimas y otros grupos enzimáticos como proteasas, aminopeptidasas, lipasas y fosfatasas en las diferentes secciones del tracto digestivo durante el proceso de lombricompostaje. Esta información es de gran relevancia para conocer su fisiología digestiva y el papel que juega en la dinámica de los nutrientes en el sistema de lombricompostaje. Ademas, la mayoria de los estudios se han realizado en climas templados por lo que no se conoce la dinámica enzimática en el tracto de esta lombriz adaptada a un clima tropical y se tiene poca información acerca de cómo transforma los residuos orgánicos bajo esas condiciones. Este es el primer estudio que aporta resultados importantes sobre la dinámica observada en el perfil enzimático a lo largo del tracto digestivo de *E. fetida* para entender mejor los PAIs y que tiene implicaciones en los PADs de las lombrices durante el proceso de lombricompostaje. El objetivo del trabajo fue describir la dinámica enzimática a lo largo de la anatomía intestinal de *Eisenia fetida*, para proponer las posibles vías catabólicas que pudieran actuar sobre los principales componentes de los residuos orgánicos durante el proceso de lombricompostaje

1.1 Objetivo general

Evaluar la dinámica enzimática a lo largo de la anatomía intestinal de *Eisenia fetida*, para proponer las posibles vías catabólicas que pudieran actuar sobre los principales componentes de los residuos orgánicos durante el proceso de lombricompostaje.

1.2 Hipótesis

Las enzimas digestivas difieren en la localización y tiempo de sus actividades en la anatomía intestinal de la lombriz *Eisenia fetida*.

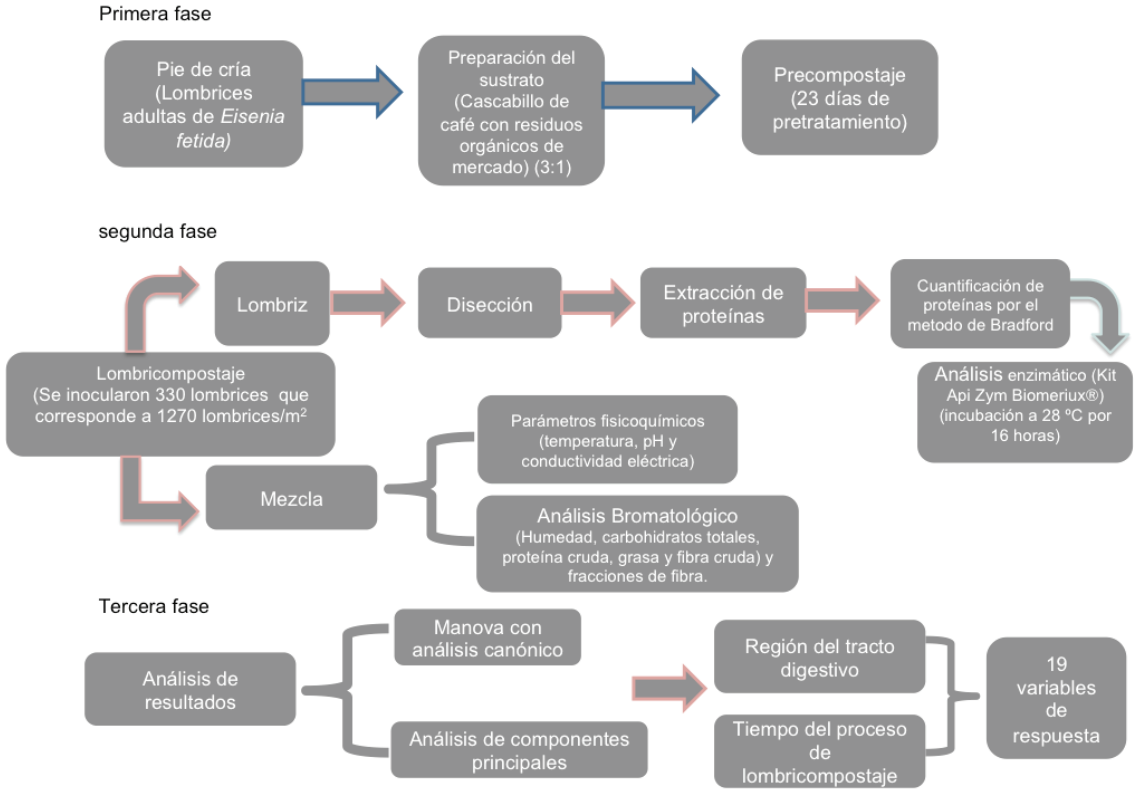
1.3 Objetivos específicos

Determinar la actividad de 19 enzimas en las tres diferentes secciones del tracto digestivo de *Eisenia fetida*.

Conocer la dinámica del proceso de degradación de los principales componentes de un sustrato.

Proponer un mecanismo metabólico de la degradación del sustrato en base a los resultados obtenidos.

CAPÍTULO II. METODOLOGÍA



CAPÍTULO III. ARTÍCULO

Enzymatic roles associated with intestinal anatomy of the earthworm *Eisenia fetida* (Savigny, 1826)

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Enzymatic roles associated with the intestinal anatomy of the earthworm *Eisenia fetida* (Savigny, 1826)

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Abstract

Epigeic worms stimulate, repress, or modify microbial communities through their digestive processes, thereby influencing the decomposition of organic matter in lumbricomposting systems. Nevertheless, the enzyme dynamics that take place within the digestive tract of the tropically adapted earthworm and the mechanism by which these enzymes transform organic compounds under tropical conditions are unknown. Therefore, the activities of 19 hydrolytic enzymes within three different sections of the digestive tract of *Eisenia fetida* (A, mouth, pharynx, crop, and gizzard; B, anterior intestine; C, middle and posterior intestine) were determined over a fasting period and at 24 hours, 30, 60, and 90 days of lumbricomposting. Moreover, proximate chemical analysis of the substrate was performed during each period. Maximal enzymatic activities were localized to section B for all enzyme groups studied. Phosphatases and glycosyl hydrolases showed greater enzymatic

activity when compared to other groups at all stages analyzed. Positive correlations were found between the maximal activity of glycosyl hydrolases in section B (coincident with the reduction of hemicellulose in the substrate) and the activity of the protease α-chymotrypsin in section C. However, it is clear that enzymatic activities change within each section of the digestive tract throughout the lumbricomposting process. Our results suggest that enzyme dynamics within the worm's intestine depend on the sequence of events taking place during digestion. This sequence is highly influenced by selective microbial enzyme enrichment, which occurs through excretion and re-ingestion of substrate and microbiota, and by the availability of nutrients throughout the lumbricomposting process.

Key words: Enzymes, microbiota, digestive tract, organic residues, lumbricomposting.

Introduction

Organic residues ingested by worms experience fundamental physical, chemical, and biological changes through their passage along the length of the digestive system (Barois and Lavelle, 1986). Lumbricomposting involves the oxidation and stabilization of organic residues by the combined activities of worms and microorganisms (Aira et al., 2007). This process consists of two primary processes: The first process involves the events associated with the passage of organic matter through the intestines (PAIs), which result in the modification of organic residues. This first process leads to the transformation of nutrients, an increase in microbial diversity, digestion of nutrients, homogenization of substrate, assimilation of nutrients, and the excretion of waste. The second process is associated with dejections (PADs) and the indirect effects of intestinal activity, such as the decomposition

of feces and the components not modified by their passage through the intestines. (Domínguez et al., 2009). The majority of the studies have focused on PADs in which earthworms increase the microbial biomass and stimulate enzymatic activities; whereas the current research on the PAIs focuses on the earthworms and microorganisms interaction from the ecological viewpoint of nutrition (Curry and Schmidt, 2007).

The digestive system or alimentary canal of the earthworm consists of a tube that extends from the mouth to the anus, formed by the oral cavity, pharynx, crop, and gizzard (food reception zone). This tube is followed by the anterior intestine, which secrets enzymes, and the posterior intestine, which absorbs nutrients (Edwards and Fletcher, 1988).

The type of digestive enzymes present in earthworms qualifies or provides information on the worm's alimentary habits and the ecological groups to which it belongs: epigeic, endogeic, and anecic (Zhang et al., 2000). Epigeic worms stimulate, repress, or modify microbial communities through their intestinal processes (Koubová et al., 2015; Gómez-Brandón et al., 2011; Monroy et al., 2008); therefore, these worms influence the decomposition of organic matter within lumbricomposting systems (Huang et al., 2013). These earthworms possess a pool of digestive enzymes that allow them to digest bacteria, protozoans, fungi, and partially decomposed vegetable matter (Zhang et al. 2000). A comparative study on the activities of digestive enzymes of epigeic worms (*Eudrilus euginae* and *E. fetida)* fed on municipal organic residues demonstrated that *E. fetida* has greater cellulase, xylanase, acid phosphatase, and alkaline phosphatase activities, while *E. euginae* has the highest cellobiose-amylase, endoglucanase, and nitrate reductase activities (Prabha et al., 2007).

E. fetida is well recognized in lumbricomposting for its manageability and tolerance to environmental factors. Studies based on 16S rDNA have revealed the structure of the

intestinal bacterial community in *E. fetida*, which may be divided into 12 different genera: *Aeromonas* (6%), *Agromyces* (3%), *Bacillus* (31%), *Bosea* (1%), *Gordonia* (6%), *Klebsiella* (6%), *Microbacteium* (7%), *Nocardia* (2%), *Pseudomonas* (10%), *Rhodococcus* (19%), *Tsukamurella* (2%), and *Streptomyces* (7%), with *Bacillus* being the dominant group (Kim et al., 2004). Toyota and Kimura (2000) characterized the autochthonous microbial community of this earthworm during fasting finding exclusively Gram-negative bacteria with a positive oxidase reaction and fermentative bacteria; one of the species identified was *Aeromonas hydrophila*. Additionally, a rapid and homogeneous change in the intestinal bacterial microbiota has been observed in response to feeding (Rudi et al., 2009).

At most of the studies of the processes occurring at the earthworm's digestive system, has been done using endogeic worms. At those studies, the worm's glycosyl hydrolases have been studied in different sections of the digestive tract *in vitro* and *in vivo* tissues to determine the origin of these enzymes, and only as studied until 7 days of the process (Garvín et al., 2000). Whereas in epigeic worms, such as *Eisenia andrei*, xylanolytic enzymes from the intestinal content have been reported, without separating it into sections (Merino-Trigo et al., 1999). Nevertheless, the role of these enzymes, as well as other enzymes groups, such as proteases, aminopeptidases, lipases, and phosphatases hasn't been studied in the different sections of worm's digestive tract throughout the lumbricomposting process. Moreover, most of the studies mentioned thus far have been carried out in temperate climates; therefore, the enzyme dynamics within the digestive tract of *E. fetida* adapted to tropical climates are not known. Therefore, the objective of this study was to evaluate the enzyme dynamics throughout the length of the intestinal tract of *E. fetida* to elucidate the catabolic pathways acting upon the main organic components during

96 lumbricomposting in tropical conditions. To our knowledge, this is the first study
97 presenting significant findings regarding the dynamics of the enzymatic profile along the
98 length of the intestinal tract of *E. fetida*, with implications on PAIs and PADs during the
99 process of lumbricomposting.

100 **Materials and Methods**

101 **Biological materials and study conditions**

102 Worms were raised adapted to tropical conditions provided by the lumbriculturing module
103 at El Colegio de la Frontera Sur, in Tapachula, Chiapas, Mexico. Pre-clitellum individuals
104 were selected (juveniles) to have a homogeneous population (adult stage) of *E. fetida*.
105 During the study, earthworms were kept under shaded conditions at an average temperature
106 of 35 ºC and under a relative humidity of 50%.

107 **Substrate preparation and pre-composting**

108 A mixed substrate of a 1:3 ratio of organic residues:coffee husk was used. Organic residues
109 from local markets were cut to fragments of 1–2 cm in size and were composed of the
110 following: cauliflower (54.7%), lettuce (19.7%), corncob (7.22%), onion and scallions
111 (7.1%), radish (7.78%), beets (2.2%), longbeak rattlebox (1.6%), and others (4.2%). Once
112 all the components were mixed, they were allowed to rest for 23 days (pre-composting);
113 additional mixing and addition of water occurred every eight days during pre-treatment
114 (Acosta et al., 2012).

115 **Lumbricomposting**

116 A population of age-matched earthworms was fasted for 48 hours by placing the worms
117 inside plastic vessels with clean water and without food, followed by several changes of
118 water to eliminate dejection residues. Worms were then placed into plastic vessels (69 x 38
119 x 30 cm) containing pre-treated substrate in a volume of 0.079 m^3 and having a density of

1270 worms/m^2. During this process, substrate samples were taken at a depth of 15 centimeters for physicochemical analysis. The sample was a mix obtained from five different places within the vessel. The first sample was obtained from the center of the vessel, and the remaining four were collected from the far sides of the vessel. The substrate was watered three times per week to maintain 70% humidity throughout the process.

Earthworm dissection

For the enzymatic analysis, four worms were used, which were collected in quadruplicate at different time points (at fasting and at 24 hours, 30 days, 60 days, and 90 days of lumbricomposting). Once the earthworms were collected, they were numbed by cooling at -20 ºC for 10 minutes, followed by dissection with methods described by Trigo et al. (1999) to separate specific regions of the digestive tract (A, mouth, pharynx, crop, and gizzard; B, anterior intestine; C, middle and posterior intestine) into separate microtubes. Each section of the intestinal tract was dissected from four worms, and fresh weight was measured. Throughout the procedure, all samples were maintained at 4 ºC. Samples were stored at -20 ºC for further enzymatic analysis.

Enzymatic analysis

Frozen samples were homogenized with a pestle in 500 µl of 0.9% sodium chloride for 5 minutes, followed by the addition of glass beads and vortexing for 20 seconds. Samples were centrifuged at 3000 *g* for 10 minutes at 4 ºC; the supernatant was recovered and diluted with 1.5 ml of 0.9% sodium chloride. Each extract was used for enzymatic analysis using an API® ZYM system for the detection of enzymes (BioMérieux, Marcy 1'Etoile, France), and total proteins were quantified by the Bradford method. The API® ZYM system is a semiquantitative method for the detection of 19 enzymes: alkaline phosphatase,

esterase, esterase lipase, lipase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, α-chymotrypsin, acid phosphatase, phosphoamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, β-glucosaminidase, α-mannosidase, and α-fucosidase. For this assay, 65 µl of enzyme extract were added to each microcuvette and incubated for 16 hours at 28 ºC. Following this incubation, colors were developed by the addition of the reagents ZYM A and ZYM B to stop the reaction following the instructions of the manufacturer; results were recorded based on the observed colors. Enzyme activity levels were scored from a value of zero to five, corresponding to the nanomoles of hydrolyzed substrate provided in the reaction for each enzyme as indicated in the reference template provided by the manufacturer. Enzyme activities were reported as nanomoles of hydrolyzed substrate per mg of soluble protein.

Physicochemical analysis

Mixed samples were collected, as previously described, every 10 days during the lumbricomposting process to measure hydrogen potential (pH) and electrical conductivity (EC). For pH determination, a ratio of 1:2.5 of the sample to distilled water was used. The sample was mixed for a few seconds and filtered, and the pH was measured with a potentiometer. To measure electrical conductivity, samples were diluted at a ratio of 1:5, allowed to rest for one hour, filtered, and used to measure conductivity; all measurements were made in triplicate. The temperature was measured every 10 days from the five different sample collection points within the vessel, and the mean value was calculated. Proximate chemical analysis and fiber content analysis of the substrate were carried out at different periods during lumbricomposting (1, 30, 60 and 90 days). For all tests, samples (100 g) were collected, stored in sealed containers to preserve their characteristics and refrigerated until further analysis.

Statistical analysis

Two factors were used for the statistical analysis: the digestive tract region in the worm (sections A, B, and C), and the time during the lumbricomposting process (fasting stage, 24 hours, 30, 60, and 90 days), with 19 response variables (each hydrolytic enzyme) to determine the difference in the activities of hydrolytic enzymes along the length of the worm's digestive tract and to determine the correlation between enzymes, using the multivariate analysis MANOVA (multivariate analysis of variance) and a Principal Components analysis. These were performed with the program R, version 3.3.0 (R Core Team, 2013).

Results

Physico-chemical parameters and composition of the substrate during the process of lumbricomposting.

At the start of the lumbricomposting process, the physico-chemical parameters showed a temperature of 29.2 °C, pH 8, and EC 0.15 (mS/cm); and it had a final temperature of 28 °C, neutral pH (7.1) and EC of 0.07 (mS/cm).

Proximate chemical analysis throughout lumbricomposting, including pre-compost mix data, had an initial 65% moisture that increased to 70% at 30 days of lumbricomposting and remained during the rest of the process. Regarding the mix components, the crude fiber had the highest percentage (73%) at the initial stage and reduced only 1% at the end of lumbricomposting process (90 days). Initial crude fibers were composed of 42% lignin, 27% cellulose, and 17% hemicellulose (data not showed); cellulose and lignin composition didn´t change, although hemicellulose had a slight decrease at the final stage of

lumbricomposting (to 14%). Organic carbon started at 36%, rised up to 39% (30 days) and 40% (60 days), and decreased to 37% at the end of the process. The remaining mix components like proteins, carbohydrates, and fat were found to be less than crude fiber and didn´t have any major changes but ashes, which percentage slight increased from 0.7 to 1.77% and 2.11% at day 30 and 60, respectively, but afterward decreased to 0.8%.

Roles of enzyme groups within each region of the intestinal tract of *E. fetida* during lumbricomposting.

During lumbricomposting, different enzyme activity patterns were observed within each region of the earthworm's intestinal tract. In Fig. 1, the different enzyme groups within each region of the digestive tract of the earthworm are shown over time (A, B, and C). The highest enzymatic activities for all enzyme groups throughout the entire process were localized to region B, in comparison to regions A and C, reaching the highest activities at the end of the study (90 days). The highest activities corresponded primarily to glycosyl hydrolases (3791 ±118 nmoles/mg protein) and phosphatases (1624 ±507 nmoles/mg protein).

In region A, the activities of glycosyl hydrolases increased by 1.3-fold, phosphatases and proteases by 1.5-fold, aminopeptidases by 1.8-fold, and esterases by 1.6-fold. In region B, the activities of glycosyl hydrolases and phosphatases increased by 1.1-fold, aminopeptidases by 1.2-fold, and esterases by 1.6-fold. In section C, only the activity of glycosyl hydrolases increased slightly by 90 days (to 1504±311 nmoles/mg protein), although this activity decreased by the 120-day stage.

The enzymatic activity behavior for each enzyme can be observed in detail in Fig. 2. At fasting to 24 hour-stage, α-chymotrypsin increased its activity in region C (from 16±33 to

79±11 nmol/mg protein), lipase activity was only present in section B (120 ± 60 nmol/mg protein), and from 30 day-stage no longer detected. From this stage until the end of the process the major activities of α-galactosidase, α-glucosidase, β-glucosidase and α-mannosidase were located only in the region B.

Comparing each enzyme activity with the respect to the section A at fasting stage, results showed differences in each section of worm intestine over time (Table 1). In this section, cystine aminopeptidase significantly increased at 60-day stage ($p = 0.043$); whereas esterase, three phosphatases, leucine aminopeptidase, valine aminopeptidase, trypsin, β-glucuronidase and β-glucosaminidase significantly increased their activities until 90-day stage ($p < 0.05$). Most enzymes in section B showed maximum activities statistically significant ($p < 0.05$.) at 60 and 90-day stages; except lipase and α-chymotrypsin. In section C all enzymes had a similar behavior ($p > 0.05$), but α-glucosidase activity was significantly higher during the fasting period ($p = 0.028$), and β-glucosidase significantly increased its activity at 90 days ($p=0.027$).

The relationships among digestive enzymes in the lumbricomposting system.

To better understand enzyme activity trends, a principal components analysis was performed, in which variables were grouped (every enzyme) based on the region of digestive tract and the sampling period (Fig. 3, for more details see supplemental Table S1). The first three components explain 85% of the total variance (71%, 7%, and 6%); among these components, the first component primarily accounts for the greater variability of results, clearly showing that region B separates from region C. Almost all enzymes correlated with each other, and maximal activity occurred within region B, when compared to region C, with the exception of two enzymes: lipase and α-chymotrypsin. The separation between regions B and C is mostly determined by the three phosphatases, β-galactosidase

239 and β-glucuronidase, which showed the same activity patterns throughout
240 lumbricomposting. More specifically, for these enzymes, no changes occurred during
241 fasting in any of the regions; at 24 hours, their activities were maintained in regions A and
242 B but decreased in region C. At 30 days, the activities of these enzymes decreased in region
243 B, and lastly, at 60 and 90 days their activities increased in regions A and B. In the second
244 component, region A separates from region C. Various enzymes were negatively
245 correlated: the three phosphatases, leucine aminopeptidase, valine aminopeptidase, and β-
246 glucuronidase, all having greater activity in region A during the last stages of the process
247 (60 and 90 days) (Fig. 3 and 5). In contrast, enzymes that were positively correlated, lipase,
248 α-chymotrypsin, α-galactosidase, and β-glucosidase, had greater activity within region C at
249 different times during the process. In the third component, regions B and C separated from
250 region A at fasting. Some enzymes that were negatively correlated, cystine aminopeptidase
251 and α-chymotrypsin, were not active during fasting; however, later in the process, their
252 activities increased in section B (cystine aminopeptidase) and section C (α-chymotrypsin).
253 In contrast, the activity of lipase was positively correlated in section B at fasting, being
254 active during that period; lipase activity was not detected during later stages.
255 **The activities of glycosyl hydrolases are associated with region B, while those of**
256 **proteases are associated with region C, in the intestine of *E. fetida*.**
257 The MANOVA (Fig. 4) showed a significative interaction between evaluated factors
258 (F=1.6103; p=0.0003409). In order to evaluate the comparisons among treatments
259 (interaction of levels in evaluated factors), a canonical analysis from the MANOVA results
260 was done with 95% confidence level areas (circles) for each treatment. The first and second
261 canonic components account for 65% of total variability, and showed significative
262 differences between groups B0, B1 and groups B2, B3, B4, while C0 separates from groups

C1, C2, C3, C4, and all A groups are statistically the same. This analysis showed that groups B2, B3, and B4 separate the following enzymes from the remaining groups: α-galactosidase, α-glucosidase, β-glucosidase, β-glucosaminidase, α-mannosidase, esterase, cystine aminopeptidase, and valine aminopeptidase; with the highest activities for these enzymes localized to region B throughout the process. Valine aminopeptidase and cystine aminopeptidase showed higher activity at 30 and 60 days, respectively. Activities of α-galactosidase, α-glucosidase, β-glucosidase, β-glucosaminidase, α-mannosidase increased from 30-day stage, while the activity of esterases increased only at 30 and 90 days, and lipase activity was present only at fasting and at 24 hours. Groups A1, A2, A3 and A4 correlate to the following enzymes: phosphatases, lipase-esterase, leucine aminopeptidase, tripsin, β-galactosidase, β-glucuronidase y α-fucosidase. C0 group enzyme is the protease α-chymotrypsin, which had high activity in region C after fasting.

Discussion

There are no previous reports of digestive enzymes in *E. fetida* adapted to tropical conditions. Hence, the data obtained in this study allowed us to know not only about its digestive physiology but also the availability of organic substrates digested by earthworms. The substrate used as a source of nutrients for these was a mix of coffee husks and organic residues from local markets mixed in a 3:1 ratio. Therefore, its fiber content was similar in composition to that of coffee pulp, albeit in different proportions (Pandey et al., 2000). Coffee husks contain anti-nutritional factors such as tannins and caffeine (Brand et al., 2000) that may be toxic to the earthworms; therefore, a period of pre-composting was usefull to allow for detoxification, making this substrate adequate for the alimentary requirements of the worms (Orozco et al., 1996). This detoxification period of 23 days also

287 favored the degradation of cellulose, likely during the thermophilic phase, mediated by
288 saprophytic fungi that produce cellulolytic enzymes (Jurado et al., 2014). Lignin remained
289 unchanged because of the variety and nature of bonds in its monomeric molecules that
290 make its degradation a challenge (Insam y Bertoldi, 2007). The same observation was made
291 with hemicellulose, it´s easier to degrade but requires different enzymes to complete the
292 task. Earthworms provided this repertoire since enzymatic activity on hemicellulose rapidly
293 increased when worms were inoculated in the substrate.
294 Once earthworms started to feed on this substrate, the highest enzymatic activities within
295 the intestines of *E. fetida* were exhibited by glycosyl hydrolases and phosphatases, in
296 agreement with the chemical composition of the substrate, consisting of a large fraction of
297 fiber. At this initial stage, earthworms would ingest microorganisms for their growth and
298 reproduction, particularly fungi, which provide nitrogen available from their proteins, since
299 initial substrate has shown to be nitrogen and fat deficient. Moreover, this deficiency is
300 consistent with the low activities of proteases, aminopeptidases, and esterases in the
301 earthworm´s intestine. In turn, the secreted intestinal mucus is ingested by microorganisms
302 that are selectively enriched inside the digestive tract. Phosphatases are known to be
303 secreted by earthworms; additionally, the presence of easily assimilated compounds in the
304 substrate increases the population of intestinal bacteria able to solubilize phosphates,
305 leading to a further increase in phosphatase activity (Wan and Wong, 2004). That agrees
306 with the evidence that this phosphatase activity present in stool comes from digested
307 microorganisms (*Bacillus* spp. y *Aspergillus* spp.) instead of earthworms' gut epithelium,
308 and contributes to phosphorous mineralization through these activities (Vinotha et al.,
309 2000). These findings are in agreement with the idea that the activities of digestive

310 enzymes reflect the nutritional characteristics of the organic matter consumed by the worms
311 (Kisilkaya et al., 2011).

312 **Enzymes associated with intestinal regions of *E. fetida***

313 The earthworm's digestive tract may be divided based on physiological functions: section
314 A is involved in the reception of nutrients; section B secretes enzymes, and section C
315 absorbs nutrients. Therefore, we proposed the hypothesis that different enzymes may be
316 associated with specific intestinal anatomical regions in the worm. Moreover, we suggested
317 that these enzymes might vary according to changes in the population of microorganisms
318 ingested.

319 In section A, there was no direct association with any of the enzymes studied, based on
320 MANOVA analysis, and enzymatic activity was generally moderate. This result suggests
321 that the majority of the digestive activity that occurs in this zone, and thus enzymatic
322 activity, may be carried out by ingested microorganisms (Prat et al., 2002).

323 In section B, the highest activities were found for five different glycosyl hydrolases (α-
324 galactosidase, α-glucosidase, β-glucosidase, β-glucosaminidase, and α-mannosidase), two
325 aminopeptidases (valine and cystine), and one esterase (Fig. 4). This result suggests that the
326 majority of the digestion of substrate components occurs within this section. As fiber
327 constitutes a main component of the substrate, it is reasonable that the activities of glycosyl
328 hydrolases are elevated in region B throughout the process. For endogeic worms, high
329 glycosyl hydrolase activity localized to the anterior intestine has been previously reported;
330 moreover, it is known that these worms produce their own cellulase (Lattaud et al., 1997).
331 Barois y Lavelle (1986) report an increment in ingested mucus as microorganisms enter the
332 anterior intestine and increase their enzymatic activities, and this is related to the high
333 glycosyl hydrolases activities.

High aminopeptidase and esterase activities were also associated with region B. Information regarding aminopeptidase activity in the worm is scarce; one report describes a leucine aminopeptidase in *E. fetida* that may be related to a physiological adaptation of the worm under stress conditions (Chen et al., 2011); although it may also participate in the catabolism of oligopeptides and proteins from the substrate. High esterase activity within section B could relate to the metabolism of phenolic esters, such as tannins from coffee husks or lignocellulosic complexes (Garcia-Conesa et al., 2004; Hermoso et al., 2004). In contrast to *Lumbricus terrestris*, where the activity of esterases has been detected within the crop, gizzard and anterior intestine (Sanchez-Hernandez and Wheelock, 2009), *E. fetida* exhibited little esterase activity.

In section C, only one enzyme (α-chymotrypsin) showed a strong association. In *E. fetida*, eight proteases with fibrinolytic activities have been reported (*Ef*P: proteases from *E. fetida*). Among these, *Ef*P-1 is similar to chymotrypsins and *Ef*P-III-1 is similar to trypsins. These enzymes are expressed in epithelial cells localized in the crop, gizzard, and anterior intestine and are thought to be involved in digesting proteins and peptides from food in these sections (Pan et al., 2010; Zhao et al., 2007). Nevertheless, we detected the highest α-chymotrypsin activity within section C, suggesting that proteases are distributed along the length of the intestinal tract to digest nutrients from food. Additionally, detection of α-chymotrypsin within section C may be related to the digestion of proteins and peptides from microorganisms that form part of the worm's intestinal microbiota, as it has been previously reported that microorganisms represent a source of essential amino acids (Pokarzhevskii et al., 1996). It is possible that these enzymes play another role, as DNAse activity was previously reported for a protease in *E. fetida* (Pan et al., 2011), although this finding has not been well explored.

Digestion of substrate during lumbricomposting

In general, the majority of the enzymes studied in the worm's digestive tract contribute to the digestion of substrate components. This analysis provided information about enzyme activities within each intestinal tract section during lumbricomposting. Therefore, we propose a more general explanation for the contribution of these digestive enzymes to the processes associated with the digestive tract:

During the first stage, worms were maintained in a fasting state for 48 hours prior to feeding. Due to the lack of nutrients, low enzymatic activities would be expected; nevertheless, we found high enzyme activity levels in all three sections of the intestinal tract. This result may be attributed to a compensatory effect in the worm triggered by the stress of the lack of nutrients, as has been described in other organisms (Cara et al., 2004). During fasting, the growth of intestinal microbiota is limited, leading to a greater variability in the microbial populations. This variability is thought to aid in counteracting the changes occurring due to the lack of nutrients (Rudi et al., 2009); moreover, enzymatic activities are induced to optimize the digestion process of any remaining nutrients present in the intestinal tract.

When the worms re-initiate substrate ingestion (24 hours), enzyme activities do not change in sections A and B, but they decrease in section C. These findings agree with two hypotheses that may explain the events taking place simultaneously during this period. First, the occurrence of priming effects due to the conditions in section A (Trigo and Lavelle, 1993), which are ideal for aerobic microorganisms due to the presence of oxygen and richness in organic matter originating from the intestinal mucus. This matter may be readily metabolized by ingested microorganisms, favoring their growth (Barois and Lavelle, 1986). Second, the role of aerobic lignocellulosic microorganisms within the worm

intestinal tract (Fujii et al., 2012) increases the capacity to degrade certain components in the substrate. Aerobic microorganisms that reach section B within the intestinal tract become inactivated due to the exposure to anoxic conditions, but their enzymatic activity persists. Simultaneously, the growth of anaerobic microorganisms is favored within this section due to a greater ingestion of intestinal mucus, leading to a greater ability to hydrolyze hemicellulose (described ahead). Next, in section C, the resultant products of digestion within section B are consumed by the worm and anaerobic microorganisms, leading to an increase in their biomass. This transition may explain the reduction of the majority of enzymatic activities in C, excepting for the activity of the protease α-chymotrypsin, which is likely released by the worm in the process of digestion of anaerobic microorganisms as a source of essential amino acids (Pokarzhevskii et al., 1997). It has been proposed that the intestinal microbiota represents a primary source of energy for the worm (Sampedro et al., 2006).

A relevant event that occurred in section B was the reduction in the activity of various enzymes at 30 days in the lumbricomposting process. This effect possibly occurred due to the loss of the most labile components within the substrate via degradation. During this period, there is a slight increase in the C:N ratio, possibly due to a proportional enrichment of more complex components in the substrate. For example, lignin has a chemical structure that is more condensed, stable and rich in organic carbon (Orozco et al., 1996). This increased ratio could potentially explain the decreased enzyme activity in the intestinal tract as the worm is not able to digest lignin.

The last event is the increase of enzymatic activities within sections A and B during the final stages of the lumbricomposting process (60 and 90 days), possibly caused by the re-ingestion of residues already excreted by the worm. Matter excreted by the worm enriches

406 the substrate with microorganisms that degrade polymers, solubilize phosphates, and fix
407 nitrogen (Raphael and Velmourougane, 2011). Therefore, dejections rich in organic carbon,
408 mineral nitrogen, and enzymatic complexes (Clause et al., 2014) is mixed with the
409 undigested substrate to be re-ingested. At this stage, the re-ingestion process increases the
410 bioavailability of substrates within the worm's digestive tract, potentially leading to the
411 enrichment of aerobic and anaerobic microorganisms within sections A and B, respectively.
412 The enrichment of the microbial population may consequently lead to an increase in the
413 variety of enzymes within these two sections of the intestinal tract at 60 days in the
414 lumbricomposting process.

415 **Digestion of hemicellulose**

416 *E. fetida* is an epigeic species that feeds primarily on plant residues (Thakuria et al., 2010),
417 a process facilitated by the microorganisms present in its intestinal tract (Zhang et al., 2000;
418 Nozaki et al., 2013). Our results suggest that these worms have difficulty degrading lignin
419 from coffee husks, although they may degrade other fractions within the lignocellulosic
420 complexes such as hemicellulose, as has been suggested from worms fed coffee pulp
421 (Orozco et al., 1996). This result is consistent with the high activities of glycosyl
422 hydrolases detected in section B and the decrease in hemicellulose content within the
423 lumbricomposting system. Hemicellulose is a heteropolymer complex formed by pentoses
424 (D-xylose and L-arabinose), hexoses (D-glucose, D-mannose and D-galactose), and
425 glucuronic acids, linked by β-1,4 linkages (Pérez et al., 2002).
426 Lignin protects other vegetable fiber fractions from the action of glycosyl hydrolases;
427 therefore, its removal by *E. fetida* would be required as a first step in the breakdown of
428 fiber, as it occurs in *Lumbricus* spp (Dempsey et al., 2013), thus exposing hemicellulose.
429 This process could occur via the combined actions of fungi, such as *Aspergillus* spp,

present in coffee husks (Ramírez et al., 2008), and via the passage of substrates through the intestinal tract of the worm (Dempsey et al., 2013). *Aspergillus* is known to produce phenolic esterases, which remove phenolic compounds from extracellular lignocellulosic complexes (Ramírez et al., 2008). Within section A of the worm's intestinal tract, the activity of fungi-derived enzymatic complexes together with that of aerobic microorganisms helps expose the hemicellulose. Once exposed, the hemicellulose may be digested in section B by the synergistic actions of various enzymes, including multienzyme complex-cellulosomes, as described in anaerobic microorganisms (Juturu and Wu, 2014; Dempsey et al., 2013; Ueda et al., 2010 and Horn et al., 2003 (Lind et al., 2002; Malherbe y Cloete, 2002; Ueda et al., 2010), and by the action of xylanolytic β-1,4-mannanase enzymes (Kim et al., 2011). The activities of β-glucosidase and α-galactosidase detected in *E. fetida* may be responsible for the breakdown of β-1,4 linkages from non-reducing ends of glucomannans and galactoglucomannans [generated by β-1,4 mannase acting upon the main chain of the hemicellulose (Moreira and Filho, 2008)] and α-1,6 linkages in the lateral chain of hemicellulose, respectively, thereby releasing glucose, mannose, and galactose residues (Puls, 1997). These free residues may be easily assimilated by the worm and microorganisms.

Conclusions

Our findings in *E. fetida* adapted to tropical conditions, suggest that enzyme dynamics within the worm's intestinal tract changes throughout the different stages of lumbricomposting. The location of certain enzyme activities is directly related to the intestine anatomy; such as the complex association of glycosyl hydrolases in section B to degrade hemicellulose and α-chymotripsin in section C to degrade peptides and proteins.

Understanding the functional role of the worm in lumbricomposting in tropical environments is of high relevance. These studies provide information regarding the routes for the decomposition of organic matter and the ecological role of the worm for nutrient dynamics within a tropical system. Moreover, findings from these studies may stimulate the development of viable alternatives to resolve contamination problems and the bioprospection of enzymes for biotechnological applications.

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Conflict to interest

The authors declare that they have no conflict of interest.

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642 Figure legends

643

644 **Fig. 1. Total activity of enzyme groups in the digestive tract of *E. fetida* during**
645 **lumbricomposting. a)** region A (from mouth to gizzard), **b)** region B (anterior intestine), **c)**
646 region C (middle and posterior intestines).

647

648 **Fig. 2. Spatial and temporal enzymatic profile of the digestive tract of *E. fetida*. a)** fasting, **b)**
649 24 hours, **c)** 30 days, **d)** 60 days, and **e)** 90 days. This is a supplemental image for Fig. 3. Region
650 A (from mouth to gizzard), region B (anterior intestine), and region C (middle and posterior
651 intestines).

652

653 **Fig. 3. Correlation of the activities of 19 enzymes in the worm's digestive tract evaluated by**
654 **principal component analysis.** Data from enzymatic profile was used for principal components
655 analysis, which was performed by grouping the variables (each type of enzyme) by the region of
656 the digestive tract and the sampling period. Each color indicates a section of the digestive tract in
657 the worm: region A, black; region B, red; and region C, green, during different sampling times: **0**,
658 fasting; **1**, 24 hours; **2**, 30 days; **3**, 60 days; **4**, 90 days. **a)** Ellipses represent 95% confidence
659 intervals, and **b)** shows the three axes (x, y, z) representing each principal component.

660

661 **Fig. 4. Comparative analysis of 19 enzymes evaluated in the worm's digestive tract via**
662 **MANOVA with discriminant canonical analysis.** Data from enzymatic profile was used to
663 perform this analysis. The size of the arrow indicates the degree of activity of each enzyme, and
664 the circle indicates the labeled groups in Fig. 2: Region A: **A0**, fasting; **A1**, 24 hours; **A2** , 30

665 days; **A3**, 60 days; **A4**, 90 days. Region B: **B0**, fasting; **B1**, 24 hours; **B2**, 30 days; **B3**, 60 days;

666 **B4**, 90 days. Region C: **C0**, fasting; **C1**, 24 hours; **C2**, 30 days; **C3**, 60 days; **C4**, 90 days.

667

Table 1. ANOVA comparison of each enzyme activity with the respect to the section A at fasting stage in each worm section throughout the time, *p<0.05. Region A: **A0**, fasting; **A1**, 24 hours; **A2**, 30 days; **A3**, 60 days; **A4**, 90 days. Region B: **B0**, fasting; **B1**, 24 hours; **B2**, 30 days; **B3**, 60 days; **B4**, 90 days. Region C: **C0**, fasting; **C1**, 24 hours; **C2**, 30 days; **C3**, 60 days; **C4**, 90 days.

	Phosphatase			**Esterase**			**Aminopeptidase**			**Protease**		**Glycosyl hydrolase**							
A0 vs	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A1	0.868	0.862	0.865	0.550	0.282	0.437	0.839	0.934	0.820	0.807	0.598	0.960	0.860	0.856	0.650	0.127	0.881	0.812	0.856
A2	0.957	0.953	0.954	0.390	0.274	0.715	0.887	0.977	0.732	0.984	0.469	0.907	0.952	0.843	0.298	0.384	0.695	0.942	0.844
A3	0.281	0.282	0.303	0.889	0.447	0.957	0.116	0.042*	0.271	0.577	0.053	0.763	0.422	0.291	0.517	0.492	0.122	0.885	0.415
A4	0.022*	0.024*	0.027*	0.038*	0.284	0.122	0.019*	0.251	0.025*	0.352	0.013*	0.747	0.087	0.025*	0.374	0.249	0.020*	0.810	0.235
B0	0.004*	0.004*	0.005*	0.445	0.370	0.062	0.059	0.284	0.297	0.175	0.103	0.088	0.004*	0.005*	0.000*	0.004*	0.006*	0.157	0.005*
B1	0.005*	0.005*	0.006*	0.313	0.270	0.037*	0.013*	0.451	0.071	0.644	0.000*	0.001*	0.004*	0.006*	0.000*	0.000*	0.006*	0.008*	0.006*
B2	0.264	0.264	0.291	0.920	0.286	0.670	0.437	0.603	0.550	0.747	0.072	0.002*	0.273	0.273	0.000*	0.001*	0.123	0.045*	0.292
B3	0.011*	0.024*	0.030*	0.018*	0.263	0.089	0.010*	0.00*	0.013*	0.878	0.004*	0.001*	0.010*	0.013*	0.000*	0.000*	0.001*	0.000*	0.013*
B4	0.000*	0.000*	0.000*	0.001*	0.285	0.001*	0.003*	0.012*	0.004*	0.523	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
C0	0.178	0.178	0.283	0.639	0.554	0.179	0.304	0.465	0.381	0.470	0.072	0.139	0.171	0.225	0.027*	0.614	0.126	0.110	0.195
C1	0.630	0.630	0.637	0.545	0.277	0.576	0.909	0.982	0.734	0.078	0.309	0.141	0.624	0.637	0.366	0.223	0.914	0.797	0.503
C2	0.616	0.546	0.554	0.443	0.348	0.464	0.663	0.647	0.514	0.195	0.324	0.141	0.609	0.622	0.103	0.170	0.994	0.744	0.617
C3	0.960	0.848	0.752	0.722	0.280	0.674	0.758	0.612	0.921	0.073	0.647	0.560	0.845	0.751	0.939	0.149	0.473	0.553	0.847
C4	0.884	0.713	0.718	0.640	0.269	0.729	0.407	0.450	0.640	0.082	0.826	0.153	0.708	0.718	0.404	0.027*	0.157	0.140	0.719

Figure 1

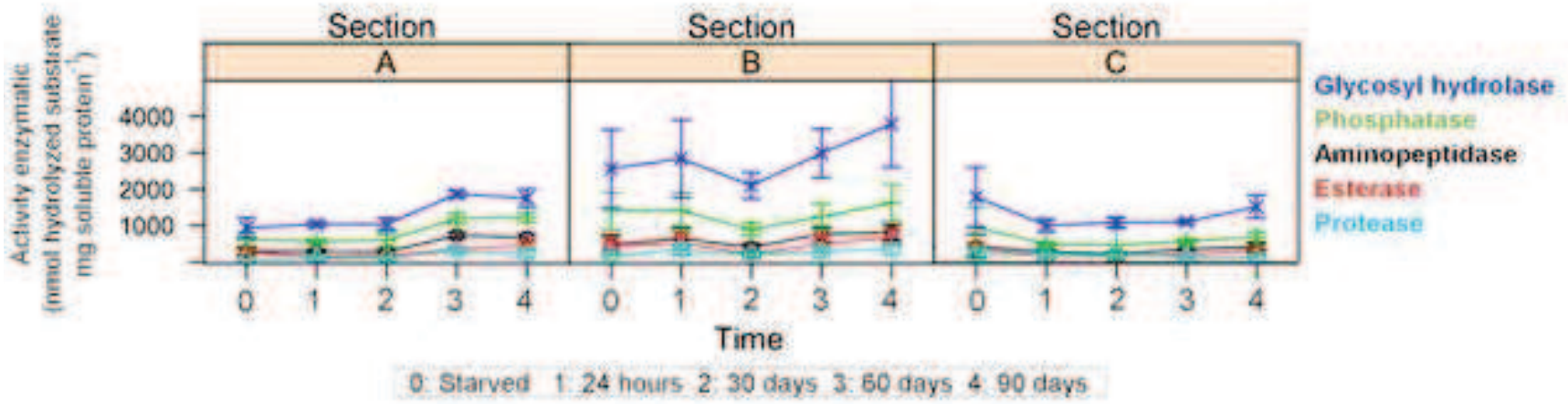

Section
A
Section
B
Section
C
Activity enzymatic
(nmol hydrolyzed substrate
mg soluble protein^{-1})
4000
3000
2000
1000
0 1 2 3 4
Time
0: Starved 1: 24 hours 2: 30 days 3: 60 days 4: 90 days
Glycosyl hydrolase
Phosphatase
Aminopeptidase
Esterase
Protease

Figure 2.

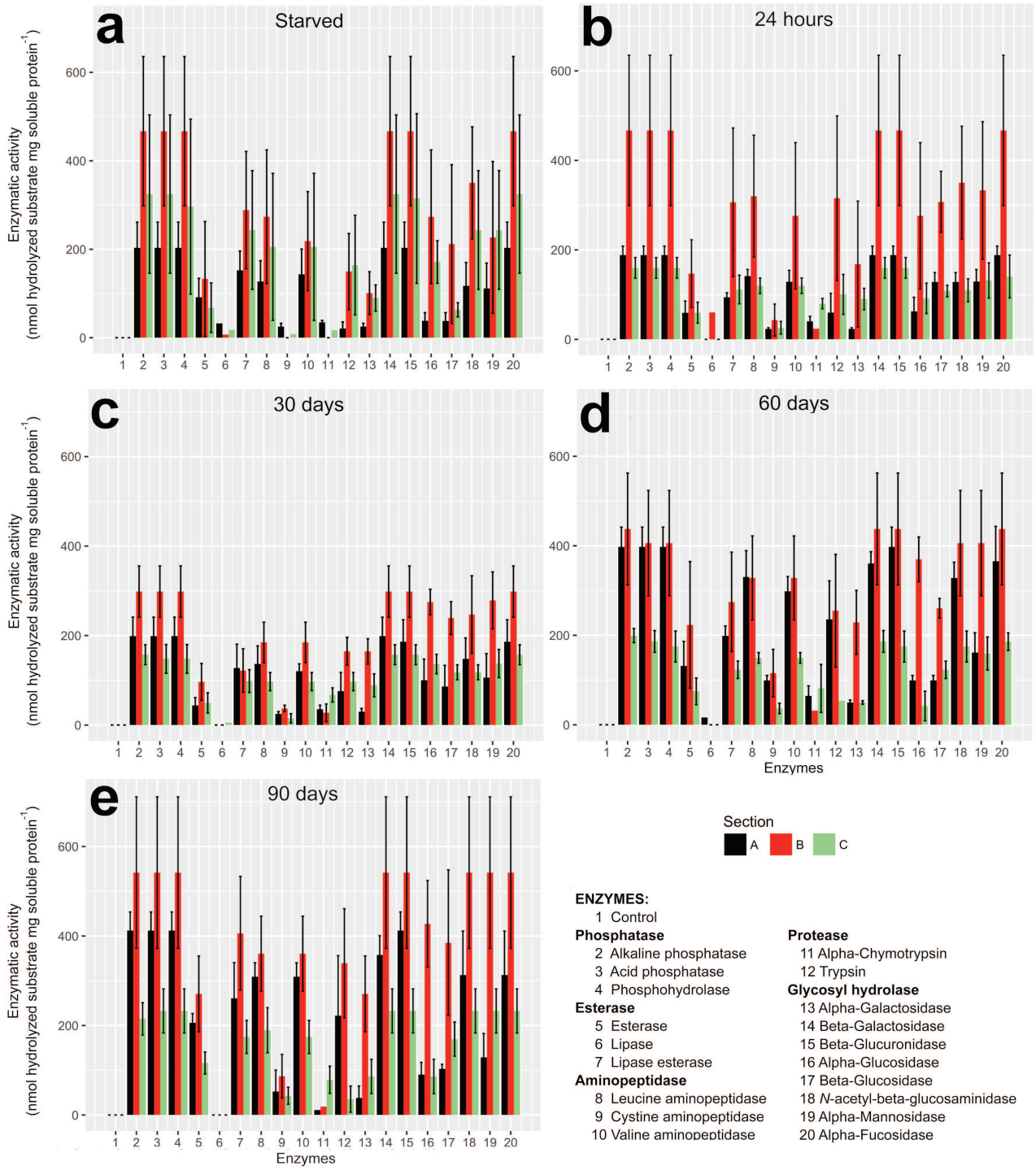

a
Starved
b
24 hours
c
30 days
d
60 days
e
90 days
Enzymatic activity
(nmol hydrolyzed substrate mg soluble protein^{-1})
0
200
400
600
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Enzymes
Section
A
B
C
ENZYMES:
1 Control
Phosphatase
2 Alkaline phosphatase
3 Acid phosphatase
4 Phosphohydrolase
Esterase
5 Esterase
6 Lipase
7 Lipase esterase
Aminopeptidase
8 Leucine aminopeptidase
9 Cystine aminopeptidase
10 Valine aminopeptidase
Protease
11 Alpha-Chymotrypsin
12 Trypsin
Glycosyl hydrolase
13 Alpha-Galactosidase
14 Beta-Galactosidase
15 Beta-Glucuronidase
16 Alpha-Glucosidase
17 Beta-Glucosidase
18 N-acetyl-beta-glucosaminidase
19 Alpha-Mannosidase
20 Alpha-Fucosidase

Figure 3.

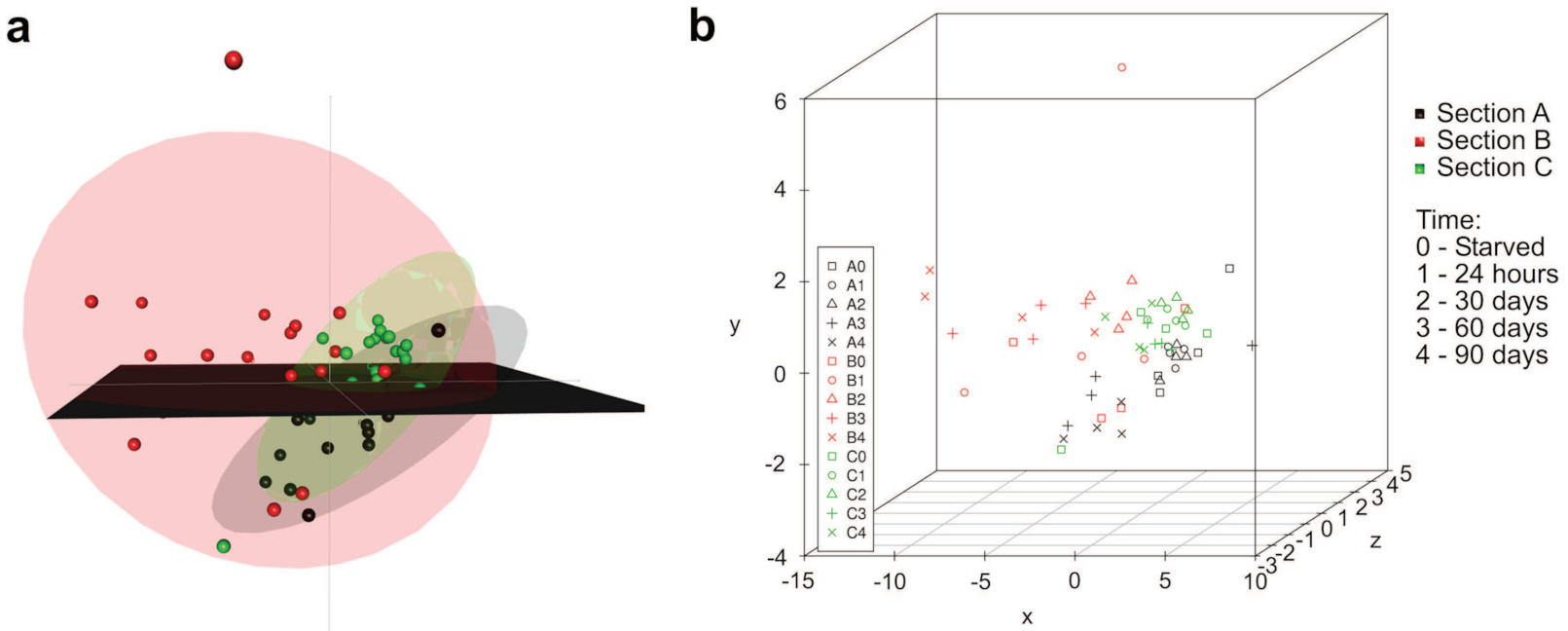
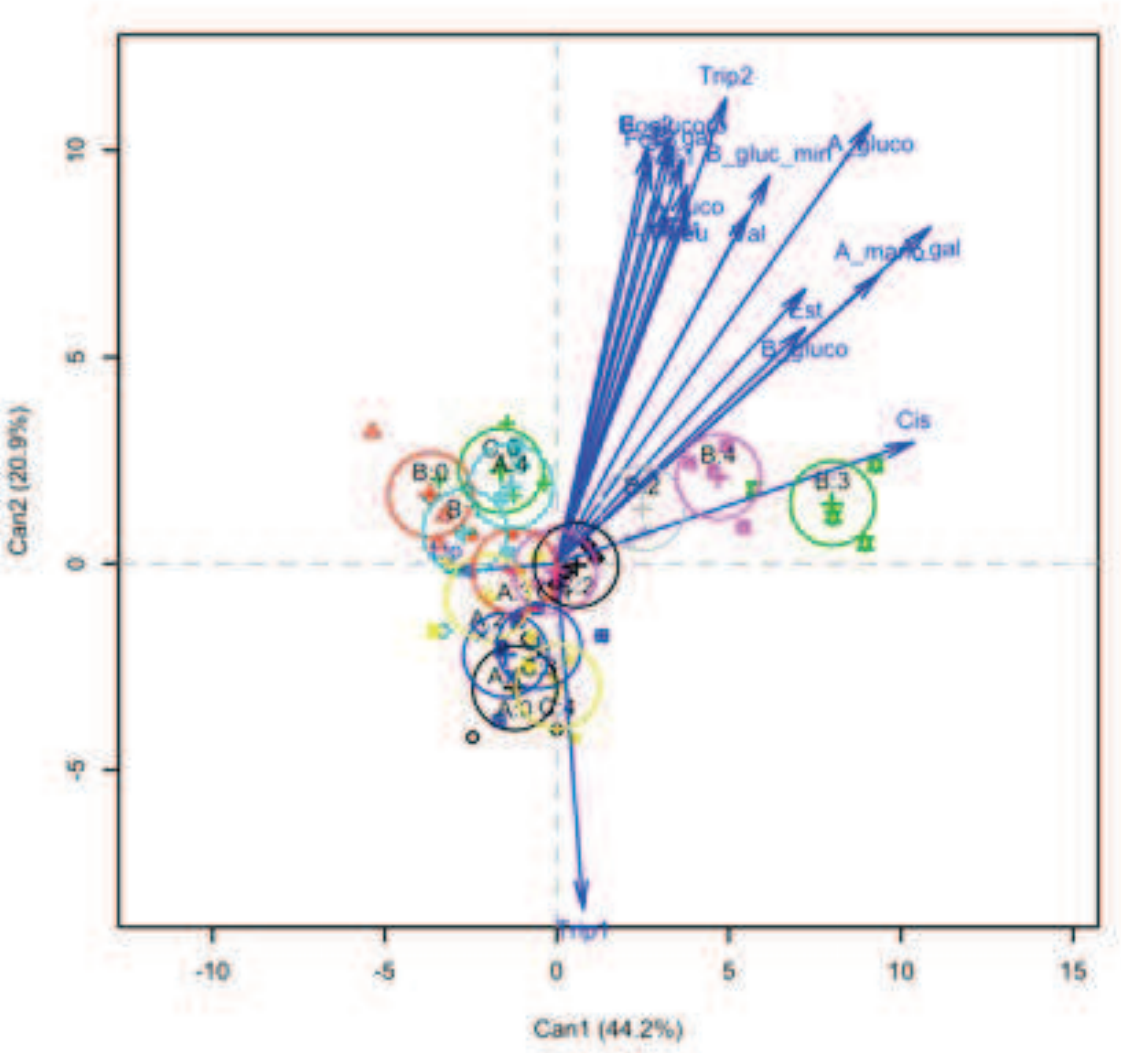

a
b
6
4
2
0
-2
-4
y
-15
-10
-5
0
5
10
x
-3
-2
-1
0
1
2
3
4
5
z
A0
A1
A2
A3
A4
B0
B1
B2
B3
B4
C0
C1
C2
C3
C4
Section A
Section B
Section C
Time:
0 - Starved
1 - 24 hours
2 - 30 days
3 - 60 days
4 - 90 days

Figure 4.


Trip2
A_gluco
A_manno.gal
Est
B_gluco
Cis
B.0
B.4
B.2
B.3
Trip1
10
5
0
-5
-10
-5
0
5
10
15
Can2 (20.9%)
Can1 (44.2%)

Supplementary material

Table S1. Principal component analysis of the enzymatic profile data grouping the 19 response variables (each hydrolytic enzyme) in worm's digestive tract by each region and stage. The first component explains 71% of variation, the second 7%, and the third one explains 6% of variation.

	Comp.1	Comp.2	Comp.3
Acid phosphatase	-0.263	-0.133	
Alkaline phosphatase	-0.263	-0.135	0.103
Phosphohydrolase	-0.263	-0.133	
Esterase	-0.231		
Lipase		0.453	0.609
Lipase esterase	-0.251		0.124
Leucine aminopeptidase	-0.249	-0.207	
Cystine aminopeptidase	-0.151		-0.586
Valine aminopeptidase	-0.248	-0.196	-0.121
Alpha-Chymotrypsin		0.303	-0.42
Trypsin	-0.233		
Alpha-Galactosidase	-0.194	0.533	
Beta-Galactosidase	-0.263		
Beta-Glucuronidase	-0.263	-0.126	
Alpha-Glucosidase	-0.229	0.211	
Beta-Glucosidase	-0.214	0.365	
N-acetil-beta-glucosaminidase	-0.262		
Alpha-Mannosidase	-0.236	0.256	
Alpha-Fucosidase	-0.261		

CAPÍTULO IV. CONCLUSIONES

Los resultados obtenidos con *E. fetida* adaptada a un clima tropical mostraron que la dinámica enzimática cambia a lo largo del tracto digestivo en el proceso de lombricompostaje. Se encontraron relaciones directas en la localización de determinadas actividades enzimáticas en la anatomía intestinal; tales como la compleja asociación de glicosilhidrolasas en la sección B para degradar la hemicelulosa y la α-quimotripsina en la sección C para digerir péptidos y proteínas de la microbiota intestinal de la lombriz.

Todas las enzimas evaluadas mostraron actividad a lo largo del proceso; aunque algunas de ellas (lipasa, cistina aminopeptidasa y α-quimotripsina) no tuvieron actividad en determinados puntos; lo que sugiere una dinámica altamente regulada.

El proceso de degradación ocurre de una manera secuencial, en donde primero se favorece el consumo los nutrientes más biodisponibles, y una vez agotados, se comienza la digestión de estructuras más complejas, como la hemicelulosa. Este proceso depende de una secuencia concertada de actividades enzimáticas que actuarían de manera sinérgica a lo largo de la anatomía intestinal de la lombriz.

El mecanismo metabólico de degradación ocurre en una consecución de eventos en el sistema digestivo de la lombriz, estrechamente relacionados. En el periodo de ayuno a 24 horas algunas actividades de las enzimas disminuyen en la sección C pero otras se incrementan, posiblemente por las condiciones fisicoquímicas en el ambiente intestinal resulta en la modificación de las poblaciones microbianas (aerobios y anaerobios) a lo largo del tracto digestivo; luego a los 30 días del proceso, las actividades de las enzimas disminuyen en la sección B probablemente por el agotamiento de los nutrientes fácilmente asimilables se estabilizan los componentes mas complejos del sustrato, esto conlleva a las adaptaciones fisiológicas de la lombriz para la asimilación de los nutrientes. Posteriormente en el periodo de 60 y 90 días se incrementan las actividades de las enzimas en las secciones A y B debido al enriquecimiento selectivo de los microorganismos para transformar el material ingerido derivado de los efectos

indirectos (la presencia de heces en el sustrato) favoreciendo la disponibilidad de nutrientes dentro del tracto digestivo de la lombriz.

Este conocimiento es de gran importancia para posibles soluciones a nivel local como el manejo agrícola, aprovechamiento de residuos orgánicos y estrategias viables a escala pequeña como el sistema de lombricompostaje.

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