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# Changes in bacterial numbers and microbial activity of pig slurry during gut transit of epigeic and anecic earthworms

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ABSTRACT

In soils, organic matter decomposition and stabilization largely occur as a result of microbial activity, although when present, earthworms are important drivers of the processes through their interactions with microflora which begin during organic matter digestion by earthworms. Here, we studied the effects of gut transit on the number of bacteria and the microbial activity in pig slurry, using three epigeic (*Eisenia fetida*, *Eisenia andrei*, *Eudrilus eugeniae*) and one anecic (*Octodrilus complanatus*) species of earthworm. Bacterial counts revealed that the effect of gut transit on microbes differed depending on the earthworm species. Thus, no changes in the number of bacteria were found in the gut contents of *E. fetida* and *E. eugeniae*, whereas large decreases were recorded in those of *O. complanatus* and *E. andrei* (2.7 and 1.3 times, respectively). We suggest that, unlike in the three epigeic earthworm species, microorganisms are preferentially utilized by *O. complanatus* to meet its nutrient requirements, because of its limited digestive capacity. Despite the decrease in bacterial numbers, there were no differences in the gut contents of the four earthworm species or undigested pig slurry in terms of dehydrogenase activity. Therefore, we suggest that after gut transit in the four earthworm species under study the potential microbial degradation of pig slurry remains unaltered.

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## 1. Introduction

Earthworms are important drivers of soil biogeochemical processes as they modify soil physicochemical properties and microbial communities by feeding, burrowing and casting activities [1]. Soil decomposition mainly occurs as a result of microbial activity, but the relationships between earthworms and microorganisms are crucial in organic matter degradation. The stimulation of microbial activity by earthworms has been related to diverse earthworm-derived processes such as alteration of soil physical structure, increase of surface attack by microorganisms through comminution of organic matter and production of mucus and excretory substances such as urea and ammonia [2], which constitute an easily assimilable pool of nutrients for microorganisms [1,3].

However, these relationships are still poorly understood, particularly the effect of gut transit on ingested microflora. In fact, stimulation of microbial activity may appear surprising, considering that microorganisms are an important part of earthworms' diets, with the major source of nutrients being fungi and protozoa followed by bacteria [4]. It has previously been shown that earthworms can digest fungi and bacteria [5,6] and that earthworm can selectively feed on particular species of fungi [7]. Although Pedersen and Hendriksen [8] found either no changes or reductions in the number of bacteria during gut transit, it has been shown that bacterial counts generally increase during gut transit [4,9,10,11].

The main difficulty in interpreting existing data regarding digestion of microorganisms by earthworms is the large variety of earthworm species and food substrates used. The composition of microflora in the earthworm gut varies depending on the species of earthworm studied, season and feeding regime of the earthworm [9]. Schönhlzer et al. [6] reported that the number of microorganisms present in the gut of *Lumbricus terrestris* depended on the substrate that the earthworm fed on; these authors found higher numbers of bacteria in earthworms fed on soil, and either no changes or higher numbers in earthworms fed on decomposed leaves, than in earthworms fed on inert substrate.

The main objective of the present study was to assess how and to what extent earthworms modify the microorganisms of substrates that they ingest. Furthermore, we tested whether there is a relationship between the different feeding and soil habits of two ecological categories of earthworms, anecic and epigeic [12] that differ in their digestive enzymatic capabilities [3] and the effect on microorganisms during digestion. For this, we performed a direct count of bacteria as a measure of microbial biomass and an analysis

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**Table 1**
Physicochemical characteristics of the fresh pig slurry used

| | |
|---|---|
| Moisture content (%) | 86 ± 10 |
| Organic matter content (%) | 86 ± 10 |
| pH | 8.3 ± 1.0 |
| Electrical conductivity (mS cm$^{-2}$) | 0.25 ± 0.01 |
| Total nitrogen (mg g$^{-1}$ dw[a]) | 24 ± 2 |
| N-$NH_4^+$ (μg g$^{-1}$ dw) | 2400 ± 100 |
| N-$NO_3^-$ (μg g$^{-1}$ dw) | 250 ± 50 |
| Total carbon (mg g$^{-1}$ dw) | 455 ± 60 |
| Dissolved organic carbon (mg g$^{-1}$ dw) | 11.1 ± 0.1 |

[a] dw: dry weight.

of dehydrogenase activity as a measure of microbial activity [13,14] in the gut contents of four earthworm species corresponding to two ecological groups or classes: the epigeic *Eudrilus eugeniae* (Kinberg, 1867), *Eisenia fetida* (Savigny, 1826), *Eisenia andrei* (Bouché, 1972) and the anecic *Octodrilus complanatus* (Dugès, 1828), which were fed with the same substrate, pig slurry, a microbial-rich substrate, with a microflora mainly composed of bacteria [15,16].

## 2. Material and methods

### 2.1. Animal manure and earthworm species

Fresh pig slurry was obtained from a pig-breeding farm near the University of Vigo, NW Spain. Pig slurry was homogenized in a slurry pit, then stored in sealed plastic containers and kept at 5 °C until use. We used the solid fraction (20% dry weight (dw)) of pig slurry in order to avoid the percolates that could be harmful to earthworms. The main physicochemical characteristics of the pig slurry are given in Table 1.

Specimens of *O. complanatus*, *E. fetida* and *E. andrei* were sampled (hand-sorted method) respectively from a corn field, a manure heap and a pig breeding farm near the University of Vigo. Specimens of *E. eugeniae* were obtained from a commercial supplier in Brazil (Minhobox). Earthworms of all four species were cultivated in the laboratory using pig slurry as breeding medium for 2 months before the start of experiments.

### 2.2. Experimental design

Each mesocosms of *E. fetida* and *E. andrei* were inoculated with 50 mature earthworms, whereas those mesocosms corresponding to *O. complanatus* and *E. eugeniae* were inoculated with 20 mature earthworms. For each of four earthworm species, we set up four mesocosms consisting of plastic containers (3 L), which were filled to 3/4 of their capacity with sieved (<2 mm) and moistened (80% moisture content) vermiculite. We chose vermiculite because it is chemically inert and does not contain any nutrients, thus obliging the earthworms to ingest the pig slurry provided. We placed a plastic mesh (5 mm pore size) over the surface of vermiculite and placed the pig slurry (ca. 200 g, fresh weight) on the mesh, to avoid mixing of the pig slurry and vermiculite and to facilitate the removal of consumed pig slurry. Mesocosms were checked twice weekly in order to replace used pig slurry and to wash the vermiculite to avoid the ingestion of casts by earthworms. The mesocosms were maintained at a constant temperature (20 °C) in a scientific incubator.

After 1 week on mesocosms, mature earthworms of similar weight from each mesocosm were taken and washed three times with sterile double-distilled water and the gut contents then released, not more than 5 mm of length, by gently pressing the bodies of intact worms from the last third to the posterior end. By doing this, we assumed that the gut content corresponding to the final section of the intestine (hindgut) was obtained [17].

Gut contents from earthworms belonging to the same species and mesocosms were pooled to obtain samples that weighed approximately 0.5 g (fresh weight). In this way, 5 earthworms of the *O. complanatus* and *E. eugeniae* species and 15 earthworms of the *E. fetida* and *E. andrei* species were needed to achieve this amount of gut content. Samples were divided into two fractions of 0.25 g each, for bacterial counts and dehydrogenase assay.

### 2.3. Analytical methods

Samples (0.25 g fresh weight) were suspended in 9 mL of filter-sterilized water (0.2 μm pore size) and fixed by adding 1 mL of filter-sterilized formaldehyde solution (37%). Ten microliters of sample suspension were then smeared evenly on a limited area (113 mm$^2$) of a glass slide and air-dried for 30 min. The spots of dried sample were then flooded with the stain solution (DTAF, 2 mg in 10 mL of a buffer solution, 7.8 g L$^{-1}$ $Na_2HPO_4$ and 8.5 g L$^{-1}$ of NaCl). The slides were then rinsed three times (20 min each) with the buffer and finally with filter-sterilized water for a few seconds. After air drying, cover slips were mounted on the slides with a drop of immersion oil and sealed with nail varnish. Counts were performed in epifluorescence microscope (Nikon Eclipse 80i) equipped with a filter set for blue light (BP 450–490 nm exciter filter, 510 nm beam splitter and LP 520 nm barrier filter) at 1000× magnification; we counted bacteria in 10 fields of view (randomly smeared) per slide [18]. We discarded the use of membrane filters because they produced a higher fluorescence background than smears in our samples complicating the analyses.

Samples (0.25 g fresh weight) were placed in Eppendorf tubes (1.5 mL) and mixed with 0.25 mL of 1.5% 2,3,5-triphenyltetrazolium chloride (TTC). The vials were closed and incubated for 24 h at 30 °C before adding 2 mL of acetone to each. The vials were shaken vigorously and the suspension was then filtered and measured in a Bio-Rad Microplate Reader 550 at 546 nm [19].

All results reported are the mean of four replicates. Data were analyzed by one-way ANOVA and post hoc comparisons were performed by a Tukey HSD test. All statistical analyses were performed using SPSS 11.5 software.

## 3. Results

The number of bacteria in pig slurry changed significantly during gut transit of earthworms (Fig. 1; ANOVA, $F_{4,15} = 7.67$; $P < 0.01$). The effects depended on earthworm species since the largest reduc-

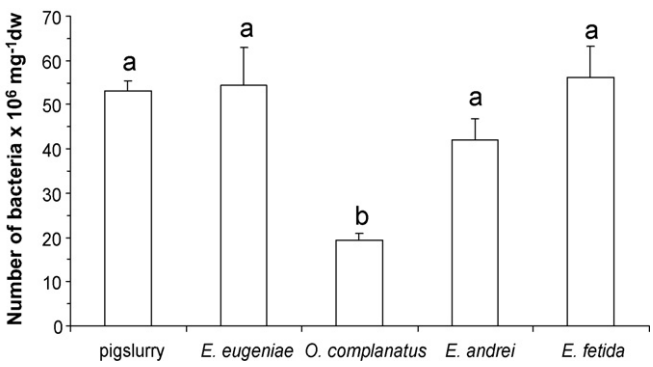


**Fig. 1.** Number of bacteria (mean ± S.E.) in fresh pig slurry and gut contents of the earthworms *Eudrilus eugeniae*, *Octodrilus complanatus*, *Eisenia andrei* and *Eisenia fetida* ($n = 4$). Different letters indicate significantly different means ($P < 0.05$, Tukey HSD test).

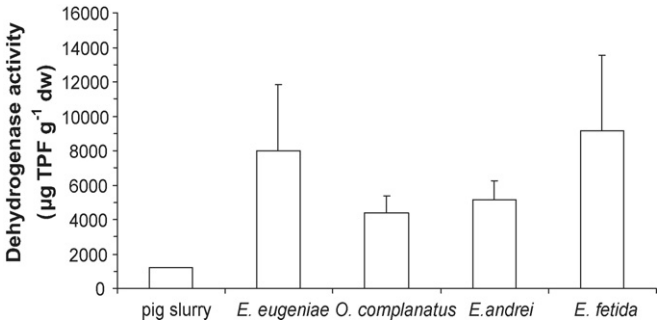


**Fig. 2.** Dehydrogenase activity (mean ± S.E.) in fresh pig slurry and the gut contents of the earthworms *E. eugeniae*, *O. complanatus*, *E. andrei* and *E. fetida* ($n = 4$).

tion in bacterial numbers (up to 2.7-fold decrease) was recorded in the gut content of *O. complanatus*; bacterial numbers in the gut content of three epigeic earthworm species did not differ from those in pig slurry (Fig. 1).

There were neither significant effects of gut transit on dehydrogenase activity (ANOVA, $F_{4,15} = 1.36$; $P = 0.29$) nor significant differences between the pig slurry and the gut contents (Fig. 2). The effect of gut transit on microbial activity was separated into two groups in terms of the dehydrogenase activity, the first included the gut contents of *E. eugeniae* and *E. fetida*, with enzymatic activities above 8000 μg TPF $g^{-1}$ dw and the second included the gut contents of *O. complanatus* and *E. andrei*, with enzymatic activities below 6000 μg TPF $g^{-1}$ dw, which was still higher than in pig slurry (Fig. 2).

## 4. Discussion

The present results clearly show that gut transit had a clear effect on ingested microorganisms of pig slurry, which differed depending on both the species and ecological classification of the earthworms. We focused on microbial counts of bacteria, despite of importance of that fungi and protozoa seems to have on diet of earthworms due to pig slurry is an enriched bacterial substrate [16,20] and fungi are mainly found as spores [15]. Although we found a significant effect of gut transit on bacterial counts it appears to be a result of the high reduction observed in the gut content of *O. complanatus* since there were no differences in bacterial counts between gut contents of three epigeic earthworms. Thus, bacterial counts in gut contents of *E. fetida* and *E. eugeniae* did not differ from those in fresh pig slurry. In contrast, we found that there was a significant reduction in bacterial counts during gut transit of the anecic *O. complanatus* and the epigeic *E. andrei*, which had no differences with those of *E. fetida* and *E. eugeniae*. However, the effects of gut transit in *O. complanatus* on microorganisms differed from those found by Schönholzer et al. [6], who reported an increase in the number of bacteria (between one and seven times higher) in the gut of *L. terrestris*, another anecic earthworm, than in uningested soils. By using the same food for the four earthworm species, we avoided masking effects in their response to ingested microflora due to differences in the nutrient quality or substrate availability [21], hence differences observed in bacterial counts between pig slurry and gut contents can be attributed to particular digestive processes of earthworms studied (i.e. food residence and passage time).

Unlike microorganisms, soil organisms are not able to assimilate mineral nutrients and in order to meet their nitrogen requirements, detritivores such as earthworms must feed on microorganisms that colonize organic matter [22,23]. This partly explains the observed trend of reduced microbial numbers, because nitrogen in pig slurry was mainly present as ammonia, thus limiting its availability to earthworms (Table 1). The reductions observed in the gut content of *E. andrei* were expected because this species has diverse and powerful enzymatic capabilities, which allow it to digest microorganisms [3]; in fact the earthworm *L. rubellus*, another epigeic earthworm, was able to digest microorganisms even when they were protected by polysaccharides and clay particles [24]. Furthermore, epigeic species may have access to a huge diversity of C and N organic sources, unlike *O. complanatus*, which seemed to be forced to digest microorganisms; however, this hypothesis cannot explain the absence of differences in the number of bacteria in the three epigeic species, two of which – *E. fetida* and *E. andrei* – are closely related. In fact, our sampling procedure, restricted to hind gut section of intestine, cannot allow us to know if in these three species could there be digestion of bacteria in the former parts of digestive apparatus (gizzard and crop) that was compensated by microbial growth during gut transit. In pig slurry, levels of dissolved organic carbon – a limiting factor for earthworm growth [25] – were sufficient for epigeic earthworms to be able to use it as an energy source, but *O. complanatus* seemed not to be capable to exploit this C source since in anecic earthworms the enzymatic apparatus is less complex [3], forcing this earthworm species to consume microorganisms. Schönholzer et al. [6] showed that mechanical breakdown of cells is produced in the crop and gizzard; these structures are more developed and muscular in anecic than in epigeic earthworms because of their lifestyle (burrowing, feeding on leaves), reinforcing the role of these two structures on digestion [3]. In addition, Schönholzer et al. [6] found that there was a reduction in bacterial size, mainly due to the break of large bacteria. Although we did not measured the change in bacterial size, this could affect the outcome of results if we have used a substrate with different microbial populations than those of pig slurry. We would therefore expect the large reduction in bacterial counts observed in the gut content of *O. complanatus*. These results indicate that microorganisms in pig slurry were preferentially used by *O. complanatus* as a nutrient pool.

Dehydrogenase assays measure intracellular catalysis and are more likely to be correlated with the activity of extant cells and it is present in all microorganisms [26]. Therefore, the assay of this enzyme is considered to be an accurate measure of the microbial oxidative activity [27]. Moreover, it has been shown that dehydrogenase assays can be used to estimate microbial activity of a huge range of anaerobic microorganisms as it was reported by Bhupathiraju et al. [28], and at the present, it was widely used to estimate the microbial activity in many presumable anaerobic environments as pressmud residues, sewage sludge, pig slurry and lignocellulosic olive wastes [29–32]. We found that dehydrogenase activity was higher in the gut content of four earthworm species than in uningested pig slurry, although it was not statistically different. This suggests that the reduction in bacterial counts maybe was not reflected by the microbial activity. This result suggests that in the case of *O. complanatus* despite of diminished bacterial counts the remaining microbes are still able to maintain a high metabolic activity.

We conclude that digestion of microorganisms differed depending on earthworm species, but the absence of any effect on microbial activity suggests that potential microbial decomposition of pig slurry remains unaltered after gut transit in the four earthworm species studied. It has additional implications in the case of the three epigeic earthworms analyzed (*E. andrei, E. fetida* and *E. eugeniae*) which are widely used in vermicomposting facilities.

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