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Selective reduction of the pathogenic load of cow manure in an industrial-scale continuous-feeding vermireactor

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ABSTRACT

Vermicomposting is a suitable technology for processing different wastes, to produce a valuable end product (vermicompost). However, the pathogenic load of the waste must be greatly reduced in order to prevent risks to human health. Although *Eisenia andrei* may reduce the levels of several pathogens, the feasibility of vermicomposting, with regard to pathogen reduction, has not been tested on an industrial scale. This work studied whether vermicomposting in a continuous feeding vermireactor, is able to reduce the pathogenic load of cow manure. The effect of *E. andrei* on pathogens depended on the type of pathogen; thus, levels of *Clostridium*, total coliforms and *Enterobacteria* were not modified, but levels of faecal enterococci, faecal coliforms and *Escherichia coli* were reduced to acceptable levels. Pathogens could have maintained their levels in continuous feeding vermireactors, as fresh layers of manure are added to the top, which allows the vertical spread of pathogens through leaching.

1. Introduction

The increasing rate at which organic residues are generated has become a problem that requires strategies for disposal and/or management. Vermicomposting, i.e. the processing of organic residues by earthworms, has proved to be a suitable technique because of its low cost and the large amounts of organic wastes that can be processed. It has been shown that sewage sludge, brewery waste, paper waste, urban residues and animal waste may be managed by vermicomposting to produce vermicomposts of different quality (reviewed in Domínguez, 2004).

One of the main problems associated to the vermicomposting process is the presence of human pathogens, the levels of which will restrict use of vermicompost as an organic fertilizer or its disposal by landfill. Unlike composting, vermicomposting is a mesophilic process (<35 °C), so that substrates do not undergo thermal stabilization that eliminate pathogens. Nevertheless, it is known that vermicomposting may reduce the levels of different pathogens such as *Escherichia coli*, *Salmonella enteriditis*, total and faecal coliforms, helminth ova and human viruses in different types of waste (Edwards, 2011; Monroy et al., 2009). However, the reduction of the pathogenic load largely depends on the earthworm species and/or the pathogen considered. Thus, Monroy et al. (2008) found that the reduction in total coliform numbers differed among four vermicomposting earthworm species (*Eisenia fetida*, *Eisenia andrei*, *Lumbricus rubellus* and *Eudrilus eugeniae*); Parthasarathi et al. (2007) found that earthworms did not reduce the numbers of *Klebsiella penumoniae* and *Morganella morganii*, whereas other pathogens such as *Enterobacter aerogenes* and *Enterobacter cloacae* were completely eliminated. It has recently been shown that vermicomposting reduced total coliform numbers after 2 weeks of processing. Moreover, the reduction was mainly due to earthworm digestive processes, which eliminated up to 98% of total coliforms, probably due to a combination of own earthworm digestive abilities, which include fine grinding of cells and several enzymes related to the degradation of bacterial cell wall (Monroy et al., 2008, 2009; Edwards, 2011). However, despite the pioneering studies of Riggle (1996) and Eastman et al. (2001), little is known about these processes in industrial-scale systems, that is, vermicomposting systems designed to deal with large amounts of wastes. Specifically, we want to test whether the results of laboratory approaches (scale and time to process the waste by earthworms) may be applied to large scale systems. Thus, we questioned whether *E. andrei*, the earthworm species most commonly used in vermicomposting facilities located in temperate regions due to its high rate of reproduction and processing rates (Domínguez, 2004), may reduce the pathogenic load of wastes in industrial-scale reactors. For this purpose, samples from a continuous feeding vermireactor, in which cow manure was applied in a similar way as in laboratory vermireactors (Aira et al., 2007), were analyzed to determine the earthworm numbers and biomass, microbial biomass, and the numbers of several pathogens, namely *E. coli*, *Clostridium*, Enterobacteria, faecal

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enterococci, total and faecal coliforms. We choose these human pathogens because we did not find *Salmonella* spp. in the initial cow manure.

2. Methods

2.1. Cow manure

Fresh cow manure was obtained from a farm near the University of Vigo, NW Spain. The manure was homogenized, stored in sealed plastic containers and maintained at 5 °C until use.

2.2. Vermireactor set-up and functioning, and sampling method

The system used was a rectangular metal pilot-scale ($4 \times 1.5 \times 1$ m) vermireactor housed in a greenhouse without any temperature control. The reactor was watered daily with an automatic watering system, to prevent desiccation. The vermireactor was established with a 10 cm layer of vermicompost (a stabilized non-toxic substrate that serves as a bed for earthworms), on which earthworms were placed, and a 5 cm layer containing 50 kg (fresh weight) of cow manure, which was placed over a plastic mesh (5 cm mesh size) to avoid sampling the bed. The initial earthworm (*E. andrei*) density was 75,000 individuals m^3. New layers containing the same amount of cow manure were added to the vermireactor as required, that was, approximately every 3 weeks (as determined by changes in the appearance of the cow manure, with the coarse fraction, such as straw, becoming more evident; Aira and Domínguez, 2008).

New layers of manure are continually processed in the vermireactor, so that at sampling the vermireactor consisted of a 25 cm layer of substrate (15 cm of manure and 10 cm of vermicompost added as a bed), with a gradient of fresh-to-processed manure from the top to the bottom. The vermireactor was divided into 12 quadrants (0.66×0.76 m), and two samples were taken at random from each quadrant with a cylindrical corer (8 cm diameter). Each core sample was divided into upper, intermediate and lower layers (each 4 cm depth) and after recovering the earthworms (to estimate the total number of earthworms and the earthworm biomass per m^3), the samples from the two corers and same layer were gently mixed for chemical and microbiological analyses.

2.3. Analytical procedures

Microbial activity was assessed by measuring the rate of CO_2 evolution from the sample during 6 h of incubation. The evolved CO_2 was trapped in 0.02 M NaOH and subsequently measured by titration with HCl to a phenolphthalein endpoint, after adding excess $BaCl_2$ (Anderson, 1982).

Microbial community structure was assessed by phospholipid fatty acid (PLFA) analysis. Briefly, the total lipidic extract was obtained from 200 mg of each freeze-dried sample with 60 mL of chloroform–methanol (2:1, v/v), following the method described by Gómez-Brandón et al. (2010) for highly organic samples. The lipid extract was then fractionated into neutral lipids, glycolipids and phospholipids with chloroform (5 mL), acetone (10 mL) and methanol (5 mL), respectively, and the fraction containing phospholipids was subjected to alkaline methanolysis to obtain the fatty acid methyl esters (FAMEs), which were analysed by gas chromatography-mass spectrometry (GC-MS). The detailed GC-MS experimental conditions are described elsewhere (Gómez-Brandón et al., 2010). The sum of all identified PLFAs (total PLFAs) was used to estimate the viable microbial biomass (Zelles, 1999). Certain PLFAs were used as biomarkers to determine the presence and abundance of specific microbial groups (Joergensen and Wichern, 2008). The sum of PLFAs characteristic of Gram-positive (i14:0, i15:0, a15:0, i16:0 and a17:0), and Gram-negative bacteria (16:1 ω 7c, 17:1 ω 7c, 18:1 ω 7c, cy17:0 and cy19:0) was chosen to represent bacterial PLFAs, and the PLFA 18:2ω6c was used as a fungal biomarker.

Microbial pathogens were determined according to standard protocols. Briefly, *E. coli* was determined by incubating the samples in Trypton Bile X-lucuronide and 5-bromo-4-clore-3-indol-β-D-glucoronate at 37 °C for 3 h, then at 44 °C for 18–24 h (EPA 821-R-97-004). Enterobacteria were determined after incubation in buffered peptone water, then transferred and incubated in plates with violet red bile agar at 37 °C for 24–48 h. Positive samples were confirmed by the glucose fermentation test (ISO 7402, 1993). Faecal enterococci were determined after incubation with Slanetz Bartley at 37 °C for 48 h (ISO 7899-2, 2001). Faecal and

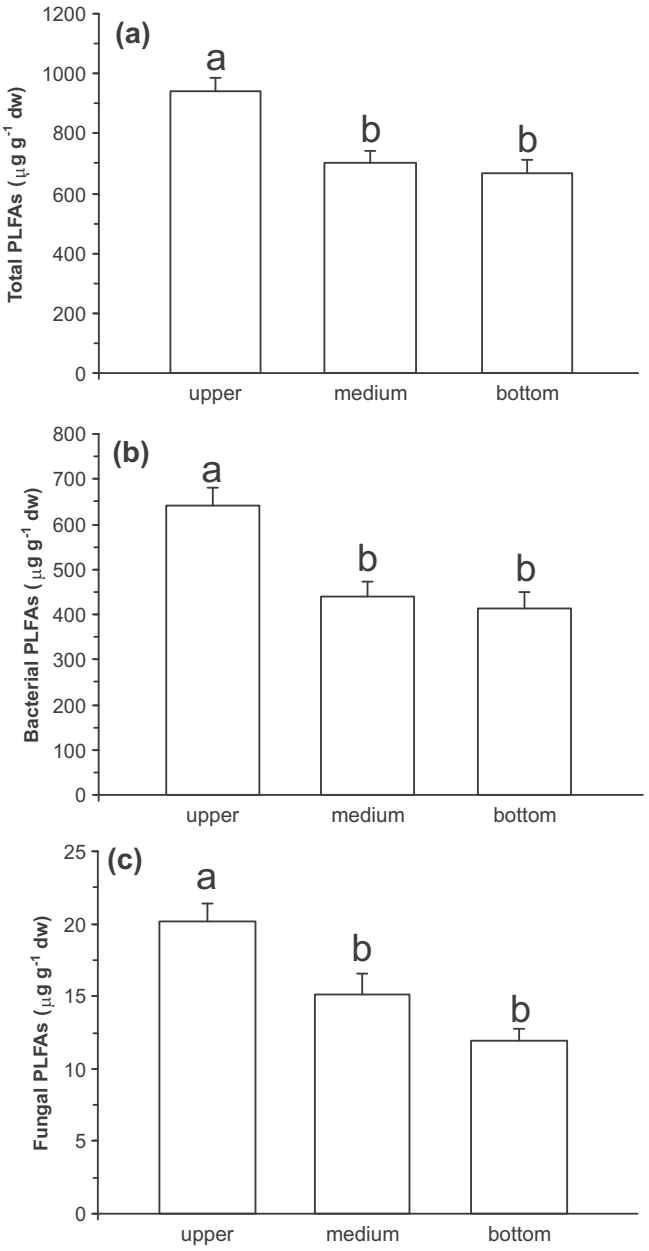

Fig. 1. Changes in: (a) total, (b) bacterial and (c) fungal PLFAs during the vermicomposting process. Upper, intermediate and lower layers representing different stages of the vermicomposting process. Different letters indicate significant differences based on post hoc test (Tukey HSD). Mean ± S.E. (n = 12).

total coliforms were determined respectively after incubation at 44 °C and 37 °C for 24 h in violet read bile agar (ISO 4832, 1991). Vegetative and sporulated forms of *Clostridium* were determined after incubating samples in sulphite polymyxin sulfadiazine agar at 37 °C for 24–48 h (Adams and Moss, 2000). All samples were analyzed per triplicate.

2.4. Statistical analysis

Data were analyzed with mixed models (function lme in the nlme library, Pinheiro et al., 2011) with depth of sampling (i.e. how processed the substrate was) as a fixed factor, and the effect of depth nested in each corer as a random factor to account for non independence of samples within the corer (Crawley, 2007). Post-hoc comparisons (Tukey HSD) between depths were performed with the glht function in the multcomp library (Hothorn et al., 2008). In order to analyze the underlying effect of the experimental factors on the microbial community structure, data from the PLFA analysis were subjected to discriminant analysis with the discriminant function in the ade4 library (Dray and Dufour, 2007). All analyses were performed with R 2.11.1 (R Development Core Team, 2010).

3. Results and discussion

At sampling earthworm numbers ($F_{2,22} = 3.21$, $P = 0.06$) and biomass ($F_{2,22} = 3.1$, $P = 0.07$) only differed between the upper (174,000 ± 40,000 earthworms m^{-3} and 7750 ± 2250 g m^{-3}) and lower layers (374,000 ± 58,000 earthworms m^{-3} and 20,000 ± 3870 g m^{-3}, Tukey HSD test $P = 0.03$ for both earthworm numbers and biomass). There were not any differences in organic matter content ($F_{2,22} = 1$, $P = 0.25$), or in pH ($F_{2,22} = 0$, $P = 0.81$), although the salt content (electric conductivity) decreased significantly ($F_{2,22} = 36.12$, $P < 0.0001$) with the level of processed manure from upper (0.41 ± 0. 03 mS cm^{-2}) to intermediate and lower layers (0.31 ± 0.03 and 0.35 ± 0.03 mS cm^{-2}, respectively). The values were higher than those in fresh cow manure (0.16 ± 0.03 mS cm^{-2}), indicating an increased level of mineralization in the processed substrate. Vermicomposting in the vermireactor followed the typical pattern of this process with slight changes in pH and a decrease in organic matter content (Aira and Domínguez, 2008; Aira et al., 2007; Domínguez, 2004).

Overall, microbial biomass decreased significantly with depth of layer ($F_{2,22} = 35.7$, $P < 0.0001$), i.e. from upper to intermediate and lower layers (1.4 decrease; Fig. 1a), although it was higher than in the fresh cow manure (580 ± 20 mg g^{-1} dw) in the three layers. The same pattern was observed for bacterial ($F_{2,22} = 24.73$, $P < 0.0001$; Fig. 1b) and fungal biomass ($F_{2,22} = 17.92$, $P < 0.0001$; Fig. 1c), and the dry weight contents of these were in the fresh cow manure 380 ± 40 and 12 ± 0.5 mg g^{-1}, respectively. Moreover, earthworms promoted a specific microbial community structure in each layer, as shown by the discriminant analysis, which accounted for 100% variance in the data (Fig. 2). Thus, differences between the upper and the other two layers were due to marked decreases in several PLFAs like 15:0, *i*15:0, 16:1*c* and *cy*17:0, whereas differences between medium and bottom layers were due to marked decreases of PLFAs *i*14:0, *a*15:0 and 18:3ω3 (Fig. 2). Despite the differences in microbial biomass and community structure, the microbial respiration was maintained throughout the three layers ($F_{2,22} = 0.82$, $P = 0.46$), at rates clearly lower (mean respiration in the three layers 1.7 ± 0.4 mg CO_2 g^{-1} dw) than in the fresh manure (3.2 ± 0.3 mg CO_2 g^{-1} dw), indicating that microbial stabilization of manure had started. Microbial properties were consistent with generally observed patterns, with the biomass of both bacteria and fungi decreasing, as previously described, although not at levels of a microbially stabilized vermicompost (Gómez-Brandón et al., 2011). On the other hand, microbial activity was maintained throughout all the samples, despite the decreased biomass, as previously reported for the initial stages of vermicomposting (Gómez-Brandón et al., 2011), which indicates the enhancing role of earthworms for microorganisms during

PLFA	Root 1	Root 2
10:00	-0.11	0.01
12:00	-0.01	-0.44
13:00	-0.44	-0.82
*i*14:0	-0.14	2.82
14:00	-0.11	1.21
14:01	0.23	-0.35
*i*15:0	0.63	-0.64
*a*15:0	-0.05	1.96
15:00	0.79	-1.28
*i*16:0	0.26	0.85
15:01	0.16	0.34
16:00	-0.05	-0.70
16:1*c*	0.49	-2.29
*a*17:0	0.39	-0.94
17:00	0.40	-0.71
*cy*17:0	0.53	-0.17
17:1*c*	-0.24	-0.74
18:00	-0.09	0.57
18:1ω9*t*	-0.15	1.24
18:1ω9*c*	0.28	-1.42
18:1ω7*c*	-0.12	1.82
18:2ω6*t*	-0.25	0.19
18:2ω6*c*	0.51	-1.96
*cy*19:0	-0.43	0.08
18:3ω6	-0.04	-0.74
18:3ω3	-0.28	0.84
20:0	0.03	1.49

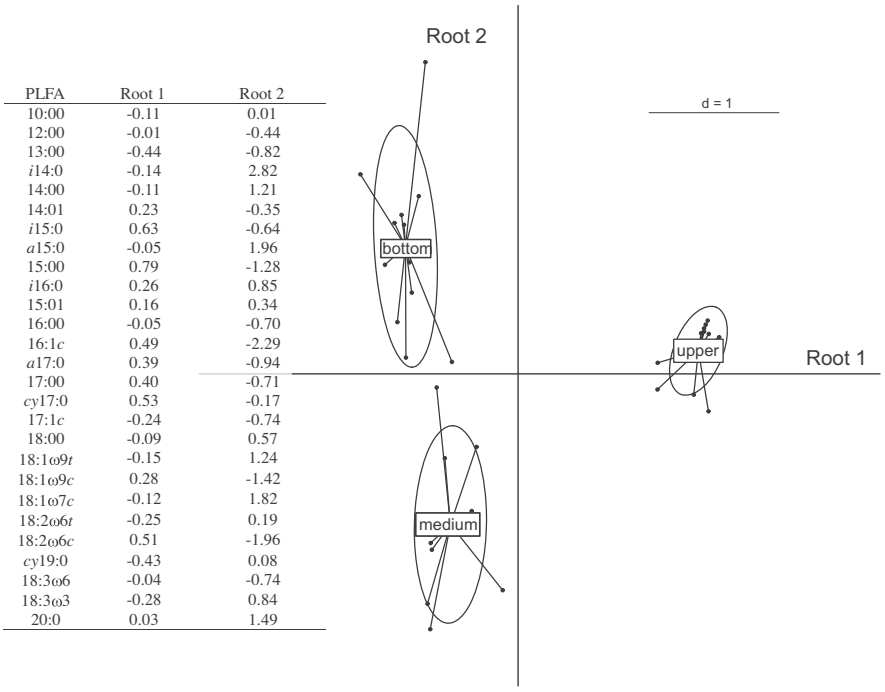

Fig. 2. Discriminant function analysis of 27 PLFAs identified from vermireactor samples, as affected by earthworms. Upper, intermediate and lower layers represent different stages of the vermicomposting process, and are labelled within their 95% inertia ellipses. The 12 points per group represent the replicates of each stage of the vermicomposting process. The table gives the values of canonical correlations of each PLFA used in the analysis with the two roots. Root 1 accounted for 56% and root 2 for 44% of the total variance.

vermicomposting (Domínguez et al., 2010). In fact, earthworm numbers and biomass were highest in the samples with the lowest contents of bacterial and fungal biomass. This enhancing process consist in comminution of organic matter after earthworm gut transit, which increases the surface available in substrate for microorganisms, and the release of earthworm mucus, an easily degradable substrate that promote microbial activity (Domínguez, 2004). Differences in microbial community structure also followed the typical pattern observed in vermicomposting, with marked changes due to decreases in different types of bacteria (Gram positive and negative), as previously reported (Lores et al., 2006; Gómez-Brandón et al., 2011).

Earthworms promoted a significant decrease in three of the six pathogens analyzed. Thus, faecal enterococci decreased significantly from upper layers (which showed levels similar to fresh manure: $8.4 \pm 1.3 \times 10^3$ CFU g^{-1} dw) to intermediate and lower layers ($F_{2,22} = 24.73$, $P < 0.0001$; Fig. 3a). The same pattern was observed for faecal coliforms ($F_{2,22} = 5.53$, $P = 0.011$; Fig. 3b) and

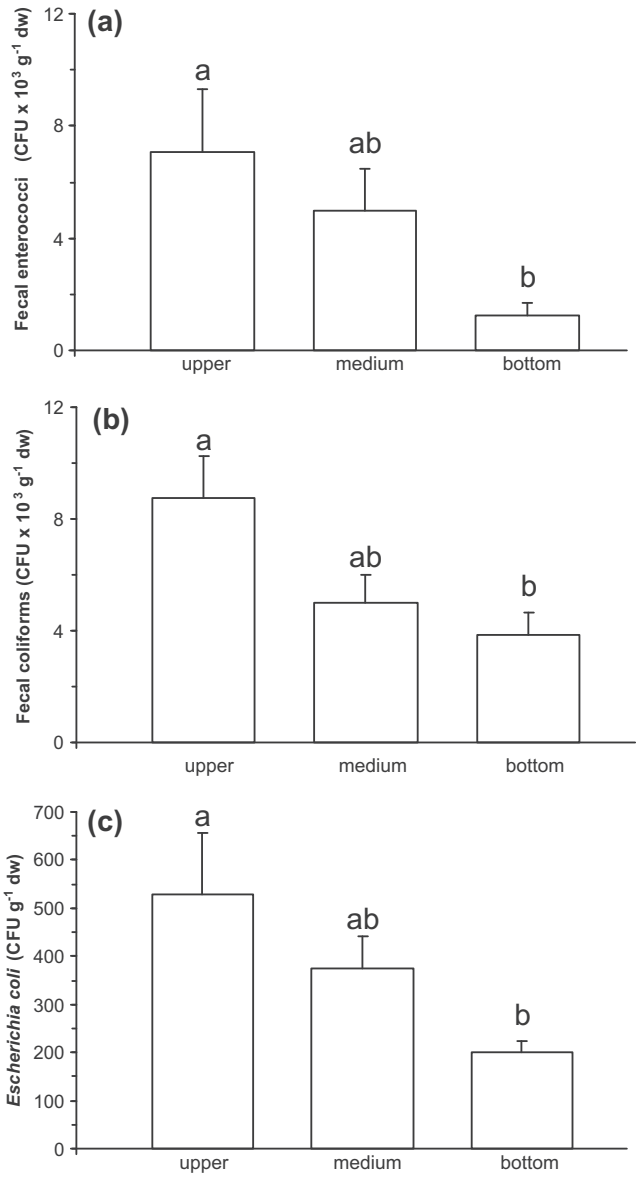

Fig. 3. Changes in numbers of (a) faecal enterococci, (b) faecal coliforms and (c) *Escherichia coli* during the vermicomposting process. Upper, intermediate and lower layers represent different stages of the vermicomposting process. Different letters indicate significant differences based on post hoc test (Tukey HSD). Mean ± S.E. ($n = 12$).

E. coli levels ($F_{2,22} = 3.73$, $P = 0.042$; Fig. 3c), with initial levels in fresh manure of $11.1 \pm 4.2 \times 10^3$ and $3.8 \pm 1.8 \times 10^3$ CFU g^{-1} dw for each pathogen respectively. Vermicomposting did not reduce the numbers of *Clostridium* ($F_{2,22} = 0.81$, $P = 0.47$), total coliforms ($F_{2,22} = 0.72$, $P = 0.50$) or *Enterobacteria* ($F_{2,22} = 2.21$, $P = 0.20$). Mean levels of the three pathogens across the three layers were 1.6 ± 0.1, 13.5 ± 2.2 and $23.1 \pm 4.3 \times 10^3$ CFU g^{-1} dw for *Clostridium*, total coliforms and *Enterobacteria*. The reduction in faecal coliforms was similar to those reported by Rodríguez-Canché et al. (2010) and Contreras-Ramos et al. (2005) working at a laboratory scale and with pre-composted sludges and different mixtures of biosolids, respectively, and using the earthworm species *E. fetida*. In fact, Contreras-Ramos et al. (2005) reported a complete elimination of faecal coliforms in some cases, whereas total coliform numbers did not change at all. Moreover, Hait and Tare (2011) found significant reductions in these pathogens reductions that did not occurred in the previous composting stage. This differential effect on pathogens indicates that earthworms not only modified the abundance of pathogens but also altered their specific composition. One possible explanation to this effect may rely on composition of cell wall of bacteria. In fact, G− bacteria possess an outer membrane composed of lipopolysaccharides, which provides them with structural integrity increasing the negative charge of the cellular membrane and protecting them against certain types of chemical attack (Vermüe et al., 1993). Previous studies involving the effects of epigeic earthworms on microorganisms have also shown that G− bacteria can survive transit through the earthworm gut (Hendriksen, 1995; Daane et al., 1997; Williams et al., 2006). The problem here is that we found reductions as expected in faecal enterococci but not in *Clostridium*, both G+ bacteria; furthermore, none of G− should be reduced due to earthworm activity but we found reductions in *E. coli* and faecal coliforms. Since none of the samples can be classified as vermicompost, as regards the organic matter content and microbial activity levels, the pathogens should not recover and their numbers should decrease even more as the vermicomposting proceeds (Monroy et al., 2009). It is difficult to ascertain what will happen with the unaffected pathogens. Thus, since a large part of the effect that earthworms exert on microorganisms occurs during gut transit, due to mechanical grinding and/or to enzymatic attack (Brown et al., 2000), we should not expect any drop in the unaffected pathogens because the high earthworm:manure ratio should ensure that most of the manure has already passed through the earthworms gut. Earthworms may indirectly lower the numbers of pathogens through changes in physical conditions, i.e. by promoting aerobic conditions during vermicomposting through casting and burrowing (Brown and Doube, 2004). This process will occur in addition to the reduction in numbers of pathogens brought about by earthworm digestion, thus hindering the recovery of pathogen numbers. In fact, aeration is one of the typical treatments to remove coliforms (total and faecal) from waste waters (Kazmi et al., 2008; Zhang and Farahbakhsh, 2007). However, the functioning of vertical continuous feeding vermireactors leads to the gradual accumulation of layers and compaction of the substrate, thus minimizing earthworm-induced aeration, so that this mechanism can be discounted as a means of diminishing pathogen levels. In fact, this process may even promote physical conditions that favour pathogens, possibly explaining the fact that some pathogens were not affected. In addition, the natural decay of pathogens in the process on an industrial scale cannot be discounted, as previously reported (Riggle, 1996; Eastman et al., 2001). Moreover, pathogens may benefit as a result of the overall decrease in bacterial and fungal biomass, which diminishes possible competition for resources. Another important factor controlling pathogen reduction in vermicomposting is the rate of application of the manure (Monroy et al., 2009). Thus, with low rates of application (20 kg m^{-2}) there were significant de-

creases in total coliforms, whereas with high rates (40 kg m^{-2}) this did not occur. In this experiment, however, rates of application were even lower than reported (c.a. 9 kg m^{-2}), so that the effect of earthworms may have been more pronounced.

Finally, pathogen reduction was sufficient to fulfil the EPA requirements, at least for *E. coli* contents, since the number of faecal coliforms, despite it decreased with vermicomposting, was still above the EPA limit of 10^3 CFU g^{-1} dw. However, the optimal reductions reported in previous studies (Riggle, 1996; Eastman et al., 2001) were not achieved, possibly because of differences in the vermicomposting system and/or experimental conditions. Although the latter studies used piles with only an initial input of waste, in the present study fresh cow manure was added continuously and the vermireactor was watered. As already mentioned, this procedure has two consequences that may mask reduction of pathogens, i.e. spread of pathogen via leachate from upper to lower layers, and compaction of substrate. Despite this, there were dramatic reductions in the numbers of three of the six pathogens analyzed. Finally, vermicompost has two stages: the active stage, during which earthworms and microorganisms jointly process the substrate, and the maturation stage, which is limited to microorganisms, and occurs once the substrate is removed from the vermireactor and where the pathogen load is finally reduced.

4. Conclusions

This is the first comprehensive study up to date describing pathogen reduction during vermicomposting on an industrial scale. *E. andrei* do not exert general effects on pathogens, with reduction depending on the microorganism studied. There were not indirect effects of *E. andrei* on pathogens via changes in physical conditions of substrate, a problem inherent to functioning of vertical continuous feeding reactors. Thus, we recommend that vermicompost from vertical continuous feeding reactors must go through a maturation stage where mechanical aeration should improve the rate of pathogen elimination.

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