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Short Communication

Role of earthworms' mucus in vermicomposting system: Biodegradation tests based on humification and microbial activity

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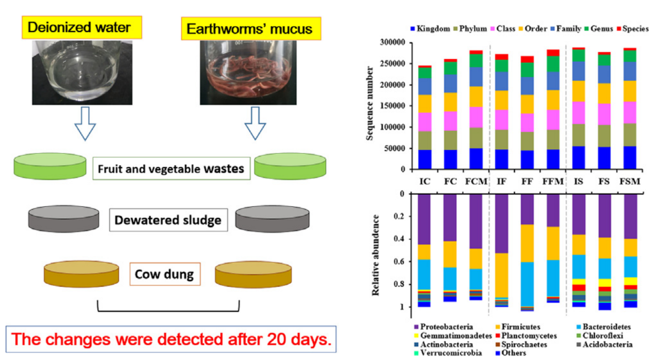
HIGHLIGHTS

- Role of earthworms' mucus in vermicomposting systems was investigated.
- Earthworms' mucus facilitated the mineralization and humification of organics.
- Mucus led to the greatest increases of microbial activity in the FVW systems.
- Mucus positively stimulated Proteobacteria, but negatively affected Firmicutes.

GRAPHICAL ABSTRACT



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ABSTRACT

During vermicomposting, the organic wastes can be recycled into high-value products as mediated by earthworms through gut digestion, burrowing, casting and mucus excretion. However, to date, few studies have been done on the role of mucus in vermicomposting system compared to the effects of the other activities. Hence, this study investigated the potential role of earthworms' mucus in the decomposition and humification of organic wastes. For this, the mucus of *Eisenia fetida* was extracted and inoculated into three vermicomposting substrates using cow dung (CD), fruit and vegetable wastes (FVW), and sewage sludge (SS). The results obtained after a 20 day experiment showed that the mucus could accelerate the mineralization and humification rates of organic components. The dissolved carbon showed 9.8%–37.5% increase in treatments containing mucus, higher than those in substrates without mucus. Moreover, the mucus significantly stimulated the microbial activity and bacterial abundance, showing the greatest increases in FVW treatments. In addition, the mucus positively stimulated growth of Proteobacteria, but negatively affected the Firmicutes during decomposition. This result suggests that the earthworms' mucus significantly accelerated the decomposition and humification of vermicomposting materials, and could even promote microbial activity, growth, and increase community diversity in vermicomposting systems.

1. Introduction

Vermicomposting is a natural process of biochemical decomposition of organic wastes through the metabolic processing of both earthworms and microorganisms, allowing the bioconversion of organic wastes into bio-fertilizer for soil improvement. As a green and environment-

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friendly technology, several organic wastes have been shown to be vermicomposted by earthworms (Lim et al., 2016). Earthworms, as a key driver, directly regulate the activity, abundance, community composition, as well as the habitat of the microbial community through their activities such as digestion, burrowing, mucus excretion, and casting, thus accelerating decomposition process (Brown et al., 2000; Domínguez et al., 2010). In addition, their metabolic activities and behavior also indirectly affect the features of microbial pools by converting the raw substrate into forms that can be readily assimilated, building a new hotspot for the microbiota (Domínguez et al., 2010; Kuzyakov and Blagodatskaya, 2015). Consequently, separately studying each earthworms' behavior in the drilosphere would allow illuminating and distinguishing the interactions and interrelations between earthworms and their microbiota.

To date, several studies have shown that the digestion, burrowing, and casting of earthworms could exert considerable influences on microbial communities in vermicomposting system (Domínguez et al., 2010; Huang et al., 2013), though the true extent of these influences are still to be ascertained. For example, after digestion in the gut of the earthworms, the total carbon and nitrogen contents decreased significantly, while the dissolved substances increased (Domínguez et al., 2010; Hussain et al., 2016). Moreover, earthworm burrows are considered as microbial hotspots, where enzyme activities and dissolved organic carbon and nitrogen tend to be higher (Parkin and Berry, 1999; Hoang et al., 2016). Similarly, earthworm casts can be enriched in mineral nitrogen, relative to the surrounding soil (Parkin and Berry, 1994; Parkin and Berry, 1999). However, compared to the other activities of earthworms in vermicomposting systems, studies on the role of the earthworms' mucus in the decomposition is still limited.

The earthworms' mucus contains a mixture of carbohydrates and protein-like substances that have multiple ecological functions in soil drilosphere. It does not only have drag-reducing characteristics in soil that benefit the movement of earthworms (Zhang et al., 2016), but also serves as nutrient source for plants (Zhang et al., 2009). Further, the earthworms' mucus had strong influences on the mobility and speciation of arsenic in contaminated soils (Sizmur et al., 2011). Accordingly, Salmon (2001) found that the earthworms' mucus could affect the community distribution of springtails in forest soils. Moreover, it has priming effect on the stimulation of microbial activity and decomposition of plant residuals in soil (Marichal et al., 2011; Bityutskii et al., 2012). To date, although some studies started investigating the effects of earthworms' mucus in the soil system, little information is available on the mucus' roles in vermicomposting systems. In contrast to the soil system, vermicomposts display much more complex characteristics with higher organic matter and microbial community diversity. Previous studies on soil systems showed that the mucus, as one of the products produced by the earthworms, may exert significant effects on the quality of vermicomposting products. Thus, to improve the quality of vermicomposts, it is of utmost importance to assess the effects of earthworms' mucus on vermicomposting system, particularly on the humification index and microbial community diversity.

This study investigated (1) the roles of the earthworms' mucus on organic matter decomposition and humification, and (2) the changes on the microbial pool when stimulated by the earthworms' mucus. Considering that different vermicomposting substrates may cause dissimilar effects, three common vermicomposting materials including fresh fruit and vegetable wastes (FVW), cow dung (CD), and dewatered sewage sludge (SS) were used as raw substrates.

2. Methods

2.1. Experimental setup

The fresh FVW, CD, and SS were separately collected from the local Hualian supermarket, cattle farm of Lanzhou Agricultural University, and Qilihe wastewater treatment plant in Anning District, Lanzhou City, respectively. All raw materials were stabilized and turned over in laboratory for one week before use. The earthworms' mucus was collected from the epigeic species of *Eisenia fetida*. After two-month culturing using dewatered sludge in the laboratory, adult earthworms with individual weights of 0.5 g were collected and then washed with tap water. To remove the gut content, the earthworms were not fed for 48 h and then rinsed with distilled water. Then, each 30 active earthworms were separately placed into 50 ml distilled water to excrete the mucus solution. In total, 600 earthworms were used to prepare the mucus. Because dying earthworms could cause sudden changes in the pH and electrical conductivity, the excretion was continued for 8 h (detailed in Supplemental Fig. S1). After which earthworms were picked out and their corresponding mucus solutions were mixed and used for subsequent experiments. Totally, 1000 ml of mucus were prepared. Physicochemical characteristics of the mucus are summarized in Supplemental Tables S1 and S2. Subsequently, 20 ml mucus was separately added into each 12 cm petri dish containing 300 g fresh substrate. For comparison, the same set-up but with 20 ml distilled water was used as the control. In this study, each treatment was carried out in triplicates. After gently mixing, all petri dishes were incubated at 25 °C in a vacuum drying oven. To maintain the moisture, 10 ml distilled water was added into each petri dish every 5 days. Since previous studies showed that the greatest effects of mucus manifested in the first 20 days (Bityutskii et al., 2012), this experiment was terminated on the 20th day, and samples were stored in −20 °C before use.

2.2. Analytical methods

The pH, electrical conductivity, dissolved phosphorous (DP), ammonia nitrogen, nitrate nitrogen, and dehydrogenase activity of the samples were measured using the methods described by Fu et al. (2016). Briefly, total carbon and nitrogen of dry samples were simultaneously determined using elemental analyzer (EuroVector, EA3000). The dissolved carbon (DC) and nitrogen (DN) were measured by TOC instrument (TNM-L, SHIMADZU) while amino acids were quantified using Cecil AA4300. Three-dimensional fluorescence excitation emission matrix spectroscopy for analyzing the organic forms of vermicomposts by the fluorescence spectrophotometers (RF-5300PC, SHIMADZU), following the methods of Huang et al. (2017).

The genomic DNA was extracted directly from the soil using the DNA isolation kit (MO BIO Laboratories, USA). Then, the abundance of bacterial 16S rDNA V3 region amplified by the primers of 341f/518r was quantified with the SYBR® *Premix Ex Taq*™ (TaKaRa) using the Thermal Cycler Dice (TP900, TAKARA), based on the methods of Huang et al. (2013). The bacterial 16S rDNA V4 region amplified the universal primers 515f/806r for pyrosequencing carried out at the Novogene Bioinformatics Technology Co., Ltd. Additional details on the PCR primers are given in the Supporting information (Table S3). The pyrosequencing was carried out in an Illumina MiSeq 2 × 250 platform, according to protocols described by Caporaso et al. (2012). High quality sequences were clustered into operational taxonomic units (OTUs) using UCLUST at 97% similarity level. The resulting data was used to calculate for alpha and beta diversities, and observed species on OTUs, as analyzed in QIIME (Version 1.7.0). Cluster analysis of the unweighted and weighted unifrac distance metrics of OTUs was performed by principal component analysis (PCA) and Principal coordinate analysis (PCoA). A student's *t*-test was used to compare differences in physicochemical properties at the P value of 0.05.

3. Results and discussion

Compared to the control without mucus, all treatments displayed lower total carbon and total nitrogen levels, but not significantly different (Table 1). In contrast, Bityutskii et al. (2012) found a significant decrease on total carbon in soil and plant residues with the addition of mucus. This difference could probably be due to the substrates used

Table 1
A summary of the physico-chemical properties of the initial substrates and final products with and without earthworms' mucus in three decomposition systems of cow dung (CD), fruit and vegetable wastes (FVW) and sewage sludge (SS). Different letters following the values represent significant differences between group (HSD test, $P < 0.05$).

	CD system			FVW system			SS system		
	Initial substrate	Final product		Initial substrate	Final product		Initial substrate	Final product	
		Without mucus	With mucus		Without mucus	With mucus		Without mucus	With mucus
pH	9.64 a	9.59 a	9.47 b	9.46 a	8.57 b	8.78 b	8.27 a	8.09 ab	7.81 b
Electrical conductivity (mS/cm)	2.29 a	2.84 b	2.98 b	0.61 a	0.39 b	0.56 ab	0.49 a	0.61 b	0.69 b
Total carbon (mg/g)	376.8 a	367.1 b	354.5 c	146.8 a	126.8 b	116.2 b	229.7 a	220.5 ab	212.3 b
Dissolvable carbon (mg/g)	37.1 a	32.6 b	35.8 a	12.6 a	11.6 a	14.5 b	24.1 a	14.4 b	19.8 c
Total nitrogen (mg/g)	12.9 a	11.4 b	11.1 b	15.5 a	14.4 a	14.1 a	21.2 a	20.2 a	19.5 a
Dissolvable nitrogen (mg/g)	8.8 a	5.4 b	6.7 c	1.8 a	0.8 b	1.4 c	12.0 a	2.4 b	3.4 c
Ammonium (mg/g)	3.51 a	2.62 b	2.83 b	0.27 a	0.23 a	0.22 a	6.75 a	1.20 b	0.97 b
Nitrate (mg/g)	0.48 a	0.65 b	0.66 b	0.27 a	0.13 b	0.27 a	0.34 a	0.20 b	0.27 c
Dissolvable phosphorous (mg/g)	3.78 a	3.55 a	4.47 b	0.61 a	0.76 ab	1.11 b	4.26 a	2.17 b	2.45 b

that already contained higher content organic matter even before the start of the experiment in this study. Interestingly, the DC in treatments containing mucus showed 9.8%–37.5% increase than in substrates without mucus. Similarly, the dissolved nitrogen and phosphorous concentrations in the substrates with mucus showed significantly higher values than those without mucus, especially in the FVW samples (Table 1). The increases in DC, DN, DP, and electrical conductivity indicate that the earthworms' mucus significantly accelerated the transformation of organic matter during the mineralization process. In addition, the earthworms' mucus could have significantly enhanced production of humic and fulvic-like substances, compared to their counterparts (Fig. 1). This finding suggests that earthworms can promote

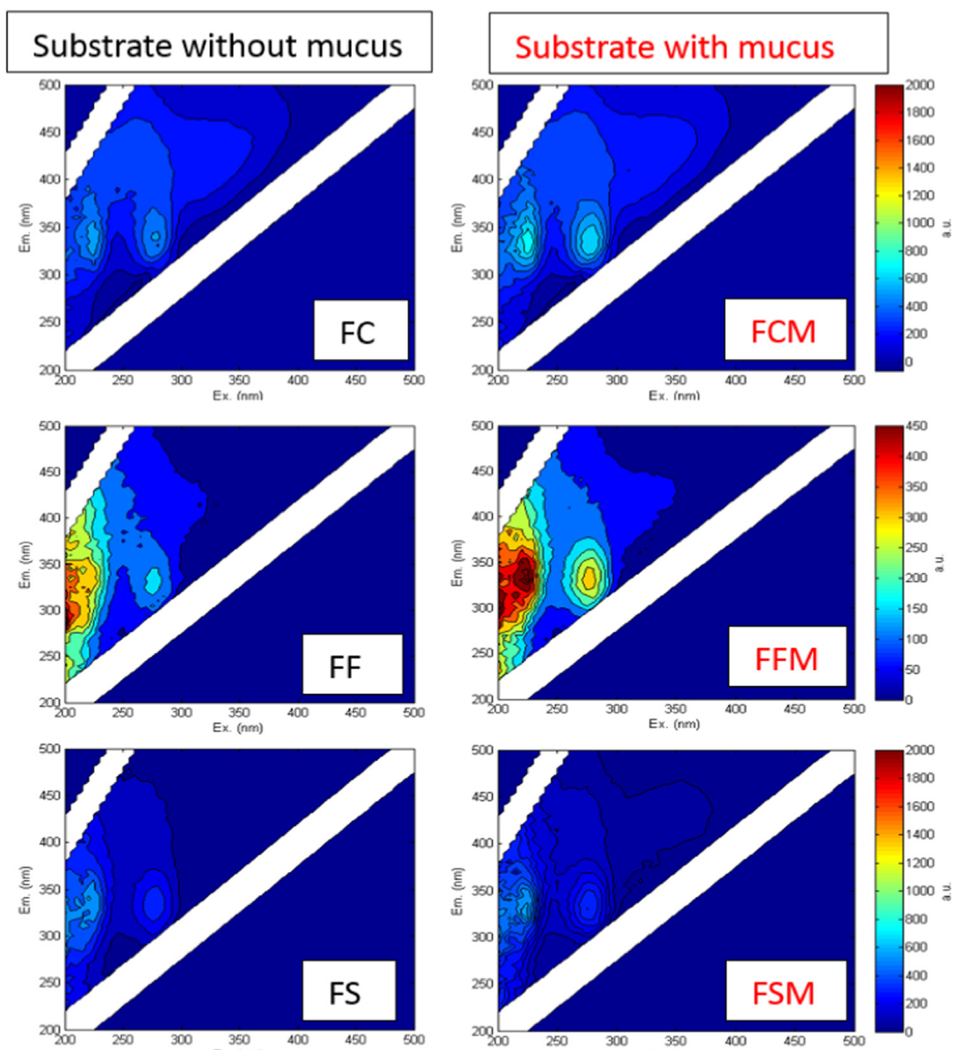

Fig. 1. Fluorescence excitation–emission matrix spectra of dissolved organic matter from final products with and without earthworms' mucus in the three decomposition systems. 'F' in each image means the final product; C, F and S pertain to the three systems of cow dung, fresh fruit and vegetable wastes and sewage sludge, respectively; 'M' represents substrates added with mucus.

humification of substrates for 20 days. Specifically, the earthworms' mucus triggered the humification of the substrate with low organic content as seen in the high ammonium concentration in mucus (Bityutskii et al., 2012). In contrast to the controls, the mineralization and humification rates were found higher in substrates with mucus. This indicates that the mucus has a positive effect on vermicomposting systems that have high organic contents.

Generally, the earthworms' mucus is high in DC and DN, amino acids. The lower carbon to nitrogen ratio however is linked with the changes of microbial communities (Brown et al., 2000; Bernard et al., 2012). Comparatively, earthworms' mucus led to an increase in the dehydrogenase activity of 9.6%, 50.9% and 20.7% in CD, FVW and SS, respectively (Fig. 2). This implies that the microbial activity was enhanced by the mucus excreted from the epigeic earthworms. Interestingly, Oleynik and Byzov (2008) reported a decrease in dehydrogenase activity in soils that have been cultured with earthworms' mucus after 3 days. The high availability of DC and DN as labile sources for microbiota easily released from organic substrates could also be responsible for the higher microbial activity. In addition, bacterial abundance in the substrates with mucus showed significant increases, 3.4 to 11 times higher than those without mucus (Fig. 2). Such finding could suggest that the earthworms' mucus provided favorable conditions for microbial growth (Hoang et al., 2017). The greatest increases in bacterial abundance and dehydrogenase activity were also found in the FVW treatment, which could be due to its rapid degradation in the beginning of vermicomposting. Furthermore, these increases indicate that the earthworms' mucus exerted critical effects on the decomposition of organic materials by promoting bacterial activity and population growth.

For the bacterial community, the total number of reads based on HTS for the substrates added with mucus was 3.3%–7.3% greater than their counterparts (Fig. 3), with the highest being in the CD system. In all taxonomic ranks (i.e. phylum, class, order, family, genus or species), the highest read abundances were observed in the substrates with mucus than in the controls. Regardless of the substrates used, the bacterial community was mainly dominated by taxa belonging to Proteobacteria, Firmicutes and Bacteroidetes in all substrates, as revealed by pyrosequencing (Fig. 3). Compared to the control, the abundance of Proteobacteria significantly increased with the addition of the mucus, which was consistent with previous reports showing that earthworms' activities could promote the abundances of Proteobacteria in vermicomposting systems particularly in treating fresh animal feces (Lv et al., 2015), FVW (Huang et al., 2013) and SS (Lv et al., 2015; Fu et al., 2016). Unexpectedly, the Firmicutes showed the lowest abundances in substrates with mucus. Spearman's correlation (Supplemental Fig. S2) further showed that the specific bacterial taxa could be associated with specific environmental factors in the substrates. For example, Proteobacteria were positively correlated with pH, total carbon, total nitrogen and ammonia, but the opposite was true for the Firmicutes. Variance partitioning analyses was then performed to assess the contributions of nitrogen and carbon availabilities on the bacterial community. Results showed that nitrogen and carbon sources independently explained 18.4% and 5.3% of the variation of microbial communities, respectively (Supplemental Fig. S3). This observation was to be expected, since the mucus inherently contains higher nitrogen concentrations than carbon that could have stimulated the changes in the diversity of the bacterial community diversity in this study.

The addition of earthworms' mucus also led to two dissimilar results in Bacteroidetes, where its abundance increased in the CD and SS systems, but decreased in the FVW system (Fig. 3). This indicates that the effect of mucus on the bacterial community may differ with the different substrates and could be due to the differences in the availability of resources in the different substrates, which are needed by the bacteria for growth. For example, CD and SS systems had higher DC and DP based, which were more needed by Bacteroidetes, but lower in the FVW system (Supplemental Fig. S2).

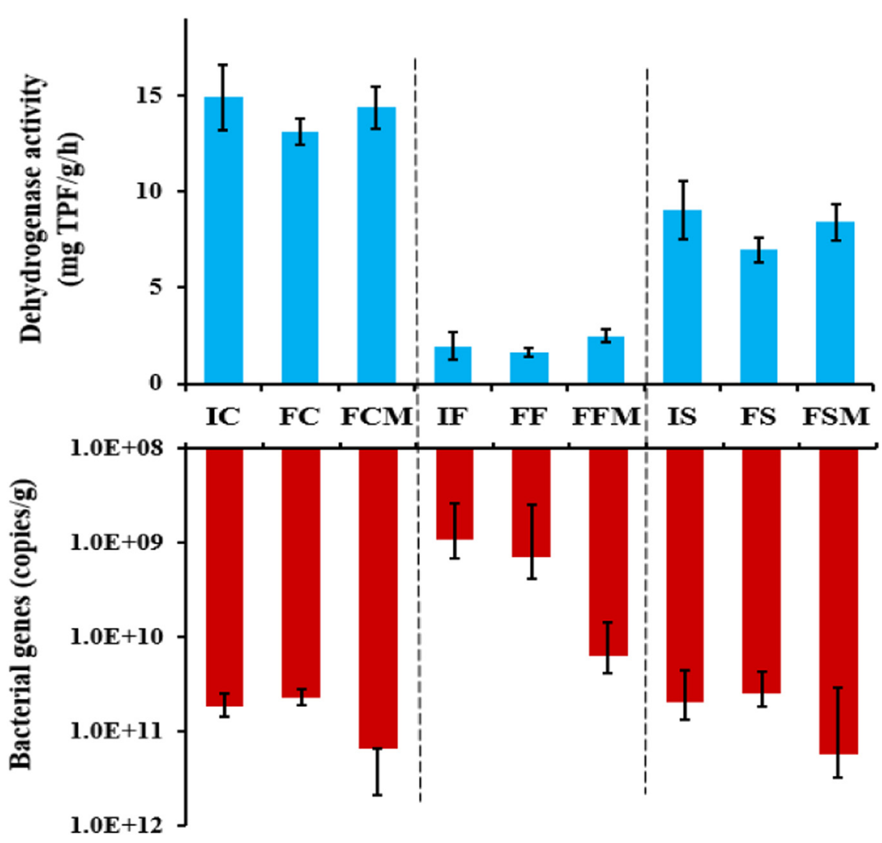

Fig. 2. Dehydrogenase activity and bacterial gene copies of initial substrates and final products with and without earthworms' mucus in the three decomposition systems. 'F' in each image means the final product; C, F and S pertain to the three systems of cow dung, fresh fruit and vegetable wastes and sewage sludge, respectively; 'M' represents substrates added with mucus.

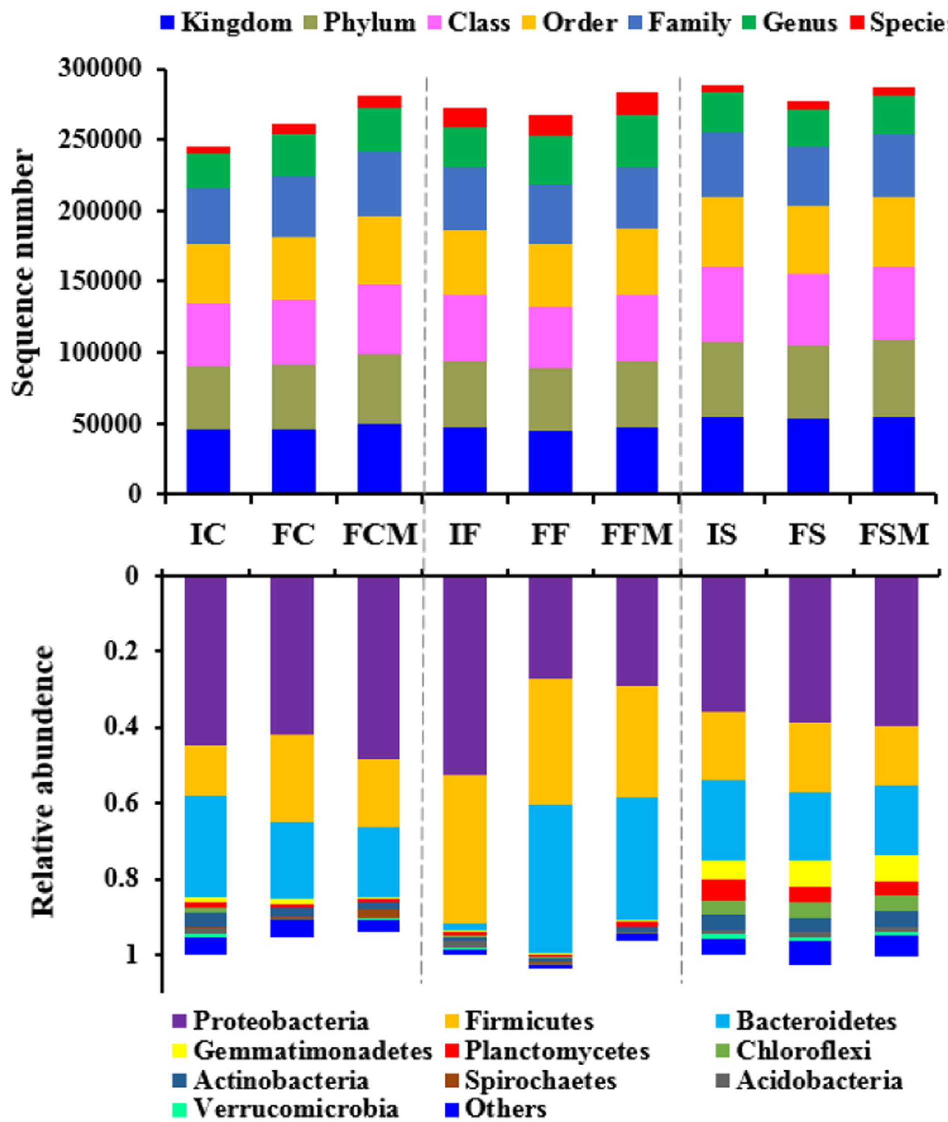

Fig. 3. Amount of bacterial taxa at different levels (top figure) and relative abundance of bacterial phyla (bottom level) for initial substrates and final products with and without earthworms' mucus in three decomposition systems, respectively. 'F' in each image means the final product; C, F and S pertain to the three systems of cow dung, fresh fruit and vegetable wastes and sewage sludge, respectively; 'M' represents substrates added with mucus.

4. Conclusions

Our results demonstrated that the mucus of epigeic earthworms could accelerate the mineralization and humification of vermicomposting materials. During the decomposition process, the bacterial community structure was strongly modified by the addition of mucus, specifically showing the greater increase abundances of Proteobacteria and a decrease in the Firmicutes. This study suggests that the mucus of earthworms could play significant roles in accelerating the decomposition and humification and modifying microbial profiles in vermicomposting system.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.08.104.

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