Difference of microbiome and antibiotic resistome between earthworm gut and soil deciphered by a continental-scale survey

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Keywords: Gut microbiome, Earthworm, Antibiotic resistome, Continental-scale survey, Mitigation

Posted Date: February 7th, 2020

DOI: https://doi.org/10.21203/rs.2.22866/v1

Abstract

Background Earthworms are globally distributed and quite capable of redistributing compounds, as well as bacteria and antibiotic resistance genes (ARGs) throughout the soil profile. The spread of medically relevant ARGs in soils has become an emerging environmental and health issue globally. However, our understanding on earthworm gut microbiome and antibiotic resistome is still lacking, especially at the large scale, and little is known about the role of earthworm in the dispersal of ARGs.

Methods We conducted a continental-scale survey, including samples (earthworm gut and soil) from 28 provinces across China, from both natural and agricultural ecosystems (arable land: 35 sites and forested land: 16 sites). The 16S rRNA amplicon sequencing and high throughput quantitative PCR were used to characterize the microbiome and antibiotic resistome, respectively. We further explored potential mechanisms behind changes in the abundance and diversity of ARGs in the earthworm gut. Then, the microcosm experiments and long-term field experiments with or without earthworms were employed to test the potential for earthworms to reduce the abundance of ARGs in soils.

Results The diversity and structure of bacterial community were observably different between the earthworm gut and soil. Firmicutes (35.7%) and Proteobacteria (34.8%) were the dominant phyla in all earthworm gut samples. A significant correlation between bacterial community dissimilarity and spatial distance was identified in the earthworm gut. The earthworm gut consistently had a lower diversity and abundance of ARGs than in the surrounding soil. We further revealed that the change of ARGs in the earthworm gut was likely a consequence of the reduction in the abundance of mobile genetic elements and dominant bacterial phylotypes that are the likely hosts of ARGs. The microcosm study and long-term field experiments provided the experimental evidence that the presence of earthworms reduced the abundance and diversity of ARGs in soils.

Conclusions Our findings highlight that earthworm gut and soil present the distinct microbiome and resistome at the continental scale, and earthworms may play an important role in the continental-scale mitigation of antibiotic resistance.

Background

Earthworms are globally distributed, and play critical roles as key ecosystem engineers by regulating soil processes and maintaining soil health [1–3]. It has been estimated that up to 10% of all existing soils pass through the gut of earthworms every year [4, 5]. Earthworms help regulate soil health by influencing microbial community structure and nutrient mineralization [6–9]. Recent work suggests that the earthworm gut microbiome is, in part, responsible for the positive effects of soil organisms on key process such as organic matter mineralization [10, 11]. Although a number of studies have examined the earthworm gut microbiome [5, 12–14], most work has been done in laboratory microcosms using model or limited earthworm species, and at the local scale. Studies evaluating the microbiome of earthworm gut at continental scales and across contrasting ecosystem types are lacking. This limits our knowledge on

the potential of the earthworm gut microbiome to regulate important soil processes such as nutrient cycling.

Terrestrial ecosystems face an emerging, global environmental and health crisis associated with the accumulation of antibiotic resistance genes (ARGs) in soils [15–20]. Accumulation of ARGs in soils has emerged as a public health concern due to their potential to further decrease the effectiveness of antibiotics [21], as shown by the increasing number of antibiotic resistant pathogens being detected in soils [17]. Drug-resistant infections currently cause the death of ~ 700,000 people per year [22]. This crisis requires immediate action to slow the dispersal and accumulation of ARGs globally, a priority for the World Health Organization [23]. This global crisis with ARGs is caused by overuse of antibiotics in the medical treatment and animal husbandry, the consequent discharge of waste waters, and application of manure to soils [15, 24]. Considering that earthworms consume tons of soils per year, and are distributed globally [5], the role of earthworms in affecting the abundance of ARGs is attracting attention. Recent studies revealed that the application of organic fertilization could increase the abundance of ARGs in the earthworm gut microbiome [25] and the gut of earthworm from the compost is a hot spot for ARGs [26]. These suggested that earthworm may have an important contribution to the dispersal of ARGs in the soil ecosystem. Despite this, little is known about antibiotic resistome in the earthworm gut, especially at the large scale. At the same time, these studies only focused on earthworm guts. Earthworm guts have special conditions (anoxia, neutral pH, high quantity/quality of substrates and native microbial competition) which may lead to a distinct resistome compared to the surrounding soil. A recent study has showed that earthworms could reduce the abundance of ARGs in composting processes [27]. However, whether earthworms can help to reduce the abundance of ARGs in soils remains unexplored.

Here, we firstly conducted a continental-scale survey to characterize the earthworm gut microbiome and antibiotic resistome. Then, we further explored potential mechanisms behind changes in the abundance and diversity of ARGs in the earthworm gut. Finally, the microcosm experiments and long-term field experiments with or without earthworms were employed to test the potential for earthworms to reduce the abundance of ARGs in soils. Because earthworm casts are difficult to collect routinely in the field, we tested earthworm gut contents. These contents are directly derived from the soil they ingest, and contribute to their casts, reflecting the potential role of earthworms in regulating the abundance and diversity of ARGs in soils. To confirm our field observations, the controlled microcosm and long-term field experiments were performed. The large-scale survey included samples from 28 provinces across China, from both natural and agricultural ecosystems (arable land: 35 sites and forested land: 16 sites). At each location, we collected soil and earthworm samples, and used high throughput quantitative PCR (HT-qRCR) with primer sets targeting the bacterial 16S rRNA gene, a panel of 285 ARGs to investigate the abundance of ARGs and 10 mobile genetic elements (MGEs) to explore the potential mechanism of ARG shifts. At the same time, bacterial communities were analyzed by high-throughput sequencing.

Methods

Sample collection

Earthworms and surrounding soil samples were collected from the top 20 cm of soil based on the depth of the cultivated horizon from 28 provinces across China, including 35 arable lands and 16 forested lands. Samples were collected from June 15 to August 20, 2017. The latitude and longitude of sampling sites are supplied in Additional file 1: Table S1, and shown in Additional file 7: Fig. S1. Except for forested land samples from Chongqi (two replicates), each collection comprised three replicates of earthworm and soil samples, thus a total of 152 earthworm samples and 152 surrounding soil samples were obtained. For collection of earthworm samples, we firstly excavated multiple rectangular block of soils (length: 50 cm, width: 30 cm and high: 20 cm) at each location, and placed them onto the clean white cotton. The soil block was broken up and all earthworms carefully collected by hand. Because different soils have different earthworm species it is not possible to find a dominant species in all soils. We collected 3–30 individuals per species in a replicate, 60–240 individuals per site and 20–80 individuals per replicate. Using morphological analysis, we identified the dominant species based on abundance. Soil samples were collected using a five-point sampling method. The collected soils and earthworms were divided into two parts. One part was used to extract DNA, and the other was used to analyze chemical properties. The samples for the chemical analysis were preserved in sealed plastic bags and transported to the laboratory at 4 ^{o}C within 24 h. Samples for DNA extraction (earthworms and soils) were immediately stored in anhydrous alcohol and kept at -20 ^{o}C in the laboratory until use.

DNA extraction and high-throughput quantitative PCR (HT-qPCR)

Earthworm samples stored in anhydrous alcohol were carefully washed twice using sterile water to remove allogenic materials from the surface, rinsed for 2 min with 0.5% sodium hypochlorite solution to sterilize the surface, and rinsed three times with sterile water at 4 °C. Earthworms were immediately dissected using sterile forceps and surgical scissors in a bacteria-free operating environment to obtain earthworm gut contents. Gut contents (0.5 g) were collected into a 2-mL Eppendorf tube, to which 0.96 mL sterile 100 mM phosphate buffer pH 7.4 was added. The FastDNA Spin Kit for soil (MP Biomedicals, Illkirch, France) was used to isolate DNA from earthworm gut contents or from 0.5 g soils. DNA extraction followed the manufacturer's instructions. The 100 μL DES (DNA elution solution) was used to elute the extracted DNA into a sterile 1.5-mL Eppendorf tube. Since gut contents often contain larger amounts of PCR inhibitors than soil, we used a universal DNA purification kit (HYK-002, Lulong Biotech, China) to purify the obtained DNA samples (earthworms and soils) before further analysis. The quality and concentration of DNA were checked using 1.0% agarose gel electrophoresis and the Quibt 3.0 Fluorometer (Invitrogen). Resulting DNA was stored at − 20 °C until further study.

Before HT-qPCR, DNA was diluted to 30 ng μL^{-1} using sterile water. Although the extracted DNA was purified, to further confirm that inhibition was negligible, we conducted a spiking test with a dilution series of the template DNA from soils and earthworm gut contents to determine recovery ratios of PCR. In the spiking test, we added three known amounts of ARGs (tetC, tetG and sul2) into soil and gut DNA extracts. At the same time, we diluted a series of DNA templates (0, 10, 100 and 1000 fold) from soils and earthworm gut contents, including three different earthworm ecotypes. The spiked and diluted DNAs were

detected using HT-qPCR. The abundance of added ARGs did not present significant difference between the spiked soils and gut DNA extracts ($P > 0.05$). No significant difference was observed in ratios of PCR from the dilution series of the template DNA between soils and gut contents ($P > 0.05$). These results indicated that the PCR inhibitors were negligible in extracted soils and gut contents.

A total of 285 primer sets (Additional file 2: Table S2) targeting resistance genes to major classes of antibiotics, 16S rRNA genes and 10 mobile genetic elements (MGEs) were used. The Wafergen SmartChip Real-time PCR system (Wafergen Inc., USA) was used to perform the HT-qPCR. Three technical repeats and a non-template negative control were included in the amplification of each primer set. The HT-qPCR amplification conditions have been previously described [28, 29]. We used SmartChip qPCR software (V 2.7.0.1) to analyze the HT-qPCR data. Data from amplifications with efficiencies outside the range of 0.9–1.1 were discarded. Only data with three positive replicates were considered as a successful amplification and used in the downstream analysis. The threshold cycle number (CT) for the detection limit was set at 31. We normalized the copy number of ARGs per 16S rRNA gene or bacterial cell to reflect the abundance of ARGs, avoiding biases arising from different bacterial abundance in each sample. Normalization was carried out using the bacterial 16S rRNA gene abundance in each sample. Calculations followed the equations below [28]:

Relative gene copy number = $10^{((31 - C_{T(detection)})/(10/3))}$

Relative abundance of ARGs = Relative ARG copy number/Relative 16S rRNA gene copy number

Normalized ARG copy number per bacterial cell = Relative abundance of ARGs × 4.1.

Based on the Ribosomal RNA Operon Copy Number Database, on average, a bacterial cell contains 4.1 copies of 16S rRNA gene, and this correction factor was used in this study [28, 29].

Real-time quantitative PCR (RT-qPCR)

To explore the relationship between bacterial abundance and ARG abundance, we used RT-qPCR with the Roche 480 system to determine the absolute bacterial 16S rRNA copy numbers of earthworm gut content and soil. Universal primers (5 ‘GGGTTGCGCTCGTTGC) and (5’ ATGGYTGTCGTCAGCTCGTG) were used to target the bacterial 16S rRNA gene region. The qPCR system and conditions were as previous studies [28, 30]. Each sample was replicated three times in all qPCR, including a non-template reaction as the negative control. The standard curve was obtained by detecting triplicate 10-fold serial dilutions of plasmid DNA with the target-gene (amplification efficiency 97 − 103%, $r^2 > 0.99$).

Bacterial 16S rRNA gene amplification, sequencing and bioinformatic analysis

To examine the biogeographic characteristic of earthworm gut microbial communities and explore the contribution of bacterial communities to the change in ARGs, we characterized the bacterial communities of earthworm gut and soil using a set of primers (515F: GTGCCAGCMGCCGCGG − 907R:

CCGTCAATTCMTTTRAGTTT) to amplify the 16S rRNA gene hypervariable V4-V5 region [28, 31]. Unique barcodes were included in each primer to distinguish different samples. The DNA was amplified and purified according to previous studies [32, 33]. We used a Qubit 3.0 fluorimeter to determine the concentration of purified products, and 24 products of equal concentration were pooled as a library. Finally, the Illumina Hiseq2500 platform (Novogene, Tianjin, China) was used for high-throughput sequencing of each library.

Quantitative Insights Into Microbial Ecology (QIIME v1.9.1) was used to analyze high-throughput sequencing data of earthworm gut and soil, following the online instructions [34]. Briefly, low-quality reads, ambiguous nucleotides and primer sequences were removed to obtain clean sequences. We assigned high quality sequences to operational taxonomic units (OTUs) according to 97% sequence similarity using UCLUST [35]. We discarded singleton OTUs to generate the final OTU table, and assigned taxonomy to OTUs based on the representative sequence using the RDP Classifier 2.2 [36]. The PyNAST aligner was used to align representative OTUs based on the GreenGenes 13.8 reference database [37]. We built a phylogenetic tree via the FastTree algorithm [38]. To minimize the error of potential sequencing, we discarded those OTUs with a total number of reads of < 100 in all samples before downstream analysis [39].

Sample analysis

Soils and earthworm gut contents were air dried in the dark and 2 mm sieved. We used soil/water (1/2.5) and gut content/water (1/5) suspensions to measure pH, and a C/N instrument (Vario MAX, C/N, Elementar) to determine total nitrogen and carbon. The clay content of soil was determined using a particle number analyzer (Malvern Instruments Ltd.). Soil types and properties were summarized in Additional file 3: Table S3.

Identification of earthworm species

After earthworm species were identified by morphological characteristics, we further used Cytochrome Oxidase I (COI) barcode gene sequencing as confirmation. DNA of earthworm tissues was isolated using the DNeasy Blood and Tissue Kit (QIAGEN, China (Shanghai) Co., Ltd) based on the manufacturer's instructions. The isolated DNA was amplified using the primers LCO1490 (5' GGTCAACAAATCATAAAGATATTGG) and HCO2198 (5' TAAACTTCAGGGTGACCAAAAAATCA) to target the COI barcode region [5]. The amplification system and conditions were as previously designed [40]. PCR products were sequenced, and the sequences submitted to NCBI to identify the species of earthworms via the Basic Local Alignment Search Tool (BLAST). Information on earthworm species and ecological group at each location is listed in Additional file 4: Table S4.

The microcosm assay and long-term field experiments

Earthworms comprise abundant species (~ 8000) and the distribution of species varies greatly in different regions. According to the distinct feeding and burrowing habits of earthworms, they are usually classified into three ecological groups (epigeic, endogeic and anecic) [14]. Epigeic earthworms mainly

ingest plant litter and live on the surface, and rarely burrow. Endogeic earthworms feed on a large number of soil food sources (e.g. mineral soil and humus), live below the surface, and commonly burrow. Anecic earthworms usually ingest organic residues, forage the surface, live in the mineral soil layer, and burrow. Earthworms of different ecotypes often have different feeding guilds, affecting the roles of earthworms in soil ecosystems. To reveal the change of ARGs among soils, earthworm gut contents and casts, we conducted a microcosm experiment using three different ecological earthworm species (epigeic: Eisenia andrei, endogeic: Amynthas robustus and anecic: Amynthas hupeiensis). The farmland soils (29°47′ N, 121°21′ E) from Ningbo were used in the microcosm experiment, and soil properties are summarized below. Three treatments were performed, including three kinds of different earthworm species representing different ecotypes. Each treatment had 15 earthworms added into 2.5 kg dried soil (75% of the field water-holding capacity) and was replicated four times. The microcosms were incubated in the greenhouse for 28 days. After 28 days, we collected soils, earthworms and casts samples, and earthworms were dissected to obtain gut contents. The obtained samples were used to detect the abundance and diversity of ARGs.

To explore the effect of earthworms on the ARGs in the soil ecosystem, we conducted the microcosm experiment with a full factorial design, including two soil conditions (control and amended with pig manure) and earthworm treatments (control and adding 15 earthworms; Amynthas sp.). Each treatment was replicated four times, and a total of 16 pots were used. Earthworm density was selected based on the following considerations. The typical earthworm density in the agricultural soil is 1–5 earthworms in 2.5 kg soil, but with the input of organic matter the number of earthworms will also increase. 15 earthworms in 2.5 kg soil can be also found in the soil, especially, which was applied organic fertilizer (e.g. pig manure). The soil (clay content = 7.35%, pH ($CaCl_2$) = 4.75, CEC = 13.76 cmol kg^{-1}, total C 32.4 g kg^{-1} and total N 3.77 g kg^{-1}) was collected from arable land (depth: 0–20 cm) at Ningbo (29°47′ N, 121°21′ E), Zhejiang province, China. Each pot contained 2.5 kg dried soil, and water content of the soil was adjusted to 75% of the field water-holding capacity. After incubating for two weeks, earthworms of similar size (~ 60 g fresh weight) and seeds of a common crop lettuce (Lactuca sativa) were added. The microcosms were incubated in the greenhouse for 75 days. We further collected soil samples from two long-term field experiments with or without earthworms, in the corn season. M. guillelmi was the dominant species of earthworm in natural fields. The two long-term field experiments were described as below: 1) Wheat-corn rotation was used. The field experiment lasted for one year. Each treatment was replicated three times. 2) Corn-vetch rotation was conducted lasting for two years. Each treatment was replicated five times. After harvest, the DNA was extracted from soil and earthworms as above, and the ARGs and bacterial communities of soil and earthworms were analyzed as described above.

Data processing and statistical analysis

The figure describing the sampling sites was produced via ArcMap 10.1. Data on ARGs and bacterial community were presented as mean ± SE. We set the significance level at 0.05 and indicated P values as follows: *P < 0.05, **P < 0.01 and ***P < 0.001. SPSS version 20.0 was selected to compare the significance of the data using the linear mixed model, paired-samples t-test and independent-sample t-

test. We used the principal coordinate analysis (PCoA) to reveal patterns of earthworm ARG profiles via RStudio with labdsv 1.8-0 and ggplot 2. The differences of shared ARGs and dominant bacterial OTUs (with relative read abundance > 0.05) between soil and earthworms were compared using STAMP software. The violin plots were conducted using the ggplot2 packages of R with version of 3.4.4, and the Venn diagram was produced via the online version of Venny 2.1. We used the vegan 2.3-1 packages of R to perform a Nonmetric multidimensional scaling analysis depicting the difference between earthworm gut and soil bacterial communities [41, 42]. Partial redundancy analysis was conducted via the vegan 2.3-1 package of R to reveal the contribution of earthworm gut bacterial communities, soil properties (bacterial community and physical and chemical properties) and gut content properties (pH and total C) to the percentage of variation in ARG profiles. We used the vegan 2.3-1 package of R to conduct the PERMANOVA (Adonis test and Anosim test), the Mantel test and the Procrustes test and to calculate the Shannon index of bacterial communities. OriginPro 9.1 was used to produce the histogram and Scatter diagram, and to perform linear regression.

Results And Discussion

Earthworm Gut vs. Soil Microbiome

The composition of microbial communities was different between soil and earthworm gut at the family level across samples taken at the continental scale (Additional file 8: Fig. S2). For example, lower diversity was observed in the earthworm gut microbiome compared with soils (paired-samples t-test, $P < 0.001$; Fig. 1a), and NMDS ordinations of bacterial community showed that samples were clustered by sample source (soil and earthworm gut) (Anosim, $P < 0.001$; Fig. 1b). These results were consistent with previous studies [5, 33]. Even so, we still found a relatively low, but significant, correlation between the microbiota of soil and earthworm by the mantel test ($r = 0.14$, $P < 0.001$), suggesting that soil microbiota contributes to the earthworm gut microbiome.

Firmicutes (35.7%) and Proteobacteria (34.8%) were the dominant phyla in all earthworm gut samples in our study. However, combined with previous studies [5, 14], we found the dominant phyla in earthworm guts were not constant, which may be influenced by many factors. At the Family level, Aeromonadaceae (8.5%), Bacillaceae (18.3%) and Pseudomonadaceae (9.5%) were the most abundant taxa in the earthworm gut samples. Almost 60% of dominant bacterial OTUs, defined as the relative read abundance > 0.05 showed a significant difference between soil and earthworm gut ($P < 0.05$; Additional file 9: Fig. S3). Land use and earthworm species had no obvious impact on the earthworm gut microbial community (Additional file 10: Fig. S4). However, in line with gut bacterial communities from other animals (e.g. fruit fly [39] and beetle [43]), earthworm gut microbiota presented significant differences between different sites (PERMANOVA, $P < 0.001$). A previous study also showed that habitat has a more important influence on earthworm gut wall-associated microbiota compared to host species [14]. A significant correlation ($P < 0.01$) between bacterial community dissimilarity and spatial distance was identified in the earthworm gut (Fig. 1c; the correlation coefficient: arable land ($r = 0.127$) < forest land ($r = 0.269$)). This indicates that the earthworm gut bacterial communities from arable land tended to be more homogeneous compared to

those in forest land, which might reflect the fact that arable ecosystems are generally more homogeneous than natural ecosystems.

In this study, the 14 different earthworm species were collected from arable and forest land. Earthworm species were not more homogeneous from arable soil. Such spatial variability might be related to changes in soil properties across locations. For example, a significant negative correlation was observed between community similarity and soil clay content ($r = -0.193$, $P < 0.05$), and significant positive relations were found between community similarity and soil nitrogen ($r = 0.193$, $P < 0.05$; Fig. 1d) and NO_3-N content ($r = 0.171$, $P < 0.05$; Additional file 11: Fig. S5), respectively.

Earthworm Gut vs. Soil Resistome

A total of 192 out of the 285 resistance genes assayed were detected in soils and earthworms from China. The three dominant ARG groups included those related to antibiotic deactivation (41.9%), cellular protection (23.6%), and efflux pumps (30.5%) (Additional file 12: Fig. S6). The result of PCoA showed the significant separation between earthworm and soil ARG profiles (PERMANOVA, $P < 0.005$; Additional file 13: Fig. S7). More importantly, our study indicated that the earthworm gut always had a lower abundance and diversity of ARGs compared to their corresponding soils (Linear mixed model including sample type as a fixed factor and earthworm species, sites and land use as random factors; $P < 0.001$). Linear mixed models also showed that land use had a significant effect on ARGs ($P = 0.022$), but earthworm species and site had no obvious influence on ARGs ($P > 0.05$). This held true for all samples, representing contrasting terrestrial ecosystems from across China (Fig. 2). We further found that earthworm guts had a lower diversity (detected number) of ARGs in both agricultural (-44.9%) and forest (-41.9%) ecosystems compared with surrounding soils (paired-samples t-test, $P < 0.001$; Fig. 2a). Similarly, earthworm guts had a lower abundance of ARGs in arable (-62.2%) and forest ecosystems (-48.1%) as compared with the soils they were found in (paired-samples t-test, $P < 0.001$; Fig. 2b). The reported higher abundance of ARGs in arable land compared with forests (Linear mixed model including land use as a fixed factor and earthworm species, sites and sample type as random factors, $P < 0.05$; Fig. 2b), is largely expected, given the higher impact of human activities on agroecosystems (e.g., addition of ARGs to soils via manure fertilization and the use of pesticides).

To confirm whether the capacity of earthworms to alter the abundance and diversity of ARGs in their guts could vary between different species of earthworms in our continental survey, we divided the collected earthworms into three ecological groups (epigeic, endogeic and anecic) based on their distinct feeding and burrowing habits. Since some earthworm species are facultative and difficult to identify to their ecological groups, we selected some typical earthworm species (epigeic: Eisenia andrei, endogeic: Amynthas robustus and anecic: Amynthas hupeiensis) to analyze the differences in the magnitude of the reduction in ARGs between earthworms of different ecologies. More ARGs were reduced in the epigeic and anecic earthworm guts compared to the endogeic earthworm gut ($P < 0.05$; Additional file 14: Fig. S8). Considering that feeding guilds of earthworm species differed in their content of ARGs, this result suggested that selective feeding on non-soil food sources (like litter, microorganisms and humus etc.) could play a role in reducing the abundance of ARGs in earthworm guts. Epigeic earthworms commonly

ingest decaying organic matter, anecic earthworms mainly feed on soil microorganisms and humus and endogeic earthworms tended to feed on soil food sources. Finally, although the magnitude of the reduction in ARGs differed between earthworms of different ecologies, there were overall fewer ARGs in gut contents than soil across all earthworm species, regardless of location or ecology.

Our results were also consistent across different types of ARGs (Fig. 2c). Common ARGs that confer resistance to aminoglycosides, sulfonamides, β-lactams, or chloramphenicol, and multidrug efflux pumps all showed lower abundance in the earthworm guts compared to surrounding soils (the ANOVA with linear mixed model, $P < 0.05$; Fig. 2c). Finally, by referring to previous studies [17, 44], we identified 12 clinically relevant ARGs which were commonly found in arable soils. Then we compared the abundance in earthworm guts and soils to evaluate the potential role of earthworms in the dispersal of these clinically relevant ARGs. These ARGs were significantly reduced in the earthworm gut compared to the soil (ANOVA with linear mixed model, $P < 0.001$; Fig. 3a and 3b). Such a result suggested that earthworms could have an underappreciated influence on the abundance and diversity of important ARGs at the continental scale.

Potential mechanisms behind changes in ARGs in the earthworm gut

We found a strong positive correlation between bacterial community composition and ARGs in both soils and earthworm guts (Procrustes $m^2 = 0.549$, $P < 0.001$; Mantel $r = 0.177$, $P = 0.001$; Additional file 15: Fig. S9), suggesting that changes to bacterial communities do influence the abundance and diversity of ARGs [24, 30]. There was a strong correlation between abundance and diversity of bacteria in soils and earthworm guts, and with those of ARGs (linear regression, $P < 0.001$; Fig. 4c and 4d). Partial redundancy analysis also showed that the dominant bacterial phylotypes present in earthworms could predict variation in gut ARGs (32.9%). These observations could not be explained by soil properties (bacterial community or physicochemical properties) or by gut properties (pH and total C) (Fig. 3c), suggesting that the link between bacterial communities and ARGs was not caused by co-variations between ARGs and bacteria with other important environmental factors.

We further examined the dominant bacterial phylotypes from soils to identify those that were largely reduced within earthworm guts. We focused on dominant bacterial phylotypes with an average relative abundance of more than 1%, and that were detected in at least 60% of earthworm and soil samples. We identified twenty-two dominant bacterial phylotypes (total read abundance of > 3.3%) (Fig. 3d) which were significantly positively associated with the abundance of at least 10 ARGs ($P < 0.05$); but were largely depleted within the earthworm gut compared with soils (paired-samples t-test, $P < 0.05$; Fig. 3d). These results suggest that reduction in abundance of selected bacterial phylotypes in the earthworm gut could be the driver behind reduction in ARGs, because these bacterial phylotypes are commonly the host of ARGs [17, 44].

Finally, our results also showed that the abundance of mobile genetic elements (MGEs) was significantly correlated with ARGs in both soils and earthworm gut contents. A significantly lower abundance of

mobile genetic elements (MGEs) was detected in earthworm gut contents compared to soil ($P < 0.05$; Additional file 16: Fig. S10). This suggests that the potential for horizontal transfer of ARGs in earthworm guts was lower than that in soils. This could also be a reason that lower ARGs in earthworm guts compared to soils.

Role of Earthworm in Mitigating ARGs in the Soil Ecosystem

Earthworm casts could provide a direct analysis of the effect that earthworm digestion has on the soil resistome, however they were very difficult to collect in the field. Thus, we used earthworm gut contents to examine the role of earthworms in mitigating ARGs in soil ecosystems. To evaluate the validity of this approach, we conducted a culturing experiment using three earthworm ecotypes. The abundance and diversity of ARGs in the earthworm gut contents were higher than those in the earthworm casts, and both were significantly lower than the soil ($P < 0.05$; Additional file 5: Table S5). This indicated that using earthworm gut contents instead of casts could actually underestimate the role of earthworms in mitigating ARG abundance. Therefore, the survey suggests that earthworms may play an important role in reducing ARGs in soils.

To provide further support for this finding, we conducted a microcosm experiment, and also collected soil samples from two long-term field experiments with or without earthworms. The results of microcosm experiment showed that reductions in the number and abundance of ARGs were observed in both soil ecosystems upon addition of earthworms (Fig. 5a and 5b and Additional file 17: Fig. S11; $P < 0.001$). Earthworms could remove ARGs associated with manures (Fig. 5c, 5d and 5e), and some dominant bacterial phylotypes were significantly reduced in soils amended with earthworms ($P < 0.001$). The two long-term field experiments also indicated that the presence of earthworms significantly reduced the diversity and abundance of ARGs in the soil ($P < 0.05$; Additional file 6: Table S6). These results further support the importance of earthworms in regulating the abundance and diversity of ARGs in the soil system, and together with the microcosm experiment, suggest that reduction in ARGs could be the result of removing those bacterial taxa that carry ARGs. To confirm this assumption, we compared bacterial diversity and abundance between earthworm gut contents and soils across all our sampling locations. Earthworm gut contents had lower diversity and abundance of bacteria than surrounding soils (paired-samples t-test, $P < 0.001$; Fig. 4), supporting the idea that reduction in the diversity and abundance of bacterial taxa in earthworm guts might be the driver behind change in the abundance and diversity of ARGs. This reduction could be the consequence of elimination of some bacterial taxa by competition and digestion [5]. Earthworms have a set of endemic bacterial species in their guts, probably mostly associated with the gut epithelium [14], and these bacteria could outcompete those acquired via feeding on soil, which are not adapted to the harsh conditions within the gut. Dominant bacteria from soil might not survive in such conditions. This is a potential mechanism for the lower abundance of ARGs in the earthworm gut compared to the soil, but would require barriers to horizontal gene transfer between soil and earthworm gut bacteria. The combined results of the field and microcosm studies suggest that earthworms may play a significant role in reducing ARGs in soils. Although earthworms have a potential ability to mitigate ARGs in soils, we still see accumulation of ARGs at the global scale. This is probably

driven by the enormous number of ARGs that are released by human activity every day [16, 17, 28], and ARGs probably accumulate faster than they can be removed under natural conditions.

Conclusions

Together, our work characterizes the earthworm gut microbiome and antibiotic resistome at the continental scale, and suggests that earthworms may play a key role in helping to reduce the abundance of ARGs in natural and agricultural ecosystems. Lower diversity was observed in the earthworm gut microbiome compared with soils, and the habitat had a more important influence on earthworm gut microbiome compared to the species. The earthworm gut always had a lower abundance and diversity of ARGs compared to their corresponding soils. We further identified a total of twenty-one dominant bacterial taxa, all positively associated with ARGs, which were selectively depleted within the earthworm gut. Reductions in the number and abundance of ARGs were observed in both soil ecosystems upon addition of earthworms. Given that earthworms are globally distributed, understanding earthworm gut processes could allow us to manipulate earthworms to regulate ARGs in the future.

Declarations

Acknowledgements

We are grateful to other members of the Yong-Guan Zhu laboratory for their help in collecting samples and Prof. Manqiang Liu and Dr. Di Wu for supplying soil samples from long-term field experiments.

Funding

This work was funded by the National Natural Science Foundation of China (41571130063), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB15020302 and XDB15020402) and the National Key Research and Development Program of China-International collaborative project from Ministry of Science and Technology (Grant No. 2017YFE0107300). M.D-B. acknowledges support from the Marie Sklodowska-Curie Actions of the Horizon 2020 Framework Program H2020-MSCA-IF-2016 under REA grant agreement n° 702057.

Author contributions: Conceived and designed the experiments: DZ and YGZ. Performed the experiments: DZ, JD and HL. Analyzed the data and prepared the figures: DZ, MDB and YGZ. Wrote the paper: DZ, MDB, JQS, MRG, JP and YGZ. Reviewed and commented on the paper: MDB, JP, MRG, JQS and YGZ. Contributed reagents/materials: DZ, JD, HL, JQS and YGZ. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All sequence data have been deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA) database under the SRA accession number SRP140455. The authors declare that the other main data supporting the findings of this study are available within this Article and in the Additional files. Extra data supporting the findings of this study are available from the corresponding author upon reasonable request. Custom codes for all analyses are available from the corresponding authors upon request.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

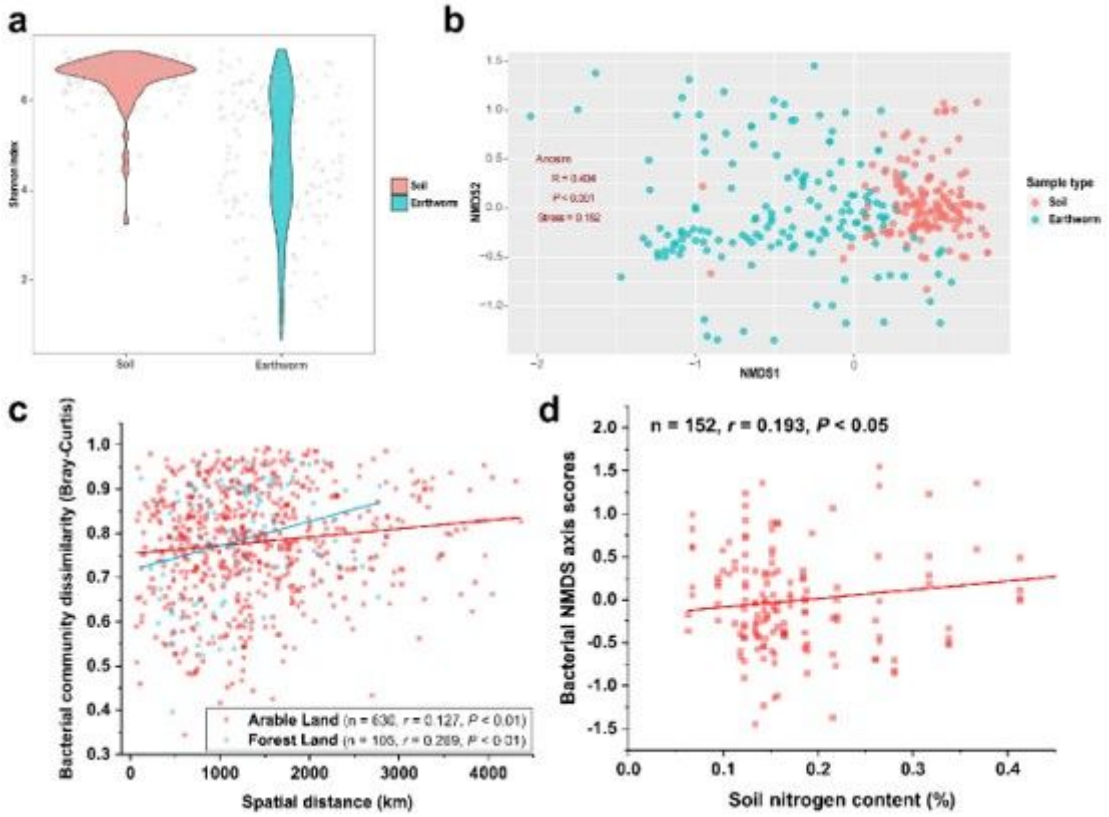

Figure 1

Earthworm gut microbiome at the continental scale. a) Violin plot showing alpha diversity (Shannon index) of soil (n = 152) and earthworm gut (n = 152) bacterial communities, and each gray point representing one sample. A linear mixed model was used to test for differences in diversity between soil and earthworm gut, with land use and sampling site as random factors (Significant level: P = 0.05). The

diversity of soil bacterial community was significantly higher than that of earthworm gut (P < 0.001). b) Nonmetric multidimensional scaling analysis (NMDS) depicting the differences in bacterial community composition between soil and earthworm gut. c) Relationships (linear regression) between earthworm gut bacterial community dissimilarity and spatial distance for samples of arable land and forest land. The slopes were significantly different in two land uses. d) Relationship (linear regression) between earthworm gut bacterial community similarity and soil nitrogen content.

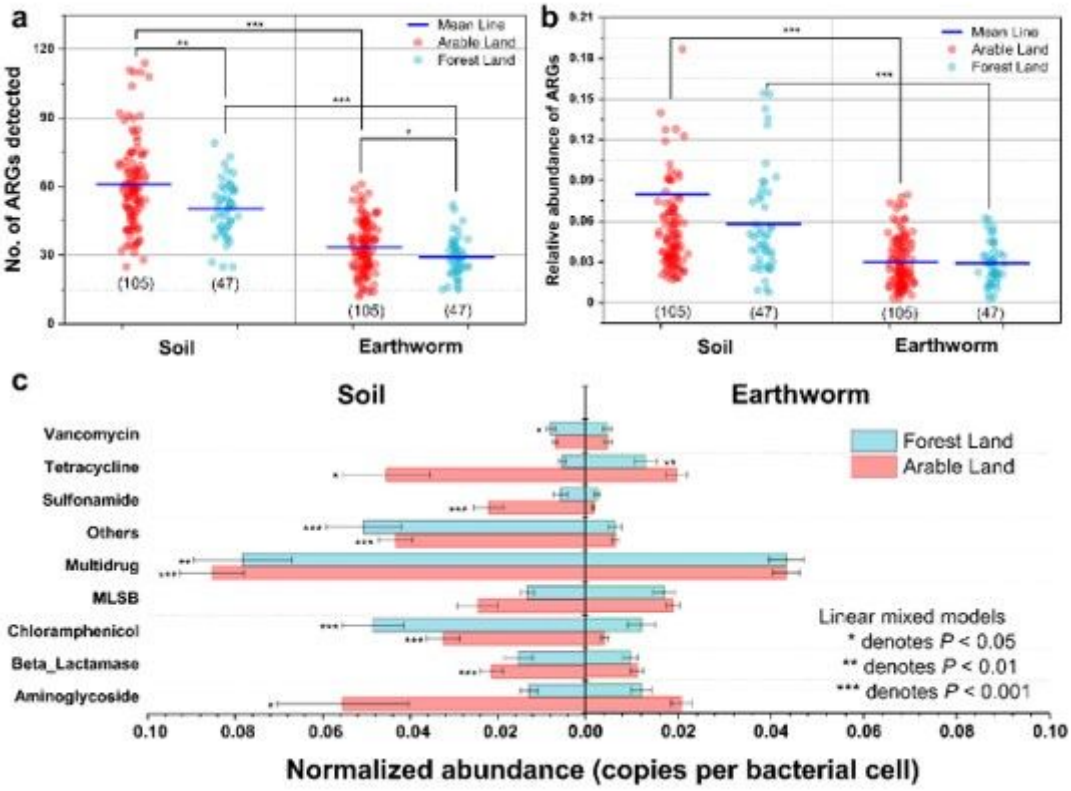

Figure 2

Continental-scale ARG profiles of soil and earthworm samples from forested (n = 47) and arable (n = 105) land. (a) Number of different antibiotic resistance genes detected in each sample; (b) Relative abundance of antibiotic resistance genes in each sample; (c) Comparison of the abundance (mean ± SE) of resistance gene types per bacterial cell between soil and earthworm gut microbiota, classified by the antibiotic class to which they conferred resistance. Linear mixed models were used to test the difference of ARGs between different treatments (Significant level: P = 0.05).

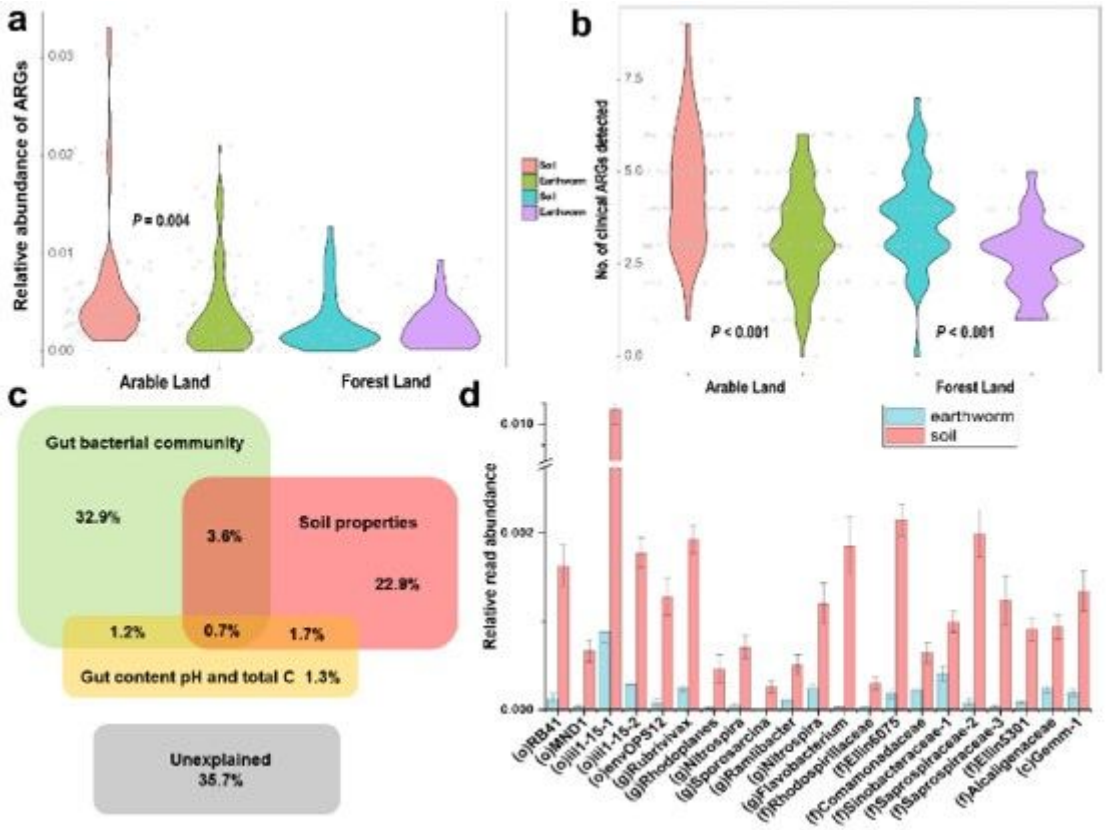

Figure 3

Contribution of bacterial communities in soils and earthworm guts to explain the continental-scale distribution of multiple ARGs. Violin plots presenting abundance (a) and number (b) of clinically relevant ARGs detected in each sample (arable land: n = 105; forest land: n = 47). Linear mixed models were used to test the differences in ARGs between different treatments (Significance level: P = 0.05). Each gray point represents one sample. c) Partial redundancy analysis identifying the percentage of variation in ARG profiles explained by earthworm gut bacterial communities, soil properties (bacterial community and physical and chemical properties) and gut content properties (pH and total C); d) Differences in the relative read abundance (n = 152, mean ±SE) of dominant bacterial phylotypes, significantly associated with the abundance of ARGs, for the earthworm gut vs. soil (P < 0.05).

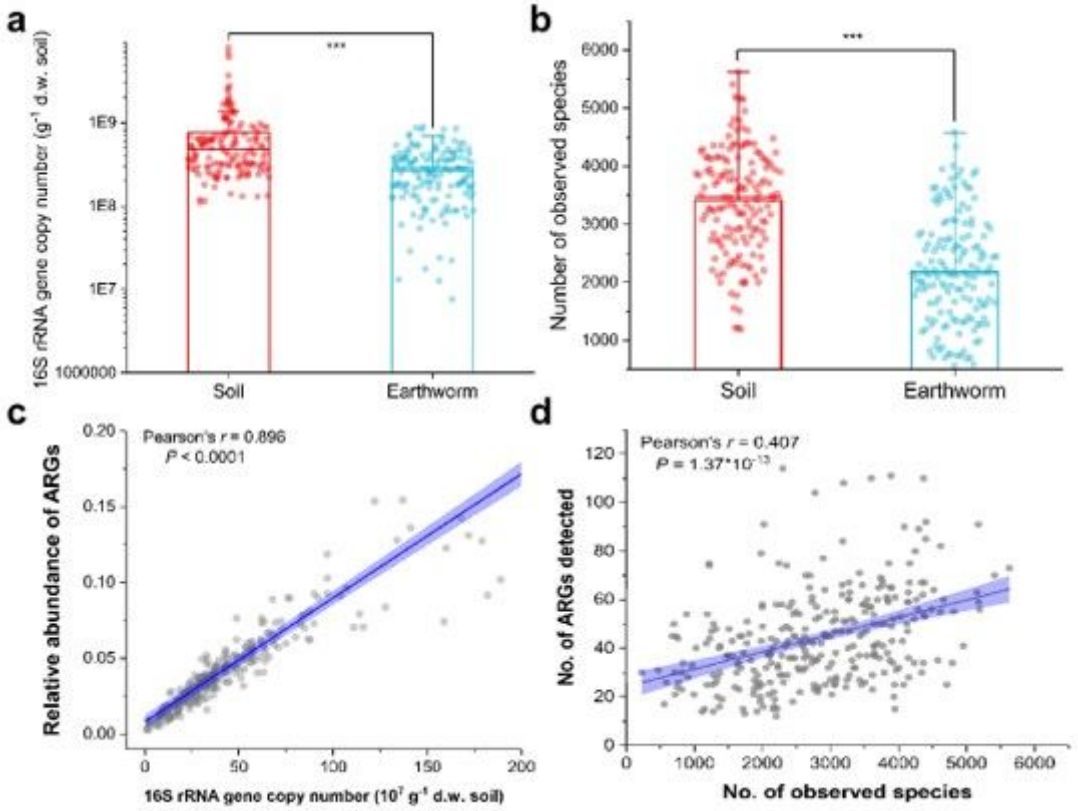

Figure 4

Bacterial abundance and diversity in soil and earthworm guts, and linear relationships between bacterial and ARG attributes. (a) Continental -scale bacterial abundance (a) and diversity (b: number of observed species per sample) in soil and earthworm gut (n = 152, mean ± SE; similar sample size (0.5 g) for soil and earthworm gut content). Each point represents one sample. The paired-sample t-test was used to test the difference in bacterial abundance and diversity (Significance level: P = 0.05). (c) Linear regression between bacterial abundance and total abundance of ARGs; and (d) Linear regression between bacterial diversity and ARG diversity. Each gray point represents one sample (n = 304). The shaded area in blue represents a 95% confidence interval.

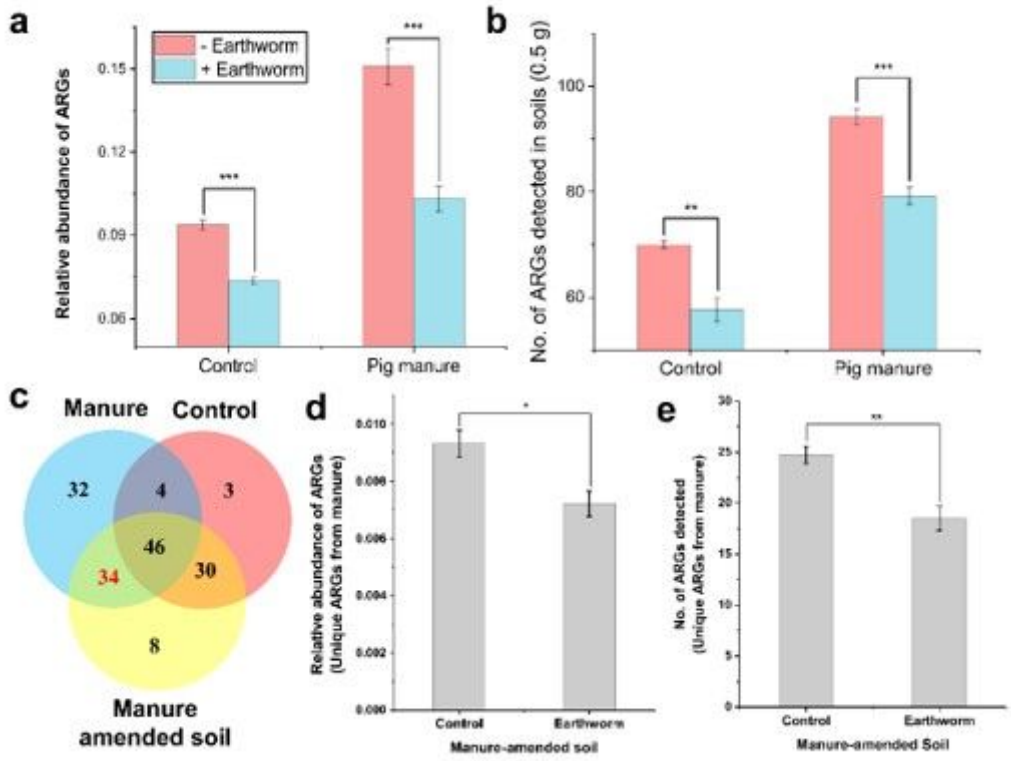

Figure 5

The effects of earthworms on (a) abundance and (b) number of ARGs detected in microcosm experiments, including soils with or without pig manure amendment (n = 4, mean ± SE). c) Venn diagram showing unique and shared ARGs among manure, controlled soil and manure amended soil. We identified 34 ARGs which were shared between manure and manure amended soil but were not found in the control soil. The effects of earthworms on (d) abundance and (e) number of these ARGs detected in microcosm experiments, including soils with or without pig manure amendment (n = 4, mean ± SE). Independent-sample t-test was used to test the difference in ARGs between different samples (Significant level: P = 0.05).

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