Gut microbial communities and their potential roles in cellulose digestion and thermal adaptation of earthworms

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**HIGHLIGHTS**

- Gut bacterial richness and diversity of earthworm reduced with increasing latitude.
- Temperature and cellulose drove gut bacterial communities and network complexity.
- Gut bacterial communities and network complexity impacted cellulase activity.
- Gut cellulolytic bacteria was in the central position of co-occurrence network.
- Gut microbiota improved adaptation of earthworms to temperature and food resources.

**GRAPHICAL ABSTRACT**

Adaptations to temperature and food resources, which can be affected by gut microbiota, are two main adaptive strategies allowing soil fauna to survive in their habitats, especially for cold-blooded animals. Earthworms are often referred to as ecosystem engineers because they make up the biggest component of the animal biomass found in the soil. They are considered as an important indicator in the triangle of soil quality, health and functions. However, the roles of gut microbiota in the environmental adaptation of earthworms at a large scale remain obscure. We explored the gut bacterial communities and their functions in the environmental adaptation of two widespread earthworm species (Eisenia nordenskioldi Eisen and Drawida ghilarovi Gates) in Northeast China (1661 km). Based on our findings, the alpha diversity of gut bacterial communities decreased with the increase of latitude, and the gut bacterial community composition was shaped by both mean annual temperature (MAT) and...
1. Introduction

Soil fauna is an important component of terrestrial ecosystems, as they participate in several soil ecosystem functions, such as litter decomposition, nutrient cycling, and energy flow (Blouin et al., 2013). As one of the most important groups of soil fauna, earthworms are widely distributed in different ecosystems and have been increasingly recognized as contributors to ecosystem health and efficient nutrient cycling (Hallam and Hodson, 2020). Successful survival and reproduction generally requires the earthworms to adapt to changing environments alongside climate conditions and food resources. To achieve this, earthworms have developed sophisticated gut systems to digest a variety of foods, predominantly plant matter which is considered to be an important food source for earthworms (Brown et al., 2004a, Brown et al., 2004b). Cellulose is regarded as one of the most basic carbon sources for earthworms to produce energy, carbon dioxide, and water, accounting for 40–50% of the dry weight of plant cell walls (Talbot and Treseder, 2012). The earthworm gut harbors millions of decomposer microbes, providing the host with physiological and ecological advantages in the digestion of cellulose-rich diets (Singleton et al., 2003; Dey et al., 2018; Sapkota et al., 2020). The complex cellulases, produced by gut microbiota, are responsible for breaking down the cellulose contained in the plant matter and converting it into a simple sugar that can be used as a source of energy for both hosts and microbes (Ravindran et al., 2015; Dvorák et al., 2016). Nevertheless, the cellulase enzymatic activities are strongly related to gut microbial community composition, and to the characteristics of substrate ingested by earthworms (Kaiser et al., 2010). Some bacteria in the gut of earthworms, such as Pseudomonas, Bacillus, and Acinetobacter, are characterized by strong cellulase activity and were found to significantly improve the digestion ability of cellulose (Jyotsna et al., 2011; Dey et al., 2018). Hence, exploring the gut microbial community composition and their response to habitat conditions can yield insight into the potential benefits of gut microbiota on the digestion of cellulose-rich foods.

Earthworms are cold-blooded animals as they are unable to maintain constant body temperature and their body temperature fluctuates based on the environment. Moreover, the material ingested by earthworms is usually a mixture of organic matter and soil. When soil particles pass through the gut of earthworms, they may affect the gut microbiota. Thus, earthworm gut microbial communities are significantly influenced by climatic variables and the environmental conditions (such as pH, soil organic carbon (SOC), total nitrogen (TN), and total phosphorus (TP) of the surrounding soil (Thakuria et al., 2010; Zhang et al., 2022). Changes in soil properties directly affect the gut microbiota of earthworms and thereby the assemblage of organisms in the earthworms’ microbiome (Ding et al., 2019). Moreover, interactions between the large numbers of microbes colonizing the earthworm gut play an essential role in regulating microbial their community structure and diversity (Cordero and Datta, 2016). Generally, more diverse and stable communities have higher and more negative correlations than less diverse ones according to network analysis (Barberán et al., 2012; Coyle et al., 2015). Most previous studies were concerned with the impacts of abiotic factors on the gut microbial communities (Thakuria et al., 2010; Wang et al., 2022; Zhang et al., 2022), but rarely focused on between gut microbes, or on potential keystone species in the communities, and whether these interaction patterns changed along an environmental gradients.

Eisenia fetida and Eisenia fetida Gates, are two abundant earthworm species, exhibiting wide distribution patterns (Shekhovtsov et al., 2020). E. fetida is the most widespread earthworm species in Siberia from the lower reaches of the Volga and Don rivers to the Arctic Ocean coast, parts of Kazakhstan, China, Korea, and Mongolia (Vsevolodova-Perel and Bulatova, 2008; Berman and Meshcheryakova, 2013). By contrast, D. ghilarovi invaded southeastern Asia following the collision of the Indian and Asian lithospheric plates in the Tertiary period (Easton, 1981; Anisimov et al., 2015). Both earthworm species have been detected in Northeast China with different topographical regions (Zhang et al., 2020). In these regions, earthworms are exposed to large temperature fluctuations (~3.3 °C-7.78 °C) and food resource variation, which are the major variables that limit earthworm growth and productivity (Fayolle et al., 1997). Gut microbiota may provide several physiological benefits to their hosts, especially in the adaptability to the temperature and food resources. However, we still lack an understanding of how gut microbial composition shifts across different habitats and whether gut microbiota beneficial to promote the adaption to the environment. Therefore, we hypothesize that (1) temperature and cellulose content exert a significant impact on gut microbial community composition, diversity, and network complexity; (2) the changes of gut microbial communities, diversity, and network complexity were associated with gut cellullase activity; and (3) gut microbiota can benefit the adaptation of earthworms to temperature and food resources through influencing cellulose degradation. The study highlights the beneficial effects of gut microbiota on the adaptation of earthworms to habitat conditions, which can provide a better understanding of the co-evolution between soil fauna and microbiota.

2. Materials and methods

2.1. Study sites and sampling

Earthworm species of E. fetida and D. ghilarovi were collected along a transect of forested habitats in Northeast China (Fig. 1 and Table 1). The transect covers latitudes from 41°38’ to 53°88’N and longitudes from 123°01’ to 129°68’E, and was 1660.9 km long. It is in the temperate continental climate zone based on Koppen-Trewartha climate classification system and experiences four distinct seasons including cold, dry, and long winters, and warm but brief summers (Baker et al., 2010). The MAT and MAP data were collected from the WorldClim global climate database (http://www.worldclim.org) for the period 1988–2017 (30-yr). >65% of the rainfall occurs between June and September. Dahurian larch (Larix gmelinii (Rupr.) Kuzen) is the dominant native tree species in this region, playing an essential role in carbon sequestration (Xiao et al., 2020; Dong et al., 2021). Other tree species, such as Pinus koraiensis, L. gmelinii, Picea koraiensis, Abies sp., Quercus mongolica, Betula platyphylla, B. dahurica, Fraxinus mandshurica, Ulmus propinquas, and Populus davidiana can be found in this region. Detailed information about the 11 study sites including name, abbreviation, latitude, longitude, altitude, mean annual temperature (MAT), and
mean annual precipitation (MAP) is outlined in Table 1. Three replicate plots of 10 m × 10 m were selected in each of the study sites along the transect (1660.9 km in length). The distance between plots was >500 m to avoid spatial autocorrelation in topographical variables. In each plot, five quadrats (0.5 m × 0.5 m) were separately chosen in each of the four corners and the center of the plot. Earthworms were collected using a spade and soil hand-sorting. At least five earthworms belonging to E. nordenskioldi or D. ghilarovi were collected from each plot. In this study, the two earthworm species were separated according to our previous studies (Zhang et al., 2020; Zhang et al., 2022). Briefly, the diagnostic features of *E. nordenskioldi* include having purple dorsally and faint yellow ventrally; the body has no stripes; the intersegmental furrows in adult individuals are faint yellow; the clitellum is faint yellow, saddle-shaped in xxvii–xxxii. By contrast, *D. ghilarovi* appears gray or brown in forest, with black or gray pigment dorsally and clitellum ring-shaped on segments X–XV. We defined the litter accumulated in the surface of the soils and plus the humus layer soil as "soil samples" (0–10 cm), because the earthworms are epigeic and mainly feed on the litter layer and humus layer soils as their food resource (Li et al., 2020). The samples from the same plot were mixed to form a composite sample. The soil samples and earthworm samples were placed into sterile ziplock bags, stored on ice at 4 °C, and transported to the laboratory for measurement within 24 h.

2.2. Soil properties

Soil samples were air-dried at room temperature for two weeks, then ground with a mortar and pestle, and passed through a 1-mm sieve. Soil pH and electrical conductivity (EC) were determined with the glass electrode method using a 1 cm conductivity cell dip-type probe (Richards, 1968). Soil total carbon (TC) was measured by dichromate oxidation and titration with ferrous sulfate (Nelson and Sommers, 1982). Total nitrogen (TN) content was determined according to the semi-micro Kjeldahl method (Bremner and Mulvaney, 1982). The nitrate nitrogen (NO<sub>3</sub>–N) and ammonia nitrogen (NH<sub>4</sub>–N) in the soil were assessed with a continuous flow automatic analyzer (Alliance-Futura, France) after filtration (Li et al., 2020). Soils were treated with HF-HClO<sub>4</sub>, and then the total phosphorus (TP) content was measured colorimetrically after wet digestion with HF-HClO<sub>4</sub> (Jackson, 1958). Available phosphorus (AP) was determined by extraction with a buffered alkaline solution of 0.5 M sodium bicarbonate (pH = 8.5). The product was quantified colorimetrically in a spectrophotometer (Hitachi, UV2300) at 660 nm (Page et al., 1982). The cellulose concentration was analyzed by High Performance Liquid Chromatography (HPLC) as described by Sluiter et al. (2008).

2.3. Gut bacterial community analysis using Illumina sequencing

The earthworms (i.e., *E. nordenskioldi* and *D. ghilarovi*) from each study site were surface-sterilized (75 % ethanol), and then rinsed three times in sterile distilled water. The earthworms were then killed by freezing and their whole body was dissected and the contents from the hindgut were collected. Earthworm gut walls were harvested according to Singleton et al. (2003). DNA was extracted from gut wall samples using an EZNA® soil DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.) according to the manufacturer’s protocols. The final DNA concentration and purification were determined with a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA), and DNA quality was checked by 1 % agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with 515F (Richards, 1968).
2.4. Isolation, screening, and identification of cellulolytic bacteria

For further analysis of cellulolytic bacteria, the hindguts of earthworms (n = 3 for each species from each study site) were excised, suspended in sterilized NaCl solution (0.9 %) containing 0.5-mm glass beads, and then mixed for 5 min with a vortex mixer. The cellulolytic bacteria were isolated using serial dilutions and pour plate techniques according to the method of Gupta et al. (2012). Specifically, 1 mL of diluted suspension was inoculated in medium I (NaNO$_3$ 2.5 g L$^{-1}$; KH$_2$PO$_4$ 2 g L$^{-1}$; MgSO$_4$ 0.2 g L$^{-1}$; NaCl 0.2 g L$^{-1}$; CaCl$_2$·6H$_2$O 0.1 g L$^{-1}$; pH 7.0) containing 0.5 % carboxymethylcellulose (CMC) as a sole carbon source. The inoculated flasks were incubated at 37 °C for two weeks.

Cellulolytic bacteria were isolated from earthworm guts using the spread plate technique and checked for cellulolytic activity by the Congo red clearing zone assay method (Teather and Wood, 1982). To identify the species of purified bacterial isolates, we amplified the 16S rDNA (E. nordenskioldi) and 806R primers in a Veriti thermal cycler (Applied Biosystems) using the following program: 95 °C for 3 min; 27 cycles of 30 s at 95 °C, annealing at 55 °C for 30 s, and elongation at 72 °C for 45 s; followed by a final extension at 72 °C for 7 min. PCR reactions were prepared in a 20 μL mixture containing 4 μL of 5 × PCR Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), and 10 ng of DNA template. The PCR products were separated on a 2 % agarose gel and purified using an AxyPrep PCR cleanup kit (Axygen Biosciences, USA) according to the manufacturer’s protocol. Gel-purified PCR products were analyzed on an Illumina Miseq platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (PRJNA717635).

2.5. Total cellulase activity assays

The earthworm gut contents and bacterial strains were cultured in medium I containing 0.5 % carboxymethylcellulose (CMC) as a sole carbon source to stimulate the induction of cellulolytic enzyme at 37 °C with shaking at 150 rpm for 48 h, separately. The culture broth was subjected to centrifugation at 4500 rpm for 10 min at 4 °C. The supernatant was collected and used as a crude enzyme for the determination of cellulase activity.

The total cellulase activities of isolated bacteria and earthworm gut contents were measured by the amount of reducing sugar using 3, 5-dinitrosalicylic acid (DNS) (Miller, 1959). The total cellulolytic activities of 0.5 mL of isolated bacteria and gut contents were tested by mixing 1 % CMC (Sigma) in a 100 mM sodium citrate buffer (pH = 4.5). Samples were incubated at different temperatures ranging from 10 °C to 60 °C for 30 min. Reactions were stopped by the addition of 2 mL DNS reagent and the samples were heated in boiling water for 10 min to allow color development. The amount of reducing sugar released in the supernatant was determined by measuring absorbance at 540 nm using a CMC control sample as a blank. The amount of released reducing sugars was determined using a standard curve recorded for glucose. One unit of total cellulase was defined as the amount of enzyme that produces 1 μmol glucose equivalent per min under the assay conditions.

2.6. Gut microbiota transplantation

This experiment was conducted to test the potential effects of gut bacterial microbiota on the degradation of food resources with different cellulose concentrations under low and high reaction temperatures. The gut contents of E. nordenskioldi (n = 3) and D. ghilarovi (n = 3) from S2 (NW) and S10 (SJJW) sites, respectively, were collected according to the method described in Section 2.3. The gut contents were then cultured in medium I at 20 °C with shaking at 150 rpm for five days, and the suspension filtered through a 5.0 μm pore size syringe filter (Millipore) was used as the donor gut bacterial microbiota. The corresponding soil samples (high-cellulose food at S2 and low-cellulose food at S10) were sterilized using radiation (γ-ray, 60Co, dose = 50 kGy) to effectively eliminate all microbes and their propagules (Buchan et al., 2012).

Earthworm microbiota transplantation was performed as previously described (Smits et al., 2013; Kim et al., 2021), with minor modifications. Fresh antibiotics (100 U/mL penicillin, 100 μg/mL neomycin, 100 μg/mL metronidazole, 100 μg/mL streptomycin, and 50 μg/mL vancomycin) were mixed with sterile water and then added into the pot containing sterilized soil samples and earthworms every two days to maintain the field capacity of 70 %. The use of mixed antibiotics prior to transplantation can deleteriously affect soil microbial activity more effectively than single antibiotic. Briefly, streptomycin, neomycin, and streptomycin are effective against a large number of both gram-positive and gram-negative bacteria, vancomycin mainly targets gram-positive bacteria, and metronidazole mainly targets anaerobic bacteria. After ten days of antibiotic treatment, the antibiotic-containing water was replaced with sterilized water. The gut microbiota from donor earthworms was transplanted by adding the suspension of gut microbiota to pots, and the acceptor earthworms were co-cultured with the donor earthworms in the donors’ pots for five days. Subsequently, earthworms were divided into three groups: gut microbiota-depleted earthworms (GMDE, the earthworms were treated with mixed antibiotics), untreated earthworms (UTE, the earthworms were treated with the same volume of sterilized water), and gut microbiota-transplanted earthworms (GMTTE, transplantation of germ-free earthworm with microbiota from the other earthworm species).

Table 1

<table>
<thead>
<tr>
<th>Study sites</th>
<th>Abbreviation</th>
<th>Latitude (°)</th>
<th>Longitude (°)</th>
<th>Altitude (m)</th>
<th>MAT (°C)</th>
<th>MAP (cm)</th>
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<tbody>
<tr>
<td>HZ (Huizhou)</td>
<td>S1</td>
<td>51.88</td>
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<td>543</td>
<td>0.96</td>
<td>44.59</td>
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<tr>
<td>WYL (Wuyiling)</td>
<td>S4</td>
<td>48.72</td>
<td>129.68</td>
<td>400</td>
<td>1.12</td>
<td>50.32</td>
</tr>
<tr>
<td>FL (Fenglin)</td>
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<td>129.22</td>
<td>391</td>
<td>0.68</td>
<td>51.95</td>
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<tr>
<td>LS (Liangshui)</td>
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<td>128.88</td>
<td>457</td>
<td>1.78</td>
<td>51.75</td>
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<td>DQH (Danqinhe)</td>
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<td>337</td>
<td>1.77</td>
<td>51.60</td>
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<td>FZ (Fanzheng)</td>
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<td>129.07</td>
<td>515</td>
<td>3.70</td>
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<td>JHH (Haangihe)</td>
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<td>127.93</td>
<td>722</td>
<td>3.16</td>
<td>54.77</td>
</tr>
<tr>
<td>SJJW (Sanjiangongwan)</td>
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<td>126.43</td>
<td>783</td>
<td>4.36</td>
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<td>BX (Benxi)</td>
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<td>811</td>
<td>7.78</td>
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</table>

MAT, mean annual temperature; MAP, mean annual precipitation.
The experiment is a $2 \times 2 \times 3$ completely random design with two types of food resources (high-cellulose food, HCF; and low-cellulose food, LCF), two levels of temperatures (low temperature of $10 \, ^\circ C$, LT; and high temperature of $35 \, ^\circ C$, HT), and three groups of earthworms (GMDE, UTE and GMTE). Each treatment had three replicates for a total of 36 pots (10 cm in height and 7 cm in diameter). Three E. nordenskioldi or D. ghillarovi from different groups were transferred into pots filled with $\gamma$-ray sterilized soil samples. The pots were well watered (70 % field capacity) during the whole experimental period. After 2 weeks, the pots were collected to measure the reduction of cellulose concentrations in the different treatments.

2.7. Statistical analysis

We firstly checked normality and homogeneity of variance using Kolmogorov-Smirnov test and Levene’s test in SPSS 21 (SPSS Inc., Chicago, IL, USA), respectively. Then, one-way ANOVA followed by Duncan’s post hoc test was performed to determine the differences in climate variables (MAT and MAP), soil properties (pH, TC, TN, NO$_3$-N, NH$_4$-N, TP, AP, Cellulose), cellulase activity, and $\alpha$-diversity (Sobs index, Chao1 index and Shannon index) of gut bacterial communities among different study sites, and also the cellulose degradation among different treatments in the gut microbiota transplantation experiment.

To obtain information about the taxonomic classification of the gut bacterial microbiota and relative abundance of taxa, we firstly quality-filtered the raw fastq files of gut microbiota using QIIME2 according to Caporaso et al. (2010). Sequences were then chimera-checked based on UCHIME to get the effective tags, and clustered into operational taxonomic units (OTUs) with a similarity threshold of 97 % using UPARSE algorithms (version 7.0.1090). The taxonomic identification was performed by the SILVA 128 database with a confidence threshold of 70 %.

The filtered sequences were normalized to 25,720 per sample to carry out the downstream analyses at the same sequencing depth. The rarefaction curves of all data sets were constructed to evaluate the sequencing depth and the bacterial communities based on OTUs defined at 97 % similarity (Fig. S1).

The $\alpha$-diversity (Sobs index, Chao1 index and Shannon index) of bacterial communities in the guts of earthworms among different study sites was calculated in R software (version 3.6.2) using the vegan package (Oksanen et al., 2013).

To test the hypothesis that the gut bacterial communities were driven by MAT and cellulose concentration, we conducted a Bray-Curtis based redundancy analysis (db-RDA) and the significance effects of MAT and cellulose on gut bacterial community composition were tested with Monte Carlo permutations (permutation = 999). Furthermore, the relationships between bacterial community dissimilarity (Bray-Curtis) and the dissimilarity (Euclidean) of MAT and cellulose were tested using Spearman’s linear regression.

To find out the most significant differences in taxa of gut bacteria, we performed an inter-group difference analysis using STAMP software with a two-sided Welch’s $t$-test and correction by Benjamini-Hochberg FDR (Lumley et al., 2001).

To decipher the composition and assembly of complex gut bacterial communities, predict potential interactions, and determine the core taxa driving interactions among gut microbiota, we conducted a co-occurrence network analysis. Briefly, the correlation (R matrix) and significance matrices ($P$ matrix) were calculated using the WGCNA package in R software (version 3.6.2), and only strong correlations (Spearman’s rank correlation coefficient, $r > 0.9$ or $r < -0.9$) were selected for co-occurrence network analysis (Barberan et al., 2015). The constructed correlation matrix was transformed into Gephi software (version 0.9.3) to generate valid co-occurrence networks. The relationships between core taxa selected based on the co-occurrence network and environmental variables were determined by the Mantel test. Besides, random forest modeling was employed to identify the most important contributors (gut bacterial phyla) to cellulase activity in R using the randomForest package (Liaw and Wiener, 2002).

3. Results

3.1. Soil properties

The selected soil properties at different study sites were assessed as shown in Fig. 2. Both pH and TC were significantly reduced in S10 and S11 soils ($p < 0.05$). The highest TC was found in S6 soils, while there were no differences in TN, NH$_4$-N, TP, and AP among study sites ($p > 0.05$). Soil pH was negatively correlated with MAT ($r = -0.721, p < 0.001$), MAP ($r = -0.721, p < 0.001$), and NO$_3$-N ($r = -0.344, p = 0.050$), while TC and cellulose had significantly negative correlations with MAT ($r = -0.391, p = 0.024$) and MAP ($r = -0.469, p = 0.006$) (Fig. S2). Moreover, there were significantly positive correlations between MAP and NO$_3$-N ($r = 0.418, p = 0.015$), pH and cellulose ($r = 0.474, p = 0.005$). Additionally, cellulase activity was significantly correlated with MAT, $\alpha$-diversity (Sobs index, Chao1 index and Shannon index), community composition, and network complexity of earthworm gut bacterial microbiota ($p < 0.01$) (Fig. S3).

3.2. Gut bacterial community diversity and composition

A total of 36 gut samples of two earthworm species were collected from 11 national nature reserves of northeast China. To evaluate the quality of the sequencing results, the raw sequences, raw tags, average length, sequencing error rate and effective tag percentage of the samples were analyzed. In the present study, raw paired-end (PE), effective tags, average length, sequence error rate and effective tag percentage of the samples were analyzed. In the present study, raw paired-end (PE), effective tags, average length, sequence error rate and effective tag percentage of the samples were analyzed.

![Fig. 2. Soil chemical properties of the study sites. TC, total carbon; TN, total nitrogen; TP, total phosphorus; AP, available phosphorus; NH$_4$-N, ammonium nitrogen; NO$_3$-N, nitrate nitrogen.](image-url)
In addition, the gut bacterial Shannon index of *D. ghilarovi* was 1.08 times higher than that in *E. nordenskioldi*, but no differences in gut bacterial Sobs ($p = 0.684$) and Chao1 indices ($p = 0.810$) were found between two earthworm species (Fig. 3 g, h, and i).

The OTUs of the samples from the different study sites were further assigned to different taxa and their relative taxonomic abundance was evaluated. In addition to some unknown groups, 62 bacterial classes were grouped into 29 phyla, while eight phyla (Proteobacteria, Actinobacteria, Firmicutes, Verrucomicrobia, Chloroflexi, Planctomycetes, Acidobacteria, Verrucomicrobia) together accounted for >90% of the relative abundance in each study site. Actinobacteria were the most abundant at a class level (mean relative abundance of 28.96%), followed by Gammaproteobacteria (14.18%) and Alphaproteobacteria (12.56%) in gut samples of *E. nordenskioldi* (Fig. 4a). On the contrary, Actinobacteria were the most abundant phylum (52.94%), followed by Alphaproteobacteria (10.00%) and Cytophagia (6.81%) in gut samples of *D. ghilarovi* (Fig. 4b). Furthermore, gut bacterial abundance varied at different study sites and many taxa were significantly correlated with latitude. Among the top10 phyla, the relative abundances of Actinobacteria ($r = 0.768, p < 0.001$), Chloroflexi ($r = 0.644, p < 0.001$), Bacteroidetes ($r = 0.660, p < 0.001$) and Saccharibacteria ($r = 0.378, p = 0.013$) had negative correlation with latitude, while the relative abundances of Proteobacteria ($r = 0.861, p < 0.001$), Firmicutes ($r = 0.533, p < 0.001$), Verrucomicrobia ($r = 0.341, p = 0.027$), and Acidobacteria ($r = 0.346, p = 0.025$) were positively correlated with latitude. Therefore, the gut bacterial abundance of earthworms was closely related to latitude.

### 3.3. Main factors driving the gut bacterial communities

Distance-based redundancy analysis (db-RDA) was used to measure the effect of environmental variables on gut bacterial community composition along the latitudinal gradient (Fig. 4c, d and e). MAT and cellulose showed a strong association with gut bacterial communities in both *E. nordenskioldi* and *D. ghilarovi*. These two variables explained 27.26% and 15.43% of the dissimilarities between the gut bacterial communities in *E. nordenskioldi* and *D. ghilarovi* at phylum level, respectively. The Bray-Curtis dissimilarity matrices demonstrated clear
clustering of gut bacterial communities for each earthworm species, with more variability detected in *E. nordenskioldi* (Fig. 4e). Gut bacteria of *D. ghilarovi* mainly gathered in the second quadrant, which were obviously positively correlated with cellulose, while the gut bacterial communities of *E. nordenskioldi* were distributed in almost all quadrants, which were mainly restricted by MAT. The Mantel test revealed that MAT (mantel $r = 0.449$, mantel $p < 0.001$), and cellulose (mantel $r = 0.144$, mantel $p = 0.013$) played vital roles in shaping the gut bacterial communities in both *D. ghilarovi* and *E. nordenskioldi* (Fig. 4f, and g). Together, these results clearly suggested that both MAT and cellulose were main environmental factors driving the gut bacterial communities of two earthworm species.

### 3.4. Core bacterial phyla contribute to the gut cellulase activity

Generally, microbial species that co-occur across study sites or perform important functions (as connectors/hubs) in the network are deemed core species. In this study, the phylum with high abundance (in the top 0.1%), high occurrence frequency (>80%), high degree and low betweenness centrality values were therefore considered as core phyla (Actinobacteria, Proteobacteria, Firmicutes, and Planctomycetes) (Fig. 5a, and b). They were also the most dominant β-glucosidase-producing microbes, playing a crucial role in the degradation of hemicellulose, cellulose, and lignin in various ecosystems. Furthermore, the Mantel test showed that MAT and cellulose exhibited the strongest effects on the communities of Actinobacteria, Proteobacteria, Firmicutes, and Planctomycetes (Fig. 5a, and b). They were also the most dominant β-glucosidase-producing microbes, playing a crucial role in the degradation of hemicellulose, cellulose, and lignin in various ecosystems. The total cellulase activity in earthworm gut had significantly negative correlation with cellulose ($r = 0.759$, $p < 0.001$) (Fig. 5d), while Actinobacteria, Firmicutes, Planctomycetes, and Bacteroidetes exhibited considerable contributions to the cellulase activity based on the random
Fig. 5. Co-occurrence networks of gut bacterial communities of *E. nordenskioldi* (a) and *D. ghilarovi* (b) are based on Spearman’s correlation analysis. The networks are colored based on gut bacterial phyla. A connection indicates a strong and significant correlation. The size of each node is proportional to the relative abundance of OTUs, and the thickness of a connection between two nodes (i.e., an edge) is proportional to the value of the Spearman correlation coefficient. Circle size represents the variable’s importance. The relationships between the communities of cellulose-degrading phyla and environmental variables based on the mantel test model (c). The relationship between cellulose activity and total cellulose activity is based on linear correlation (d). The contribution of gut bacterial phyla to total cellulose activity based on random forest model (e). TC, total carbon; TN, total nitrogen; TP, total phosphorus; AP, available phosphorus; NH$_4^+$-N, ammonium nitrogen; NO$_3^-$-N, nitrate nitrogen; MAT, mean annual temperature; MAP, mean annual precipitation.
forest model (Fig. 5e).

3.5. Isolation of cellulase-producing bacteria from earthworm gut

To compare the adaptability of cellulase enzymes in the guts of *E. nordenskioldi* and *D. ghilarovi* to temperature and food resources, we screened cellulase-producing bacteria with the highest degradation rate of Congo red in a solid medium with CMC. Finally, a representative subgroup of 14 isolates, representing the community of cellulolytic bacteria obtained in earthworm gut, was analyzed by 16S rRNA sequencing. Most of the isolates were identified as members of the phyla Proteobacteria (7 isolates), Actinobacteria (2 isolates), Firmicutes (2 isolates), and Bacteroidetes (3 isolates). Six isolates from gut of *E. nordenskioldi* were affiliated with Proteobacteria and Actinobacteria, while the other two isolates were grouped with Firmicutes and Bacteroidetes. The Proteobacteria and Bacteroidetes were the dominant groups, with the Proteobacteria accounting for 50% of relative abundance of cellulolytic bacteria. The gut isolates belonged to the genera *Pseudomonas*, *Enterobacter*, *Xanthomonas*, *Sphingomonas*, *Bacillus*, *Cellulosimicrobium*, *Mycobacterium*, *Sporocytophaga*, *Cytophaga*, and

![Figure 6](image-url)

**Fig. 6.** The profile of cellulase activity in earthworm gut to temperature and substrate. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of isolates (a). The sequences identified from the gut of *D. ghilarovi* (DG) and *E. nordenskioldi* (EN) are shown in bold letters. Sequences of reference strains obtained from GenBank DNA databases are indicated in italic and accession numbers are given in parentheses. Cellulolytic bacterial isolates outlined in the top, middle, and bottom groups belong to the phylum Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes, respectively. The scale represents 0.02 substitutions per nucleotide position. Bootstrap values are based on 1000 replicates. The influence of temperature on total cellulase activity of gut content (b) and bacterial isolates (c) in *D. ghilarovi* and *E. nordenskioldi*. The cellulose degradation among different treatments (d and e). Different letters indicate that there is a significant difference between parameters (p < 0.05).
Flavobacterium (Fig. 6a). All isolates obtained from the medium had the ability to degrade cellulose as determined by the Congo Red test.

3.6. Cellulose degradation of gut microbiota depends on temperature and cellulose

The temperature profile of total cellulase activity showed that the cellulose substrate (CMC) as a carbon source could be utilized by the isolated bacterial strains and gut microbiota in the tested temperature range (Fig. 6b and c). The optimal temperature of cellulase was around 25–30 °C for *E. nordenskioldi* and 40 °C for *D. ghilarovi*, indicating that gut bacteria of *E. nordenskioldi* maintained relatively high cellulase activity under low temperature compared with *D. ghilarovi* (Fig. 6b and c).

To explore the beneficial effects of gut bacteria on the adaptation of earthworm to temperature and food resources, we conducted a gut microbiota transplantation (Fig. 6d and e). The results showed that gut microbiota-depleted (GMD) earthworms had the lowest cellulose degradation rate (1.07%) compared with the control (7.94%) and gut microbiota-transplanted earthworms (15.27%) under all treatments. The *E. nordenskioldi* with the original gut microbiota (EN) had 1.84 times higher cellulose degradation rate of high cellulose food than *D. ghilarovi* with the original gut microbiota (DG) under 10 °C treatment, while DG exhibited larger cellulose degradation rate compared with EN under 35 °C treatment. After gut microbiota transplantation, DG was more sensitive to high temperature and had a stronger ability to degrade low cellulose food, while EN became insensitive to temperature and showed a stronger ability to degrade high cellulose food (Fig. 6d and e).

4. Discussion

To our knowledge, this is the first ecological work on *D. ghilarovi* in China, and one of the first that seriously explore its ecological adaptability anywhere in the world, although some ecological works have focused on cold hardiness and issues surrounding the phylogenetic status of different morphs of *D. ghilarovi* and *E. nordenskioldi* (Shekhovtsov et al., 2020; Zhang et al., 2020). No ecological works have addressed the gut microbiota of either of these species, or the potential links of gut microbiota to their environmental adaptations. In this regard, the current study was conducted to compare the gut bacterial communities between two earthworm species with different latitudinal distribution patterns, explore the key environmental factors shaping the composition and structure of the gut bacterial communities, and determine the potential role of the gut bacterial microbiota in the adaptation of earthworms to temperature and food resources. Therefore, insights gained from this study should allow a better understanding of how these species representing the most cold-tolerant and/or most northerly distributed members of their respective taxonomic and phylogenetic families, are capable of living in what must be considered extreme environments for earthworms.

In eleven natural forest sites, spanning a latitudinal gradient from 41.38 to 51.88°N and 1660.9 km in length, the gut bacterial richness (Sobs index and Chao1 index) and alpha-diversity (Shannon index) of *E. nordenskioldi* were significantly higher than that of *D. ghilarovi* (Fig. 3i). Among the environmental factors analyzed according to db-RDA and mantel test, MAT and cellulose were dominant factors to explain the variations in the gut bacterial communities of both *D. ghilarovi* and *E. nordenskioldi* (Fig. 4c, d, e, f, and g). MAT is accepted as a key factor of biodiversity due to its substantial influence on soil properties and plant growth, which subsequently impact the quantity and quality of organisms’ food resources (Zhang and Wang, 2015). On the other hand, MAT could regulate the bacterial assemblage in the litter because microorganisms are generally sensitive to climate conditions, leading to a variation in bacterial species ingested by earthworms during their daily feeding behavior. Remarkably, our study indicated that gut bacterial communities could largely be predicted by cellulose content of potential food resources (Fig. 4c, d, e, and g). Cellulose is a dominant plant cell wall component, comprising 20–30% of the dry weight of the primary wall and accounting for 40–90% of the dry weight of the secondary wall (Klemm et al., 2005; Keegstra, 2010). As earthworms mainly feed on soil and plant remains, cellulose comprises an important dietary component. After ingestion by earthworms, food is then ground to a pulp in their gizzard, broken down, and digested by their gut bacteria (Zhang et al., 2013). To take up nutrients effectively, earthworms might establish symbiotic associations with specialized bacteria living within their gut to help digest cellulose. The decomposition of cellulose within the gut can provide carbon energy for gut microorganisms, which in turn affect the various metabolic processes and the performance of their hosts.

Knapp et al. (2009) and Thakuria et al. (2010) suggested that the gut microflora was mainly soil- or food-derived. Different food resources not only varied in their chemical composition, but also differed in the attached microbiota (Krišttauk et al., 1992). The variation in litter quantity, quality, and physiological traits could generate a strong selection pressure to maintain a distinct bacterial community structure in the gut of earthworms. In the present work, we found that the cellulose concentration changed across the latitudinal gradient, and there were significantly higher cellulose concentrations in the habitat of *E. nordenskioldi* compared with that of *D. ghilarovi* (Fig. 4f). Cellulose concentration of food resources is presumably a key environmental factor influencing the adaptability of earthworms to their habitat.

To explore the possible links between gut bacterial communities and cellulose concentration in habitats, we conducted a multi-group comparison of gut bacteria based on relative abundance among study sites using the Kruskal-Wallis test. We found that the family of gut bacteria most different between *E. nordenskioldi* and *D. ghilarovi* was the Cytophagaceae (Fig. S4a and S4b). The positive correlation between the abundance of Cytophagaceae and cellulose indicated that members of this family prefer environments with higher availability of cellulose (Fig. S4c and S4d). Cytophagaceae is one of the largest families in the phylum Bacteroidetes and its members have long been known as efficient cellulose metabolizers (Taulier et al., 2018). We additionally defined high-cellulose and low-cellulose bacterial clusters in the gut of earthworms based on the positive and negative correlation between the abundance of bacterial families and cellulose concentration (*r* > 0.6 or *r* < 0.6), respectively. The relative abundance of high-cellulose clusters increased with the increase of latitude and cellulose (*Fig. S4e* and *S4f*), which is consistent with our predicted environmental preferences of gut bacteria. Cytophagaceae, Svat0725, and Comamonadaceae can generate chitinase, cellulases, hemicellulases, and lignin-modifying enzymes (Chen et al., 2017; Dahal and Kim, 2018; Budroni et al., 2020), were abundant in high-cellulose regions, while Parachlamydiaceae, KD4-96, and Halomonadaceae preferred low-cellulose environments. These findings provide useful information for predicting the environmental preferences and ecological functions of particular gut bacterial taxa and certain soil fungi that may contribute to the decomposition of cellulose in earthworm guts.
themselves improving the understanding of the links between gut microbiota and cellulose degradation.

It has been widely accepted that Actinobacteria, Proteobacteria, Firmicutes, and Planctomycetes are the most dominant β-glucosidase-producing microbes, playing a major role in the degradation of hemi-cellulose, cellulose, and lignin (Yarwood, 2018; Jiang et al., 2020). The potential links between the capabilities of gut bacteria for cellulose degradation were revealed based on mantel test models (Fig. 5c). The results showed that the communities of Actinobacteria, Proteobacteria, Firmicutes, and Planctomycetes were strongly correlated with MAT and cellulose concentration, which was presumably due to their sensitivity to temperature and the quantity of food resources.

To validate the links between gut microbiota and their ability to break down the food resources, we compared the potential metabolic pathways of cellulose degradation of gut bacteria by assessing the relative abundance of enzymes involved in cellulose metabolism. According to the prediction of PICRUSt2, gut bacterial functions related to cellulose degradation were in relatively high abundance (Fig. S5). Interestingly, the enhanced capability in cellulose degradation was favorable for cellulose accumulation, while the relative abundances of cellulase (EC 3.2.1.4), 1,4-beta-cellobiosidase (EC 3.2.1.9), beta-glucosidase (EC 3.2.1.21), 1,4-beta-xylanase (EC 3.2.1.31), and 1,4-beta-xylosidase (EC 3.2.1.8) being higher in the gut of *D. ghiaro*vi than in *E. nordenskiöldi*. The high temperature in the habitats of *D. ghiaro*vi could partly explain the high relative abundance of cellulolytic enzymes in its gut. To a certain extent, molecules come together more frequently and with greater energy at high temperatures, which can promote enzyme activity and result in a relatively high sequence number of genes encoding the enzymes. In addition, low temperatures in HZ (−3.33 °C) and NWH (−1.71 °C) study sites greatly inhibited the enzyme activity in the gut of *E. nordenskiöldi*. On the other hand, the high diversity of plant species in LS, DQH, and FZ study sites, providing diverse food resources, presumably enhanced the cellulolytic enzyme activity in the gut of *D. ghiaro*vi. The relationship between the relative abundance of cellulolytic enzymes and cellulose concentration exhibited the same pattern in the guts of *D. ghiaro*vi and *E. nordenskiöldi*, suggesting that the gut microbiota likely evolved similar metabolic functions that enables hosts to adapt to habitats across cellulose gradients.

To underpin the potential effects of gut bacteria on the environmental adaptation of earthworms to temperature and food resources, we further assessed the temperature profiling of cellulose activity of gut content and gut cellulose-decomposing bacteria. A gut microbiota transplantation experiment was also conducted to test the potential influence of gut bacteria on the feeding behavior of earthworms in response to high- and low cellulose food resources. The optimal temperatures for cellulase in the gut of *E. nordenskiöldi* and *D. ghiaro*vi were around 25-30 °C and 40 °C, respectively (Fig. 6b and c). The cellulase activity in the gut of *E. nordenskiöldi* could adapt to lower temperatures compared with that of *D. ghiaro*vi, indicating a long-term co-evolution between *E. nordenskiöldi* and a cold climate at relatively high latitudes. In addition, *E. nordenskiöldi* presented a stronger ability to decompose high cellulose food than *D. ghiaro*vi (Fig. 6b and c). In contrast, the decomposing ability for low cellulose food was higher for *D. ghiaro*vi. The gut microbiota transplantation experiment further demonstrated that the gut bacteria from *D. ghiaro*vi could significantly increase the low cellulose food decomposition in the gut of *E. nordenskiöldi*, and vice versa. Home-field advantage (HFA) in soil ecology refers to the idea that decomposer communities are specialized to break down the substrates they associate with, resulting in the prediction that plant litter is decomposed faster than expected underneath the plant from which it originates than underneath other plants (Panin et al., 2021). We therefore found evidence for a home-field of earthworm gut bacteria for cellulose decomposition, suggesting that decomposers are more efficient in decomposing the plant litter they most frequently encounter (Ayres et al., 2009). Differences in litter decomposition might be attributed to the match between a litter type and its environment due to the “local adaptation” of the gut cellulose-decomposing bacteria (Lin et al., 2020). The different adaptability of gut bacteria to food resources (high- and low cellulose litter) and temperature between *E. nordenskiöldi* and *D. ghiaro*vi potentially contribute to their environmental adaptation through influencing feeding habits and food resource acquisition.

Co-occurrence network analysis placed higher values for the topological features of abundant gut bacterial phyla (Actinobacteria, Proteobacteria, Firmicutes, and Planctomycetes) than other phyla, suggesting that the four phyla were more often located in central positions. Therefore, abundant gut bacteria taxa, such as Actinobacteria, Proteobacteria, Firmicutes, and Planctomycetes, had closer relationships and a more important influence on microbial interactions. The findings are supported by previous studies suggesting that Actinobacteria and Proteobacteria play a leading role in cellulose degradation and decomposition of plant remains (Wang et al., 2016; Wang et al., 2021). A more complicated network structure of the gut bacterial communities in *E. nordenskiöldi* than that in *D. ghiaro*vi (Table S1) could be attributed to the relatively higher heterogeneity of climate conditions in habitats of the former. This was also supported by the results based on the random forest model suggesting that gut bacterial interactions were mainly driven by MAT, MAP, and cellulose (Fig. S6). Generally, the complicated networks have a higher efficiency of resource and information transmission compared with simple networks, and thereby supporting more functions (Wagg et al., 2019; Jiao et al., 2022). Furthermore, relatively high network complexity could lead to stable microbial communities, which promote their resistance to the harsh environments (Qiu et al., 2021). Presumably, the stronger ability of *E. nordenskiöldi* to adapt to low temperatures and its higher efficiency to decompose high cellulose food is due to its more complicated gut bacterial interactions and the higher ability of its gut bacteria to sustain multiple ecosystem functions, especially for cellulose composition (Fig. 5). Additionally, the percentage of positive or negative links in co-occurrence networks can reveal the cooperation and niche overlap and competition and niche separation among species (Deng et al., 2016). In this study, a larger percentage of positive links was observed in gut bacterial networks of *E. nordenskiöldi* compared with that of *D. ghiaro*vi (Fig. 5a and Table S1). Cooperation of its gut bacterial communities could have improved the tolerance of *E. nordenskiöldi* to the harsh environmental conditions in cold regions with high latitudes (Hock et al., 2016). The central position of the most efficient cellulose-degrading phyla Actinobacteria, Proteobacteria, Firmicutes, and Planctomycetes in the bacterial communities of the earthworm gut (Fig. 5c and e) suggests that cellulose degradation is the most important ecological process governing interactions among bacterial species in the earthworm gut.

5. Conclusions

This study provides insights into the distribution patterns of gut microbial communities along latitudinal gradients and their potential importance in the adaptation of earthworms to changing environment. We found that the richness and diversity of gut bacterial communities were decreased with increasing latitude. The gut bacterial community composition of both *E. nordenskiöldi* and *D. ghiaro*vi could be well accounted for by MAT and cellulose based on db-RDA, mantel test, and random forest model. Actinobacteria, Proteobacteria, Firmicutes, and Planctomycetes were categorized as core phyla in the co-occurrence networks, suggesting their essential roles in regulating interactions of gut bacteria communities. Phylogenetic analysis indicated that all the isolated cellulase-producing bacteria were affiliated with these four phyla, uncovering their important role in cellulose decomposition. Furthermore, diversity, composition, and network complexity of earthworm gut bacterial communities were the main driving factors of cellulase activity. A gut microbiota transplantation experiment further revealed the beneficial effects of gut microbiota on adaptation of earthworms to temperature and food resources. This study provides a
foundation for understanding the biotic strategies adopted by soil biota when invading new habitats, with gut microbiota being central to food digestion and thermal adaptability for new habitats.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

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Author contributions

Yurong Yang and Donghui Wu designed and conceived the experiment. Yurong Yang, Mac A. Callaham Jr., Xuefeng Wu and Yufeng Zhang carried out the experiments and collected the empirical data. Yurong Yang and Xuefeng Wu performed the data analysis. Yurong Yang and Donghui Wu wrote the paper with contributions from Mac A. Callaham Jr. Deli Wang contributed to the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.166666.

References


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Author contributions

Yurong Yang and Donghui Wu designed and conceived the experiment. Yurong Yang, Mac A. Callaham Jr., Xuefeng Wu and Yufeng Zhang carried out the experiments and collected the empirical data. Yurong Yang and Xuefeng Wu performed the data analysis. Yurong Yang and Donghui Wu wrote the paper with contributions from Mac A. Callaham Jr. Deli Wang contributed to the final version of the manuscript.

Appendix A. Supplementary data

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