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Title page

Termite mound formation reduces the abundance and diversity of soil resistomes

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Originality-Significance Statement

By comparing the patterns of resistome between termite mounds and bulk soils, we provide new insights into the role of termite nesting activities in regulating soil antimicrobial resistance (AMR). Our results provide evidence that termite nesting activity could reduce the diversity and abundance of soil antibiotic resistance genes (ARGs) by increasing soil pH and nutrient availability. Given that termite mounds constitute an important compartment of natural ecosystems, especially in the savanna ecosystems, termite nesting activities could potentially help to mitigate the spread of AMR in soil. Our study highlighted the roles of soil faunal activities in regulating AMR dissemination in terrestrial ecosystems in the changing environment.

Summary

Termites are pivotal ecosystem engineers in tropical and subtropical habitats, where they construct massive nests ("mounds") that substantially modify soil properties and promote nutrient cycling. Yet, little is known about the roles of termite nesting activity in regulating the spread of antimicrobial resistance (AMR), one of the major Global Health challenges. Here, we conducted a large-scale (> 1,500 km) investigation in northern Australia and found distinct resistome profiles in termite mounds and bulk soils. By profiling a wide spectrum of ARGs, we found that the abundance and diversity of antibiotic resistance genes (ARGs) were significantly lower in termite mounds than in bulk soils ($P < 0.001$). The proportion of efflux pump ARGs was significantly lower in termite mound resistome than in bulk soil resistome ($P < 0.001$). The differences in resistome profiles between termite mounds and bulk soils may result from the changes in microbial interactions owing to the substantial increase in pH and nutrient availability induced by termite nesting activities. These findings advance our understanding of the profile of ARGs in termite mounds, which

is a crucial step to evaluate the roles of soil faunal activity in regulating soil resistome under global environmental change.

Introduction

Antimicrobial resistance (AMR) has been recognized as a major health issue of global concerns in the 21st century (Pruden et al., 2006; WHO, 2019). Currently, the annual death toll related to AMR has exceeded 700,000 globally. If no action is taken, the number of AMR-related death is projected to increase to 10 million per year by 2050 (WHO, 2019). The uncontrolled AMR could also, directly and indirectly, impact food production and trading, and the AMR-related economic damage is comparable to the catastrophic global financial crisis during 2008-2009 (WHO, 2019). Therefore, the influence of AMR on human, animal, and plant health and welfare, as well as its ecological and economic consequences, has reinforced the need for a concerted effort to track and control its emergence and dissemination. Recently, the 'One Health' concept that focuses on the role of geographically interconnected environments in the dissemination of ARGs has been developed to fight against AMR (Hernando-Amado et al., 2019).

As an intrinsic phenomenon of the soil microbiome, antibiotic resistance genes (ARGs) have existed in the environment for an extended time (Martínez, 2008; Hu et al., 2018). In natural environments, ARGs are critical to several metabolic processes that are essential for the survival of bacteria (Allen et al., 2010; D'Costa et al., 2011; Martínez, 2008). Currently, the factors driving the soil resistome, the collection of all ARGs in both pathogenic and non-pathogenic bacteria in a community (Wright, 2007), in various ecosystems have been explored in a large number of studies. Abiotic factors, including soil pH, soil nutrient availability, and soil salinity, are demonstrated to be crucial in shaping the soil resistome profile (Li et al., 2020; Sun et al., 2020; Xiao et al., 2016; Zhang et al., 2019). A recent study proposed that the relative abundance of ARGs in global topsoil could be a

robust indicator for the intensity of inter-kingdom microbial antagonisms (Bahram et al., 2018). Despite the longstanding interest in soil resistome, the impacts of soil fauna, a critical component of soil food webs, on the dissemination and evolution of ARGs, as well as the contribution of the environmental factors to those impacts, remain largely unresolved.

Termites are soil-dwelling macrofauna that is among the most abundant organisms on Earth, and they are widely distributed in tropical and subtropical regions (Marynowska et al., 2020). Termites have close relationships with soil microbes (i.e., bacteria, archaea, fungi, and/or protists) which enable them to digest cellulose and thus become the major ecosystem engineers that decompose dead woods and leaf litter (Jouquet et al., 2011; Ashton et al., 2019). Some termite colonies construct unique nest structures ("mounds") with soil materials, which change soil physical and chemical compositions through activities such as decomposition of soil organic matter and bioturbation. Therefore, termite mound environments are usually substantially different from surrounding bulk soils (Bonachela et al., 2015; Nishimura et al., 2020; Chen et al., 2021a). As "islands of fertility", termite mounds harbour a broad range of microbial communities that play essential roles in regulating multiple ecosystem functions related to the ongoing global changes (Bonachela et al., 2015; Ashton et al., 2019Chiri et al., 2020). It is estimated that termites produce ~ 1 to 3% of global greenhouse gas methane (CH_4), half of which was filtered in termite mounds before emission to the atmosphere (Nauer et al., 2018). This "hidden biofilter" process is mediated by methanotrophic bacteria dwelling in termite mound walls, for which an internal intricate network of tunnels can facilitate CH_4 transport (Nauer et al., 2018). A recent study demonstrated that the microbial community assemblies in termite mounds were driven more by deterministic selection rather than stochastic forces, suggesting that soil properties and mean annual temperature were the major selection forces for termite mound dwelling microbes (Chen et al., 2021b). By comparison, the AMR related to termite mounds remains

largely unknown, and a detailed comparative study of the resistome profiles in termite mounds and bulk soils would be imperative to bridge the knowledge gaps of the impacts regarding soil faunal activities on soil resistome.

In this study, we investigated the occurrence and abundance of 285 ARGs in 134 termite mounds collected from an over 1,500 km transect in northern Australia using high throughput quantitative PCR (HT-qPCR) array. To our best knowledge, this study represents the first attempt to assess the resistome profile in termite mounds across a large spatial scale. Compare with local or regional scale studies, large-scale studies can better reveal the spatial patterns of resistome profile, and provide more insights into the underlying mechanisms of the development and spread of environmental AMR. We aimed to (i) explore how the nesting activity of termites influences the soil resistome profiles in natural terrestrial ecosystems and (ii) to identify the main determinants shaping the resistome profiles in termite mounds and bulk soils. We hypothesized that termite nesting activities would increase soil nutrient availability and alter soil pH, which would lead to alternations in resistance mechanisms of ARGs and shifts in ARG abundance.

Results

Physiochemical properties of termite mounds and bulk soils

Termite mounds formation led to significant alternations in soil physicochemical properties. Termite mound pH, which ranged from 5.29 to 7.86 (with an average of 6.66) was significantly higher (Wilcoxon rank-sum test $P < 0.001$) than the pH of bulk soils, which ranged from 4.93 to 7.36 (with an average of 6.22; Fig. 1). In addition, dissolved organic carbon (DOC) of termite mounds, which ranged from 139.02 to 2821.5 mg per kg (with an average of 572.78 mg per kg) was significantly higher than that of bulk soils (Wilcoxon rank-sum test $P < 0.001$), which ranged from 43.57 to 166.3 mg per kg (with an average of 87.48 mg per kg; Fig. 1). Dissolved organic nitrogen (DON) of termite mounds ranging from 5.26

to 310.50 mg per kg (with an average of 83.41 mg per kg), was also significantly higher than that of bulk soils (Wilcoxon rank-sum test $P < 0.001$) ranging from 1.64 to 39.21 mg per kg (with an average of 14.13 mg per kg; Fig. 1).

Microbial properties of termite mounds and bulk soils

Termite mounds were characterized by significantly higher bacterial and fungal abundances but significantly lower ratios of bacterial to fungal abundances in comparison to bulk soils (Wilcoxon rank-sum test $P < 0.001$; Fig. 1). Bacterial abundances ranged from 2.78×10^8 to 3.51×10^{10} copies/g (with an average of 5.61×10^9 copies/g) and 1.18×10^8 to 9.56×10^9 copies/g (with an average of 3.13×10^9 copies/g) for termite mounds and bulk soils, respectively (Fig. S2). Fungal abundances ranged from 2.87×10^5 to 2.66×10^{10} (with an average of 1.24×10^9 copies/g) and 1.37×10^6 to 1.00×10^9 copies/g (with an average of 9.41×10^7 copies/g) for termite mounds and bulk soils, respectively (Fig. S3).

Resistome profiles of termite mounds and bulk soils

The HT-qPCR results revealed that termite mounds harboured significantly less abundant and diverse ARGs than bulk soils (Wilcoxon rank-sum test, $P < 0.001$, Fig. 2). The absolute abundance of ARGs (copies / g sample) in termite mounds and bulk soils ranged from 3.17×10^7 to 3.00×10^9 (with an average of 1.14×10^8) and 2.59×10^7 to 6.97×10^8 (with a average value of 1.43×10^8), respectively (Fig. S4). To minimize the variations in background bacterial abundances, the ARG absolute abundances were normalized to 16S rRNA gene copies as ARG copies per 16S rRNA gene. The relative abundance of ARGs detected in termite mound and bulk soil samples ranged from 1.20×10^{-3} to 1.72×10^{-1} (with an average of 2.28×10^{-2}) and 7.03×10^{-3} to 3.02×10^{-1} (with a median value of 7.28×10^{-2}), respectively (Fig. S5).

We further assessed the overall profiles of the resistomes in the termite mound and bulk soil samples and found there was a clear separation of ARGs depending on the sample

type (Adonis test $P < 0.001$; Fig. 2B). In addition, the resistome compositions based on the antibiotic resistance mechanisms were distinct between termite mounds and bulk soils. Particularly, the proportion of efflux pump genes was significantly lower in termite mound resistome than in bulk soil resistome (Wilcoxon rank-sum test $P < 0.001$). The efflux pump genes accounted for 68.51% vs. 43.98% of the abundance of total ARGs detected in the bulk soil samples and termite mound samples, respectively (Fig. 2C). The ARGs shared between the termite mound and bulk soil samples and those detected exclusively in each type of sample were visualized using bipartite network analysis (Fig. 2D). A total of 190 ARGs were shared between termite mounds and bulk soils, 67 ARGs were detected only in termite mounds, and 14 ARGs were detected only in bulk soils.

Relationships between environmental factors and ARG abundances

To gain further insights into the potential drivers shaping the termite mound and bulk soil resistome profiles, we analysed the relationships between ARG abundances and selected environmental factors. The results showed that pH was one of the most important determinants of ARG abundances in both termite mounds and bulk soils. Spearman's rank correlation, as well as first and second-order polynomial regression models, revealed that pH was significantly negatively correlated with ARG abundances for both types of samples ($P < 0.001$; Fig. 3). Nutrient availability, as represented by DOC and DON, oppositely influenced the ARG abundances in termite mounds and bulk soils (Fig. 3). According to the correlation and regression analyses, the ARG abundance in termite mounds was positively correlated with DOC and DON. In contrast, the ARG abundance in bulk soils was negatively correlated with DOC and DON.

Discussion

By using the large-scaled investigation, our study improved the understanding of the profiles of ARGs in widely distributed termite mounds. Consistent with our hypothesis, the

results showed that compared with bulk soils, termite mounds harboured a lower level of ARG abundance and diversity, which may potentially result from the increases in pH and nutrient availability during the nesting processes (Fig. 4). In the following discussion, we will focus on the potential impacts of pH and nutrient availability on termite mounds ARGs.

Impact of pH on the profiles of ARGs in termite mounds

The quantitative results revealed that termite mound formation led to significant reductions in soil ARG abundance and diversity (Fig. 2). It is interesting to note that the relative abundance of ARGs in our bulk soil samples was comparable to those in urban soils as reported previously (Yan et al., 2019). The relatively high levels of ARGs in the bulk soil samples indicate that soils in natural habitats with no or limited anthropogenic impact do not necessarily contain lower levels of AMR than soils in habitats with continuously intensive anthropogenic impacts. These findings indicated that as an intrinsic component of soil microbiota, ARGs could be abundant in the environment, independent from selection pressures imposed by human activities such as overuse of antibiotics and waste discharge (Surette and Wright, 2017). The significantly lower ARG abundances in termite mounds than in bulk soils is likely related to the significant increases in pH values due to the termite nesting activities (Fig. 1), as pH value was significantly and negatively correlated with ARG abundances (Fig. 3). Such strong correlations between ARG abundances and pH could be a consequence of the close relationships between pH and soil bacteria, the potential hosts of ARGs. According to previous studies, most soil bacterial taxa exhibit a narrow pH optimum (Rousk et al., 2010; Xiao et al., 2016). Minor deviation from the optima pH (i.e., ~ 1 pH unit) could greatly decrease the bacterial growth and substantially result in the bacterial community being rapidly outcompeted by the fungal community whose growth is far less sensitive to pH change (Rousk et al., 2010). The impacts of pH change on the competitive interactions between bacterial and fungal communities could also be revealed by the

observation that the bacterial to fungal abundance ratio was significantly lower in termite mounds than in bulk soils (Fig. 1).

In addition, termite mound formation led to substantial alternations in the resistance mechanisms and classifications of ARGs. The clear separation in the termite mound and bulk soil resistomes (Fig. 2B) might be associated with the differences in resistome compositions, particularly the significantly lower proportion of efflux pump genes in termite mounds than in bulk soils (Fig. 2C). Efflux pumps are evolutionarily ancient and the most common antibiotic resistance mechanism in pristine ecosystems, such as the Tibetan plateau, deep ocean, and undomesticated old bog ecosystems (Chen et al., 2013; Chen et al., 2016; Obermeier et al., 2020). The multi-protein pumps encoded by the efflux pump genes typically confer resistance to several unrelated substances including solvents, heavy metals, and endogenous compounds produced by the bacterial hosts (Martinez et al., 2009). Their physiological roles also involve regulating intracellular pH, transporting quorum sensing molecules of cell-to-cell communications, and enhancing bacterial pathogenicity (Martinez et al., 2009). Resistance to antibiotics commonly used in clinical and agricultural sectors mediated by efflux pump genes is considered to be a by-product of the physiological functions of these genes that allow bacteria to survive in their ecological niches (Piddock, 2006). Consequently, the much higher proportion of efflux pump genes in the bulk soil resistome than in the termite mound resistome indicates that ARGs in the two habitats may evolve due to different reasons. Compared to termite mounds, bulk soils are nutrition poor and less suitable for the growth of an abundant bacterial community. Therefore, ARGs, especially the efflux pump ARGs, in bulk soils are likely evolved as a strategy of the bacteria to survive. The possible differences in evolutionary mechanisms between ARGs in termite mounds and bulk soils are further supported by the results of shared and unique ARGs in these two habitats. The finding that 67 ARGs were detected exclusively in termite mounds

(Fig. 2D) suggests that there might be a termite mound endogenous resistome which is not originated from bulk soils.

Impacts of nutrient availability on the profiles of ARGs in termite mounds

Previous studies have shown that the evolution of resistomes in natural ecosystems without anthropogenic selection pressures is mainly driven by the inter-kingdom interactions among microbial communities within their environmental habitats (Bahram et al., 2018; Obermeier et al., 2020). Negative interactions among microbes, particularly the antagonism between bacterial and fungal communities, are commonly associated with an elevated level of ARGs, as many bacterial and fungal cells can produce antibiotics as weapons when competing with each other. These antibiotics can select against bacterial cells not encoding ARGs (Bahram et al., 2018). We assume that the distinct resistome profiles in termite mounds and bulk soils might result from the shifts in the forms of bacterial-fungal interactions which are consequences of the substantial increases in nutrient availability in termite mounds in comparison to bulk soils (Fig. 1). The stress gradient hypothesis (SGH) provides a framework to predict whether the interactions among microbes from different kingdoms are positive or negative in an environment (Hammarlund and Harcombe, 2019; Piccardi et al., 2019). The SGH states that nutrient availability is one of the most important factors that govern the direction of microbial interactions. In a benign environment with abundantly available nutrients, negative interactions among microbes should be more common. In contrast, in a stressful environment where resources essential for microbial growth are lacking, the interactions among microbes would switch to facilitation (Hammarlund and Harcombe, 2019; Piccardi et al., 2019). Due to the foraging activities of termites that involves the accumulation of plant litter and invertebrates in their mounds and the ability of termites in facilitating soil nutrient cycling (Whitford and Eldridge, 2013), termite mounds are unique nutrient hotspots with significantly more abundant DOC and

DON than bulk soils (Fig. 1). Therefore, it is likely that the ARGs in termite mounds are mainly evolved due to the selection pressures associated with the antagonism between bacterial and fungal communities. However, ARGs in bulk soils are mostly evolved as a strategy for the bacterial to survive in the stressful habitat. Our hypothesis is supported by the observation that DOC and DON oppositely impacted the ARG abundances in termite mounds and bulk soils and is further strengthened by the results that the abundance of ARGs in termite mounds but not in bulk soils was significantly correlated with the ratio of bacterial to fungal abundances (Fig. 3). However, to verify this hypothesis, more targeted analyses with additional data from different geographic locations and evolution experiments will be required.

Conclusions

Taken together, these findings improve our understanding of the resistome in termite mounds and provide evidence that the lower level of ARG abundance and diversity in termite mounds than in bulk soils could potentially result from the increases in pH and nutrient availability during the nesting processes. Given that termite mounds constitute an important soil compartment (up to ~10%) especially in the savanna ecosystem, termite nesting activity might potentially mitigate the soil antibiotic resistance. Our results highlight the necessity to consider the roles of soil faunal activities in regulating AMR in terrestrial ecosystems, which would facilitate the development of strategies in fighting against AMR dissemination in natural ecosystems under the changing environment.

Experimental procedures

Termite mounds and soil sample processing

Termite mound and bulk soil samples were collected in May 2019 from 16 locations in northern Australia (133.36 °E to 140.36 °E, 22.98 °S to 19.25 °S; Fig. S1). Geographic coordinates of the sampling locations were recorded using a hand-held GPS device (eTrex

Venture, Garmin, Olathe, KS, USA). Mean annual temperature (MAT) of the sampling region ranged from 21.36 to 26.54 °C. Mean annual precipitation (MAP) ranged from 254 to 583 mm. At each location, we established a 50 × 50 m plot and collected eight to ten samples of termite mound samples. Three bulk soil samples were collected by mixing five cores (0-10 cm) for each sampling plot. In total, 134 termite mounds and 44 bulk soil samples were collected. All samples were sieved through a 2 mm mesh and divided into two portions. One portion was frozen at -80 ºC for molecular analyses and the other portion was stored at 4 ºC for soil physiochemical analyses.

Molecular analyses

Total genomic DNA was extracted from the termite mound and bulk soil samples using a DNeasy PowerSoil Kit (QIAGEN Pty, Ltd., Hilden, Germany) *as per* the manufacturer's instruction. The bacterial 16S rRNA gene and fungal ITS region were quantified on a CFX96 Touch™ PCR Detection System (Bio-Rad, Hercules, USA). Primers set 515FmodF (GTGYCAGCMGCCGCGGTAA) and 806RmodR (GGACTACNVGGGTWTCTAAT) were used for the amplicon of the bacterial 16S rRNA gene (Walters et al., 2016). Primer set ITS1F (TCCGTAGGTGAACCTGCGG) and ITS2R (TCCGTAGGTGAACCTGCGG) was used for the amplicon of the fungal ITS region (White et al., 1990). The bacterial and fungal quantification was conducted in a 20 μL reaction system which contained 10 μl Sensimix SYBR NO-ROX reagent (Bioline, London, UK), 0.8 μL each primer (10 μM), and 2 μL DNA template. Amplicon conditions for the 16S rRNA gene and ITS region were as follows: an initial enzyme activation at 95 °C for 10 min, followed 40 cycles of 95 °C for 20 s, 53 °C for 30 s and 72 °C for 45 s and a final 72 °C for 10 min.

The abundances of ARGs were measured using HT-qPCR on the Wafergen SmartChip Real-time PCR system (Wafergen, Inc., CA, USA) as previously described (Hu et

al., 2016). The HT-qPCR array contained 285 primers targeting ARGs conferring resistance to all major classes of antibiotics (Table S1). Amplification of ARGs was conducted in a 100 nL reaction system. The amplicon condition was as follows: an initial enzyme activation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. The melting curve was generated by the Wafergen SmartChip qPCR software (V2.7.0) automatically. Only data with a single melting peak and an amplification efficiency between 1.8 to 2.2 were retained. A cycle threshold (C_T) of 31 was used as the detection limit. The relative copy numbers of ARGs were calculated according to the following equation: Relative gene copy number = $10^{(31-Ct)/(10/3)}$ (Stalder et al., 2014).

Characterization of abiotic determinants of ARGs

Climatic attributes including MAT and MAP of each sampling site were obtained from the WolrdClim database version 2 (https://www.worldclim.org/) based on the spatial geographical coordinates (Fick and Hijmans, 2017). The obtained climatic data were processed with the "sp" and "raster" packages in R (V 3.6.2) (R CoreTeam, 2020; Pebesma and Bivand, 2005, Hijmans et al., 2015) and are shown in Table S2. Soil physicochemical properties including soil pH, DOC and DON were characterized using standard methods (Rayment and Lyons, 2011). Briefly, soil pH was measured in a 1:2.5 mass: volume of soil and water suspension using a pH meter (Thermo Scientific Inc. Waltham MA, US). DOC and DON were extracted with MilliQ water and measured with a TOC analyzer (Shimadzu, Kyoto, Japan). Detailed information about the physicochemical properties of each termite mound and bulk soil samples is provided in Table S3.

Statistical analyses

All statistical analyses were performed in the R platform (V 3.6.2) (R Core Team, 2020). Shannon diversity index of the detected ARGs in the termite mound and bulk soil samples was calculated with the "vegan" package (Oksanen et al., 2013). The differences in

soil microbial properties and physicochemical properties, as well as ARG abundance and diversity were determined using the Wilcoxon rank-sum test with the "stat" package (Field et al., 2012). The overall profiles of resistome in the termite mound and bulk soil samples were determined using a Bray-Curtis dissimilarity matrix and illustrated in a principal coordinate analysis (PCoA) plot with the "ggplot2" package (Wickham, 2016). The significance in the difference of termite mound and bulk soil resistomes was determined with the Adonis test with the "vegan" package (Oksanen et al., 2013). The shared and unique ARGs in termite mounds and bulk soils were visualized using the bipartite network constructed with the Gephi software (V 0.9.2). Spearman's rank correlation coefficients between the maim factors and ARG abundances were calculated and visualized with the "corrplot" package (Wei et al., 2017). The relationships between the ARG abundances and selected environmental factors were further explored with the first order and second-order polynomial regression models. The goodness of fits of the models was estimated based on the corrected Akaike information criterion (AICc) (Burnham et al., 2011) and the better fit models were selected and presented.

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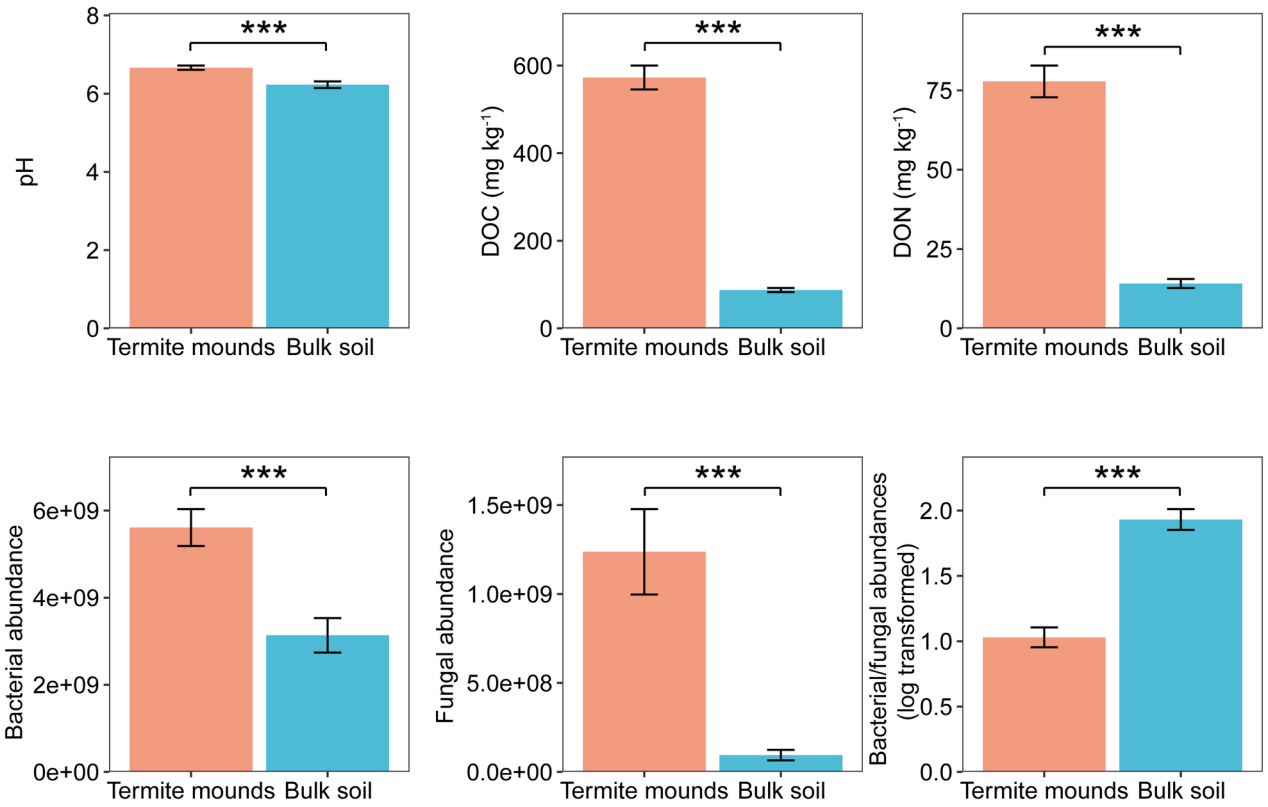
Figure legends

Fig. 1. Comparison of the microbial and physicochemical properties between termite mounds and soil samples. *** indicates Wilcoxon rank-sum test $P < 0.001$. DOC: dissolved organic carbon. DON: dissolved organic nitrogen.

Fig. 2. Profiles of the resistomes in termite mounds and soil samples. (A) Relative abundance and Shannon index of antibiotic resistance genes (ARGs) in termite mounds and bulk soils. Error bars represented as mean ± standard deviation. *** indicates Wilcoxon rank-sum test $P < 0.001$. (B) Principal coordinate analysis (PCoA) based on the Bray-Curtis distance showing the overall distribution patterns in the termite mounds and soil. (C) Classification of the ARGs detected based on the mechanisms of the resistance. (D) Bipartite network analysis revealing the shared and unique ARGs in termite mounds and soil.

Fig. 3. Main determinants of the relative abundance of antibiotic resistance genes (ARGs) in termite mounds and soil. (A) Spearman's correlation matrix. (B) Significant ($P < 0.05$) and the best fit (determined based on the corrected Akaike Information Criterion) polynomial fits of ARG relative abundance of selected determinants. Red and black lines represent first- and second-order polynomial fits, respectively. DOC: dissolved organic carbon; DON: dissolved organic nitrogen; MAT: mean annual temperature; MAP: mean annual precipitation.

Fig. 4 Schematic summary of the impact of termite mound formation on soil antibiotic resistance genes (ARGs). Termite nesting activity led to increases in soil pH and nutrient availability, which could alter the inter-kingdom interaction between bacteria and fungi and shift the soil resistome. The figure was created in BioRender.com.



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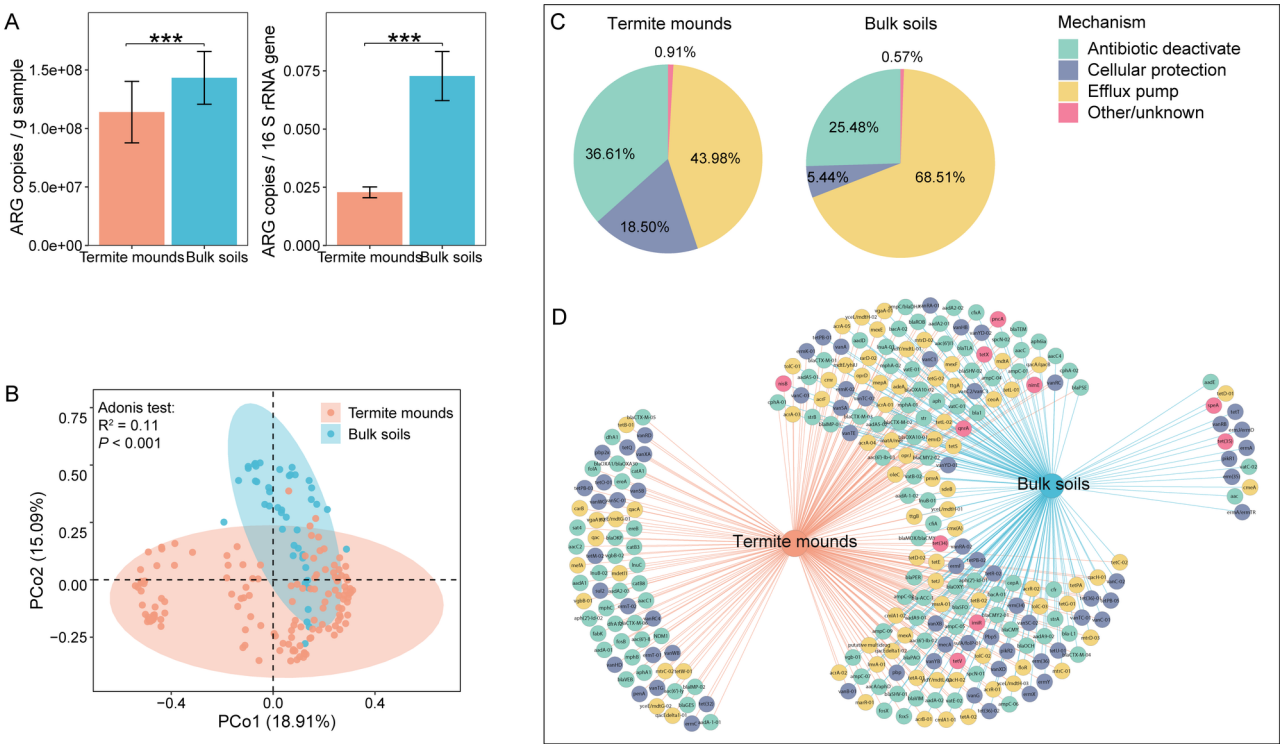

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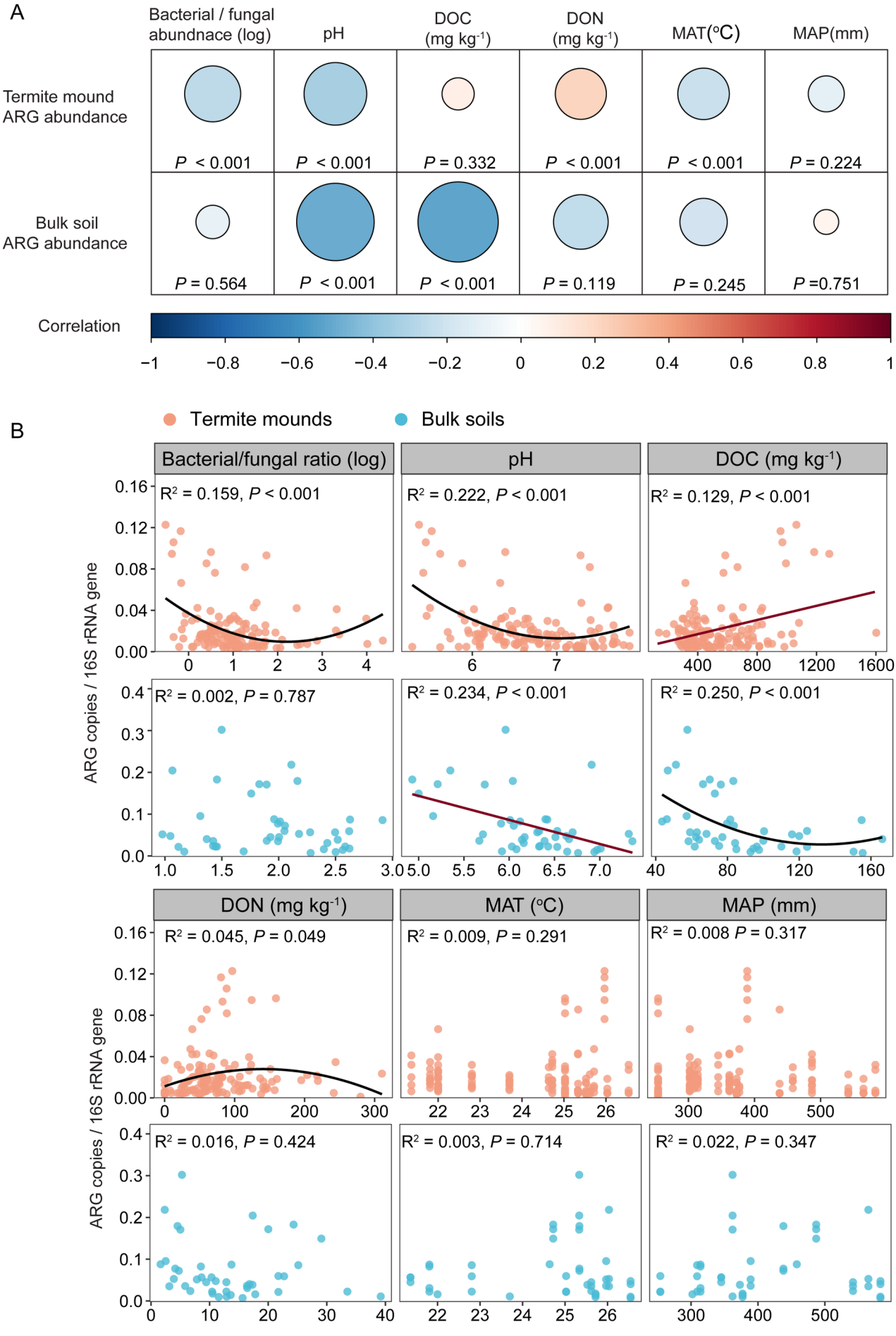

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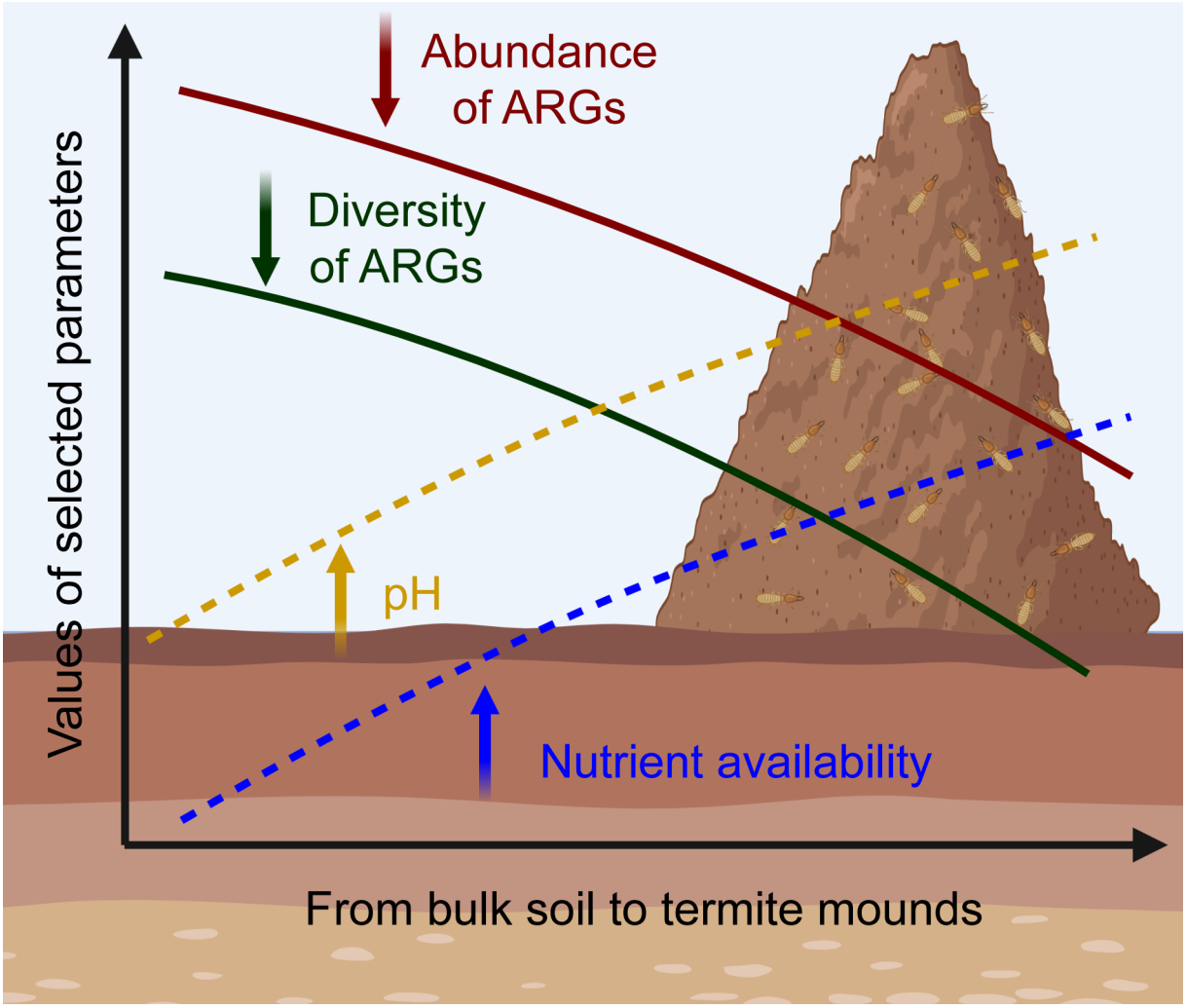

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