



Rhizosphere suppression hinders antibiotic resistance gene (ARG) spread under bacterial invasion.

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ABSTRACT

The rhizosphere is an extremely important component of the “one health” scenario by linking the soil microbiome and plants, in which the potential enrichment of antibiotic resistance genes (ARGs) might ultimately flow into the human food chain. Despite the increased occurrence of soil-borne diseases, which can lead to increased use of pesticides and antibiotic-producing biocontrol agents, the understanding of the dynamics of ARG spread in the rhizosphere is largely overlooked. Here, tomato seedlings grown in soils conducive and suppressive to the pathogen *Ralstonia solanacearum* were selected as a model to investigate ARG spread in the rhizosphere with and without pathogen invasion. Metagenomics data revealed that *R. solanacearum* invasion increased the density of ARGs and mobile genetic elements (MGEs). Although we found ARGs originating from human pathogenic bacteria in both soils, the enrichment was alleviated in the suppressive soil. In summary, the suppressive soil hindered ARG spread through pathogen suppression and had a lower number of taxa carrying antibiotic resistance.

1. Main

Genes conferring resistance to antibiotics (antibiotic-resistance genes, ARGs) occur naturally [1]. ARGs can be easily exchanged among organisms and surrounding environments [2,3]. For instance, ARGs present in the soil can enter the food chain through rhizosphere dwelling microorganisms [4], the accumulation of which can dramatically increase the health risks of the exposed population. In fact, the acquisition and transfer of ARGs imposes significant harm to human health by generating multi-resistance human pathogens [5]. Therefore, strategies to reduce the amount of ARGs and hinder their spread in soils are crucial.

The accumulation of ARGs in the environment is closely related to artificial interference. Many studies have reported that exogenous contamination, such as the discharge of urban wastewater, manure and sludge from agricultural animals increases ARGs in soil, and the invasion of ARG-rich microorganisms in these wastes is directly linked to the

higher human risk [6–8]. In agriculture, intensive cropping and conventional mineral fertilizer application usually increase soil pathogen density [9] and ARG abundance [8,10]. In the presence of mobile genetic elements (MGEs), these ARGs can be easily transferred between microbial community members. However, the subsequent interaction among the coaccumulation factors (pathogens, ARGs and MGEs) around crops remains unclear. After all, soil microbiome under biotic and abiotic stress seem to change their associated microenvironment and consequently leads to the enrichment of ARGs [11,12]. In this context, it is crucial to understand the ARG dynamics of the rhizosphere microbiome of disease-induced soils, as pathogen invasion could promote the spread of ARGs.

We hypothesize that the biotic pressure imposed by pathogens can lead to an increase in ARG-rich taxa, but that the response will depend on the health status of the soil. Conducive soils, which are more susceptible to disturbances and prone to pathogen colonization, would pose a higher risk of antibiotic resistance. The proliferation of ARG-rich

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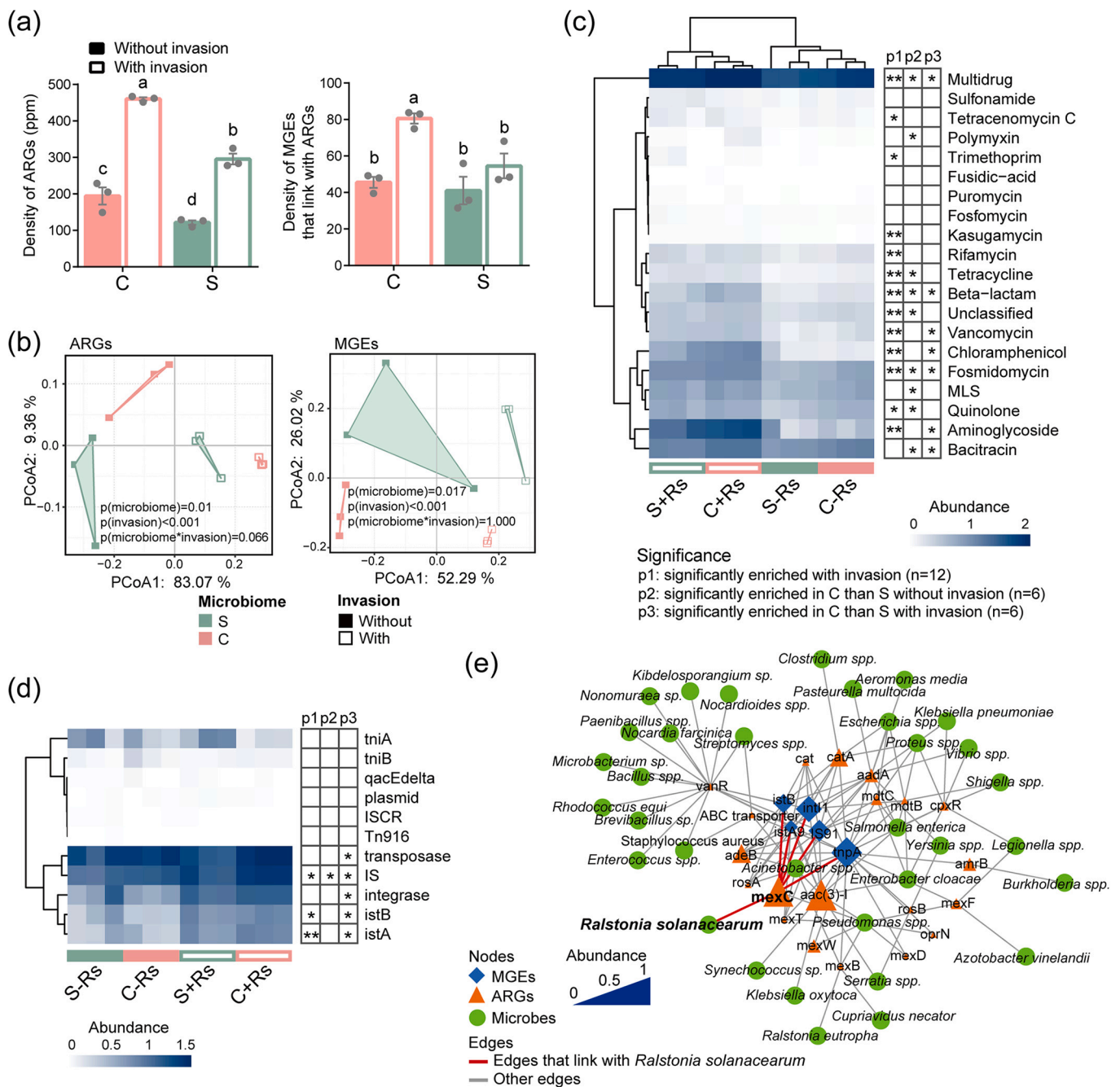


Fig. 1. Abundance and composition of ARGs and MGEs that are linked to ARGs in the rhizosphere. (a) Relative abundance of ARGs and MGEs among the four treatments; (b) PCoA of rhizosphere ARG and MGE composition based on Bray-Curtis distance. The p values were calculated through PERMANOVA based on Bray-Curtis distance; (c) clustered heatmap of ARGs at the type level; (d) clustered heatmap of MGEs at the type level; (e) the network among significantly enriched MGE subtypes and their associated ARGs and the assigned taxonomy of the ARGs. The value of ARG abundance was transformed through \log_{10} transformation. The size of the MGE and ARG nodes was normalized from 0 to 1 based on their abundance. “C”, conducive rhizosphere derived from soil following the long-term application of chemical fertilizer; “S”, suppressive rhizosphere derived from soil following the long-term application of bio-organic fertilizer; “-Rs”, rhizosphere soil without *R. solanacearum* invasion; “+Rs”, rhizosphere soil after *R. solanacearum* invasion. Different letters indicate significant differences among the treatments as defined by Tukey’s test ($p \leq 0.05$). Asterisks indicate significant differences (Mann-Whitney U test, * $p \leq 0.05$, ** $p \leq 0.01$).

pathogens, such as *Ralstonia solanacearum* which could cause severe bacterial wilt [13], in intensive agriculture, presents an interesting model to access the link between pathogen invasion and ARGs. Here, we experimentally examined how pathogen (*R. solanacearum*) invasion affects ARGs and MGEs in rhizosphere soil derived from soils collected from two treatments (continuously applied chemical fertilizer or bio-organic fertilizer) at a long-term field experiment site. The soil disease incidence was higher than 60% in the chemical fertilizer treatment and

<30% in the bio-organic fertilizer treatment, which led to the designation of conducive (C) and suppressive (S) soils, respectively [14]. After allowing plant root colonization by the S or C microbiome, seedlings were transferred to new pots with germ-free substrate and subsequently inoculated with or without *R. solanacearum* (Rs). This approach allowed us to identify the potential dynamics of ARGs and MGEs in the rhizosphere, which harbor different suppressiveness levels, under bacterial invasion. The method details are shown in the supplementary file.

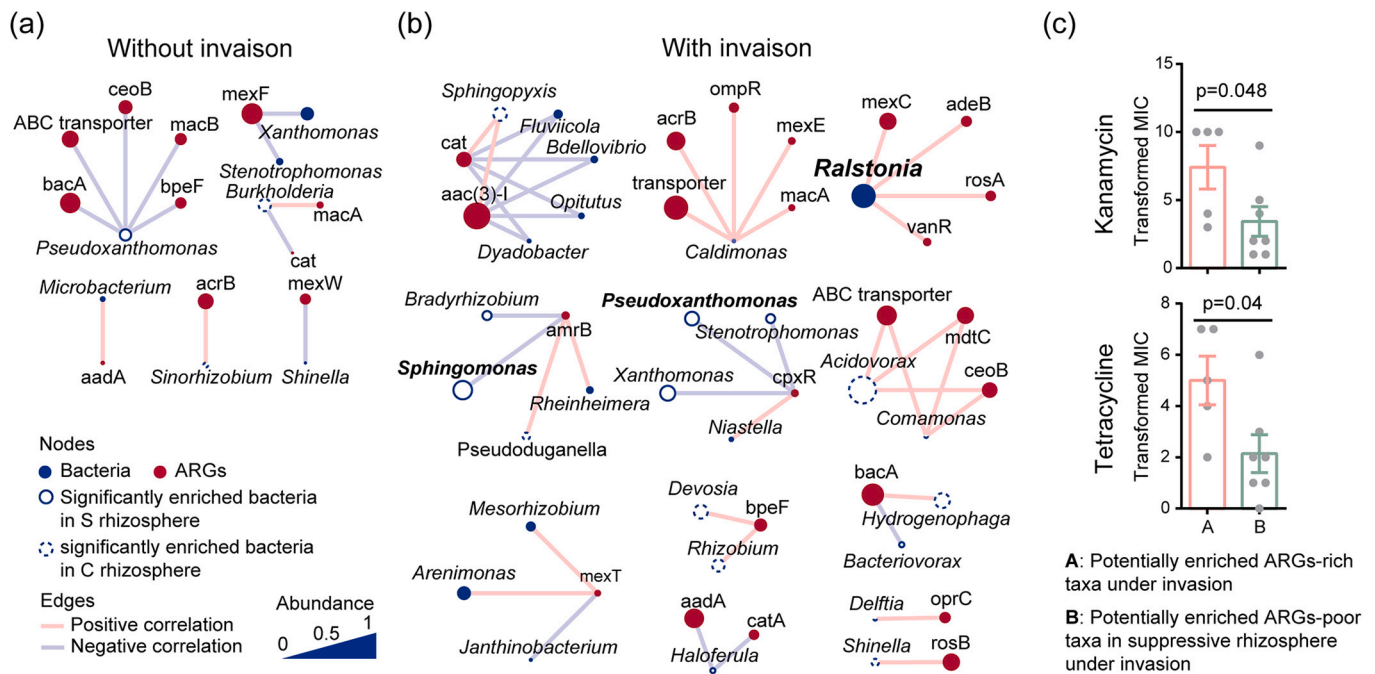


Fig. 2. Potential interaction between bacteria and ARGs. (a) Networks based on the samples without *R. solanacearum* invasion; (b) networks based on the samples with *R. solanacearum* invasion. The abundances of ARGs and bacteria were standardized to a scale of 0–1. The red circles represent ARG subtypes, and the blue circles represent bacterial genera. The pink lines represent the positive correlations between ARGs and bacteria, and the blue lines represent the negative correlations between ARGs and bacteria. Only Spearman correlations with $r > 0.06$ and $p < 0.05$ were reserved. The solid line circles and the dashed lines represent the significantly enriched bacteria in the suppressive and conducive rhizospheres, respectively. (c) MIC comparisons between responsive taxa groups with potentially rich and poor ARGs. The taxa group of A (potentially enriched ARG-rich taxa under invasion) contains *Staphylococcus aureus* and *Pseudomonas* spp., and B (potentially enriched ARG-poor taxa in the suppressive rhizosphere under invasion) contains *Pseudoxanthomonas* spp. and *Sphingomonas* spp. All the MIC values were transformed using the formula $(\log_{2x}) + 2$, where x indicates the MIC value. The p values were calculated through two-sample Mann-Whitney U tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We observed a lower abundance of ARGs in the rhizosphere derived from the suppressive than from the conducive soil, regardless of pathogen invasion. However, *R. solanacearum* invasion significantly enhanced this value (Fig. 1a). For MGEs, although the whole MGE abundance in the rhizosphere decreased after pathogen invasion (Fig. S1), the abundance of MGEs linked to ARGs increased following *R. solanacearum* inoculation, and only the conducive rhizosphere showed significant enrichment (Fig. 1a). Moreover, although microbiome origin and pathogen invasion significantly determined the ARG and MGE composition, invasion exerted the most powerful influence (Fig. 1b). Specifically, 13 of the 20 ARG types and 3 of the 11 MGE types significantly increased following pathogen invasion. In addition, the comparison between S and C soils before invasion (S-Rs with C-Rs) revealed that 9 ARG types and 1 MGE type were significantly higher in the conducive rhizosphere. The comparison of the same soils after invasion (S + Rs with C + Rs) showed that 7 ARG and 5 MGE types were significantly higher in the conducive rhizosphere (Fig. 1c, d). The response of aminoglycoside-, chloramphenicol-, and vancomycin-type ARGs and transposase-, integrase-, istA-, and istB-type MGEs following invasion enhanced the difference between the two kinds of rhizosphere microbiomes, which indicates that in comparison to the suppressive rhizosphere, these ARGs and MGEs show more significant responses in the conducive rhizosphere (Fig. 1c, d, and S2a).

Next, we traced dominant ARGs to their contributors. The results showed that almost all ARG contributors in the conducive rhizosphere were more abundant than those in the suppressive rhizosphere following invasion (Fig. S2b). Similar to the abundance of ARGs in *R. solanacearum* (Fig. S2c), some biocontrol agents, such as *Streptomyces* and *Pseudomonas*, and many human conditional pathogens, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, which contain multidrug-type ARGs, were significantly enriched in the conducive

rhizosphere (Fig. S2b). To understand the patterns associated with MGEs following invasion, their associated ARGs, and the potential hosts of the ARGs were visualized as a network (Fig. 1e, S3a, and S3b). The results showed that the pathogen mainly contributed to the multidrug resistance gene *mexC*, which was potentially connected with various MGEs, such as *tnpA*, *IS91*, *istB*, *int1*, and *ista9* (Fig. 1e, S3c and S3d). These MGEs were also potentially linked with many ARGs belonging to other microbial hosts, including some of the human conditional pathogens (Fig. 1e).

We next constructed co-occurrence networks to examine the interaction between the rhizosphere microbiome and ARGs (Fig. 2). Importantly, *Ralstonia* was abundant following invasion and was significantly positively correlated with ARG density (Fig. 2b), which indicates that pathogen invasion affected ARG enrichment. More specifically, the network of interactions between bacteria and ARGs was simpler in the absence of invasion (Fig. 2a) but increased in complexity in the presence of the pathogen (Fig. 2b). Most of these resistance genes are multidrug-resistant, followed by aminoglycoside, chloramphenicol, fosmidomycin, and macrolide-resistant (Fig. S2a). Some bacteria, such as *Sphingomonas*, *Pseudoxanthomonas*, and *Stenotrophomonas*, were significantly enriched in the suppressive rhizosphere and negatively correlated with ARGs. In contrast, the bacteria enriched in the conducive rhizosphere showed the opposite trend (Fig. 2b). Finally, an in vitro test demonstrated that the antibiotic resistance of these taxa (*Pseudoxanthomonas* spp. and *Sphingomonas* spp.) was significantly less enriched in the suppressive rhizosphere than the potentially responsive ARG contributors (*Staphylococcus aureus* and *Pseudomonas* spp.) that were enriched in the conducive rhizosphere (Fig. 2c, S2, and Table S1).

Intensive agricultural cropping contributes to the buildup of pathogenic microorganisms, further adding biotic pressure to the plants and their associated microbiomes, directly influencing “one health” [15]. To

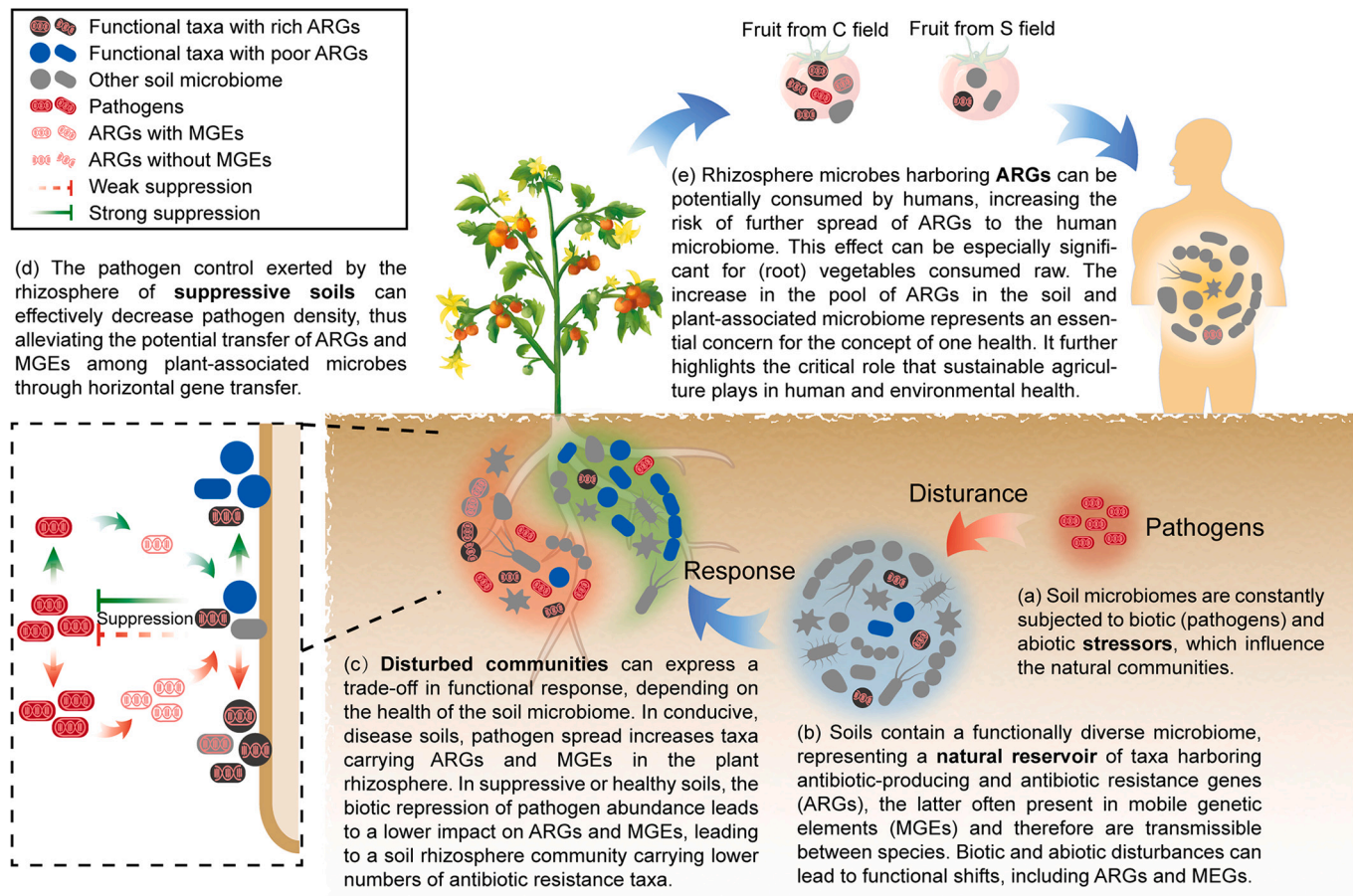


Fig. 3. Conceptual model of ARG spread under biotic disturbance caused by pathogen invasions.

compete with pathogen spread, the production of antibiotics by the resident microbiome could significantly increase the level of antibiotics in soil, potentially contributing to the diversity and abundance of ARGs [11,16]. In this scenario, this selective pressure could result in soil enriched with antibiotic resistant human pathogenic bacteria [12], substantially increasing the health risk to humans. However, soil microbial communities with different levels of suppression may have distinct responses following invasion by exogenous microbes [17,18]. Thus, sustainable agricultural practices that promote a decrease in plant pathogen densities can contribute to the “one health” concept by alleviating the risk of ARG spread while promoting plant growth and reducing the environmental footprint of agricultural production.

Our results support the overlooked potential link between pathogen incidence and the increase in antibiotic resistance spread risk. In general, *R. solanacearum* invasion increased ARG and MGE abundances in both rhizospheres, indicating that the biotic pressure imposed by the pathogen boosted the abundance of ARG-rich taxa. However, ARG enrichment was relatively moderate in the suppressive rhizosphere soil, which occurs in soil following long-term (bio)-organic fertilization, confirming that the response depends on the health status of the soil. It is widely known that soil suppressiveness can be primed by organic amendments through microbiome manipulation [14,19], which can also decrease the soil ARG level [20]. Our previous study showed that rhizosphere soil derived from suppressive soil featured more Sphingomonadaceae and Xanthomonadaceae than that from conducive soil. And these suppressive indicator taxa were also showed the ability to suppress bacterial wilt in rhizosphere [14]. Interestingly, the results here suggest that ARG-poor taxa such as *Sphingomonas* and *Pseudoxanthomonas*, belonging to Sphingomonadaceae and Xanthomonadaceae, respectively, were stimulated by pathogen invasion in suppressive soils. In

suppressive rhizosphere soil, these species can control bacterial wilt, contributing to lower pathogen density while potentially alleviating the ARG horizontal transfer risk. In contrast, conducive soils, which are more susceptible to disturbances and prone to pathogen colonization, pose a higher risk of ARG. Therefore, we hypothesize that the high suppression induced by organic management is a promising agricultural mode in plant-associated disease control.

Antimicrobial resistomes in the soil can be absorbed by plants and then migrate into the food chain, serving as an additional risk to human health [4]. Therefore, reducing the amount of ARGs in agricultural soil, especially in plant-associated soil, is particularly important for human health. We propose a conceptual model for ARG spread following biotic stress based on our results. First, the functional microorganisms in suppressive soil limit the migration and colonization of *R. solanacearum* and directly inhibit the horizontal and vertical transfer of its ARGs; second, the resident response of the ARG-poor microbiome reduces the space of microorganisms with a high ARG microbiome, thus indirectly limiting the abundance of ARGs in indigenous communities, and reducing the risk to human health (Fig. 3).

The results of this study highlight the intricate yet unknown dynamics of the soil ARG spread mode that is influenced by the rhizosphere microbiome with different suppressiveness levels. We postulate that the invading pathogen leads to a shift in microbiome composition and activation of ARG-rich functional taxa to restrict biotic stress and inhibit pathogen propagation. Specific ARG-poor taxa that respond to pathogen invasions in suppressive soil may be a promising solution for this scenario. Notably, more work in soil-borne disease control should consider ARGs when estimating the “one health” contribution to humans in future studies.

Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required for this specific study.

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CRediT authorship contribution statement

Yuchan Li: Visualization, Methodology, Validation, Writing – original draft. **Xuhui Deng:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Funding acquisition. **Na Zhang:** Methodology, Investigation, Data curation. **Zongzhuan Shen:** Methodology, Writing – review & editing. **Rong Li:** Conceptualization, Writing – review & editing, Funding acquisition. **Qirong Shen:** Conceptualization, Funding acquisition. **Joana Falcao Salles:** Writing – review & editing.

Declaration of Competing Interest

The authors have no conflicting interests related to this manuscript.

Data availability

The raw sequence data for the metagenomics of all samples were submitted to the NCBI Sequence Read Archive database (<https://www.ncbi.nlm.nih.gov/>) with the accession number SRP319842.

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Not available.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.onehlt.2023.100481>.

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