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Abstract: The emergence, evolution and spread of antibiotic resistance genes (ARGs) in the environment represents a global threat to human health. Our knowledge of antibiotic resistance in human-impacted ecosystems is rapidly growing with antibiotic use, organic fertilization and wastewater irrigation identified as key selection pressures. However, the importance of biological interactions, especially predation and competition, as a potential driver of antibiotic resistance in the natural environment with limited anthropogenic disturbance remains largely overlooked. Stress-affected bacteria develop resistance to maximize competition and survival, and similarly bacteria may develop resistance to fight stress under the predation pressure of protists, an essential component of the soil microbiome. In this article, we summarized the major findings for the prevalence of natural ARGs on our planet and discussed the potential selection pressures driving the evolution and development of antibiotic resistance in natural settings. This is the first article that reviewed the potential links between protists and the antibiotic resistance of bacteria, and highlighted the importance of predation by protists as a crucial selection pressure of antibiotic resistance in the absence of anthropogenic disturbance. We conclude that an improved ecological understanding of the protists-bacteria interactions and other biological relationships would greatly expand our ability to predict and mitigate the environmental antibiotic resistance under the context of global change.

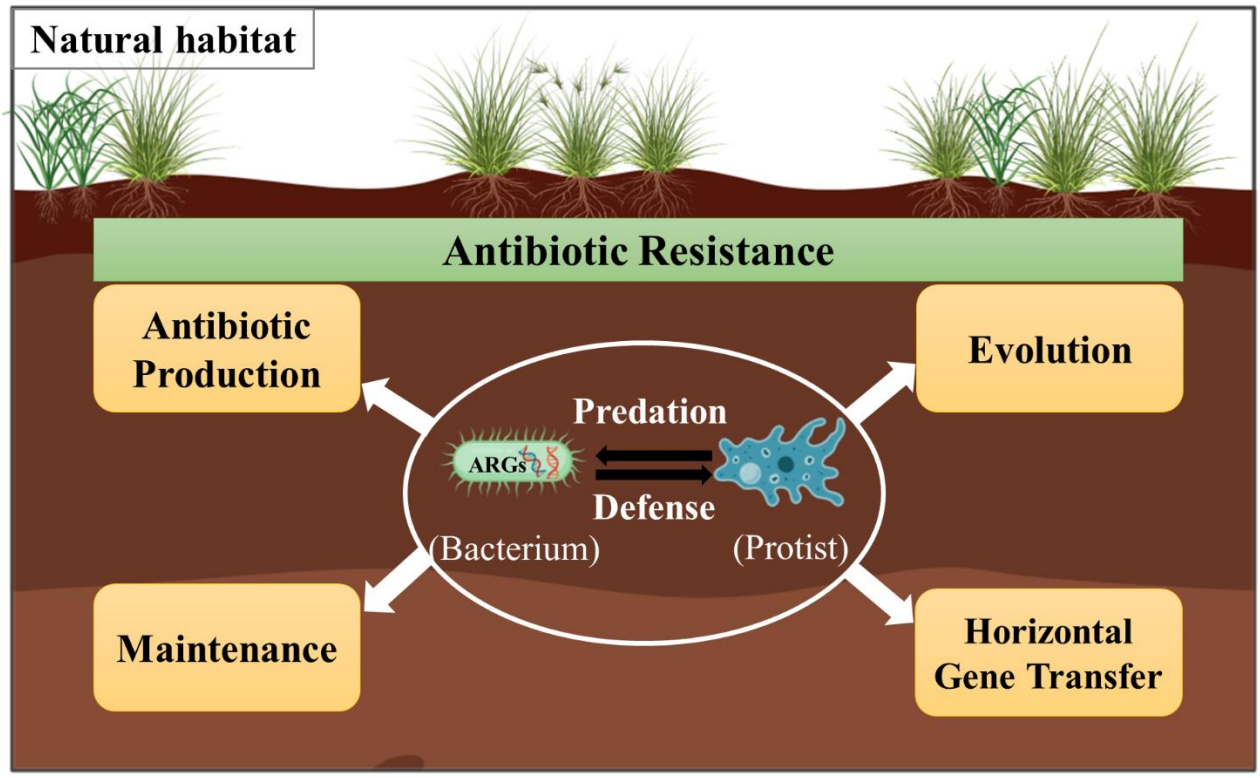
Response to Reviewers: Responses to Editor and Reviewers' comments on STOTEN-D-19-14714

Editor's comments (Jay Gan):
The revised manuscript reads well and is much improved. Figure captions are abnormally long and detailed. Please simplify as much as possible - some information could be placed in the text. A typical figure is one or two lines.

Dear editor Prof Jay Gan,
We would like to thank you for your positive comments and this opportunity to resubmit the manuscript. We have carefully reduced the length of figure captions into 1 or 2 lines throughout the manuscript.
Hang-Wei Hu, on behalf of all co-authors

*Graphical Abstract

Graphical abstract



Highlights

- Antibiotic resistance is a natural phenomenon on Earth
- Biological interactions trigger antimicrobial production and antibiotic-resistant strategies of bacteria in nature
- Protists might be important factors for the origin, evolution, spread and maintenance of antibiotic resistance
- Understandings of protist-bacteria interactions is important to mitigation of the natural antibiotic resistance

Title page

Microbial regulation of natural antibiotic resistance: understanding the protist-bacteria interactions for evolution of soil resistome

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Abstract

The emergence, evolution and spread of antibiotic resistance genes (ARGs) in the environment represents a global threat to human health. Our knowledge of antibiotic resistance in human-impacted ecosystems is rapidly growing with antibiotic use, organic fertilization and wastewater irrigation identified as key selection pressures. However, the importance of biological interactions, especially predation and competition, as a potential driver of antibiotic resistance in the natural environment with limited anthropogenic disturbance remains largely overlooked. Stress-affected bacteria develop resistance to maximize competition and survival, and similarly bacteria may develop resistance to fight stress under the predation pressure of protists, an essential component of the soil microbiome. In this article, we summarized the major findings for the prevalence of natural ARGs on our planet and discussed the potential selection pressures driving the evolution and development of antibiotic resistance in natural settings. This is the first article that reviewed the potential links between protists and the antibiotic resistance of bacteria, and highlighted the importance of predation by protists as a crucial selection pressure of antibiotic resistance in the absence of anthropogenic disturbance. We conclude that an improved ecological understanding of the protists-bacteria interactions and other biological relationships would greatly expand our ability to predict and mitigate the environmental antibiotic resistance under the context of global change.

Keywords:

Antibiotic resistance genes; Protist-bacteria predation; Biological interactions; Selection pressure; Horizontal gene transfer; Anthropogenic disturbance

1. Antibiotic resistance in the natural environment

The growing proliferation of antibiotic resistance genes (ARGs), collectively known as resistome (Wright, 2007), is one of the major challenges to human health in the 21st century. Antibiotics, organic fertilizers (animal manures, digestates or sewage sludge), heavy metals, quaternary ammonium compounds and biocides have been recognized as key selection pressures that can contribute to the emergence, acquisition and dissemination of environmental ARGs (Han et al., 2016; Hu et al., 2016; Hu et al., 2017; Ye et al., 2017; Smalla et al., 2018). However, antibiotic resistance in the biosphere is considered to emerge and evolve long before the use of synthetic antibiotics by humankind since 1940s (Allen et al., 2009; D'Costa et al., 2011; Wright and Poinar, 2012). Recent studies have demonstrated that antibiotic resistance is ancient and ubiquitous in the natural environment and intact ecosystems with no or limited anthropogenic disturbance (Segawa et al., 2013; Pawlowski et al., 2016; Hu et al., 2018; Shen et al., 2019). The ubiquity of ARGs in the natural environment might be attributed to (1) antibiotics or their derivatives antibiotics produced by living organisms in soils (Fleming, 1929; Wright et al., 2014), which have exerted selection pressure on the soil microbiome, and (2) biotic interactions between bacteria and other microorganisms, for example, antagonism of fungi and bacteria directly affects the bacterial community composition and the abundance of ARGs (Bahram et al., 2018). However, a full-spectrum understanding of the underlying mechanisms for the resistance in natural ecosystems is still lacking, and in particular, we have limited knowledge of the role of the biological interactions among bacteria and other microorganisms in the resistance development.

ARGs have been widely detected in ancient permafrost samples with the age of thousand to million years old in polar areas in recent reports (Figure 1; Table 1). In 2003, as one of the earliest evidences of antibiotic resistance, a mobile genetic element (MGE) was found in a bacterial strain *Pseudomonas* sp. A19-1 from 8000~10000-year-old Siberian permafrost samples

(Kholodii et al., 2003). Later in 2005, bacterial strains isolated from Siberian three-million-years-old permafrost samples are resistant to many modern antibiotics (ampicillin, chloramphenicol, erythromycin, streptomycin and tetracycline) (Ponder et al., 2005). In addition, the ancient resistome conferred resistance to β-lactam, tetracycline and glycopeptide were detected in 30,000-year-old permafrost cores in Yukon, Canada (D'Costa et al., 2011; Wright and Poinar, 2012). In particular, one of these ancient genes, a variant of *vanA* encoding an ATP-dependent D-alanyl-D-lactate ligase, was synthesized and its three-dimensional structure is similar to that of present VanA ligases conferring resistance to vancomycin. Different layers of at-least-5,000-year-ago Arctic permafrost in another site in Canada also harbored diverse ARGs, which encoded resistance against many modern antibiotics such as β-lactam, tetracycline, macrolides and aminoglycosides (Perron et al., 2015).

In the absence or with limited anthropogenic disturbance, ARGs have been identified in diverse ecosystems across most of continents on Earth, including ocean bed, forests, mountains, and even polar regions (Figure 1; Table 1). In 2009, ancient β-lactamases and soil microbes conferring resistance against β-lactam were found in remote forest soils in Alaska (Allen et al., 2009). This is an early and important indication of the ARGs' presence in pristine environments, followed by the detection of 160 resistance genes against eight main antibiotic groups in forest soils (Hu et al., 2018). Additionally, a four-million-year cave and indigenous people from an isolated jungle are also habitants of ARG-carrying bacteria that may be resistant against many common antibiotics (Bhullar et al., 2012; Clemente et al., 2015; Pawlowski et al., 2016). In the deep ocean bed, ARGs resistant to macrolides and polypeptides are abundant in the relatively intact sediments (Chen et al., 2013). Furthermore, a wide-range investigation into ice and snow samples across the globe found that several ARGs with potential agricultural or clinical origins were detected from various glaciers in Arctic, Central Asia, North and South America and Africa (Segawa et al., 2013). In recent reports, numerous ARGs encoding resistance to eight main

antibiotic groups were identified at different soil sampling sites in Antarctica (Wang et al., 2016; Van Goethem et al., 2018).

In general, the origin of antibiotic resistance is ancient before the antibiotic era, but the global distribution of ancient ARGs and the mechanism of the antibiotic resistance in natural environments remains largely unknown. There is little knowledge about the factors influencing the antibiotic resistance in ecological systems with no or limited anthropogenic disturbance. Compared to the ever-growing information of abiotic and anthropogenic factors, less is known about the biotic factors as potential natural selection pressures and how they drive the origin and evolutionary processes of antibiotic resistance. In this article, we review the recent advances in our understanding of the role of biological elements in the emergence and dissemination of ARGs in the natural environment.

2. Biological factors as selection pressures of natural antibiotic resistance

To decipher the selection pressures of antibiotic resistance in nature, a mechanistic understanding of ecological purposes of bacteria in using antibiotics is a paramount key to open the locked closet. In comparison to synthetic antibiotics used for pathogen controls in human and animals, the biological functionalities of antibiotics in natural environments remain poorly understood.

2.1 Potential functionalities of antibiotics in natural ecosystems

In the natural environment, microorganisms must cope with competition, predation and other environmental stressors, and they cannot have enough energy, capacity and time to combat those stressors separately (Dragosits et al., 2013; Święciło, 2016; Cruz-Loya et al., 2019). Hence, production of antibiotics and/or the development of the antibiotic resistance system are considered as strategies of bacteria to survive under stressful conditions. It is supposed that

specific bacteria produce antibiotics for multifunctionalities in natural ecosystems, such as (1) competition for space or nutrients with other organisms (e.g. fungi) (Martínez, 2008; Bengtsson-Palme et al., 2017; Bahram et al., 2018); (2) predation on other bacteria for nutrients (Leisner et al., 2016); (3) defense against predation of protists or others (Jousset and Bonkowski, 2010; Song et al., 2015); (4) plant pathogen control regulated by plant exudates or protists (Geisen et al., 2018; Liu et al., 2019); or (5) signaling molecules for regulation of cellular responses or interactions within microbes or plants at low levels (Linares et al., 2006; Yim et al., 2007). Not all bacterial strains can produce antibiotics, but both antibiotic and non-antibiotic producers can develop resistance mechanisms to protect themselves from toxicity of their own antibiotic products or from other species (Munita and Arias, 2016). For instance, bacteria evolutionally developed resistance mechanisms with permeability barrier and efflux transporter systems to pump hazardous or antibiotic compounds out of their cells (Nikaido, 2001; Lubelski et al., 2007). This is an example of bacterial responses to environmental stressors.

2.2 Proposed selection pressures of natural resistome

Based on above-mentioned potential functionalities of antibiotics in nature, we propose that fundamental biotic interactions among microorganisms, namely competition, predation, antipredation, and/or interactions with plants, might be important factors for the production of antibiotics and the evolution of antibiotic resistance in nature (Figure 2). These biological factors dramatically influence the community structure of soil microorganisms and their mediated ecosystem services (Cairns et al., 2018). Firstly, competition among bacteria and other microorganisms, an essential ecological process in all biomes, stimulates bacteria to suppress or kill their antagonists by releasing toxic antimicrobial compounds (Fig. 2A) (Hibbing et al., 2010; Gonzalez and Mavridou, 2019). Bacteria, fungi and other microbes are competitors of bacteria in soil, and they compete nutrients, space and other resources for the maintenance of population. It

128 can be a great leverage for bacteria to evolve the antibiotic resistance mechanisms to outcompete
129 their competitors for survival. Secondly, predation of bacteria on other microbes by utilizing
130 antibiotics for cell lysis is hypothesized to form a significant pressure to enhance resistance
131 development in the microbial community (Fig. 2B). In 1941, *Bacillus brevis* produced an
132 antibiotic tyrocidine to destroy cellular structures of bacterial strains *Escherichia coli* and
133 *Staphylococcus aureus* (Dubos and Hotchkiss, 1941). The lytic phenomenon of susceptible
134 strains *E. coli* and *S. aureus* was examined under microscopic analysis. It is assumed that
135 antibiotic producers feed on other bacterial cells by producing antibiotics to lyse cells of
136 susceptible preys (Leisner et al., 2016), and then both antibiotic producers and other antibiotic
137 resistant bacteria may take advantage of the lysed cells as nutrients. Thirdly, the predation of
138 protists and other soil predators (mainly nematodes and microarthropods) is postulated as a
139 pivotal selection pressure on the antibiotic-resistance evolution of bacteria (Fig. 2C). Protists
140 mainly graze on bacteria and fungi. To escape from protistan grazers, the evolution of anti-
141 predatory systems is a prerequisite for bacteria to repel or escape protists' attracts (Matz and
142 Kjelleberg, 2005). As one of crucial predators in soil microbiomes, protists regulate the
143 composition and activities of bacterial and other microbial communities in soil ecosystems
144 (Geisen et al., 2018; Gao et al., 2018). Nonetheless, direct and indirect impacts of soil predators
145 on soil microbial activities and antibiotic resistance in nature are still remarkable knots needed to
146 be disentangled.

147 Finally, bacteria – plant interaction to suppress plant pathogens by biological agent
148 production of bacteria is a considerable contributor to the development of natural resistome (Fig.
149 2D). In the rhizosphere, plants can manage the composition of rhizosphere-associated bacteria by
150 excreting root exudates to suppress or promote microbial activities and populations (Berendsen
151 et al., 2012; Liu et al., 2019). Intriguingly, when plants are attacked by pathogens, beneficial
152 bacteria are recruited by volatile organic compounds from plant roots and induced to produce

pathogen-inhibitory compounds for plant protection (Berendsen et al., 2012; Liu et al., 2019). A number of these inhibitory compounds are known as antibiotics (Rudrappa et al., 2008; Mavrodi et al., 2012; Haas and Défago, 2005), and they can activate the resistance systems and select antibiotic resistant microorganisms in soils. Therefore, the interactions between bacteria and plants may potentially promote the availability of natural antibiotics, thereby further enhancing the dissemination of resistance among soil microbiomes.

3. Protists and their interactions with bacteria

Protists, primarily microscopic and single-celled eukaryotes, are an important group of microbial predators that regulate key ecological functions and services (Hiltunen et al., 2012; Geisen et al., 2018). They are highly diverse and ubiquitous in terrestrial and aquatic ecosystems (Adl and Coleman, 2005; De Vargas et al., 2015) including harsh conditions such as North pole (De Jonckheere, 2006), the edge of a volcanic fumarole (De Jonckheere et al., 2011), and desert (Rodriguez-Zaragoza et al., 2005). Protists are an integral regulator on microbial diversity and functions through predation on bacteria (Rønn et al., 2002) and fungi (Geisen et al., 2016), but other protists and soil biota can be also their foods (Yeates and Foissner, 1995; Geisen et al., 2015; Dumack et al., 2019). In ecology, they govern functionalities, compositions and evolution of microbes via predation activities (Rosenberg et al., 2009; Cairns et al., 2016), and form an important factor influencing the biogeochemical nutrient cycling in the soil food web (Bonkowski et al., 2000; Jassey et al., 2015; Trap et al., 2016; Creevy et al., 2016). The intimate interaction between protists and root-associated microbiomes has significantly direct and indirect influences on the health, development and tolerance of plants to environmental stressors in various ways (Geisen et al., 2018; Gao et al., 2018). Furthermore, the response of protists to different agricultural management practices, particularly applications of pesticides (Imparato et al., 2016) and fertilizers (Lentendu et al., 2014; Zhao et al., 2019), might consequently influence other soil microbes under the context of overpopulation and climate change.

Soils are the richest and most diverse habitats for microorganisms and other living organisms that are central to the functioning of our planet (Nielsen et al., 2015). However, the majority of soil protists and their diversity are still in a black box. Protists and bacteria have close interactions in the food web, such as parasitism and mutualism and predation (Sherr and Sherr, 1994; Davidson, 1996). Bacterivorous protists selectively target bacteria based on bacterial morphological traits (Gonzalez et al., 1990; Kinner et al., 1998; Baltar et al., 2016), excreted metabolic products (Matz et al., 2004a) and volatile compounds (Kai et al., 2009; Schulz-Bohm et al., 2017). The bacterial preys evolved to develop antipredation strategies to fight against their predators (Matz and Kjelleberg, 2005). There are possibly five main defensive mechanisms, including (1) morphological alteration into inedible forms to avoid recognition (Jürgens et al., 1999; Pfandl et al., 2004); (2) biofilm formation to create a protective fence (Matz et al., 2004b); (3) motility enhancement to escape (Šimek et al., 2001; Matz and Jürgens, 2005); (4) production of toxic secondary metabolites to kill or repel attackers (Jousset and Bonkowski, 2010; Song et al., 2015); and (5) symbiosis with protists to avoid other protistan grazers (Monteil et al., 2019). Among these strategies, the biosynthesis of secondary metabolic compounds is regarded as an immense strategy to defend against grazing, and most of these antimicrobial agents, such as violacein (Matz et al., 2004a), 2,4-diacetylphloroglucinol (Jousset and Bonkowski, 2010) and massetolide (Mazzola et al., 2009), are antibiotics used as anti-predatory weapons. The grazing activity of protists is probably an important factor causing the release of antibiotics by specific bacteria in natural settings.

4. Protist predation induces production of antimicrobial and anti-protist agents

Although protists play important roles in multiple ecological functions, our knowledge of protists and their relationships with bacterial antibiotic resistance in the environment remains limited. Many previous studies have indicated the relevance of protists to the emergence of biological antibiotics and the evolution, dissemination and/or maintenance of antibiotic

resistance through *in vitro* or *in vivo* experiments (Table 2). We argue that the predation of protists may directly drive the production of antimicrobial and anti-protist agents and the evolution of antibiotic resistance in nature. However, there is a lack of knowledge about the exact impacts of protistan predators on the antibiotic resistance within microbial communities in natural settings.

Bacteria are frequently encountered the predatory activity of protists in all biomes, which impose a selective pressure on the bacterial community. Bacteria must build up their defense mechanisms to escape the capture of protists, and the production of antibiotics as anti-protists agents is considered as one of an advanced methods for them to fight against their grazers (Jousset et al., 2006). Susceptive bacteria may be easily consumed by protists. For resistant strains, recent studies have indicated that each resistant bacterium can release one or few antibiotics for anti-predatory purposes (Table 3). Bacteria may selectively decide types of the anti-predatory compounds by sensing the grazing habit, species or other characteristics of protists. Jousset et al. (2006) identified that the co-predation of three different protists induced a single bacterial strain *Pseudomonas fluorescens* CHA0 to produce an arsenal of antibiotics and other compounds (hydrogen cyanide and exoprotease) under culture conditions. The bioactivity of the excreted antibiotics effectively inhibited the protist growth (Jousset et al., 2006). Under the attack of the same grazer, *Pseudomonas fluorescens* strain SS101 and *Pseudomonas fluorescens* strain SBW25 produced particular antibiotics massetolide and viscosin, respectively, to protect their cells (Mazzola et al., 2009; Song et al., 2015). The biological action of 2,4-diacetylphloroglucinol was also found in *Pseudomonas fluorescens* Q2-87 as a biological weapon to defend against their predator (Jousset and Bonkowski, 2010). On the other hand, same antimicrobial agents can be produced by different bacterial preys. Two freshwater isolates *Janthinobacterium lividum* CM37 and *Chromobacterium violaceum* ATCC 31532 excreted violacein, an antibiotic compound, to toxify three different nanoflagellated protists in *in vitro*

experiments (Matz et al., 2004a). Flagellate cells quickly died and were lysed by the action of violacein (Matz et al., 2004a).

In general, the predator-prey interaction can activate essential defensive actions of bacteria at risk of being consumed (Matz and Kjelleberg, 2005). Although only Jousset et al. (2006, 2010) mentioned that the compounds for anti-predatory activities are antibiotics, all products excreted by bacteria to defend protists in Table 3 are considered as antibiotics in previous studies, including violacein (Fang et al., 2015; Im et al., 2017), 2,4-diacetylphloroglucinol (DAPG), pyoluteorin and pyrrolnitrin (Haas and Défago, 2005; Jousset et al., 2006; Jousset and Bonkowski, 2010), viscosin and massetolide (De Bruijn et al., 2007). Therefore, the protist predation might be a crucial factor for the regulation of antibiotic production and bacterial community activities in natural habitats. Nevertheless, the majority of previous laboratory-based studies were conducted on one or few model and non-indigenous species. Despite soils being renowned as a huge reservoir of ARGs, there is a shortage in research on soil protists (Gao et al., 2018), which necessitate community-level investigations to decipher the chemical communication between protists and other microbiomes.

5. Protists impact the evolution and maintenance of antibiotic resistance

Although protists impose a selective pressure on the bacterial production of antibiotics, probably at low concentrations, recent laboratory investigations pointed out that the predation of heterotrophic protists associated with low-level antibiotics has potential effects on the evolution and maintenance of resistant bacteria (**Table 2**). The evolution of preys *Pseudomonas fluorescens* SBW25 gained the highest fitness during their adaptation to a gradient of an antibiotic gentamicin and two predators *Tetrahymena pyriformis* and *Chilomonas paramecium* under culture conditions (Friman et al., 2015). Evolved preys can grow better upon stresses of antibiotics and their enemies. Notably, the evolutionary fitness of bacterial preys is strengthened

252 in the presence of co-evolved protists (Hiltunen and Becks, 2014). Furthermore, the horizontal
253 gene transfer of the antibiotic resistance plasmid under the predatory action maintained the
254 availability of antibiotic resistance elements and survival of the bacterial community (Cairns et
255 al., 2016), thus the protistan grazing is suggested to foster the evolution of bacterial preys and
256 ultimately antibiotic resistance in ecosystems.

257 By the consumption of bacteria and other microbes, protists excrete nitrogen and
258 phosphorus sources (Bonkowski et al., 2000; Trap et al., 2016) and other micronutrients in the
259 environment (Gao et al., 2018). Microorganisms and plants can take advantage of these nutrients
260 to promote their growth, and some opportunistic bacteria conferring resistance against protists
261 can utilize available nutrients and rapidly develop their population. Gao et al. (2018) also
262 suggested that the predation of protists enforces the evolutionary dynamics and abundance of
263 protist-resistance microbiomes in ecosystems. Therefore, we speculate that antibiotic resistant
264 bacteria that produce antibiotic agents against protists may comprise a main component of the
265 microbial community under the grazing pressure.

266 Apart from the selection pressure imposed by protist predation, protists can be possible
267 vectors of antibiotic resistance transmission to soil fauna and higher trophic levels. Many living
268 antibiotic-resistant bacteria were identified in digestive tracts of protists after capture, and they
269 transferred ARGs to other un-resistant species (Schlimme et al., 1997; McCuddin et al., 2006).
270 Notably, while protists serve as a food source of soil fauna (e.g. nematodes) in the soil food web,
271 a number of bacterial symbionts that colonize within protists' bodies (Monteil et al., 2019) may
272 transfer to soil fauna. Through grazing activities of soil consumers, bacteria might be transferred
273 from protists to soil fauna, other organisms, and plants via rhizosphere systems. Soil protists
274 might be potential vectors to spread ARGs and antibiotic resistant bacteria to larger spatial scales
275 and higher trophic consumers and human. Therefore, we argue that protists may be an important
276 factor for the persistence of resistant bacterial populations. Eventually, these can raise concerns

on promoting the evolution, spread and maintenance of antibiotic resistant pathogens and antibiotic resistance.

6. Protists may promote horizontal gene transfer among bacteria

Bacterial ARGs can be transferred among microorganisms and/or pathogens by horizontal gene transfer (HGT), facilitated by MGEs consisting of plasmids, integrons, and transposons (Xie et al., 2018). HGT is a key process rendering the widespread distribution of ARGs in the environment (Bengtsson-Palme et al., 2017). Some previous investigations indicated the HGT occurrence of ARGs among bacteria inside protist's bodies, especially gastrointestinal tracts (**Table 2**). In 1997, Schlimme and his colleagues found that digestive vacuoles and fecal pellets of protists *Tetrahymena pyriformis* are sites for HGT events of a conjugative plasmid RP4, harboring resistance to antibiotics ampicillin, kanamycin and tetracycline, between *Escherichia coli* strains. The model predators *Tetrahymena pyriformis* enhanced the intensity of the resistance gene dissemination within the bacterial community (Schlimme et al., 1997). In addition, the HGT mode conjugation of ceftriaxone-resistance genes within two bacterial isolates *Klebsiella* and *Salmonella*, isolated from adult cattle and goats, was identified inside rumen protists (McCuddin et al., 2006). Bacterial isolates *Klebsiella* shared ARG-carrying plasmids to susceptible strains *Salmonella* for survival within protist cells. In the presence of protist inhibitor, there was no observation of ceftriaxone-resistant *Salmonella* strains (McCuddin et al., 2006) . Likewise, under *in vivo* conditions, the resistance gene transfer among *Enterococcus faecalis* waterborne pathogens still occurred in the protistan digestive system, as a conjugative platform, after being ingested by protist grazers (Olanrewaju et al., 2019). Therefore, the digestion or capture of protists might stimulate the HGT of ARGs among these bacterial strains, and protists' bodies might be a hotspot of HGT and bacterial evolution.

Furthermore, the protist predation contributes to the spread of ARGs within selective model strains through HGT. The predatory activity of ciliated protists *Tetrahymena thermophila*

CCAP 1630/1U provoked the spread and maintenance of resistant factors among bacterial pathogens *Serratia marcescens* (Cairns et al., 2016). Upon the protist grazing, the conjugative transfer of plasmid RP4 encoding kanamycin, ampicillin and tetracycline resistance was surged to promote pathogenic survival. Cairns et al. (2018) indicated that multiple ecological factors including a low-level antibiotic kanamycin, predation and spatial structure also affected the community composition and HGT of antibiotic resistance plasmid in a 62-strain artificial bacterial assemblage. Therefore, we suppose that protists are a potential contributor to the evolution and dissemination of antibiotic resistance. To date, the direct roles of protists in the HGT process of ARGs in bacterial community have not been addressed, especially in soil and water systems.

7. Protists and natural resistome under the context of global changes

In the environment, protistan or bacterial species do not live alone in their own "kingdom" but always interact with their supporters, enemies or neutrals. It can be supposed that soil biota, plants, animals and humans form "a closed system" (Figure 3), in which shifts in one component can affect others. Soils, a huge reservoir of natural resistome, are the richest habitat of microorganisms as one of the original hosts of clinical ARGs that can be transferred to human pathogens (Wright, 2010; Forsberg et al., 2012). Interactions of soil biota, significantly impacted by plants and nutrient resources, can form crucial factors in the emergence and development of resistome and antibiotic resistance in complex soil matrix.. In microbial assemblages, protists play crucial roles as consumers, which directly drive bacterial and fungal functions and affect microbial communities, and also act as preys of soil fauna (e.g. nematodes). However, there is a paucity of knowledge about protists - bacteria interaction on resistome in pristine environments, influences of predatory protists on bacteria – bacteria, and bacteria – fungi competitions that are key drivers of the antibiotic resistance (indicated in red arrows in Figure 3). By the capture and

symbiotic associations with bacteria, protists are also carriers of antibiotic resistant bacteria and ARGs that are potentially migrated to soil fauna's bodies through grazing, and to plant tissues through root uptake, air, soil biota or insects. ARGs and the resistant bacteria in soils can be transferred to larger spatial scales and higher trophic consumers and human bodies via food chains, direct contact, or the surrounding environment (Zhang et al., 2019a; Chen et al., 2019). Unexpectedly, animal manures and human wastes (sewage sludge, wastewater and biosolids) also bring large inputs of ARGs and resistant microorganisms to belowground systems, and this exacerbates the dissemination of ARGs in soils (Lillenberg et al., 2010; Bouki et al., 2013; Cycoń et al., 2019). Recent studies reported that land application of animal manures for crop production dramatically enhanced the occurrence of ARGs and significantly changed microbial communities in soils (Zhang et al., 2017) and plant tissues (especially rhizosphere and phyllosphere) of vegetables such as lettuces (Zhang et al., 2019a) and cherry radish (Zhang et al., 2019b). Humans can be in risk of being infected by ARG-carrying pathogens via food chain (meats, vegetables or fruits) or direct contacts with animals, food materials, soils and other people.

Protists and other microorganisms are vulnerable to global changes including climate change, environmental pollution and land use changes (Nielsen et al., 2015). Diversity and abundance of protists mostly depend on seasons, soil pH, moisture, and nutrient resources (Bates et al., 2013; Tedersoo et al., 2016; Maritz et al., 2019). Climate change can alter temperature and rainfalls on our planet, and ultimately threatens biodiversity of soil protists and their preys (e.g., bacteria, fungi and others) and impede their capacity to facilitate plant growth and other ecological processes (Cavicchioli et al., 2019). In addition, anthropogenic activities, such as environmental pollution, land use changes, and agricultural management practices (Nielsen et al., 2015), cause loss of microbial diversity and habitants, and further impose strong stressors on the evolution and spread of ARGs. Therefore, global changes are supposed to directly influence the

holistic system, damage the balance of natural resistome, and boost evolution and proliferation of antimicrobial resistant bacteria in both clinical and natural settings.

8. Concluding remarks and future perspectives

Protists, a crucial regulator of bacterial structure and functionalities, and their intimate predator-prey interactions with bacteria might exert certain selection pressures on the resistance events related to antibiotic agents. Such biological interactions and competition are supposed to be potential mechanisms by which microbiomes maintain their antibiotic resistance in the environment. We argue that more attention should be paid to the biological interactions among microorganisms to understand the emergence and evolution of resistance, and the roles of protists and other soil organisms on ARGs in the environments with limited human-impacts (Outstanding questions). Additionally, investigations into relevant biotic (e.g., plant and soil fauna), abiotic (e.g., soil nutrients, soil pH, and climate) and human factors (e.g., land-management practices) are important to have a system-level knowledge about the dissemination of antibiotic resistance in both pristine and clinical habitants (Outstanding questions). Further investigations on community levels are necessary to understand the bacterial sensing and excretion of antimicrobial agents in response to soil protists. In this review, we proposed a framework to quantify the importance of protists on the evolution of ARGs in terrestrial ecosystems (Figure 4). In the proposed framework, indigenous protists and bacteria in soils are suggested to be investigated on community levels in field studies or laboratory-based microcosm experiments with intensive analyses, including (1) molecular analyses (high-throughput sequencing, quantitative PCR and high-throughput quantitative PCR) to estimate levels of impacts via the relative abundance and diversity of protists, bacteria and ARGs; (2) biochemical and molecular analyses (metatranscriptome, metaproteomics and metabolomics) to identify cellular activities and the metabolized products of protists and bacteria; (3) proximity-ligation

method to detect the origin of metagenomic materials, e.g. ARGs; and (4) measurements of edaphic properties to determine structure and distribution of soil microorganisms and ARGs. In brief, a mechanistic understanding of microbial interactions will provide fundamental insights into the prediction and management of the ARG evolution and dissemination, and eventually will facilitate mitigation and controls of antibiotic-resistant pathogens in future.

Outstanding Questions:

- In the absence of anthropogenic disturbance, what environmental factors and processes affect the biological interactions and where the HGT events take place in the environment?
- What are the underlying mechanisms for bacterial sensing and excretion of antimicrobial agents in response to soil protists and their chemical communication?
- How does global changes, such as climate change, environmental pollution, nitrogen deposition and land-use changes, influence the protist-bacteria interactions and its consequences for the evolution and dissemination of antibiotic resistance?
- What is the role of protist as vectors in the dissemination of ARGs in the soil food web, and from soil microbiome to the plant microbiome?
- What is the relative contribution of protist-bacteria predation to the prevalence of antibiotic resistance in the environment? How to quantify the proportion of total antibiotic resistance in a soil explained by biological factors?

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742 **Table 1** Evidences of antibiotic resistance in environmental samples before synthetic antibiotic use or
743 with no or limited anthropogenic disturbance.

Categories	Sampling Sites	Samples	Main Findings	References
Before synthetic antibiotic use	n/a	A bacterial strain	An enzyme penicillinase to destroy penicillin action in 1940	(Abraham and Chain, 1940)
	The polar region of the Kolyma-Indigirka lowland, near the East Siberian Sea	8000~10000-year-old Siberian permafrost sample	A mobile genetic element, a Tn21-related mercury resistance transposon, Tn5060, was detected from a bacterial strain *Pseudomonas* sp. strain A19-1.	(Kholodii et al., 2003)
		Permafrost core samples up to 3 million years	Siberian permafrost isolates are resistant to many antibiotics, but the resistance is different among isolated bacterial strains.	(Ponder et al., 2005)
	East of Dawson City, Yukon, at the Bear Creek (Canada)	Floral and faunal samples collected from 30,000-year-old permafrost cores.	Ancient resistome conferred resistance to β-lactam, tetracycline and glycopeptide was identified. The three-dimensional structure of the enzyme vancomycin resistance element *VanA* is similar as modern VanA ligases.	(Wright and Poinar, 2012)
		30,000-year-old permafrost sediments		(D'Costa et al., 2011)
	Eureka, Ellesmere Island, Nunavut, Canada	Different layers of the at-least-5,000-year-ago Arctic permafrost.	+ In the ancient permafrost core, eight ARGs confer resistance against aminoglycoside, β-lactam, tetracycline, and particularly an unoriginal microbial antibiotic amikacin. + In bacteria sampled from the overlaying active layer, 10 ARGs were resistant to all six antibiotics including aminoglycoside, β-lactam and tetracycline variants.	(Perron et al., 2015)
No or limited human impacts	Bonanza Creek Experimental Forest, Alaska	Alaskan soil	Ancient β-lactamases were found soil, and soil microbiomes in this remote area conferred resistance against the antibiotic β-lactam.	(Allen et al., 2009)
	China	Soils in pristine forests ranging over 4000 km.	160 genes resistant to eight main groups of antibiotics were found	(Hu et al., 2018)

		in forest soils, and β-lactamase is the most common gene.	
Centralia, Pennsylvania, United States	Soils above the underground coal seam fire for 57 years of burning operation.	17 ARGs spanning most of main groups of antibiotics in Centralia. Soil temperature impacted soil microbiome and rendered shifts in diversity and abundance of some ARGs.	(Dunivin and Shade, 2018)
Deep ocean bed, the South China Sea	Relatively pristine deep ocean sediments	The most prevalent and abundant ARGs conferred resistance to macrolides and polypeptides.	(Chen et al., 2013)
Lechuguilla Cave (isolated for more than 4 million years), New Mexico.	Bacterial isolates from a cave isolated for more than 4 million years.	An isolated bacterium *Paenibacillus* sp. LC231 is resistant against most common used antibiotics. The intrinsic resistome of the bacterial strain is preserved over four million years.	(Pawlowski et al., 2016)
		Bacterial species obtained have resistance against many different commercial antibiotics, and several isolates were resistant to up to 14 antimicrobial drugs.	(Bhullar et al., 2012)
Terra Nova Bay, southeast Antarctica.	62 soil samples near Research stations in Antarctica	73 detected ARDs encode resistance to eight main antibiotic groups but most at very low concentrations. There was no increasing trend on the relative abundance of ARGs within four years of the study.	(Wang et al., 2016)
Arctic, Central Asia, America, Africa, Antarctica	54 snow and ice samples	ARGs originated from agriculture and clinics were detected from various glaciers in Central Asia, North and South America, Greenland and Africa.	(Segawa et al., 2013)
High Orinoco area, Amazonas state, Venezuela	Human bodies of people in a jungle.	Bacterial strains isolated from indigenous people were identified with many ARGs and MGEs that are related to resistance against many commercial antibiotics.	(Clemente et al., 2015)
Mackay Glacier, Antarctica	17 Antarctic soils	177 ARGs mostly conferring resistance to aminoglycosides,	(Van Goethem et al., 2018)

			chloramphenicol and β-lactam antibiotics were detected.

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746 **Table 2** Studies on the protist-bacteria interactions related to antibiotic resistance

Categories of Research	No. of Protists	No. of Bacteria	Conditions	References
Predation-Antibiotic Production	1	1	*In vitro*	(Jousset and Bonkowski, 2010)
Predation-Antibiotic Production	1	2	*In vitro* and *in situ*	(Mazzola et al., 2009)
Predation-Antibiotic Production	3	1	*In vitro*	(Jousset et al., 2006)
Predation-Antibiotic Production	3	Several isolates	*In vitro*	(Blom and Pernthaler, 2009)
Predation – ARG release	4	1	*In vitro*	(Bien et al., 2017)
Predation - Evolution	3	5	*In vitro*	(Saleem et al., 2012)
Predation - Evolution	2	1	*In vitro*	(Friman et al., 2015)
Predation - Co-evolution	1	1	*In vitro*	(Hiltunen and Becks, 2014)
Predation - Antibiotic Activity	1	2	*In vitro*	(Song et al., 2015)
Predation - Antibiotic Activity	3	2	*In vitro*	(Matz et al., 2004a)
Predation - HGT	1	2	*In vitro*	(Schlimme et al., 1997)
Predation - HGT	1	62	*In vitro*	(Cairns et al., 2018)
Predation - HGT	1	2	*In vitro*	(Cairns et al., 2016)
HGT	1	5	*In vitro* and *in vivo*	(McCuddin et al., 2006)
HGT	2	2	*In vitro* and *in vivo*	(Olanrewaju et al., 2019)

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749 **Table 3 Bacteria producing antibiotics under the predation of protists**

Bacterial Strains	Antibiotics	Protists	Conditions	References
Janthinobacterium lividum CM37, *Chromobacterium violaceum* ATCC 31532	violacein	*Ochromonas sp.*, *Spumella sp.*, *Bodo saltans*	*In vitro*	(Matz et al., 2004a)
Pseudomonas fluorescens CHA0	2,4-diacetylphloroglucinol (DAPG), pyoluteorin and pyrrolnitrin	*Vahlkampfia* sp. and12, *Neobodo designis*, *Colpoda steinii*	*In vitro*	(Jousset et al., 2006)
Pseudomonas fluorescens strain SS101	massetolide	*Naegleria americana*	*In vitro* and *in situ*	(Mazzola et al., 2009; Song et al., 2015)
Pseudomonas fluorescens strain SBW25	viscosin			
Pseudomonas fluorescens Q2-87	2,4-diacetylphloroglucinol	*Acanthamoeba castellanii*	*In vitro*	(Jousset and Bonkowski, 2010)

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Figure 1 The prevalence of antibiotic resistance genes in the environment before or with limited human disturbance. Note: Locations are approximate to depict detected sampling sites.

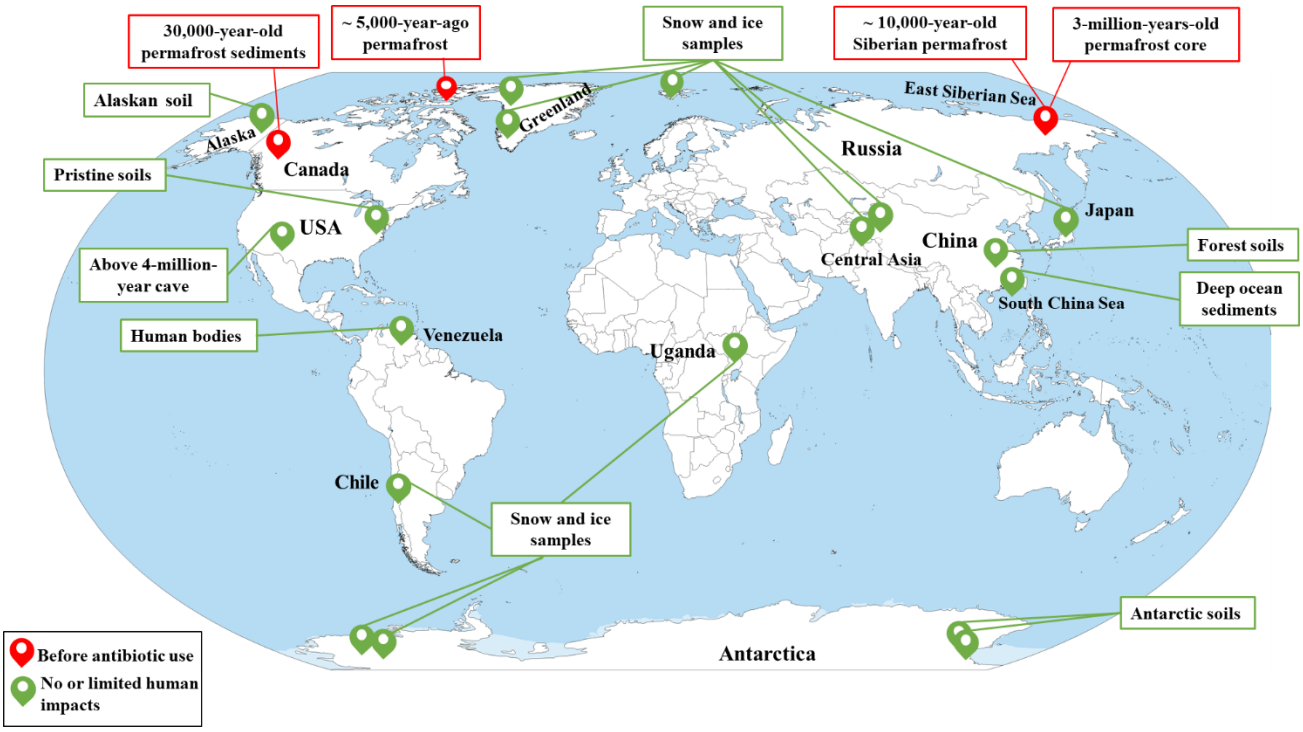


Figure 2 Potential selection pressures of natural resistome: (A) Competition between bacteria and microbes; (B) Predation of bacteria on microbes; (C) Predation of predators on bacteria; and (D) Bacteria-plant interactions.

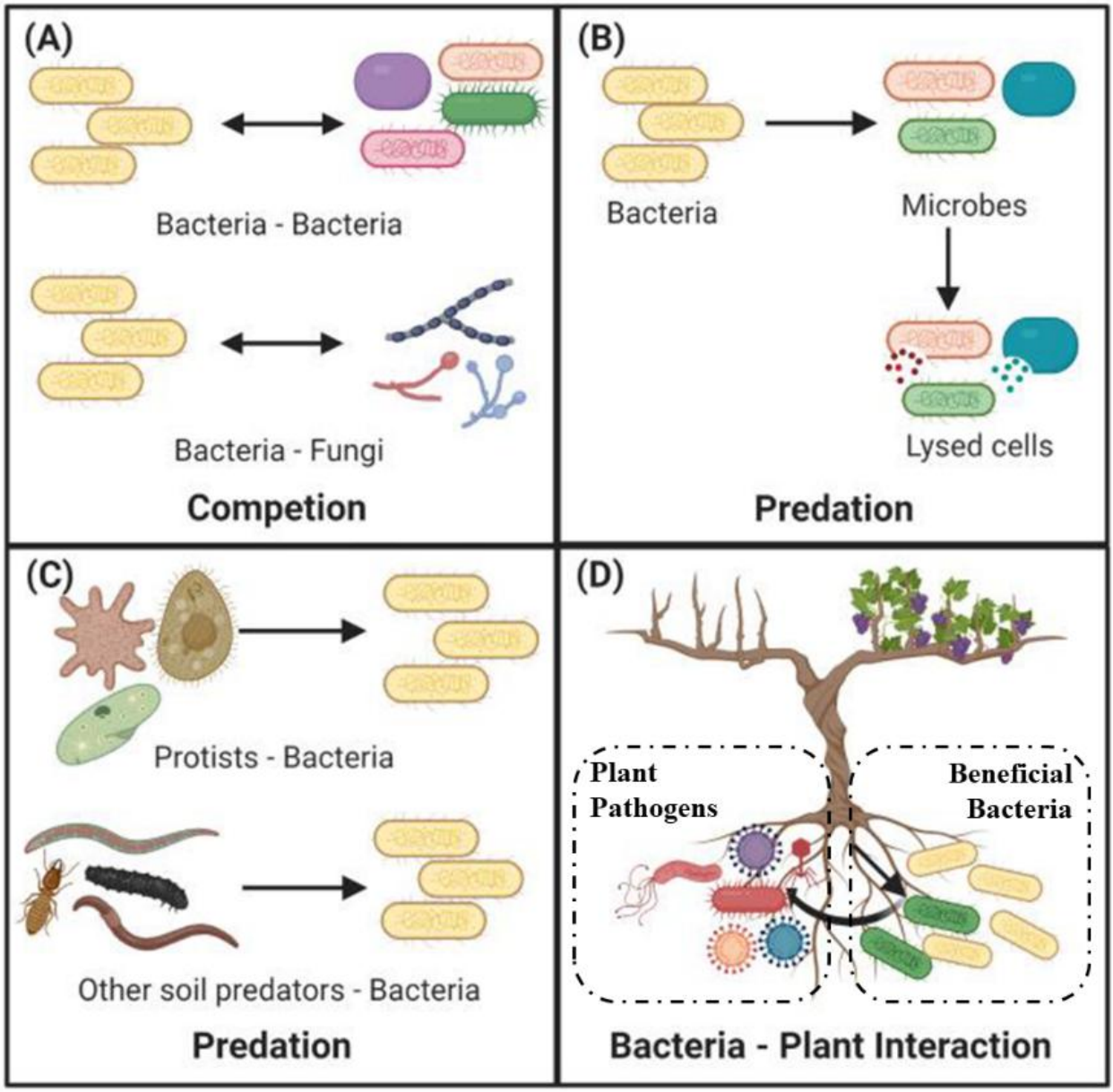


Figure 3 The closed complex system under the context of global changes. Global changes can significantly affect the equilibrium of natural resistome and the development and evolution of antibiotic resistance.

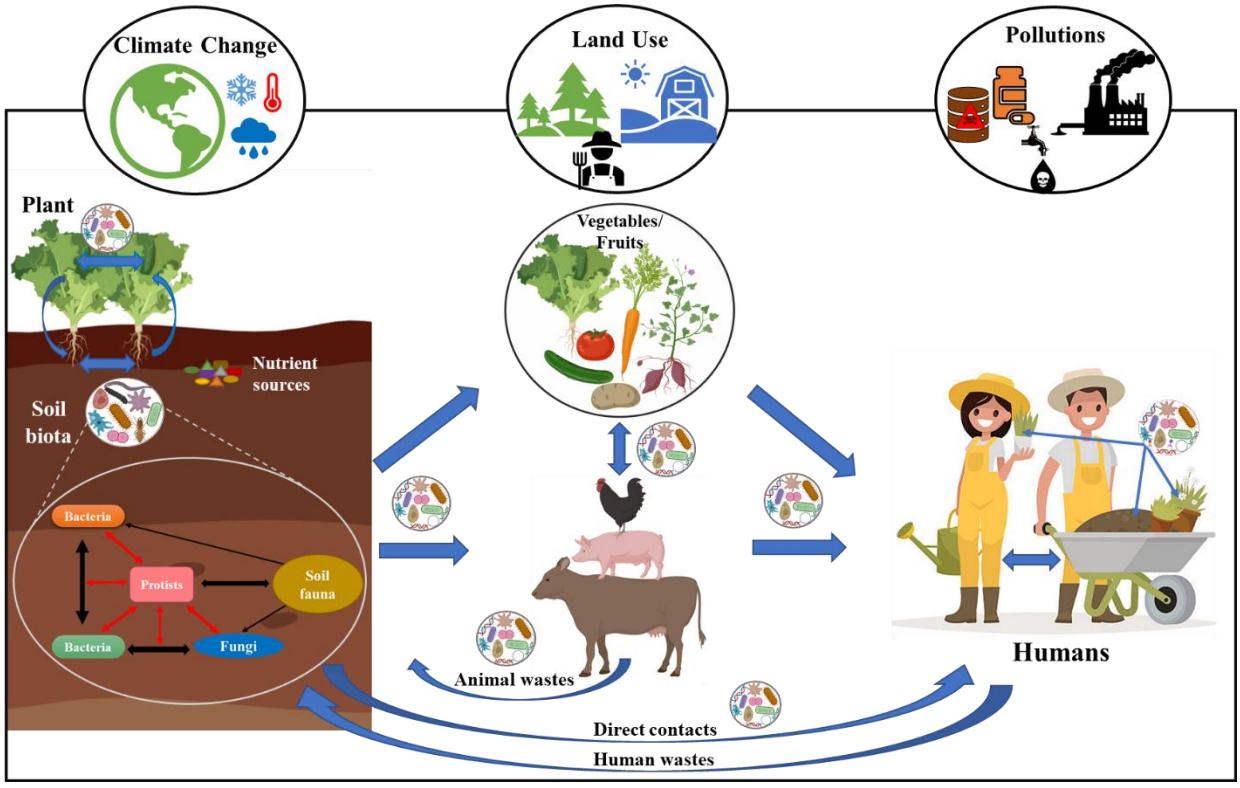
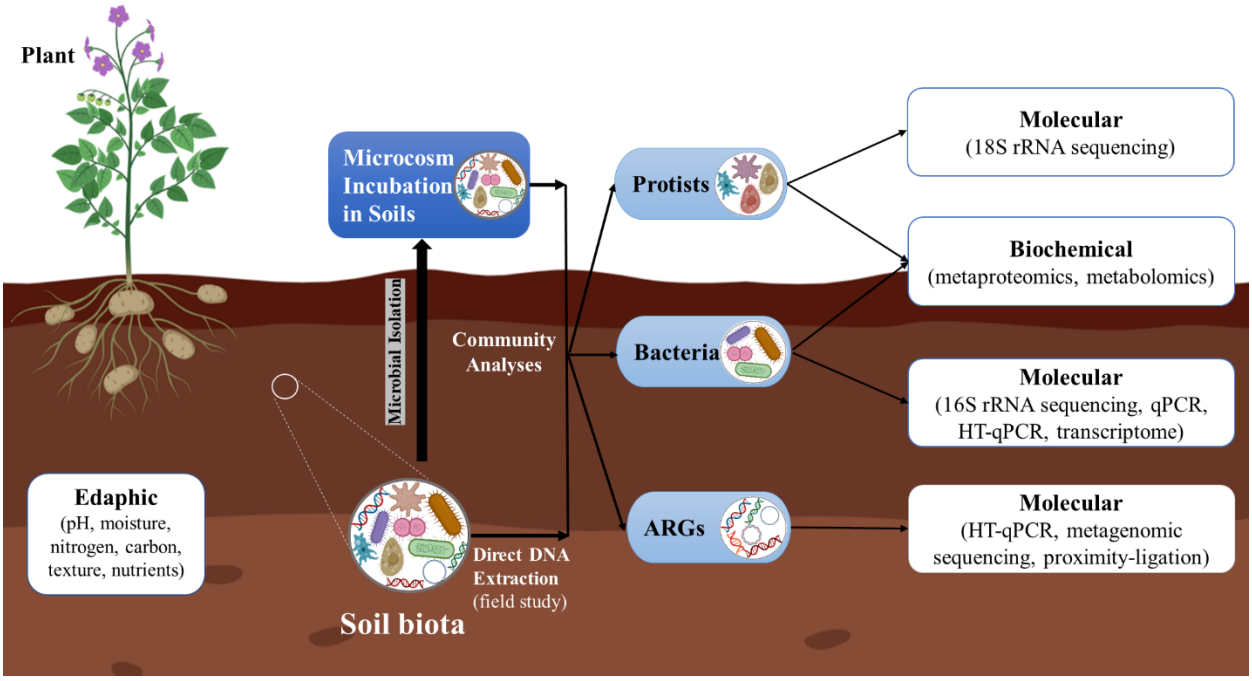


Figure 4 A proposed framework to quantify the importance of protists on the evolution of ARGs and bacterial dynamics in soils.



*Conflict of Interest Statement

The authors declare no conflict of interest.