

## COMMENTARY

## Thoughts &amp; Opinion

# A case for the importance of following antibiotic resistant bacteria throughout the soil food web

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## Funding information

MCIN/AEI/10.13039/501100011033,

Grant/Award Number:

PID2020-116495RB-I00; Basque

Government, Grant/Award Number:

IT1578-22; JRL Environmental Antibiotic Resistance

## Abstract

It is necessary to complement next-generation sequencing data on the soil resistome with theoretical knowledge provided by ecological studies regarding the spread of antibiotic resistant bacteria (ARB) in the abiotic and, especially, biotic fraction of the soil ecosystem. Particularly, when ARB enter agricultural soils as a consequence of the application of animal manure as fertilizer, from a microbial ecology perspective, it is important to know their fate along the soil food web, that is, throughout that complex network of feeding interactions among members of the soil biota that has crucial effects on species richness and ecosystem productivity and stability. It is critical to study how the ARB that enter the soil through the application of manure can reach other taxonomical groups (e.g., fungi, protists, nematodes, arthropods, earthworms), paying special attention to their presence in the gut microbiomes of mesofauna-macrofauna and to the possibilities for horizontal gene transfer of antibiotic resistant genes.

## KEYWORDS

Antibiotic resistance, gut microbiomes, horizontal gene transfer, manure, microbial ecology, organic farming, soil biota

## COMMENTARY

Antibiotic resistance is a global health problem of the utmost importance. The study of the emergence, dissemination, and evolution of antibiotic resistant bacteria (ARB), harbouring antibiotic resistance genes (ARGs), is often claimed to require a One Health approach. Then, in the last decades, much research has been done on the links between the human resistome and the environmental resistome (the resistome is the collection of all the ARGs and their precursors present in pathogenic and non-pathogenic bacteria). It is important to emphasize that microorganisms (e.g., bacteria, fungi) have used antibiotics, for competition purposes or as signalling molecules that facilitate intra- or interspecies interactions within microbial communities,<sup>[1]</sup> for millions of years. Therefore, ARB and ARGs are often isolated from pristine environments, not subjected to antibiotic residues derived from human

usage, indicating that the resistome is an integral part of the microbial ecology of the different environmental compartments.

Due to the use of antibiotics in livestock, there are many studies on the potential consequences of fertilization with animal manure for the introduction of ARB and ARGs, the latter in many cases associated with mobile genetic elements (MGEs), in agricultural soils, with concomitant risks for human health via the consumption of crops, especially fresh horticultural produce.

In approximately the past decade, as a result of the remarkable development of advanced sequencing technologies and bioinformatics tools, most of these studies on the impact of fertilization with animal manure on the soil resistome (the resistance determinants present in the soil) have been focused on the use of next-generation sequencing (NGS), particularly 16S rRNA amplicon sequencing and metagenomics (via, e.g., shotgun sequencing with Illumina, Oxford nanopore or PacBio

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technologies), to determine the presence and abundance of ARGs and MGE-related genes in soils, as well as their potential relationship with bacterial taxonomic groups (i.e., to establish possible links between functional and structural-taxonomic-phylogenetic information). For the detection and quantification of these functional genes, high-throughput qPCR has also been used extensively.

Although there is no doubt that molecular technologies and NGS in particular have brought great progress and novel insights to the soil microbial ecology field (most of all, the discovery of the extremely high richness of soil microbial communities), primarily thanks to the possibility of overcoming the limitations of cultivation-based techniques, lately, it is often pointed out that, because of the current gene-centric approach, many of the recent soil microbial ecology studies (e.g., soil resistome studies) are of a descriptive, correlation-based, and/or pattern-seeking nature, and have seldom contributed to significant advances in knowledge and understanding of the ecology of soil microorganisms, nor have substantially increased our capacity to predict soil microbial community composition, dynamics, and resilience, nor unravelled the consequences of community composition for soil process rates and ecosystem functioning.<sup>[2]</sup> Then, the field of soil microbial ecology in general, and the topic of the impact of the application of animal manure on the soil resistome in particular, need to address key ecological questions and perform hypothesis-based, mechanism-based studies that contribute to the advance of microbial ecology theory and applications.

Although, by definition, the microbial “ecology” field must incorporate an ecological and ecocentric approach, in order to attract attention to a given field of knowledge, it is often very useful to point out its potential links with human health and/or well-being from an anthropocentric point of view. In this respect, given the magnitude and relevance of the antibiotic resistance crisis, without doubt it can be stated that the soil resistome and its potential links with the human resistome is one of the soil topics of more interest from an anthropocentric perspective. After all, antibiotics, antibiotic residues that remain bioactive, ARB, and ARGs are not only environmental emerging contaminants of abiotic and biotic nature, respectively, but are putting at risk one of the most important cornerstone of modern medicine, that is, antibiotics.

On the other hand, the food web (the complex network of interconnecting and overlapping food chains in an ecosystem) is a key ecological concept of paramount importance for the understanding of ecosystem functioning. Food webs represent complex ecological networks whose topologies have been widely used in ecology to illustrate the structural and functional properties of biological communities and ecosystems. Explicitly, food webs are representations of species and their feeding relationships. The nodes are often termed “trophic level species or trophospecies” and the links that reflect the direction of interactions “trophic interactions” (e.g., who is the prey and who is the predator). Although a discussion on the vast complexity of the soil food webs and the ongoing debates on their structure and topology is not within the scope of this commentary (for a highly simplified representation of the soil food web, see Figure 1), from the pivotal work of Hunt and colleagues,<sup>[3]</sup> they are often represented as webs compartmentalised into bacterial-based and fungal-based energy channels (with

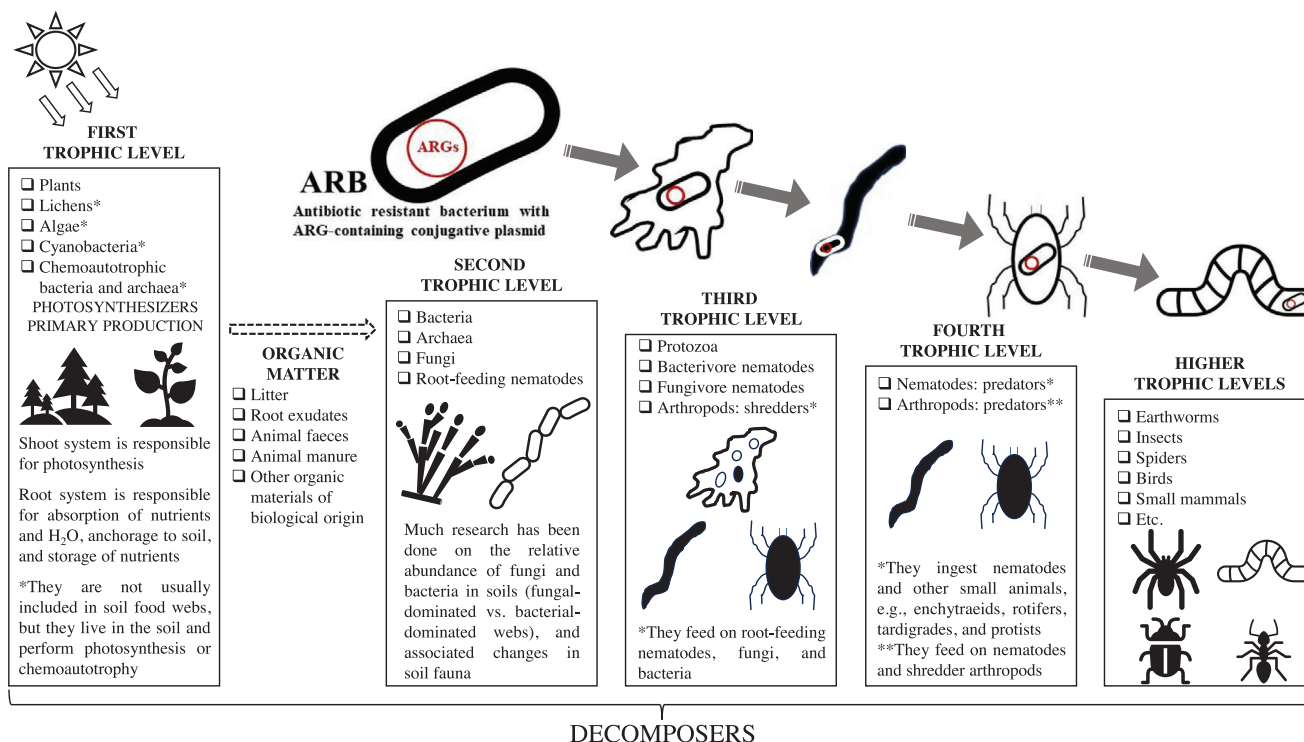
some webs dominated by bacteria and others by fungi) united at higher trophic levels occupied by protists-protozoa, nematodes, arthropods, earthworms, insects, small vertebrates, and so forth.

When ARB harbouring ARGs enter the soil ecosystem during the application of animal manure as fertilizer (an agricultural practice being encouraged, due to its many proven benefits, under a variety of agricultural systems such as organic farming, regenerative agriculture, ecological agriculture, and agroecology), they can be viewed as “invasive species” whose ecological success will depend on a variety of abiotic and biotic factors (e.g., their ecological valence under the specific soil physicochemical conditions, their ecological fitness and competitiveness, their capacity for acclimation and adaptation, their dispersal abilities, and so on). In this respect, in a study on the barriers that microbial diversity can provide against microbial invasion by ARB, soil microbial diversity was found to be negatively correlated with the abundance of ARGs and, then, the authors concluded that a high microbial diversity in soil can act as a biological barrier against the spread of antibiotic resistance.<sup>[4]</sup> Likewise, clinical class 1 integrons, MGEs that have been widely recognized as major contributors to the evolution and dissemination of antibiotic resistance, have been reported as invasive species<sup>[5]</sup> with a decisive role in the antibiotic resistance crisis. These integrons have invaded many bacterial taxa, their hosts, and, critically, all environments associated with human activity.<sup>[5]</sup>

Another useful conceptual approach to the investigation of the introduction of ARB in soil via animal manure is the study of community coalescence (the mixing of different biological communities),<sup>[6]</sup> a process with many ramifications for community structure and function, shaped, among many other factors, by the types of interactions and by various processes of community assembly such as selection, drift, dispersal, and diversification.

When entering agricultural soil, among other possibilities, the ARB initially present in the manure can (i) die owing to, for instance, lack of adaptation to the new environmental conditions, poor ecological competitiveness, predation, and so forth; (ii) survive and then inhabit the inanimate component of the soil matrix, living as free living cells in the soil pores or, more often, within biofilms attached to the soil aggregates, in active or dormant forms; and (iii) survive after being ingested by, or associated with, members of other taxonomical groups of the soil food web; in this respect, in many cases, they end up inhabiting the gut microbiome of species belonging to the soil mesofauna or macrofauna.

From all of the above, in this commentary article, we propose that, regarding the soil resistome, it is critical to complement the myriad of gene-centric NGS studies with ecology-centred studies. In particular, among these ecology-centred studies, we propose that those dealing with the dissemination of the ARB initially present in the animal manure used for agricultural fertilization should ideally follow over time all possible biological-ecological routes of propagation throughout the soil food web, by means of ARB identification and study in soil fungi, protozoa, nematodes, arthropods, earthworms, insects and so forth. Of course, this proposal does not exclude the study and monitoring of the fate of those same ARB in the soil matrix itself, regarding their transformation into bacteria that will live in the pores and, more often, within biofilms attached to soil aggregates.



**FIGURE 1** Simplified representation of the soil food web. The biomass of each level decreases because a considerable amount of energy is lost (e.g., dissipated as heat) with each transfer as entropy increases. It is proposed to follow antibiotic resistance bacteria throughout the soil food web.

Furthermore, we propose to pay special attention to (i) the possibility of horizontal gene transfer (HGT) among the different taxonomical groups of the soil food web, as this phenomenon of lateral gene transfer does not only occur among bacteria or between bacteria and archaea, but also between prokaryotes and eukaryotes; and (ii) the presence of ARB in the gut microbiomes of the soil mesofauna and macrofauna. For example, HGT studies can be performed *in vitro*, using available cultivation-dependent classical and/or high-throughput conjugation assays, with the isolated environmental ARB as donor and recipient bacteria (environmental perspective), or alternatively with the isolated ARB as donors and potential human pathogens (e.g., *Escherichia coli*) as recipients (anthropocentric perspective).

Unlike many studies that are focused on the presence of ARB and, above all, ARGs in the gut microbiomes of specific taxa of the soil mesofauna or macrofauna at a given point in time, we propose to study over time the propagation of ARB in all or, failing that, in as many as possible taxa of the soil food web in the same soil, in an attempt to achieve a comprehensive, temporal, and dynamic representation of the different soil biological-ecological routes that ARB can follow once they have been introduced into agricultural soil.

We are certainly aware of the fact that this proposal can prove a somewhat daunting task but, at the same time, we do reckon that this ecological-ecocentric approach, as compared to the gene-centric approach, has the potential to generate much needed microbial ecology knowledge, as well as to bring out important scientific questions and hypotheses for the soil microbial ecology field in relation to (i) the links between the soil resistome and the human resistome, a topic of much value and interest from a One Health perspective; and (ii) the sur-

vival and ecological implications of the introduction and propagation of ARB in agricultural soils, a subject of much relevance for the biodiversity, functioning, resistance, and resilience of the soil ecosystem, whose health is critical to the provision of essential services and, in general, to the functioning of terrestrial ecosystems.

On the positive side, it must be stated that new developments in molecular probes, fluorescent probes, flow cytometry, advanced microscopy (e.g., Raman micro-spectroscopy, FT-IR micro-spectroscopy), and other analytical techniques are facilitating the tracking and monitoring of bacteria in soil. On the negative side, it must be mentioned that soil food webs are extremely complex, often with five trophic levels, and not well understood. Predators in soil food webs appear to be generalists (even with omnivorous feeding habits), thus being able to ingest organisms from various trophic levels, which means that they can acquire bacteria from organisms belonging to different trophic levels.

Finally, a few studies related to the topic under consideration here are briefly presented. Since bacteria and fungi are the dominant groups of soil biota in terms of both abundance and diversity, it is not surprising that they constantly interact, physically and metabolically, with each other. Fungal hyphae provide a habitat for bacterial communities that can form biofilms on them (here, it must be remembered that within biofilms, the HGT of ARGs is often intensified), but, most importantly, fungal hyphae serve as vectors for bacteria (e.g., ARB) to travel across the soil, the so-called hyphal highways along which bacteria can swim in the water film that coats the hyphae. Alternatively, bacteria can travel passively by settling at the tip of the growing hyphae.<sup>[7]</sup>

Pertaining to protists, although soil protozoa are known to feed on bacteria, many bacteria engulfed in the vesicles of protozoa have the capacity to avoid being digested, thus opening up opportunities for cell-to-cell contact among bacteria present in the vesicles, resulting, for instance, in some protozoa enhancing the frequency of ARG conjugation among them.<sup>[8]</sup>

Moreover, ARGs have been found to be diverse and abundant in nematode gut microbiomes and, most importantly, to have the capacity to be transferred along belowground food chains.<sup>[9]</sup>

In relation to arthropods, it has been reported that antibiotic exposure can alter the gut microbiome of the common soil collembolan *Folsomia candida* and significantly increase the diversity and abundance of ARGs in the collembolan gut.<sup>[10]</sup>

Interestingly, antibiotic-producing *Streptomyces* (a genus of soil actinobacteria well-known for their capacity to produce antibiotics and other secondary metabolites used in human medicine) and other actinobacteria can live as symbionts of soil invertebrates, where they play a protective role by producing antibiotics that are used to defend the hosts against bacterial infections.<sup>[11]</sup> These symbioses in which the host feeds and protects the actinobacteria and, in return, the actinobacteria produce antibiotics to protect the host from pathogens are most interesting for the study of the spread and evolution of ARB, as *Streptomyces* cells carry resistance to their own antibiotics and, under selective pressure, their ARGs can spread to other bacteria via HGT.<sup>[11]</sup>

Although the role of earthworms in the spread of antibiotic resistance is still controversial, different studies have shown that manure fertilization can increase the abundance of ARGs in earthworm gut microbiomes,<sup>[12]</sup> a fact which must be interpreted taking into close consideration the role of earthworms as ecosystem engineers that can influence the movement of other soil organisms, including bacteria.

To finish, we do hope this commentary promotes the development of more ecologically relevant studies on soil microbial ecology and, in particular on the topic of antibiotic resistance in soil.

#### AUTHOR CONTRIBUTIONS

Itziar Alkorta: Conceptualization, writing – original draft, writing – review and editing; Carlos Garbisu: Conceptualization, writing – original draft, writing – review and editing.

#### ACKNOWLEDGEMENTS

This work was supported by MCIN/AEI/10.13039/501100011033 (PID2020-116495RB-I00), Basque Government (IT1578-22), and Euskampus – JRL Environmental Antibiotic Resistance.

#### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

#### DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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**How to cite this article:** Garbisu, C., & Alkorta, I. (2023). A case for the importance of following antibiotic resistant bacteria throughout the soil food web. *BioEssays*, 45, e2300153. <https://doi.org/10.1002/bies.202300153>