INTERACTIVE FEEDBACKS BETWEEN SOIL FAUNA AND SOIL PROCESSES

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EDITORIAL
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Editorial: Interactive Feedbacks Between Soil Fauna and Soil Processes

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Editorial on the Research Topic

Interactive Feedbacks Between Soil Fauna and Soil Processes

Soil fauna plays a significant role at all trophic levels of the soil food web and regulates processes that are crucial for soil functioning, such as nutrient cycling, immobilization and/or degradation of toxic compounds, formation of soil structure, greenhouse gas emissions, and C turnover. Yet, the functional contribution of soil fauna to many soil processes is not well-understood due to methodological limitations and the high complexity of interactions at various spatiotemporal scales (Briones, 2014; Frouz, 2018). For example, some effect such as aggregate formation may cumulate over time and finally contribute to the formation of whole soil profiles, which serve as a framework for other soil processes such as water movement, decomposition, etc. This complexity can only be disentangled through multidisciplinary efforts, which to this day have remained a major challenge.

In spite of its relevance, soil fauna has received far less scientific attention than bacteria and fungi (and lately archaea) in soil studies and has been regularly ignored in global biogeochemical models, the only exception, to some extent, being earthworms (Blouin et al., 2013; Briones, 2014; Grandy et al., 2016). However, recent studies are raising awareness of the influence of soil fauna on ecosystem dynamics (Filser et al., 2016). For instance, earthworms exert a strong influence on C stabilization (Frouz, 2018), and they promote the degradation of organic contaminants, such as PAHs or PCBs (Hickman and Reid, 2008). In laboratory studies, they have been found to be major players in N_2O emissions from soils (Lubbers et al., 2013) although their impact under field conditions remains practically unknown (MOU KEYSOME, 2014). Less studied, ants and termites have been found to increase crop productivity in drylands (Evans et al., 2011), and different lifeforms of Collembola have been shown to impact microorganisms in various ways over time, thereby potentially affecting C and N cycles within farming systems (Filser, 2002). Recently, the role of soil fauna on root-associated microbiome and its interactions with plants has rapidly emerged as a potential new field of research, which is still practically unexplored.

In this general context of potentially extremely relevant, yet very much downplayed, effects of soil fauna on a wide range of soil processes, we believe that this Research Topic makes significant contributions to the current literature. It gathers a collection of studies that investigate (or reflect on) the interactive feedbacks between soil fauna and soil processes, and it also addresses the question of how the increasing human pressure affects soil fauna biodiversity, with associated consequences on soil functioning and resilience. A total of eight articles have been published, including two reviews and six original research articles.

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In the first review, Briones reflects on the intrinsic difficulties encountered when describing and classifying soil biodiversity, and on the necessity to expand our knowledge about the interactions between soil fauna, soil microorganisms, and plant roots. She critically examines novel concepts arising in soil fauna research and proposes to redirect the focus toward advanced experimental ideas in order to better understand the complex interactions between soil fauna and soil processes.

Earthworms are, by far, the most studied organisms from all soil fauna and the review by Medina-Sauza et al. synthetizes the current knowledge about their influence on the structure and function of soil microbial communities, and how this indirectly affects soil processes in the rhizosphere. The study pays special attention to the effect of earthworms on signal molecules promoting plant growth.

Two of the original research articles focus on earthworms. In a state-of-the-art mesocosm study, Vidal et al. combine advanced spectroscopic techniques (TEM, NanoSIMS) with classical, robust bulk measurements (13C-CPMAS-NMR and EA-IRMS) to follow the fate of C from plant residues to earthworm casts. They clearly demonstrate the role of earthworms in the formation of organo-mineral associated organic matter in soil. The second article on earthworms describes a field study in Ivory Coast, where Tondoh et al. investigate the role of earthworms on soil health changes over a 25-year chronosequence from forest to rubber tree plantations. Surprisingly, they show no statistical differences in soil physical characteristics, soil organic carbon, earthworm density, species richness and soil health indices between forest and longstanding (>12 years) rubber tree plantations, which suggests a restorative trend after the initial soil health deterioration.

Macrofauna (earthworms, termites, ants, beetles) are important agents of bioturbation in soils. Their foraging and burrowing activities modify soil physical structure, which impacts relevant soil functions, such as water infiltration and retention, gas exchange and soil organic matter dynamics (Bottinelli et al., 2015). In a fascinating study, Cheik et al. use X-ray computed tomography to quantify soil macroporosity and relate it to soil saturated hydraulic conductivity in a sloping area in northern Vietnam. Their study shows that X-ray CT is a promising tool to quantify macroporosity in soil and verifies the positive impact of soil fauna on water infiltration. They also demonstrate that aboveground biostructures and macropore properties are not necessarily related.

Two articles focus on Collembola. In an original contribution, Menta et al. investigate the feeding preferences of springtails, which were offered a choice between 12 different species of truffle. The study demonstrates the ease of springtails to modify their feeding habits, and it also shows that natural feeding preferences do not always lead to the best fitness (in terms of survival and reproduction at least in the short term). In a second inspiring article, Coulibaly et al. examine how two natural assemblages of Collembola affect microbial communities using PLFA markers, enzyme activities and C mineralization rate. They demonstrate that the influence of Collembola differs depending on their ecological traits, with varying effects on microbial community abundance, structure, and activity.

The last manuscript, a very comprehensive study by Ayuke et al., shows empirical evidence of the benefits of conservation agriculture on soil fauna diversity in three field trials in Kenya. At a time when anthropogenic pressure through deforestation, agriculture intensification, habitat fragmentation, and climate change threatens soil biodiversity, the experimental work presented by these authors should help bridge the gap between agro-ecological principles and practical agronomic applications, hopefully paving the way for a transition toward biodiversity-promoting agriculture.

We hope this Research Topic will stand as a small but solid and appreciated contribution to the field of soil biodiversity and that the articles published will be supportive and stimulating not only to researchers interested in this field, but also to others who may not have been particularly sensitive until now to the very significant role played by soil fauna in a wide variety of soil processes. Finally, we are very thankful to all the authors who submitted their manuscripts for consideration in this Research Topic, as well as all the reviewers for their efforts, which certainly improved the quality of the manuscripts.

AUTHOR CONTRIBUTIONS

MC and PB drafted a first version of this editorial. All authors contributed to and approved the final version.

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REVIEW
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The Serendipitous Value of Soil Fauna in Ecosystem Functioning: The Unexplained Explained

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Soil fauna is crucial to soil formation, litter decomposition, nutrient cycling, biotic regulation, and for promoting plant growth. Yet soil organisms remain underrepresented in soil processes and in existing modeling exercises. This is a consequence of assuming that much of the below-ground diversity is just ecologically "redundant" and that soil food webs exhibit a higher degree of omnivory. However, evidence is accumulating on the strong influence of abiotic filters (temperature, moisture, soil pH) and soil habitat characteristics in controlling their spatial and temporal patterns. From this, new emerging concepts such as "hot moments," "biological accessibility," and "trophic cascades" have been coined to enable plausible explanations of the observed faunal responses to environmental changes. Here, I argue that many of these findings are indeed "happy accidents" (i.e., "eureka discoveries") that remain disjointed between disciplines, impeding us from making significant breakthroughs. Therefore, here I provide some new perspectives on soil fauna research and highlight some experimental approaches to better explore the great variety of organisms living in soils and their complex interactions. A more comprehensive and dynamic holistic approach is needed to couple soil pedological and biological processes and to combine current experimental and theoretical knowledge if we aim to improve our predictive capacities in determining the persistence of soil organic matter and soil ecosystem functioning.

Keywords: soil ecology, plant-soil interactions, soil fauna-microbial interactions, soil food web, non-trophic interactions, functional diversity

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INTRODUCTION

Soils are complex systems and their complexity resides in their heterogeneous nature: a mixture of air, water, minerals, organic compounds, and living organisms. The spatial variation, both horizontal and vertical, of all these constituents is related to soil forming agents varying at different scales (from micro- to macro-scales; Lin et al., 2005). Consequently, the horizontal patchy distribution of soil properties (soil temperature, moisture, pH, litter/nutrient availability, etc.) also drives the patchiness of the soil organisms across the landscape (Berg, 2012), and has been one of the main arguments for explaining the great diversity observed in soil communities (Nielsen et al., 2010). Furthermore, because soils also show vertical stratification of their elemental constituents

(along the soil profile) as result of microclimate, soil texture, and resource quantity and quality differing between soil horizons, soil communities also change in abundance and structure with soil depth (Berg and Bengtsson, 2007).

In addition, because the majority of these organisms are aerobic, the amount of porous space, pore-size distribution, surface area, and oxygen levels are crucial to their life cycles and activities. The smallest creatures (microbes) use the micropores filled with air to grow, whereas other bigger animals require bigger spaces (macropores) or the water film surrounding the soil particles to move in search for food. Therefore, soil textural properties together with the depth of the water table are also important factors regulating their diversity, population sizes, and their vertical stratification. Ultimately, the structure of the soil communities strongly depends not only on the natural soil forming factors but also on human activities (agriculture, forestry, urbanization) and determines the shape of our landscapes, in terms of healthy or contaminated, pristine or degraded soils.

Since all these drivers of biodiversity changes also operate above-ground, it is expected that there must be some concordance of mechanisms regulating the spatial patterns and structure of both above- and below-ground communities. In support of this, a small-scale field study revealed that the relationships between environmental heterogeneity and species richness might be a general property of ecological communities (Nielsen et al., 2010). In contrast, the molecular examination of 17,516 environmental 18S rRNA gene sequences representing 20 phyla of soil animals covering a range of biomes and latitudes around the world indicated otherwise, and the main conclusion from this study was that below-ground animal diversity may be inversely related to above-ground biodiversity (Wu et al., 2011).

The lack of distinct latitudinal gradients in soil biodiversity contrasts with those clear global patterns observed for plants above-ground and has led to the assumption that they are indeed controlled by different factors (Bardgett and van der Putten, 2014). For example, Lozupone and Knight (2007) found that salinity was the major environmental determinant of bacterial diversity composition across the globe (rather than extremes of temperature, pH, or other physical and chemical factors). Similarly, in another global scale study, Tedersoo et al. (2014) concluded that fungal richness is causally unrelated to plant diversity and is better explained by climatic factors, followed by edaphic and spatial patterns. Global patterns of the distribution of macroscopic organisms are far poorer documented. However, the little evidence available appears to indicate that, at large scales, soil metazoans respond to altitudinal, latitudinal or area gradients in the same way as those described for above-ground organisms (Decaëns, 2010). In contrast, at local scales, the high diversity of microhabitats commonly found in soils provides the required niche portioning to create "hot spots" of diversity in just a gram of soil (Bardgett and van der Putten, 2014).

Not only spatial patterns of soil biodiversity are difficult to explain, but also its potential linkages to many soil processes and the overall ecosystem functioning remains under debate. For example, while some studies have found that reductions in the abundance and presence of soil organisms results in the decline of multiple ecosystem functions (e.g., Wagg et al., 2014), others concluded that above-ground plant diversity alone is a better predictor of ecosystem multi-functionality than soil biodiversity (Jing et al., 2015). Soil organisms exhibit a wide array of feeding preferences, life-cycles and survival strategies and they interact within complex food webs [reviewed by Briones (2014)]. Consequently, "species richness" *per se* has very little influence on soil processes and "functional dissimilarity" can have stronger impacts on ecosystem functioning (Heemsbergen et al., 2004). Therefore, besides the difficulties in linking above- and below-ground diversities at different spatial scales, gaining a better understanding of the biotic effects on ecosystem processes might require incorporating a great number of components together with several multi-trophic levels (Scherber et al., 2010) as well as the much less considered non-trophic interactions (e.g., phoresy, passive consumption; Goudard and Loreau, 2008). In addition, if soil systems are indeed self-organized, and soil organisms concentrate their activities within a selected set of discrete scales with some form of overall coordination (Lavelle et al., 2016), there is no need for looking for external factors controlling the assemblages of soil constituents. Instead we might just need to recognize the "unexpected" and that the linkages between above-ground and below-ground diversity and soil processes are difficult to predict.

The last three decades of soil ecology research has evidenced that the initial focus on distributions of specific faunal groups has turned significantly into understanding their activity roles, plant-soil interactions, and ecosystem functions. In addition, the studies accumulated so far clearly illustrate that, as soil ecologists, we have been very efficient in gathering information and proposing new hypothesis and ideas. Therefore, can we then assume that we have thoroughly explored all the possible research questions arisen when trying to obtain a more complete view of how the soil systems are organized, how their different components interact and how they respond to changes in the belowground environment but also to those in the one above?

The most recent literature seems to indicate that further advances will emerge from studying sub-organism level responses and thus environmental DNA (Thomsen and Willerslev, 2015) and various "omics" approaches (mainly metagenomics, metatranscriptomics, proteomics, and proteogenomics) are rapidly advancing, at least for the microbial world (Nannipieri, 2014 and references therein). Furthermore, recently "metaphenomics" has been proposed as a better way to encompass the entire omics and the environmental constraints (Jansson and Hofmockel, 2018). Should macroscopic organisms then follow?

In this overview, I argue that before we become overly involved with these new promising tools, which we do not know what they exactly do or to what extent they can be applied to bigger organisms, soil ecology might benefit from looking at available information from a different perspective, re-interpreting and integrating what we have learnt. There are many basic physiological and behavioral aspects of soil organisms, interactive biotic relationships (below-below and above-below ground), functional roles and responses to the abiotic environment that are consistently ignored or less

explored, despite being aware of their existence. By bringing the available information (old and/or new) together and breaking the bridges with other disciplines, we could start fitting all these puzzle pieces together and aim at a "eureka moment" in which a more complete picture of the importance of soil fauna in ecosystem functioning becomes revealed.

DESCRIBING SOIL BIODIVERSITY: FROM BROAD GROUPS TO MOLECULES

The vast array of different organisms inhabiting the soil makes it very difficult to establish broad groups where they can be lumped in and in turn, make life easier for soil ecologists and modelers (Briones, 2014). Despite the great progress achieved in taxonomical diversity thanks to advances in molecular techniques (e.g., DNA sequencing and fingerprinting of different organisms, but also direct DNA extraction from a soil sample, i.e., environmental DNA or e-DNA), their applicability to soil ecology studies remains difficult. This is due to the existence of relic DNA that could overestimate the amount of biodiversity (Carini et al., 2016), the lack of standardization in these methodological procedures (Orgiazzi et al., 2015), the limited information that they provide on the activity and viability (dead or alive) of many groups (Cangelosi and Meschke, 2014), and the high number of habitats and animal groups that still remain undersampled.

The broad classification into micro-, meso-, and macrofauna, although having some conceptual advantages [e.g., it is assumed that the bigger the animal the bigger the effects on soil processes, e.g., Bradford et al. (2007), and that the bigger the animal the most susceptible to environmental perturbations (e.g., Tsiafouli et al., 2015; Briones and Schmidt, 2017)], also poses some inconveniences. Among them, the difficulty in placing many organisms into a specific group, partly because many of them can vary considerably in size. For example, several mesofaunal groups (such as mites, collembolans, enchytraeids) include species that span from small specimens (around microfauna body width values) to large ones (close to the values observed for macrofauna). In addition, the methodologies to collect these organisms are not group-specific and for example, protozoa (1–2 μm) are still extracted from the soil using microbiological techniques. Not only that, from the systematic point of view, should protozoa be considered as fauna, when they actually consist of several phyla that do not belong to the Animal kingdom? Or would it be better to lump them with microflora (since most species are $<$50 μm in size)? And what about considering fungi as "microflora" when their mycelium can extend over kilometers? The same can be said about trying to link this classification to specific microhabitats and functional roles, such as the traditional description of "mesofauna" as those organisms that cannot create their own biogenic structures, when for example enchytraeids can tunnel the soil profile and even reach soil depths beyond the capabilities of many earthworm epigeic species ("true ecosystem engineers").

One alternative option is the use of functional classifications and functional traits, i.e., instead of putting the focus on the morphology of the soil organisms to split them into different groups, the target would be to quantify their functional role in the ecosystem (e.g., decomposition processes, soil physical structure maintenance) or their responses to changes in the environment (e.g., behavioral or life-history traits). Trait-based approaches are becoming more widely used in soil community ecology and standardized protocols are now available for the most representative taxa (Moretti et al., 2017). These might help us to gain a better understanding of why taxonomic diversity and functional diversity do not often show the same responses to habitat changes (Pey et al., 2014). Importantly, both functional and trait-based approaches are based on activities rather than on the presence of a certain organism and therefore, enabling us to identify who are the true players, what they exactly do and to what extent. Although, in many cases, some of these features can be linked to morphology and taxonomical identity, it avoids the inclusion of inactive states (e.g., cocoons, cysts, letarged/diapaused specimens, carrion) that will be unavoidably extracted in a DNA sample. Furthermore, the fact that the bioturbation activities of some organisms can also re-distribute the genetic material through the soil matrix (Prosser and Hedgpeth, 2018) also pose more difficulties to the interpretations derived from environmental DNA analyses.

PLANTS TALKING AND RHIZOSPHERIC FAUNA RESPONDING

In many ecological studies, the term primary production is typically associated to above-ground plant biomass, usually referred to "net primary production" or NPP, and completely ignores "below-ground plant productivity." This is due to plant productivity being commonly referred to in agriculture context. Consequently, for several decades, research on rhizospheric fauna mainly concentrated on agricultural pests (Bonkowski et al., 2009), and only from around 1990s onwards a more complex invertebrate community was included (Lavelle, 1996). However, the role of plant roots in soil processes cannot be dissociated from the vast array of organisms that proliferate around them. These include, besides parasites, herbivores and predators, free-living microbes feeding on root exudates, and microbial grazers such as nematodes, collembolans or worms.

While the association between plant roots and mycorrhiza is known to be very old since they co-evolved together, the interactions between soil fauna and plant roots are just starting to be revealed and seem to be more complex than anticipated (Bonkowski et al., 2009; Puga-Freitas and Blouin, 2015; Xiao et al., 2018). Plants produce a variety of secondary metabolites, such as iridoid glycosides (through root exudates) and volatile organic compounds (emitted by green leaves and roots) for above-ground (e.g., to attract pollinators; Dudareva and Pichersky, 2000) and below-ground communication (e.g., to deter herbivores; Wurst et al., 2010). Accordingly, plants are not just merely suppliers of litter for decomposers and instead, they play an active role in attracting beneficial soil invertebrates (e.g., attracting entomopathogenic nematodes to kill the herbivore), providing bacterial inoculum, disturbing the communication between harmful bacteria and also, in modifying rhizodeposition

and root architecture (for a more detailed description of the intimate interactions of soil fauna with plant roots see Bonkowski et al., 2009). All these chemical signals released by the plants are directed to benefit their own growth and increase their viability and vigorousity.

However, not only below-ground herbivore suppression can indirectly result in a positive feedback on plant performance, predator-induced shift in detritivore habitat can also help to increase plant biomass. For example, the presence of a carabid beetle (*Agonum impressum*) that commonly feeds on earthworms resulted in a vertical movement of the prey from the upper to lower soil layer, leading to improved soil properties and enhanced plant biomass (Zhao et al., 2013). Interestingly, the positive effects of this predator-driven response were only significant for above-ground plant biomass but not for root biomass (although a non-significant positive trend toward higher values in the treatment with predators was detected). Hence, the next question will be why despite the actions occurring below-ground the positive response is only being detected above-ground? Could plants become more efficient in taking up nutrients without increasing their root surface area?. According to the reported results, the predators did not significantly affect the overall earthworm densities, but the proportion of the "larger" species (*Pheretima aspergillum*) present in the top layer. This is an anecic species that lives in permanent vertical burrows (Chang et al., 2009) and hence, it could simply retreat to bottom end of its burrow to avoid predation. In contrast, the burrow system of the second species investigated here (*Aporrectodea nocturna*) comprises a few long (>100 mm) vertical burrows, exhibiting few branches and low sinuosity (Capowiez et al., 2015). Since this latter species do not possess a "located" home, it will find refuge anywhere in the soil profile. Burrow temporal stability is known to affect not only the amount of organic matter deposited in the lining of the walls (e.g., Hoang et al., 2016), but also the activity of other organisms using these tunnels (Butt and Lowe, 2007; Han et al., 2015) and intra-specific competition (Grigoropoulou et al., 2009). Therefore, are the observed results a reflection of a higher burrowing activity of the "smaller" species or to a greater inactivity of the "larger" one?

One clue to solve this puzzle is the fact that plants are more efficient in foraging N in earthworm casts than in the bulk soil (Agapit et al., 2018) and that root growth could be limited through large increases in soil bioturbation as a result of increased earthworm activity (Arnone and Zaller, 2014). From this, it could be concluded that plant roots would benefit from earthworm casting but not from their burrowing, which is exactly what the predatory beetle achieved.

From this, it would be interesting to know whether plants could stimulate a similar response without relying on an above-ground predator and release any kind of "alarming secretions" that would encourage soil organisms to produce more casting material or to tunnel less, or even more in some cases, so they can access nutrients or water more easily. A new promising tool, which enables recording the acoustic signals emitted by plant roots growing and earthworms burrowing (Lacoste et al., 2018), might help us to decipher whether plant bioturbating activities could also drive soil fauna responses.

SOIL FAUNA PASSING BY AND MICROORGANISMS WAKING UP

Soil microbial activities are hampered by the fact that they are strongly limited by C and N availabilities and their low dispersal abilities prevents them from moving to a more favorable patch with a better nutrient supply. The concept of the "sleeping beauty paradox" coined by Lavelle et al. (1995) perfectly describes the discrepancy between potentially high metabolic capabilities and slow turnover rates by stating that microbial communities are largely dormant and need a "Prince Charming," either a macroorganism, a physical process or an environmental factor, which "awakens them" by facilitating their contact with the nutrient pools.

As a result, new microsite areas (biopores, aggregates) are created, where soil processes occur at a much faster rate at least during short periods (hours to days) while the food resources last. From this, another new concept in soil ecology has emerged, "hot spots and hot moments" described by Kuzyakov and Blagodatskaya (2015). This close link between these pulses of microbial activity and nutrient availability explains the contradictory estimates of active microorganisms in the soil obtained in the laboratory and in the field. This is a consequence of the use of indirect techniques that rely on substrate additions and bioassays or that are based on static approaches (for a full discussion of the current methods see Blagodatskaya and Kuzyakov, 2013). This together with the high temporal and spatial heterogeneity exhibited by soil microbial communities, both across latitudes and vertically in the complex soil matrix, clearly demonstrate that soil ecology urgently requires more advances in this field.

Furthermore, the roles played by different soil invertebrates in the dispersal of soil microorganisms deserve further consideration. Both mesofauna (microarthropods) and macrofauna (earthworms) are known to carry cells, spores and mycelium attached to their bodies and in their guts and then released out again via egestion in their feces. While phoresy will help transported microorganisms in colonizing new areas, gut passage could result in either activation or destruction of the microbial cells (e.g., Schoënholzer et al., 1999; Renker et al., 2005; Buse et al., 2014). In other cases, although spore/propagule viability is retained, germination might be delayed (Talbot, 1952). Whether these dispersal mechanisms are stochastic (awaiting for a passing by invertebrate) or there is some attraction mechanism involved is another interesting aspect that deserves more experimental research. For example, earthworm skin secretes mucus, a rather attractive source of labile C for microbes, which could stimulate microbial activities and accelerate the mineralization of soil organic matter (Scheu, 1991; Bernard et al., 2012). "Fecal attraction" on earthworm casts and middens or microarthropod fecal pellets is another way of congregating a high number of microorganisms (Bohlen et al., 2002; Tagger et al., 2008), and bacteria living in feces can serve a way of intra-specific communication among certain insects (Wada-Katsumata et al., 2015). On the other hand, microorganisms can also attract soil invertebrates, and it has been shown that fungal odor attracts collembolans (Bengtsson et al., 1988) which

might enhance the dispersal of the fungi, but also the movement of the fungivorous collembolan (Bengtsson et al., 1994). From these studies, it is clear that many of these faunal-microbial interactions are not always random and that finding how and when a macroorganism can "switch on" a hot moment could enhance our understanding of ecosystem functioning.

Furthermore, it would be interesting to know if any of these mechanisms could also represent a hot spot for gene transfer as it has been demonstrated that fungal hyphae are useful infrastructures for bacteria to move toward more accessible food sources but also for horizontal transfer of genes between differing bacteria (Berthold et al., 2016). Could a soil invertebrate wake up a fungal species and as a result, facilitate the movement of bacteria through the soil and consequently, their functional attributes (e.g., gene expression for nitrification or denitrification)?

SOIL FOOD WEBS REVISITED: DINING AT THE SOIL RESTAURANT

The first description of a topological food web appeared in 1912 and was produced by Pierce and Cushman (1912), who were investigating the insect enemies of the cotton boll weevil. This seminal work progressed by linking detrital biotic interactions with other components of the aquatic and terrestrial ecosystems [reviewed by Pimm et al. (1991)]. Unfortunately, our current understanding on trophic interactions is far from complete and we need a more refined picture of the number and identity of the potential consumers at the different trophic levels. For example, soil viruses and enchytraeids are usually omitted in these food web simulations and nematodes and mites are the only groups that might be subdivided into different feeding groups, whereas the different ecological groupings of earthworms or the feeding guilds of collembolans are typically ignored. This is important because the inclusion or omission of a certain trophic level or food source could change the overall interpretation of the soil food web. For example, omitting the grazing activities of certain groups, such as protozoa and nematodes could result in underestimations of total N mineralisation rates, with reductions of 28 and 12%, respectively (De Ruiter et al., 1993).

However, even in the case of a well-defined trophic classification, we can also find some species "breaking the rules." For example, in the case of nematodes, by looking at their head structures (i.e., presence/absence of stylets, teeth, etc.) we can obtain information about their feeding habits (i.e., whether they typically feed on bacteria, fungi, plants, other nematodes or on a mixture of food sources); however, some species from any of these groups can switch to cyanobacteria (Yeates, 1998). Similarly, collembolans, which are generally considered to be fungivorous, include species feeding on nematodes (Chamberlain et al., 2005). And what about coprophagy exhibited by mites, isopoda, enchytraeids and earthworms? This particular case represents a special way of soil fauna interacting with microorganisms and functions as an "external rumen" (Swift et al., 1979) allowing many soil organisms to obtain extra nutrients from suboptimal foods (Ponge, 1991). All these behavioral patterns complicate their placement into a particular trophic/functional group.

Furthermore, the number of trophic levels in terrestrial food webs rarely exceed three levels (Hairston and Hairston, 1993), due to the low efficiency of their trophic groups in assimilating their preferred food and transferring energy from one level to the next (i.e., fraction of the food below that is contributing to the biomass production of the trophic level above). This has led to the suggestion that complex soil food webs are not stable, which contrasts with the pioneering work by earlier researchers (Svensson and Rosswall, 1980; Parker et al., 1984; Hunt et al., 1987; De Ruiter et al., 1995) and more recent work (Digel et al., 2014; Van Altena et al., 2016) that described soil food webs with 4–8 trophic levels. For example, Digel et al. (2014) analyzed 48 forest soil food webs ranging from 89 to 168 taxa and found 729 to 3344 feeding interactions. The results from these studies indicate that long and dynamically stable soil food webs are possible. The key variables controlling the functioning of these more complex web structures are the "number of species involved," the "degrees of connectedness" and the "strengths of species interactions," which were identified by May (1972) and tested by De Ruiter et al. (1995). They found that the omnivorous links from the higher predators in the web were crucial in terms of preserving stability, confirming May's theory that the most densely connected species, that is where the trophic connections were most complex, were crucial for their stability [see also review by (Manne and Pimm (1996)]. Indeed, compared to other food webs, soil food webs are characterized by exhibiting a higher degree of omnivory, with a high number of species feeding on different trophic levels, as well as cannibalism or "intra-guild predation" (Digel et al., 2014).

Omnivory can represent a problem when classifying organisms according to their feeding habits and in quantifying the effects of predation on the biomass of those organisms placed at lower trophic levels. This, in turn, has implications on the magnitude and extent of "trophic cascades" (*sensu* Scheu and Setälä, 2002 and Wardle, 2002) and/or on the overall dominance of "top-down vs. bottom-up regulation processes" (*sensu* Moore et al., 2003). In relation to this, Neutel et al. (2002) described the unequal effects of top-down regulations exhibited by predators that feed on preys that belong to different trophic levels and showed that prey density could determine their preferential feeding on a particular prey and consequently, have a significant effect on the trophic level where that consumed prey is concentrating its activities.

However, is it really a omnivory/generalist feeding behavior that characterizes the trophic relationships between soil organisms or do soil animals actually rather prefer to choose what to eat by looking at the whole menu, instead of just merely going for their local basic food source? That is what I have called "feeding flexibility" (Briones et al., 2010) to better describe how soil mesofauna could switch from one diet to another in response to changes in the environmental conditions (abiotic or biotic). This concept differs from "biological accessability," which has been proposed as a better predictor of soil organic matter turnover than recalcitrance (Dungait et al., 2012). The accessibility of the organic sources to decomposers could be mediated by physical (e.g., by a macroorganism facilitating

the close contact) or chemical factors (e.g., by microbial pre-conditioning of the plant material, as an "external rumen"). I argue that besides cooperation between soil fauna and microorganisms, some invertebrates are more selective than currently assumed and they could feed on more labile or more recalcitrant substrates depending on what is available on the menu or what is easier to get under certain circumstances. For example, the observed priming effects on soil fauna (e.g., Nieminen and Pohjola, 2014; Eck et al., 2015) explain how organisms that typically feed on more humified organic matter (e.g., enchytraeids and endogeic worms) could suddenly find a labile substrate irresistible. However, this does not mean that their diet naturally consists of a mixture of substances of different nature (omnivory) because this variety of foods is not always available and hence, they cannot rely on their regular supply nor spend their energy in searching insistently for them. Intra-specific competition can also drive changes in feeding behaviors, and a beautiful example of this can be extracted from the work by Anderson (1975), who showed that when two species of oribatid mites were grown in isolation they preferred to feed on similar sources, but when put together in competition they changed their feeding habits by moving to a different layer (litter or fermented). Similarly, if the soil at the surface becomes too wet or too dry and the organisms are able to escape from those adverse conditions by migrating down, their survival could only be guaranteed if they can feed on any of the food choices available at the deeper below-ground menu (and this might lead to "compensatory feeding," as it has been seen in root herbivores in response to lower nutrient quality of the source; Johnson et al., 2014). Perhaps, these feeding choices are "context-dependent" and, under different abiotic and biotic pressures, the same species could exhibit different feeding strategies.

Other factors that are currently impeding us from gaining a full understanding of the functioning of the soil food webs are: (i) redundancy (several species feeding on the same resource) and complementarity within functional groups (Setälä et al., 2005), which will also have implications on top-down and bottom-up relationships; (ii) the fact that some soil organisms feed on different diets or exhibit different feeding rates during their lifetime (e.g., Briones et al., 2005) and hence, their tissue turnover and feeding efficiencies/diets might also change with age, and (iii) density-dependent effects on their feeding activities, which could lead to positive or negative (direct and indirect) effects on their prey (Kaneda and Kaneko, 2008). They all need to be integrated in food web analyses to provide a more realistic (dynamic) quantification of energy flows across the different trophic levels.

Can we therefore conclude that at least some soil invertebrates are selective (eating their preferable food when available), but others are opportunistic (eating whatever is abundant at that particular moment) or generalist feeders (eating whatever is easy to obtain in order to avoid competition)? Could the soil food webs also exhibit temporal "feeding pulses" during "hot moments" (at one or several trophic levels), with a measurable effect on the trophic levels below ("cascade trophic effects")?

SOIL FAUNA LINKAGES TO SOIL PROCESSES AND ECOSYSTEM FUNCTIONING

Despite our yet limited knowledge on the identity of the different organisms inhabiting our soils, what is the usefulness of having such a huge diversity? Is it truly necessary? Do every existing species have a role in their lives? And, more importantly, is this high richness always accompanied by a better performance of the ecosystems where they live?

It could be expected that in those extreme environments (such as cold and arid ecosystems) where nutrients are scarce and/or supply discontinuous, due to environmental pressures (climatic, soil conditions, etc.), soil biodiversity will be low and food chains short. Under these conditions, any nutritional surplus will lead to a "hot moment" (*sensu* Kuzyakov and Blagodatskaya, 2015) in which the soil food web will re-activate and hence, soil processes rates will increase (**Figure 1**). In contrast, those systems with a regular supply of nutritious substrates will be able to sustain a higher number of different taxonomical and functional entities. In this case, not only a greater variety of trophic niches will be available, but also the co-existence of several groups feeding on the same sources could be maintained as long as nutrient availability persist. This is expected to result in a higher number of "hot spots" (*sensu* Kuzyakov and Blagodatskaya, 2015) across the soil matrix (**Figure 1**). The resulting increases in foraging activities and reproductive rates will "cascade" up and down along the soil food web bringing other pressures into action (predation, competition) which will modulate the initial responses to increased nutrient availability. On the other hand, this also means that those "hot spots" in the more limiting arid and cold ecosystems could be more intense, and thereby exponentially increase the role of soil fauna and trophic cascades in those systems.

Since feeding is a primary need, it is not surprising that soil organic matter quantity and quality has always been considered the main driver of decomposition and nutrient cycling. Indeed, much research focus has been placed on C resource quality (recalcitrance and chemical protection), following the works by Hooper et al. (2000) and Ponge (2003, 2013). However, another important aspect that should be considered here is that besides the "chemical heterogeneity" of the C substrates deposited by the primary producers, there is also considerable "physical heterogeneity" in the soil systems that needs to be accounted for. The existence of physical gradients (pH, moisture, aggregates, porosity, etc.) together with the horizontal patchy distributions exhibited by soil organisms [even at local (plot) level] hinders any attempt to link all attributes present in soils, i.e., soil biodiversity, soil properties, and soil functions. Therefore, despite the success in finding a gradient of increasing biodiversity with increasing humification of soil organic matter (Ponge, 2003), different outcomes can be anticipated depending on the influence of environmental filters and anthropogenic forces acting upon decomposition rates (Zanella et al., 2017).

This could explain why litter decomposition rates show such a great variability across biomes, elevations and soil types,

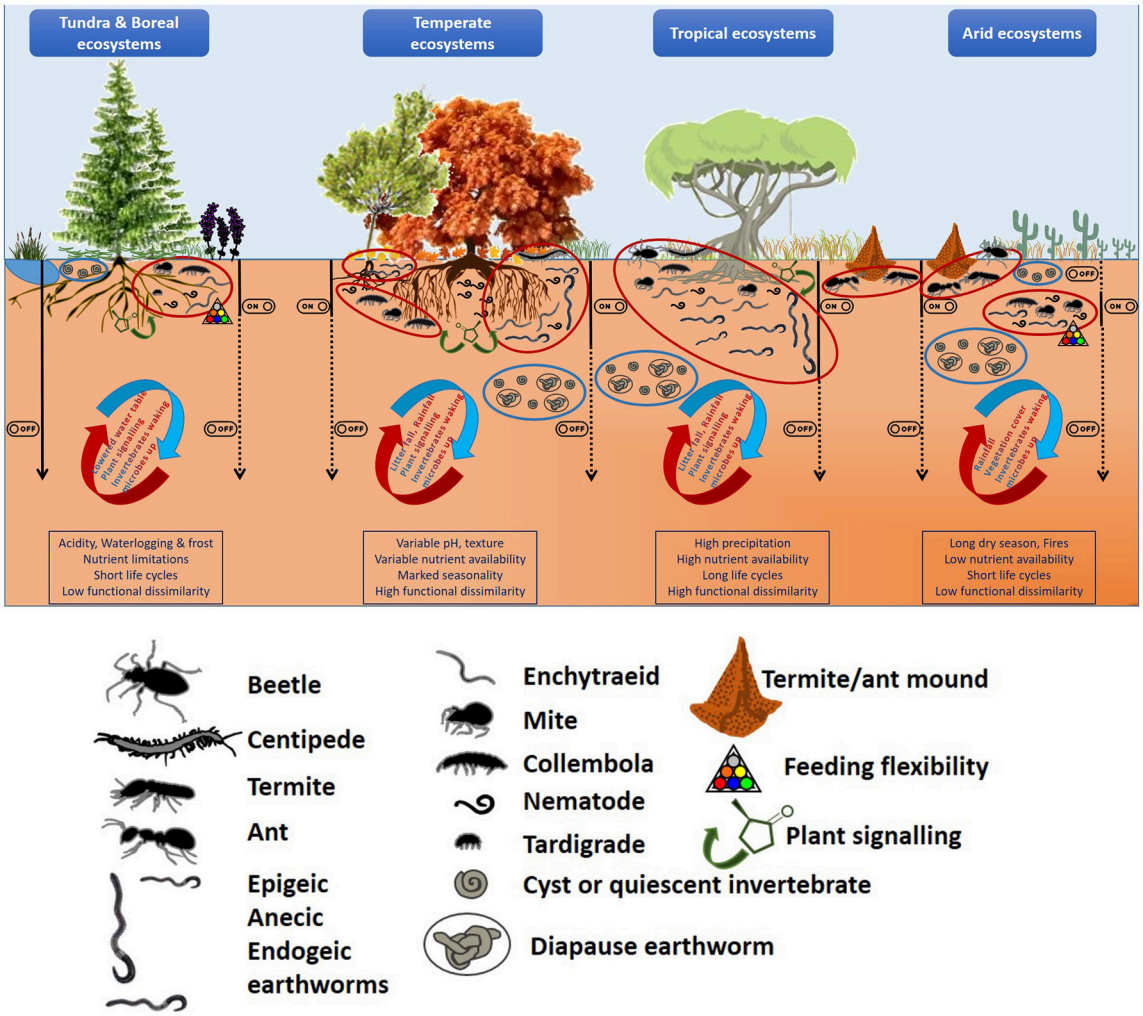

FIGURE 1 | Linking hotspots and hot moments of soil fauna to climatic gradients and soil heterogeneity: Historical factors (climate, parent material) shape our landscapes (both above- and below-ground), but the regional/local abiotic conditions constraint biological activities. These operate at different spatial and temporal scales and can switch on and off different organisms at different microsites resulting in a hot moment in a particular hotspot. Since each of their responses can have effects on others, their effects could then cascade up and down in the food web. Soil invertebrates are depicted not in scale, just for illustrative purposes (see pictorial legend for major taxonomical groups). Ellipses indicate hot (red) or cold spots (blue), with the curved arrows giving some examples of the factors that could switch on/off a hot moment and the straight black arrows (continuous black line = on, dashed = off) showing the implications for soil processes along the soil profile. In the boxes, the main ecosystem characteristics are listed.

highlighting that, besides temperature and moisture, there must be other important factors controlling decomposition rates. Sadly, despite being widely known that decomposition has been intimately associated to soil fauna since prehistoric times (Labandeira et al., 1997), they are continuously ignored in recent modeling and perspective studies (e.g., Schmidt et al., 2011) or lumped together in a big box, whereas bacteria and fungi are given their own individual compartments and full roles. As a soil fauna ecologist, I can only stress the need to increase their visibility and fight for "their own rights" so they become considered as least at the same level of prokaryotes. In support of this, a global meta-analysis showed that soil fauna consistently enhanced litter decomposition across biomes by 27% (García-Palacios et al., 2013). In addition, the classical work by Coûteaux et al. (1991) nicely demonstrated that increasing soil web complexity (by adding invertebrates at higher trophic levels) accelerates litter decomposition more than expected from a simple sum of their individual activities, so their omission is unforgettable.

Similarly, the majority of the so-called "soil quality indexes" are merely based on soil properties (phosphorus runoff potential, nitrogen availability, metal contamination) or microbial activities (C_{mic}/C_{org}, soil respiration, litter decomposition, etc.), and completely ignore those based on soil fauna parameters, such as the eco-morphological index (EMI) proposed by Parisi (2001) and the abundance-based fauna index (FAI)

proposed by Yan et al. (2012). For example, the inclusion of a soil biodiversity index that included measures of richness and abundance of functional guilds has allowed to relate soil community characteristics to ecosystem multifunctionality (Wagg et al., 2014) and another study concluded that higher diversity at different trophic levels is necessary to maintain ecosystem multi-function performance (Soliveres et al., 2016). More advances in linking the responses of species assemblages, biotic interactions and ecosystem processes to global change are envisaged from recent efforts in standardizing protocols measuring ecological traits in soil invertebrates (Moretti et al., 2017). This will also allow us to rationalize the use of the soils for cultivation/urbanization and for allowing a better use of the natural sources (such as zero- carbon and circular economies).

IS FURTHER RESEARCH NEEDED?

Soil fauna ecologists have profusely explored soils around the world trying to determine the number of species, abundance, and temporal patterns of the organisms that live in it, the interactive roles of plant-soil-fauna on soil processes and ecosystem functioning. Here, I provide several stimulating ideas that might lead to a more refined understanding of the heterotrophic component of the soil system or help in looking at the information available from a different perspective:

1. Only a proportion of all the species living in soils have been described and, although advances are expected thanks to rapid DNA sequencing, very little is known about their community structure and dynamics across different ecosystems. If we are unable to clearly relate ecosystem functions to ecosystem diversity, then the relationships found in several studies might not be causal correlations and hence, much of the diversity present might just be "redundant." To answer this question, we might need not only further refinement of our experimental approaches and more taxonomical efforts and ecological work on soil biota, but also "activity proxies" and "response traits" rather than abundance and biomass ones.
2. If soils sustain a high diversity, how could the little attention they receive compared to plants or birds be justified? Measures to preserve and promote higher biodiversity in soils should come into force, including the implementation of management policies at National and International levels. Biodiversity is protected under various directives and international commitments (e.g., Habitats Directive, Natura 2000, Convention on Biological Diversity, CITES), but without explicit mention of soil biodiversity. For organisms living above-ground, the current species' extinction rates have prompted the need for preserving endangered species. Thus, in the case of plants, seed banks have been built in selected parts of the world to keep unique and valuable plant species (e.g., Svalbard Global Seed Vault). Similarly, cells, tissues and embryos of different vertebrates have been frozen to open the possibility of "resurrecting" extinct species or saving nearly extinct species in the future (e.g., Frozen Zoo® in San Diego). Large culture collections of fungi (including yeasts), bacteria and plasmid exist (CBS-KNAW Collections in The Netherlands) for commercial value. Should not a "soil biota zoo" be constructed to safeguard our soil biodiversity and serve as a home to a World Data Archive?
3. Re-defining soil quality/soil health goes along with including soil biological indicators that integrate parameters measuring soil biodiversity functionality. For example, the humus index developed by Ponge et al. (2002), besides providing a framework to integrate soil biodiversity, soil conditions, and humus forms, also allows a quantitative assessment of soil formation and development and plant-soil biodiversity co-evolution across different ecosystems.
4. Feeding preferences of soil organisms are not yet clearly established, and in the particular case of burrowing forms that move through the soil by ingesting it, can we say that they are selective feeders or just ingesting accidentally? Moreover, if they do select what they eat, what are the implications for soil functioning? For example, some studies have shown that earthworms and collembolans can be highly selective when grazing on fungi (Moody et al., 1995; Jørgensen et al., 2005) and, in some cases, decrease the crop damage caused by fungal infections (Stephens et al., 1994; Sabatini and Innocenti, 2001). Obviously, spreading or reducing the incidence of fungal disease in successive crops will depend on the survival through the gut passage (Moody et al., 1995), which might be different for different species. Answering these questions may open new research options and, for example, elucidating the level of specificity of the grazer (i.e., major groups or species-specific selection) and assessing the overall impacts of grazing in shaping the fungal communities (e.g., increases in fungal diversity as a result of reducing the presence of the dominant fungus; for more impacts of fungi grazing fauna see McGonigle, 2007) could have different implications for crop performance. And finally, could these aspects be extended to other soil organisms (pseudoscorpions, predatory mites and beetles) and hence, by inoculating certain species (or species combinations) reduce the effect of soil-borne pathogens (not only fungi, also bacteria and viruses)?
5. When linking soil biodiversity and soil processes we also need to re-define "recalcitrance" beyond merely chemical terms and "biological accessibility" beyond microbial attack. An additional difficult task will be to combine the spatial and temporal heterogeneity of food substrates together with the "functional dissimilarity" of soil organisms (Heemsbergen et al., 2004) in modeling exercises. Importantly, can we provide mathematical formulations of "seasonal pulses of litter fall and nutrients" and "hot spots of biological activities," including hyphal horizontal and vertical transfers between leaf litters and soil layers together with microsites of preferential nutrient flow paths and high biological densities that could lead to immobilization or mobilization of certain elements?
6. Since in the last three decades soil ecology research has turned its interest to the functional role of soil organisms, can we say we have identified and/or accounted for all their possible roles? The current literature is dominated by descriptions of the processes that occur at the root-soil interface. However, there are other soil fauna effects that can modulate the breaking down of organic inputs and yet, are

very rarely included in ecological investigations: "zoological weathering" (i.e., mobilizing inorganic elements from rocks by the action of soil organisms), "zoological retarding" (e.g., the presence of a peritrophic membrane that encapsulates mite's feces and that slows down their degradation), "zoological bioturbation" (movement of organic and mineral particles and other organisms by soil fauna), "zoological bonding" (chemical binding of C and P induced by soil fauna). Could they become emerging concepts in future soil ecology studies?

7. A final challenge is the integration of abiotic (climate, soil texture) and biotic factors (litter quality and biological accessibility) influencing decomposition across spatial and temporal scales and to understand how their effects could change under environmental perturbations such as land use and climate changes. The fact that many of these factors do no work in isolation, understanding the interactions between soil and climatic factors, plant and soil organisms, microbes and soil fauna, and among them all is crucial.

These are only few examples that illustrate the complex nature of soil communities living in a heterogeneous soil matrix, consisting of a mosaic of microsites with different soil conditions and resource availabilities. The structure and degree of connectivity between these patches together with their temporal dynamics determines the number and composition of species assemblages. However, because they do not conform to closed loops and their responses to short-term changes in soil abiotic conditions are usually stochastic, it is difficult to underpin the mechanisms that allows their shelf-organization (*sensu* Lavelle et al., 2016). Soil biodiversity is at the core of the International agendas (GSBI, IPBES), and in the UN Sustainable Development Goals, and only with a more refined view of all the potential contributions of soil organisms to soil processes their full integration in sustainable management and climate change mitigation policies will be possible.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Quantification of Three Dimensional Characteristics of Macrofauna Macropores and Their Effects on Soil Hydraulic Conductivity in Northern Vietnam

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Soil bioturbation is associated with the production of soil macropores that influence numerous ecological functions such as those associated with water infiltration and the generation of runoff water. This impact is especially important on sloping lands in the tropics that are highly susceptible to erosion. In this study, we questioned the influence of soil biodiversity on soil macropore properties (>20 mm^3) and saturated hydraulic conductivity (*Ksat*) on sloping land in northern Vietnam. Biostructures found at the soil surface (casts, sheetings, and soil excavated on the ground) were used to identify areas colonized either by earthworms, termites or dung beetles, respectively. The influence of soil macrofauna on *Ksat* was measured *in situ* using the Beerkan method below bioturbated zones and compared to the surrounding soil without visible biostructures at the soil surface. Undisturbed soil columns were afterwards sampled and scanned by X-ray computed tomography (X-ray CT). Properties of macropores below each biostructure depicted a large variability, revealing the complexity of the macropore network. Further, galleries made by termites, dung beetles, and earthworms were manually isolated from the rest of macroporosity. Galleries made by beetles, termites and earthworms were clearly differentiated on the basis of their diameter, verticality, sphericity, tortuosity, length and number of branches and the fraction of galleries in the top part of the column. *Ksat* was most increased by dung beetles (45-fold), then by termites (30-fold) and to a lesser extent by earthworms (16-fold). Relationships between total macropore properties and *Ksat* showed that the most important properties explaining *Ksat* were (i) the volume of percolating macropores, (ii) the diameter, (iii) the critical macropore diameter, and (iv) the number of macropores. In conclusion, this study confirmed not only the interest in using X-ray CT for the quantification of macroporosity but also the absence of a clear relationship between aboveground biostructures and macropore properties and functional impacts.

Keywords: soil, X-ray computed tomography, soil macrofauna, galleries networks, saturated hydraulic conductivity

INTRODUCTION

Soil structure regulates many key ecological processes in soils, such as those influencing the habitat of soil organisms, the growth of roots, the protection of carbon, the release of mineral nutrients or the infiltration and diffusion of water in soil. In a recent review, Rabot et al. (2018) differentiated two complementary approaches for understanding the dynamic of soil structure: the solid and pore perspectives. From the solid-phase perspective, the dynamic of soil structure is considered through the organization and dynamic of soil aggregates. This perspective is useful for understanding the habitat of microbes and the dynamics of carbon and nutrients in soil (e.g., Six et al., 2004). Conversely, the pore-phase perspective considers soil architecture through its voids and the properties of the soil pore network (Young et al., 2001), in particular their influence on the water dynamic (e.g., Beven and Germann, 1982; Jarvis, 2007; Luo et al., 2010). Although the dynamic of soil aggregates has long been debated (e.g., Tisdall and Oades, 1982; Oades and Waters, 1991), the importance and dynamic of soil porosity on the water dynamic in soil have only recently gained in knowledge with the development of non-destructive and non-invasive scanning techniques by X-ray computerized tomography (X-ray CT). During the last decades, X-ray CT has been applied in many different studies exploring the architecture and functions of soils. The application of X-ray CT has expanded rapidly, now covering the characterization of pore space and bulk density for different land use and management systems (e.g., Anderson et al., 1990; Luo et al., 2010; Capowiez et al., 2011; Larsbo et al., 2014; Naveed et al., 2016; Jarvis et al., 2017). Furthermore, X-ray CT has been used widely to non-destructively quantify earthworm bioturbation in repacked soil cores (Joschko et al., 1991; Jégou et al., 1997; Langmaack et al., 1999; Capowiez et al., 2001, 2011; Bastardie et al., 2003) or undistributed natural soil cores (Pierret et al., 2002; Bastardie et al., 2005). The interest in X-ray CT relies on its description of the pore size distribution, connectivity, continuity, tortuosity and length, which are all considered to influence soil hydraulic properties (Perret et al., 1999; Vogel, 2000; Pierret et al., 2002).

The influence of soil biota on the properties of soil aggregates has been largely considered, especially with roots, earthworms and the production of casts or termites and the production of mounds and sheetings (e.g., Six et al., 2004; Bottinelli et al., 2015). Information about the influence of soil biodiversity on soil porosity and thus on the dynamic of water in soil remain, however, very limited to studies that have mainly been carried out in controlled conditions with earthworms (e.g., Capowiez et al., 2015; Bottinelli et al., 2017). Therefore, a clear dearth of information exists on how the other soil bioturbators influence soil porosity and the water dynamic in non-perturbed environments, which justifies the need to describe the properties of galleries produced by soil fauna. Hence, the objectives of this study were to use X-ray CT to (i) provide quantitative data of the galleries made by the most important soil engineers (*sensu* Jones et al., 1994, 1997) in tropical soils, namely, termites, beetles and earthworms (e.g., Lavelle et al., 1997; Jouquet et al., 2006; Filser et al., 2016) and (ii) determine how their macropores impacted water infiltration in soil.

MATERIALS AND METHODS

Study Site

This study was carried out at the M-Tropics long-term observatory (46 ha) located in Dong Cao Village in the northeast of Vietnam, approximately 60 km southwest of Hanoi (20° 57′N, 105° 29′E). The annual rainfall ranges from 1,500 to 1,800 mm, and 80–85% of total rainfall is concentrated during the rainy season from April to October. The humidity is always high between 75 and 80% (Jouquet et al., 2008a). The mean daily temperature varies between 15 and 25°C (Jouquet et al., 2008b). Soils derive from the weathering of volcanic sedimentary schists of the Mesozoic age and are mainly described as Acrisols (WRB, 1998) or Ultisols (Podwojewski et al., 2008; Soil Survey Staff, 2014). The soils are dominated by clay particles (>50%, mainly kaolinite) and contain ∼12 and 40% of sand and silt, respectively (Jouquet et al., 2008b). The vegetation is a deciduous forest dominated by *Vernicia montana* (Euphorbiaceae) and *Brachiaria ruiziziensis* (Poaceae) (De Rouw, unpublished data). The itinerant pasture of buffaloes in the watershed leads to the production of buffalo dung that is very attractive for dung beetles (Scarabaeidae). The study site is also characterized by high activity of earthworms (mainly *Amynthas khami*) and termites (mainly fungus-growing termites) (Jouquet et al., 2012). The experiment took place during the rainy season in September 2017 when the activity of soil macrofauna is considered to be the most important.

Soil Macrofauna Diversity

Soil macrofauna (>2 mm in size) were collected using the TSBF method (Anderson and Ingram, 1993) below the soil excavated by dung beetles (DB), termite sheetings (TS), and earthworm casts (EC) and in the control surrounding soil environment without visible trace of soil macrofauna (Ctrl). Soil fauna were removed by hand sorting from 25 × 25 cm wide and 30 cm deep blocks ($n = 3$). Individuals were preserved in 70% alcohol before counting. Regarding their occurrence, individuals were classified into 4 taxonomic groups: beetles, termites, ants, and earthworms.

Soil Hydraulic Conductivity

Saturated hydraulic conductivity (*Ksat*) was measured *in situ* using the Beerkan method (Lassabatère et al., 2006) below DB, TS, and EC and in the Ctrl ($n = 3$ per treatment). PVC cylinders (14 cm height and 13 cm in diameter) were positioned at the soil surface and inserted to a depth of approximately 1 cm to avoid lateral loss of the ponded water at the soil surface. A fixed volume of water (100 ml, corresponding to a water layer of 1 cm) was poured into the cylinder, and the time needed for the water to infiltrate was measured. The procedure was repeated between 7 and 10 times to reach a steady state of infiltration. Soil cores (100 cm^3) were used to determine the soil bulk density and the initial water content in the surrounding soil (0–5 cm depth). The results were analyzed with the original BEST algorithm (Lassabatère et al., 2006) in order to estimate *Ksat*.

Quantification of Macropores and Galleries

After measuring water infiltration, soil cores were excavated by gently inserting the PVC pipes into the soil to a depth of 10 cm ($n = 3$). All cores were scanned using medical X-ray CT (Siemens Somatom® Definition Flash) at the Bach Maï hospital (Hanoï, Vietnam) to obtain a set of 0.6 mm thick images with a pixel size of 0.3 mm. The X-ray beam was operated at 93 mA and 120 kV. Images (16-bit DICOM format, 512 × 512 pixels) were transformed into 8-bits TIFF format and rendered isotropic with a resolution of 0.3 mm. Prior to segmentation, a 3-D Median filter with a radius of two voxels size was applied in order to reduce noise and scatter. Since the gray-level of histograms was bi-modal, the automatic Otsu thresholding method was used (Otsu, 1979). Image processing and quantification were conducted with the open-source software ImageJ version 1.51 (Schneider et al., 2012).

After the images were preprocessed, soil macrofauna macropores inside each core were selected by removing pores <20 mm^3 in order to reduce noise and exclude roots. Characteristics of total macropores were then described based on their number, volume (largest volume, volume of the pores connected to the surface, volume of the pores connected to the bottom and the percolating volume), diameter (the mean diameter and the critical diameter of the percolating macropores) and global connectivity (Γ), which reflects the probability of two randomly chosen pore voxels to belong to the same macropore cluster (Renard and Allard, 2013). Macropores were then reconstructed and visualized using AvizoFire 8.1.

Field observations revealed that beetles produced larger galleries (∼5–6 mm in diameter) than termites (<3 mm in diameter), while earthworms produced intermediate galleries (∼3–4 mm). Further, anecic earthworm species are also well-known to make large and vertical burrows open to the surface (Capowiez et al., 2011). From these observations, galleries made by earthworms, beetles, and termites were manually isolated from the total macroporosity based on their body size and shape (**Figure 1**) and using the option "volume edit" in the Avizo 8.1 software. Galleries were then described by measuring their (i) diameter, (ii) verticality (orientation or angle between the maximum Feret diameter of the object and the XY plane), (iii) tortuosity (the ratio between the actual branch length >10 mm of the object and the Euclidean distance along the skeleton), (iv) sphericity (the ratio between the volume and surface of the object), (v) total length of galleries (sum of branches with length >10 mm after skeletonization), (vi) number of branches (number of branches with length >10 mm after

TABLE 1 | Abundance of soil macrofauna (ind m^{-2}) ($n = 3$) collected below termite sheetings (TS), dung beetles (DB), and earthworm casts (EC) and in control (Ctrl) treatments.

Treatments	Ants	Termites	Earthworms	Beetles
Ctrl	56.3 (±5.3)	7.3 (±5.2)	2.33 (±1.2)	0.0 (±0.0)
DB	20.1 (±6.2)	0.0 (±0.0)	0.0 (±0.0)	7.3 (±0.0)
TS	9.2 (±5.0)	40 (±8.2)	0.0 (±0.0)	0.0 (±0.0)
EC	18.1 (±8.1)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)

Data are the mean ± standard error, n = 3.

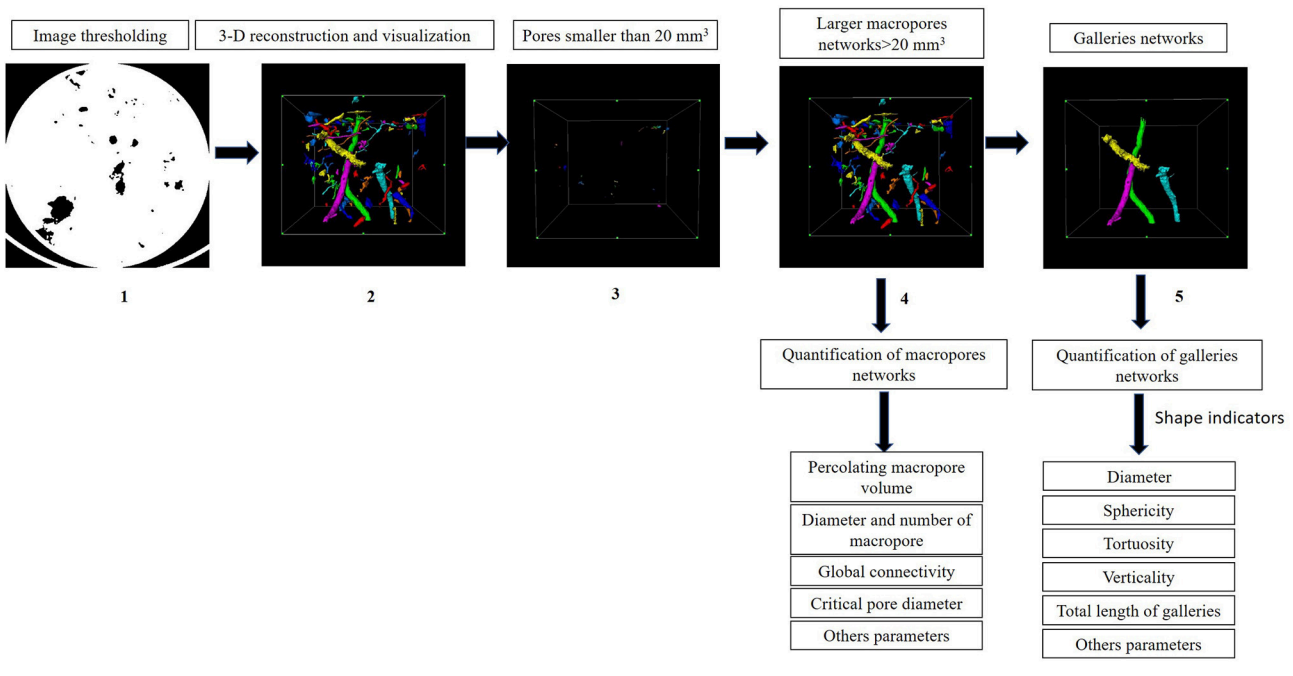

FIGURE 1 | Procedure used for image analysis and quantification of macropores below soil macrofauna biostructures and the differentiation of macropores and galleries. (1) Segmentation of the image. (2) Reconstruction and visualization of the total macroporosity. (3) Representation of the pores <20 mm^3 that were discarded to reduce noise. (4) Representation of macropores >20 mm^3. (5) Galleries manually isolated from macropores.

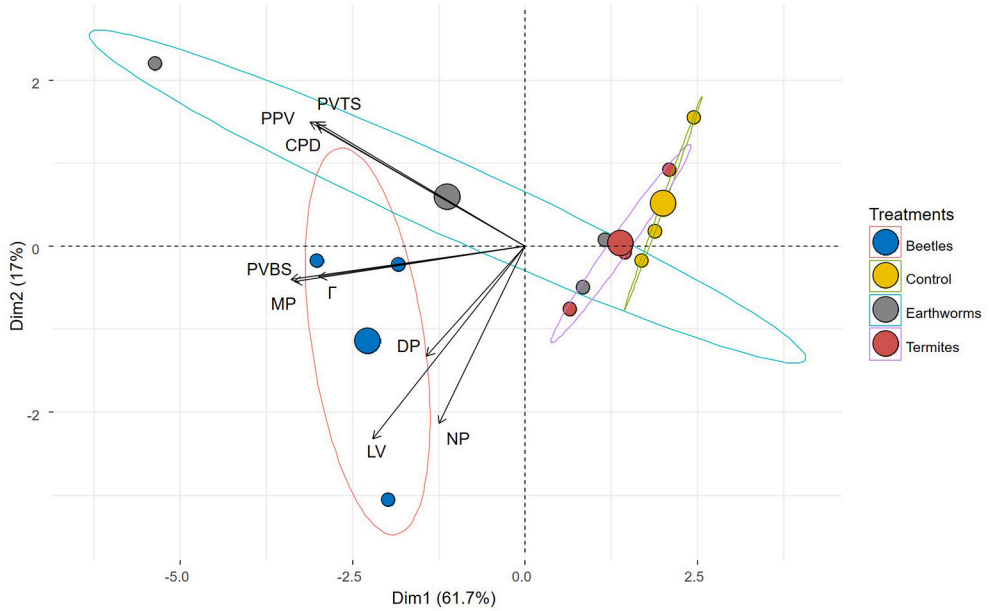

FIGURE 2 | Biplot showing the principal components analysis (PCA) from variables describing soil macroporosity for the different treatments (control in yellow, termite sheetings in red, dung beetles in blue and earthworm casts in gray) in the plane defined by axes 1 and 2 of the PCA. Variables are number of macropores (NP), macroporosity (%) (MP), pore diameter (mm) (DP), macropore volume connected to the surface (mm^3) (PVTS), macropore volume connected to the bottom surface (mm^3) (PVBS), percolating macropore volume (mm^3) (PPV), largest volume (mm^3) (LV), critical pore diameter (mm) (CPD), and connection probability (Γ).

skeletonization), and (vii) fraction of galleries volume in upper part (fraction of the galleries volume in the top part of the column).

Statistical Analyses

Prior to analysis, the homogeneity of variances was inspected using Levene's test, and data were log-transformed if needed. One-way analysis of variance (ANOVA) and least significant difference (LSD) tests were performed to assess differences between means. To visually resume the information from the soil total macroporosity and galleries properties, an ordination method (principal component analysis, PCA) was applied using "ade4" packages in R. Partial least squares regression (PLSR) analysis was performed to predict important total macropores variables associated with soil hydraulic conductivity using the "pls" package (Mevik and Wehrens, 2007). All statistical calculations were carried out using R version 3.5.1. Differences among treatments were declared significant at the <0.05 probability level.

RESULTS

Soil Macrofauna

Four dominant soil macrofauna groups were identified across the study area, namely, earthworms, beetles, ants and termites. The abundance of soil macrofauna was influenced by the different treatments (**Table 1**). Ants were in all the treatments and in large numbers, especially in the Ctrl treatment, although they could not be clearly associated with any specific galleries. Termites were mainly found in the TS treatment and to a lesser extent in the Ctrl treatment. Termites belonged to soil-feeding termites in Ctrl, while they belonged to the fungus-growing termite taxon in TS (subfamily Macrotermitinae, *Odontotermes* spp.). Beetles were exclusively found below DB, while endogeic earthworms (small-sized and non-pigmented) were only found beneath EC. In total, 62.4, 29.1, 4.2, and 4% of the total number of individuals ($n = 165$) were ants, termites, beetles or earthworms, respectively.

Visualization and Quantification of the Macropore Network

The three-dimensional visualization of the macroporosity within the columns is shown in **Supplementary File 1**. Macropore characteristics were obviously different among the different treatments. We observed also different macropores with different origins. **Figure 2** shows the PCA obtained from the properties of the macropores (data used for computing the PCA are shown in **Table 2**). Treatments were mainly differentiated along the first axis of the PCA that explained 61.7% of the total variability, while variability within treatment was mainly evident on the second axis of the PCA (17% of the total variability). The DB treatment was clearly differentiated from the Ctrl treatment, while overlaps were observed among EC, TS and Ctrl treatments. From the different variables, only two were significantly influenced by the treatments (i.e., the volume of the total macroporosity and the volume of the largest pore) (ANOVA test, $P < 0.05$ in both cases). The volume of the total soil macroporosity was highest in DB and EC ($P > 0.05$ between the two) and lowest in Ctrl, while an intermediate value was reached in TS ($P > 0.05$ with EC and

TABLE 2 | Influence of the treatments [termite sheetings (TS), dung beetles (DB), earthworm casts (EC) and control (Ctrl)] on soil macroporosity.

Treatments	NP	MP (%)	DP (mm)	PVTS (mm^3) ($\times 10^3$)	PVBS (mm^3) ($\times 10^3$)	PPV (mm^3) ($\times 10^3$)	LV (mm^3) ($\times 10^3$)	CPD (mm)	Γ
DB	121 (±6.03)^a	5.0 (±0.005)^a	4.42 (±0.55)^a	31.87 (±26.91)^a	56.60 (±11.47)^a	29.05 (±0.01)^a	45,945 (±18,312)^a	0.69 (±0.45)^a	0.43 (±0.19)^a
EC	107 (±14.18)^a	4.8 (±0.027)^{ab}	4.70 (±0.66)^a	32.81 (±45.39)^a	38.61 (±41.76)^a	28.82 (±0.01)^a	10,138 (±4,735)^b	0.85 (±0.71)^a	0.28 (±0.38)^a
TS	92 (±31.75)^a	2.0 (±0.005)^{bc}	4.35 (±1.47)^a	6.36 (±3.53)^a	12.81 (±11.53)^a	0.01 (±0.01)^a	6,833 (±1,549)^b	0.29 (±0.00)^a	0.17 (±0.10)^a
Ctrl	101 (±35.68)^a	1.6 (±0.006)^c	2.69 (±0.58)^a	2.86 (±5.16)^a	3.02 (±1.41)^a	0.01 (±0.01)^a	2,681 (±2,461)^b	0.29 (±0.00) a	0.10 (±0.01)^a
F-value	0.94	32.05	2.45	1.01	3.55	1.15	13.09	1.41	1.25
p-value	0.691	0.042*	0.139	0.4	0.07	0.391	0.002**	0.309	0.35

*The results of the ANOVA are given for each variable. The number in parentheses is one standard error of the mean. The letters after the parenthesis indicate the significance test of mean difference among treatments at $P < 0.05$. Variables are number of macropores (**NP**), macroporosity (%) (**MP**), pore diameter (mm) (**DP**), macropore volume connected to the surface (mm^3) (**PVTS**), macropore volume connected to the bottom surface (mm^3) (**PVBS**), percolating macropore volume (mm^3) (**PPV**), largest volume (mm^3) (**LV**), critical pore diameter (mm) (**CPD**), and connection probability (**Γ**).* $^{**}P < 0.01$, $^{*}P < 0.05$.

Ctrl). The largest pores were also measured in DB in comparison with those in the other treatments ($P > 0.05$ between them).

Geometrical Properties of Soil Macrofauna Galleries

Galleries made by beetles, termites and earthworms are shown in **Figure 3**. Treatments were clearly differentiated along the first and second axes of the PCA, which explained 62.3 and 16.1% of the total variability, respectively (**Figure 4**). Galleries made by beetles were characterized by their large diameter (5.8 mm on average) and their verticality (52° on average) (**Table 3**). Conversely, TS galleries were relatively small (∼2 mm in diam.). Galleries were also markedly horizontal (∼32°). Finally, earthworm galleries had intermediate size with a diameter of 4 mm on average and were markedly vertical (51°) in comparison with those made by termites. The total length of galleries network and the number of branches were calculated based on their skeletons. Galleries of beetles were longer with more branches than those of termites and earthworms. No significant difference was found among treatments in terms of sphericity, tortuosity and fraction of the galleries volume contained in the top part of the column ($P > 0.05$ in both cases).

Impact of Soil Macrofauna on Ksat

Figure 5 shows that *Ksat* was significantly influenced by soil macrofauna activity [ANOVA test, $F_{(3, 8)} = 6.39$, $P = 0.03$], and *Ksat* increased by 45 and 30-fold in DB and TS in comparison with that in Ctrl ($P < 0.05$). Earthworm activity also increased *Ksat* by 16-fold in comparison with that in Ctrl, although this difference was not significant ($P > 0.05$). The best model was obtained when only the diameter and the number of pores, the volume of the percolating pores and the critical pore diameter were considered (RMSEP = 0.55, $Q^2 = 0.68$) (**Table 4**).

DISCUSSION

Influence of Soil Macrofauna Activity on Soil Porosity

Anecic earthworms and fungus-growing termites produce specific casts and sheetings on the ground in the Dong Cao watershed (Jouquet et al., 2008b, 2009, 2012), while dung beetles excavate an important quantity of soil. Our study showed that these soil biogenic aggregates (*sensu* Bullock, 1985) were associated with complex macropore networks in soil. Despite specific signatures on the PCA, treatments were characterized by an important variability, most likely due to the low number of replicates ($n = 3$) and the presence of galleries that could be attributed to a variety of soil organisms. Since only macropores >20 mm^3 were considered in this study, it is unlikely that these macropores corresponded to roots, while they could result from ant, termite and earthworm activities, which were abundantly found in soil. Although the morphological properties of ant nest chambers have been previously described (e.g., Mikheyev and Tschinkel, 2004), a clear lack of information exists concerning the shape of their galleries. The morphological properties of ant galleries remain unknown and most likely difficult to differentiate from those made by earthworms and termites in the field.

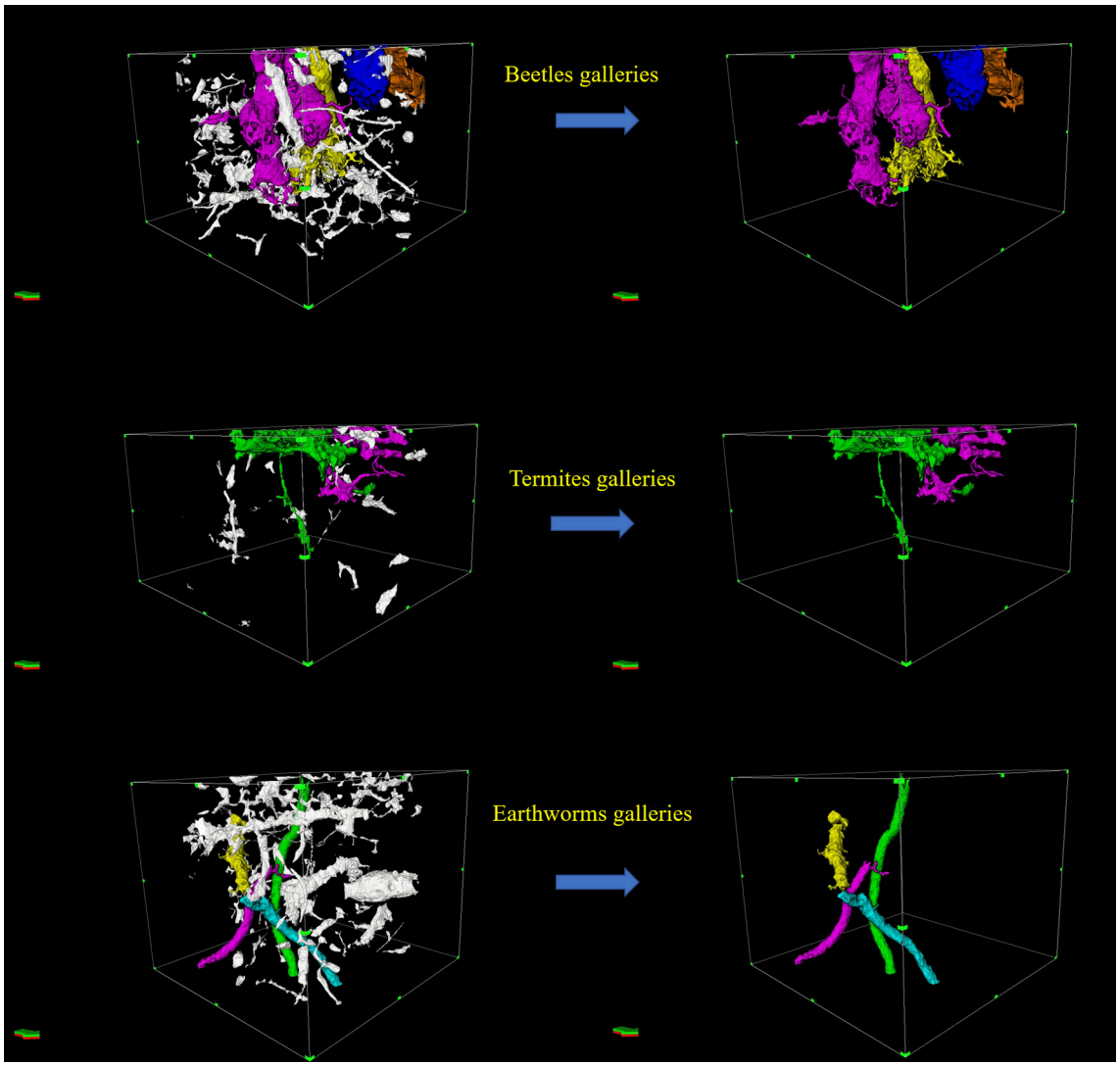

FIGURE 3 | Examples of three-dimensional images of galleries made by beetles, termites and earthworms (**Right**) manually isolated from macropores (in white: **Left**).

Moreover, as highlighted by Cheik et al. (2018b), the lifetimes of galleries made by soil invertebrates are difficult to estimate, making it difficult to estimate the origin, age and functional impact of the numerous macropores that were observed in our study.

Regarding the variability of the macropore networks, galleries made by earthworms, beetles and termites were visually distinguished from the rest of the macroporosity and manually extracted from the images. Although the accuracy of this approach is likely to be site-dependent, and the approach probably minimizes the influence of these soil invertebrates on soil architecture, a clear distinction was revealed among the gallery types. Beetle galleries were significantly larger than those of the others (∼6 mm in diameter) and marked by their verticality. Beetles had also the longest galleries networks and the highest number of branches. Although the size of their galleries is likely to vary depending on the size and functional group of beetle species (e.g., Slade et al., 2007), our result is in line with that of Mikus and Uchman (2013) who found that beetles make vertical galleries ranging from 6 to 11 mm in diameter in temperate ecosystems. Conversely, termite galleries were more connected to the upper part of the soil column with small galleries (∼2 mm in diameter) mainly markedly horizontal. Although our study is the first to quantify the complexity of termite galleries using X-ray CT technology, our findings are in line with those of Kooyman and Onck (1987) who manually measured in the field gallery diameters ranging from 2 to 5 mm. Our study also confirms results obtained by Léonard and Rajot (2001) who found that galleries made by *Odontotermes* sp. (Macrotermitinae) are mainly horizontal and shallow within the first cm of soil in west Africa. The complexity of termite galleries was especially important in comparison with that of earthworm galleries, which

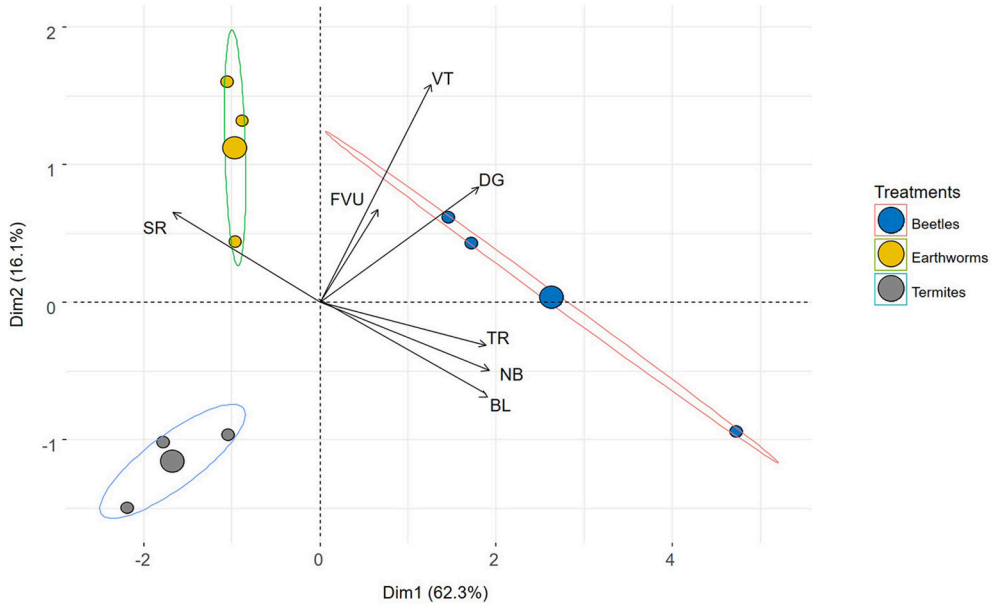

FIGURE 4 | Biplot showing the PCA made from the 3-D characteristics of galleries made by beetles, termites and earthworms. Variables include the diameter of galleries (mm) (DG), their tortuosity (–) (TR), sphericity (SR) (–), verticality (°) (VT), total length of galleries 10 (mm) (BL), number of branches 10 (NB) (–), and fraction of galleries volume in upper part (FVU). The correlation circle is also given on the right side. Ordination of the samples is in the plane defined by axes 1 and 2 of the PCA.

TABLE 3 | Influence of the treatments on the morphological characteristics of galleries created by beetles, earthworms and termites derived from X-ray CT image analysis.

Treatments	VT (°)	DG (mm)	SR (–)	TR10 (–)	BL10 (mm)	NB10 (–)	FVU (–)
Beetles	52.40 (±0.15)^{a}	5.8 (±0.01)^{a}	0.3 (±0.14)^{a}	1.53 (± 0.11)^{a}	586.13 (±177.70)^{a}	48.0 (±9.87)^{a}	0.86 (±0.01)^{a}
Earthworms	51.03 (±1.50)^{a}	4.1 (±0.01)^{b}	0.3 (±0.00)^{a}	1.32 (±0.08)^{a}	261.08 (±74.66)^{b}	7.0 (±15.33)^{b}	0.86 (±0.17)^{a}
Termites	31.76 (±1.02)^{b}	2.2 (±0.01)^{c}	0.3 (±0.00)^{a}	1.31 (±0.08)^{a}	141.14 (±5.98)^{b}	15.0 (±6.67)^{b}	0.77 (±0.25)^{a}
F-value	385.5	1,629	1.06	4.01	12.82	36.86	0.17
p-value	$P < 0.001^{***}$	$P < 0.001^{***}$	0.354	0.076	0.007^{**}	$P < 0.001^{***}$	0.849

*The results of the ANOVA are given for each variable. The number in parentheses is one standard error of the mean. The letters after the parenthesis indicate the significance test of mean difference among treatments at $P < 0.05$. Variables are diameter of galleries (**DG**) (mm), tortuosity (**TR**) (–), sphericity (–) (**SR**), verticality (°) (**VT**), total length of galleries (mm) (**BL**), number of branches (–) (**NB**), and fraction of galleries volume in upper part (–) (**FVU**). Verticality of the galleries in degrees (°). Tortuosity 10, total length of galleries 10, number of branches 10 are, respectively, the mean tortuosity for galleries with length >10 mm, total sum of branches for galleries after skeletonization with length >10 mm and number of branches after skeletonization with length >10 mm $^{***}P < 0.001$,$^{**}P < 0.01$.*

were larger (∼4 mm in diameter), mainly vertical and with the highest elongation index. Earthworm galleries were produced by *A. khami*, which was not found during the soil macrofauna sampling. This species might be very long (up to 70 cm) and goes down very quickly to the deep soil layers (>1 m, Jouquet pers. com.). This species is also considered to belong to the anecic functional group because its globular casts have similar isotopic signatures to those of the litter (e.g., Hong et al., 2011). Consistent with the properties of its casts, our study showed that its galleries are also characteristic of the anecic earthworm functional group, with vertical and percolating galleries open on the soil surface, as shown in laboratory conditions (e.g., Bastardie et al., 2003; Capowiez et al., 2015; Bottinelli et al., 2017).

Influence of Soil Macrofauna on Water Infiltration

Despite high variability in soil macroporosity, our treatments led to significant differences in *Ksat* with the highest values below

TABLE 4 | Coefficient values from the most relevant variables used for the PLSR describing the evolution of soil hydraulic conductivity at saturation (*Ksat*).

Variables	Coefficients
Percolating pore volume	31.28
Diameter of pores	28.84
Critical pore diameter	2.99
Number of pores	1.64

DB (45-fold) followed by those of TS (30-fold) and EC (16-fold) in comparison with those in Ctrl. These results underline the importance of differentiating the influence of macropores made by earthworms, termites and beetles on water dynamic in our study site. The highest *Ksat* values measured for DB can be explained by the largest size of the galleries, most likely because of the harder and larger body diameter of beetles than that of earthworms and termites.

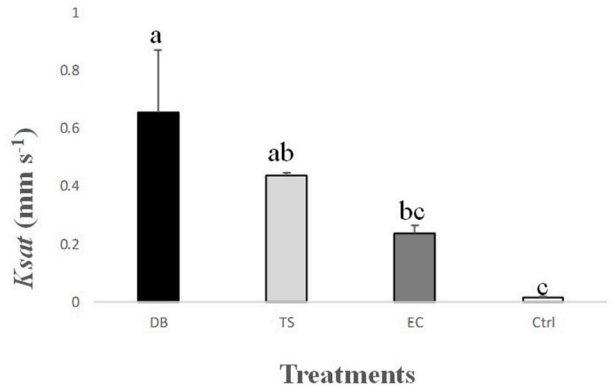

FIGURE 5 | Influence of the treatments on *Ksat* (mm s^{-1}). Treatments are "DB" for dung beetles, "TS" for termite sheetings, "EC" for earthworm casts, and "Ctrl" for control. Error bars represent the standard error of the mean. Histograms with the same letters are not significantly different at $P = 0.05$, $n = 3$.

The positive influence of termites on water infiltration is mainly evident in dry environments such as in west Africa in comparison with the control surrounding environment (1.5- to 10.5-fold) (e.g., Mando et al., 1996, 1999; Léonard et al., 2001, 2004; Kaiser et al., 2017) and more recently in India (3–12-fold, Cheik et al., 2018a,b). Our findings showed that termite foraging activity also increased water infiltration in the humid tropical environment of Southeast Asia. However, these results have to be considered in light of another study carried out in the same study site by Jouquet et al. (2012), who showed that the fragmentation of termite sheeting on the ground by the rain leads to the production of soil crusts that reduce water infiltration and increase soil erosion. Consequently, it can be concluded that the impact of termite foraging activity on water infiltration results from a balance between two antagonistic processes (increasing water infiltration through the production of galleries vs. reducing water infiltration through the production of soil sheetings and then soil crusts on the surface), making any simple conclusion on the functional impact of termites difficult to establish.

Finally, our study confirmed the positive impact of anecic earthworm galleries on water infiltration (e.g., Fischer et al., 2014; Andriuzzi et al., 2015). Although the positive influence of earthworms on *Ksat* has been previously demonstrated, especially in temperate environments (van Schaik et al., 2014) or more specifically in our study site (e.g., Jouquet et al., 2008b, 2012), our study showed that earthworms only slightly improved *Ksat* in comparison with beetles and termites. Hydraulic conductivity strongly depends on the number and diameter of connected flow pathways. The results from the PLSR showed that four variables were used for the prediction of *Ksat*: the diameter and the number of pores, the volume of the percolating pores and the critical pore diameter. Interestingly, these variables were not influenced by the treatments, and a higher *Ksat* would have been expected with EC than that with TS because of the larger, more vertical and more elongated galleries of earthworms than those of termites. We explain these results by the fact that galleries made by termites and earthworms represented only a small proportion of the efficient macroporosity. However, regarding the importance of earthworm activity in our study site and the comparatively low and sporadic activity of beetles and termites, we assume that the earthworm species *A. khami* plays a very important role in favoring water infiltration and then reducing soil erosion in the watershed (Podwojewski et al., 2008).

CONCLUSIONS

Properties of the soil macroporosity and galleries made by beetles, termites, and earthworms were studied using X-ray CT, thereby providing evidence of the impact of soil invertebrate biodiversity on soil architecture. A conclusion of this study is that most of the macroporosity in soil can be viewed as a heritage of the activity of many other soil invertebrates, such as ants or endogeic earthworms, which do not leave traces of activity on the soil surface. We confirmed the positive impact, although taxon-specific, of soil fauna on water infiltration (beetles $\geq$ termites $\geq$ earthworms), and we confirmed that macroporosity measured by X-ray CT provides an accurate prediction of *Ksat* (Rachman et al., 2005; Kim et al., 2010; Luo et al., 2010). This finding confirms also the interest in this approach for quantifying the impact of soil fauna on the dynamic of water in soil and highlights the need for a better understanding of the dynamic of these galleries in terms of production and degradation.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because the datasets generated during and/or analyzed during the current study are available from the corresponding author upon request. Requests to access the datasets should be directed to sougueh.cheik@ird.fr.

AUTHOR CONTRIBUTIONS

SC, NB, and PJ conceived and designed the research and analyzed the X-ray CT images, analyzed and interpreted the data, and wrote the manuscript. TM and TD provided access to the laboratory, medical scanner, and the study site. SC, PJ, and NB wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2019.00031/full#supplementary-material

Supplementary File 1 | Three-dimensional images of soil macropores (>20 mm^3) obtained below treatments DB: "dung beetles," EC: "earthworm casts," and TS: "termite sheetings" and in Ctrl: control.

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Earthworm Cast Formation and Development: A Shift From Plant Litter to Mineral Associated Organic Matter

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Earthworms play a major role in litter decomposition, in processing soil organic matter and driving soil structure formation. Earthworm casts represent hot spots for carbon turnover and formation of biogeochemical interfaces in soils. Due to the complex microscale architecture of casts, understanding the mechanisms of cast formation and development at a process relevant scale, i.e., within microaggregates and at the interface between plant residues, microorganisms and mineral particles, remains challenging. We used stable isotope enrichment to trace the fate of shoot and root litter in intact earthworm cast samples. Surface casts produced by epi-anecic earthworms (*Lumbricus terrestris*) were collected after 8 and 54 weeks of soil incubation in mesocosms, in the presence of ^{13}C-labeled Ryegrass shoot or root litter deposited onto the soil surface. To study the alteration in the chemical composition from initial litter to particulate organic matter (POM) and mineral-associated organic matter (MOM) in cast samples, we used solid-state ^{13}C Nuclear Magnetic Resonance spectroscopy (^{13}C-CPMAS-NMR) and isotopic ratio mass spectrometry (EA-IRMS). We used spectromicroscopic approach to identify plant tissues and microorganisms involved in plant decomposition within casts. A combination of transmission electron microscopy (TEM) and nano-scale secondary ion mass spectrometry (NanoSIMS) was used to obtain the distribution of organic carbon and δ^{13}C within intact cast sample structures. We clearly demonstrate a different fate of shoot- and root-derived organic carbon in earthworm casts, with a higher abundance of less degraded root residues recovered as particulate organic matter on the short-term (8 weeks) (73 mg·g^{-1} in Cast-Root vs. 44 mg·g^{-1} in Cast-Shoot). At the early stages of litter decomposition, the chemical composition of the initial litter was the main factor controlling the composition and distribution of soil organic matter within casts. At later stages, we can demonstrate a clear reduction of structural and chemical differences in root and shoot-derived organic products. After 1 year, MOM clearly dominated the casts (more than 85% of the total OC in the MOM fraction). We were able to highlight the shift from a system dominated by free plant residues to a system dominated by MOM during cast formation and development.

Keywords: carbon isotopic labeling, root and shoot litter, microorganisms, NanoSIMS, TEM, ^{13}C-CPMAS-NMR

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INTRODUCTION

Plant residues represent the main contributor to soil organic matter (SOM), followed by microorganism biomass. Among plant residues, the distinction between above (leaves and shoots) and below ground (dead roots and rhizodeposits) inputs is crucial. It is now commonly recognized that roots decompose at a slower rate than shoots and root-derived carbon represents a larger pool of carbon in soils (Balesdent and Balabane, 1996; Puget and Drinkwater, 2001; Lu et al., 2003; Angst et al., 2016). However, whether the slower root decomposition depends on chemical composition, physical or physico-chemical protection, remains unclear, due to the initial location of root in the soil (Rasse et al., 2005).

Biotic factors driving plant residue decomposition encompass the litter quality, as well as the activity of soil fauna and microorganisms (Oades, 1988; Cortez and Bouché, 1998). Soil fauna fragments, transports and partly decomposes residues (Lavelle et al., 1993), and microorganisms decompose and transform organic compounds (Kuzyakov and Blagodatskaya, 2015). Earthworms, ants and termites are considered as the main ecosystem engineers having a significant impact on their environments, under suitable living conditions (Lavelle, 2002; Hastings et al., 2007). In temperate regions, earthworms account for the main invertebrate biomass in soils (Lee, 1985; Edwards, 2004). These saprophageous invertebrates ingest both organic (plant litter, SOM and microorganisms) and mineral soil particles. During ingestion, residues are fragmented and the preexisting soil microstructures destroyed. Organic elements are mixed with mineral particles, complexed with mucus, partly assimilated and mineralized, and mainly released at the soil surface in the form of biogenic organo-mineral aggregates called casts (Lee, 1985; Six et al., 2004). Within a few weeks, the presence of earthworms increases the proportion of macro and microaggregates that are more stable compared with non-biogenic aggregates (Six et al., 2004; Bossuyt et al., 2005; Zangerlé et al., 2011). The mutualistic relationship maintained between earthworms and microorganisms enhance litter decomposition during the gut transit and in casts (Brown et al., 2000). This results in higher microbial activity within casts compared to bulk soil at the scale of days and weeks (Frouz et al., 2011), inducing hotspots of microbial activity (Decaëns, 2010; Kuzyakov and Blagodatskaya, 2015; Athmann et al., 2017). However, casts are among the most complex and dynamic structures in soil (Lee, 1985) and were recognized as potentially favoring long-term carbon protection (Martin, 1991; Bossuyt et al., 2005; Frouz et al., 2009; Sánchez-de León et al., 2014). The occlusion of SOM within microaggregates, which can be found in casts, tends to protect organic carbon (OC) from decomposition (Chenu and Plante, 2006; Lützow et al., 2006; Dignac et al., 2017).

Many relevant processes in earthworm casts happen at a fine spatial scale, i.e., within microaggregates and at the interface between plant residues, microorganisms and mineral particles. As the gut passage leads to a fine scale mixing of mineral and organic soil constituents together with earthworm-derived mucus and bacteria, the resulting casts show a highly complex microscale architecture (Vidal et al., 2016b). To gain a more fundamental understanding of the processes at the biogeochemical interfaces at the relevant process scale within the casts, the use of spectromicroscopic imaging techniques allowing for high spatial resolution is necessary. The visualization of OM within undisturbed cast microstructures using spectroscopic and microscopic methods can improve our understanding of plant tissue degradation and their association with mineral particles and microorganisms, at these soil biology hot spots. Due to methodological difficulties, this scale of study has often been left out for large scale investigations (Hastings et al., 2007), and studies depicting the role of earthworm casts in the formation and transformation of SOM at the fine scale are scarce (Barois et al., 1993; Pey et al., 2014; Vidal et al., 2016b).

Existing studies focused on revealing cast constituents at a given date, without discerning possible differences with respect to the processing of different substrate materials (Barois et al., 1993; Pey et al., 2014; Vidal et al., 2016b). As main plant-derived SOM constituents, roots and shoots represent a major source for OM during the buildup of cast-rich soils. However, while it is recognized that roots and shoots have contrasted fates is soil, little is known on the ability of earthworms to ingest and transform roots (Curry and Schmidt, 2007; Zangerlé et al., 2011; Cameron et al., 2014), and its impact on root decomposition in casts. The physico-chemical processing of plant residues during the gut passage, coupled to the intense microbial activity and the formation of organo-mineral associations in casts compared with soil, questions the different decomposition processes depicted for roots and shoots in soils. This could significantly influence the carbon cycling and storage in soils, considering that casts might account for at least half of the surface soil layer in natural conditions (Ponomareva, 1950; Lee, 1985).

The present study aimed at highlighting the transfer of plant-derived C and the stage of decomposition of incorporated residues into casts over time. We hypothesized that the chemical characteristics of shoot residues will drive their rapid degradation compared to root residues, and that associations between organic and mineral particles within cast will develop over time. Cast samples, produced in the presence of ^{13}C-labeled shoots and roots, were collected 8 and 54 weeks after the beginning of a mesocosm experiment. The change in the amount and isotopic composition of OM from initial plant residues to particulate organic matter (POM) and mineral-associated OM (MOM) was determined by isotope ratio mass spectrometry (EA-IRMS). Particulate OM and MOM were studied separately to differentiate the free from partly occluded plant residues, respectively. The alteration of the chemical composition of the POM and MOM derived from the casts with time was determined using solid-state ^{13}C cross polarization magic angle spinning nuclear magnetic resonance spectroscopy (^{13}C CPMAS NMR). So as to follow the decomposition processes in intact cast samples at the microscale, we combined elemental and isotopic information obtained with nano-scale secondary ion mass spectrometry (NanoSIMS) with high-resolution information on the arrangement of organic and mineral constituents obtained with transmission electron microscopy (TEM). In previous works, we focused on the method development and technical requirements of the micro-scale analyses (Vidal et al., 2016b) and demonstrated soil alteration due

TABLE 1 | Characteristics of initial soil, as well as Cast-Control, Cast-Root and Cast-Shoot samples over time, before fractionation in POM and MOM (numbers in parentheses indicate the standard deviation, $n = 3$).

		Initial soil	Cast-control		Cast-shoot		Cast-root	
			8 weeks	54 weeks	8 weeks	54 weeks	8 weeks	54 weeks
OC	$mg.g^{-1}$	12.1	19.0 (2.2)	16.8 (2.5)	34.9 (1.2)	20.6 (1.6)	55.0 (0.8)	22.2 (0.6)
N content	$mg.g^{-1}$	1.30	1.97 (0.2)	1.73 (0.2)	3.33 (0.1)	2.03 (0.21)	3.77 (0.1)	2.10 (0.1)
C/N		9	10 (0.5)	10 (0.3)	11 (0.1)	10 (0.3)	15 (0.3)	11 (0.2)
$\delta^{13}C$	‰	−28.1	−25.3 (1.3)	−28.6 (0.1)	938 (3.0)	168 (1.9)	673 (25)	127 (8.2)
Litter-derived C	%	–	–	–	58.1 (0.2)	11.8 (0.1)	51.8 (1.8)	11.5 (0.6)

to earthworm activity using bulk measurements and molecular analyses (Vidal et al., 2016a, 2017). We now use the developed methods and bulk analyses in addition to fractionation and NMR analyses to demonstrate the fine scale mechanisms of litter degradation through time. This approach reflects the increasing cognition in environmental science for the need to combine imaging with classical bulk measurements to gain a deeper understanding of biogeochemical processes (Mueller et al., 2013; Baveye et al., 2018).

MATERIALS AND METHODS

Experimental Setup

Three mesocosms were filled with ∼75 L of a loamy-sand soil (clay, 19%; silt, 25%; sand, 56%) collected on permanent grassland in North of France (Oise, France). The soil characteristics are described in **Table 1** and available in Vidal et al. (2017) and Vidal (2016). Mesocosms were placed in a greenhouse where soil humidity and temperature were maintained at 23% and 13°C, respectively. Six *Lumbricus terrestris* earthworms were deposited onto each mesocosm.

Plants of Italian Ryegrass (*Lolium multiflorum*) were artificially labeled in ^{13}C at the PHYTOTEC platform of the Alternative Energies and Atomic Energy Commission (CEA) in Cadarache (France). Plants were grown under a controlled and constant $^{13}CO_2$ enriched atmosphere (2.6% $^{13}CO_2$). The mean $\delta^{13}C$ values were 1,632 ‰ (±16) and 1,324 ‰ (±42) for shoots and roots, respectively. Shoots and roots were separated, dried and subsequently homogenized separately during 40 s with a laboratory blender (Waring Commercial) in order to obtain small fragments with millimeter size. We deposited 250 g of shoots and roots (∼0.9 g OC.kg $soil^{-1}$) on the soil surface of the two mesocosms, respectively. Although the design does not reflect the real condition of *in situ* root systems, both roots and shoots were voluntarily deposited onto the soil surface, under the same conditions, in order to consider the sole effect of the chemical composition of litter (without any initial physical contact of the roots with the soil particles) on its incorporation and decomposition in earthworm casts. No litter was applied on the third mesocosm, which served as control. After 8 and 54 weeks of experiment, around 10 earthworm cast fragments were randomly collected on the soil surface of each mesocosm using a spatula and combined to form a composite sample of around 50 grams for each time step. The present work aimed at improving the understanding of fundamental processes by combining bulk chemical and imaging techniques, which together provide a more complete view on small scale soil functioning. As we combined all used techniques on one sample per treatment each, no replication could be achieved due to time concern. The time points of sampling were selected according to the contrasted isotopic and molecular composition measured on bulk samples in previous works (Vidal et al., 2016a, 2017). For example, the shift from more than 50–12% of litter-derived carbon in casts from 8 to 54 weeks (**Table 1**) showed a clear differentiation into a first and second decomposition phase which led to the two chosen sampling dates. Casts were distinguished from the bulk soil due to their round shape and smooth texture (Velasquez et al., 2007). After 8 weeks of experiment most casts were fresh when collected, while after 54 weeks, casts started to age and dry. A sub-sample of 5 grams, made of around three cast fragments, was directly processed for TEM and NanoSIMS analyses after sampling. The rest of the sample was dried, ground and subsequently fractionated into POM and MOM physical soil fractions. The obtained SOM fractions were analyzed for OC, N and $\delta^{13}C$. The chemical composition of the SOM fractions was analyzed using ^{13}C CPMAS NMR spectroscopy. For the cast collected in the mesocosms containing roots, shoots and no litter, we will refer to Cast-Root, Cast-Shoot and Cast-Control, respectively.

Separation of POM and MOM Fractions

In order to differentiate between plant residue dominated and mineral-associated OM, dry and ground cast samples were fractionated to separate POM and MOM. Briefly, 4 g of cast sample were saturated with 50 mL sodium polytungstate solution with a density of 1.8 (TC Tungsten compounds, Grub am Forst, Germany). After settling overnight, the floating free POM was collected using a vacuum pump, washed to remove excess Sodium Polytungstate (conductivity < 3 µS) using pressure filtration (22 µm filter) and freeze-dried. The mineral fraction containing the MOM was washed to remove salts (conductivity < 50 µS), centrifuged (3,000 g, 30 min) and freeze-dried. The density fractionation resulted in a mean recovery of 94 ± 2.9% of the initial sample mass. In the present study, the POM fraction is considered as the particulate plant residues, which are extractable by floatation in a dense liquid, while the MOM fraction comprises the organo-mineral associations.

Bulk Elemental and Isotopic Analyses

All POM and MOM fractions were analyzed (Helmholtz Zentrum, Munich, Germany) for organic carbon, nitrogen and $\delta^{13}C$ using IRMS (delta V Advantage, Thermo Fisher, Dreieich, Germany) coupled to an Elemental Analyzer (Euro EA, Eurovector, Milan, Italy). An acetanilide standard, calibrated against several suitable international isotope standards (IAEA; Vienna), was used for calibrating. Prior to organic carbon and $\delta^{13}C$ analyses, MOM fraction samples were decarbonated adding 20 µl of HCl 2N to 1–5 mg samples for 10 h and drying overnight at 60°C. Additional samples were prepared (10–40 mg) for nitrogen analyses.

The labeled litter-derived carbon in POM and MOM fractions of earthworm casts was expressed according to equation 1:

$$\text{Litter-derived C}(\%) = [(\delta^{13}C_{sample} - \delta^{13}C_{control})/(\delta^{13}C_{litter} - \delta^{13}C_{control})] \times 100 \quad (1)$$

Where $\delta^{13}C_{sample}$ is the $\delta^{13}C$ value of the POM or MOM fraction samples isolated from casts incubated with labeled roots or shoots, $\delta^{13}C_{control}$ is the $\delta^{13}C$ value of the POM or MOM fraction samples isolated from control casts incubated without litter, $\delta^{13}C_{litter}$ is the $\delta^{13}C$ values of the labeled roots or shoots.

The percentage OC of the MOM or the POM fraction compared to the total OC contained in both POM and MOM isolated fractions (% OC bulk) was also calculated.

Nuclear Magnetic Resonance Spectroscopy

The ^{13}C-CPMAS-NMR analyses were performed (Chair of Soil Science, TUM, Freising, Germany) on initial litter, as well as POM and MOM fractions of cast samples, using a Bruker AvanceIII 200 spectrometer (Bruker BioSpin GmbH, Karlsruhe, Germany). The NMR was operated at a ^{13}C-resonance frequency of 50 MHz, with a spinning speed of 6.8 kHz and according to the carbon content, a recycle delay time of 2 or 0.4 s, for initial litter and other samples, respectively. From around 1,000 to up to 200,000 scans were accumulated for initial litter and other samples, respectively. The spectra were processed with a line broadening from 0 to 50 Hz, followed by phase adjustment and base line correction. The chemical shift regions were obtained by dividing the NMR spectra as followed: 0–45 ppm (alkyl-C), 45–110 ppm (O-N-alkyl-C), 110–160 ppm (aromatic-C) and 160–220 ppm (carboxyl-C) (Kögel-Knabner et al., 1992). It has to be noted that the 160–220 ppm chemical region also include carbonyl-C, but that the carboxyl-C are by far dominant. The ratio between alkyl-C and O-N-alkyl-C was used as an indicator of organic matter degradation. A higher alkyl-C/O-N-alkyl-C ratio generally reflects a higher OM degradation, as alkyl carbon chains tend to be less degradable compared with carbohydrates and proteins (source of O-N-alkyl-C) (Baldock et al., 1997). Although these values cannot be considered as absolute ones (due to overlapping signals and potential differences in relaxation times between the different types of C), they can be used for comparison purposes.

Ultrastructural Analyses by TEM

The materials and methods used to prepare undisturbed samples for TEM and NanoSIMS analyses were identical to those described in Vidal et al. (2016b). In brief, osmium tetroxide was used to chemically fix cast samples (2 g for each sample) and initial litter parts. To avoid sample disruption, cast structures were physically preserved with agar (Watteau et al., 2006). Cast samples were cut into cubes of few mm^3 (around 10 for each sample), dehydrated in graded acetone series, and embedded in epoxy resin (Epon 812). Ultrathin sections (80–100 nm) were sliced using a Leica Ultracut S ultramicrotome, stained with uranyl acetate and lead citrate and analyzed with a JEOL EMXII transmission electron microscope operating at 80 kV (LSE, Nancy, France).

Nano-Scale Isotope Analyses by NanoSIMS

Ultrathin twin sections of 100–200 nm were sliced from the same blocks prepared for TEM analyses, allowing the comparison between NanoSIMS and TEM images. Samples were gold coated and images were acquired using the NanoSIMS 50 (Cameca, France) located at Museum national d'Histoire naturelle in Paris, France. The sample surface was sputtered by a 1.5 pA Cs^+ beam to obtain 24 ×24 µm images (256 × 256 pixels) of $^{12}C^-$, $^{12}C^{14}N^-$, $^{13}C^{14}N^-$, and $^{28}Si^-$ secondary ions. The images were processed using the L'IMAGE® software (L. Nittler, Carnegie Institution, USA). Secondary ion images of $^{12}C^{14}N^-$ and $^{28}Si^-$ were used to distinguish organic structures from mineral particles. The ^{13}C isotopic images, named as $\delta^{13}C$ in the following, were generated using the $^{13}C^{14}N^-/^{12}C^{14}N^-$ ratio relative to the PDB standard. The heterogeneity of the $\delta^{13}C$ values observed on similar organic structures on NanoSIMS images can either reflect a methodological bias (variable contribution of C from epoxy resin) or a natural process (variable extent of C recycling), both leading to variable degree of isotopic dilution. Given these approximations, $\delta^{13}C$ values obtained in the present study were considered as indicators of the occurrence of labeled OC, and not taken as representative of accurate isotopic enrichment values.

Statistical Analyses

A principal component analysis (PCA) was performed with the R statistical software (package "FactoMinerR") on the 12 fraction samples (POM and MOM) using the 4 NMR chemical shift regions as variables. Root and shoot litter samples were implemented as illustrated samples in the PCA. The variables were normally distributed, as tested by the Shapiro-Wilk test.

RESULTS

C and N Elemental and Isotopic Composition, and Distribution in POM and MOM Fractions

At 8 weeks, the mass proportion of POM fraction was higher in Cast-Root compared to Cast-Shoot (73 vs. 44 $mg.g^{-1}$, respectively) (**Table 2**). Particulate OM and MOM (Cast-Shoot

TABLE 2 | Organic carbon, nitrogen, $\delta^{13}C$ and chemical characteristics of particulate organic matter (POM) and mineral associated organic matter (MOM) fractions isolated from earthworm casts.

			Cast-control		Cast-shoot		Cast-root	
			8 weeks	54 weeks	8 weeks	54 weeks	8 weeks	54 weeks
POM	Mass proportion of fraction	$mg.g^{-1}$	14	11	44	15	73	8
	OC	$mg.g^{-1}$	95	105	306	171	317	139
	Total OC	*mg*	5	5	54	10	93	5
	%C of bulk		9	11	42	15	43	7
	N content	$mg.g^{-1}$	7	7	21	12	14	9
	C/N		15	15	14	14	23	16
	$\delta^{13}C$	‰	−28	−28	1,177	87	935	185
	Litter-derived C	*%*	–	–	73	7	71	16
	Alkyl-C/O-N-alkyl-C		0.43	0.40	0.25	0.46	0.11	0.35
MOM	Mass proportion of fraction	$mg.g^{-1}$	952	900	917	955	879	886
	OC	$mg.g^{-1}$	15	11	21	15	35	17
	Total OC	*mg*	57	39	76	58	124	60
	%C of bulk		91	89	58	85	57	93
	N content	$mg.g^{-1}$	1	1	2	1	2	1
	C/N		12	13	12	11	15	13
	$\delta^{13}C$	‰	−28	−28	754	198	846	190
	Litter-derived C	*%*	–	–	47	14	65	16
	Alkyl-C/O-N-alkyl-C		0.42	0.50	0.38	0.49	0.17	0.38

The total organic carbon (Total OC) values were calculated using POM or MOM fraction masses. The % OC of bulk corresponds to the percentage of OC in POM or MOM fractions compared to the sum of the OC in POM and MOM fractions.

and Cast-Root) fractions contained around 40 and 60% of the total OC isolated, respectively (**Table 2**). Cast-Control POM and MOM fractions contained around 10 and 90% of the OC of bulk casts, respectively. In both Cast-Shoot and Cast-Root fraction samples, at least 50% of OC was litter-derived, with a higher percentage in Cast-Root MOM fraction (65%) compared to Cast-Shoot MOM fraction (47%). At 54 weeks, the mass proportion of MOM fraction slightly increased compared with 8 weeks and more than 85% of the total OC isolated was contained in the MOM fractions of both Cast-Shoot and Cast-Root. In both POM and MOM fractions, the litter-derived carbon dropped to 15%, with a minimum of 7% in the Cast-Shoot POM fraction. The C/N ratio decreased, compared with 8 weeks, of 30% and 13% in the Cast-Root POM and MOM fractions, respectively.

Chemical Characterization of Cast POM and MOM Fractions

Initial roots and shoots presented similar NMR spectra clearly dominated by carbohydrates (O-N-alkyl-C) (**Figure 1**). In initial roots, the relative abundance of alkyl-C was lower, while the relative abundance of aromatic-C was slightly higher than shoots (**Figures 1B,C** and **Table S1**). At 8 weeks, spectra for the POM fraction of Cast-Root and Cast-Shoot presented similar characteristics as the initial litter (**Figures 1B,C**), while those of MOM fraction spectra were broader (**Figures 1E,F**). At 54 weeks, a general broadening of spectra was observed for both POM and MOM fractions (**Figure 1**). A PCA was carried out to highlight the chemical characteristics of organic matter in POM and MOM isolated from earthworm casts after 8 and 54 weeks of experiment (**Figure 2**). The two factors (F1, F2) generated by the PCA explained 97% of the variance. F1 clearly separated Cast-Control samples and 54-week samples from Cast-Shoot and Cast-Root samples collected at 8 weeks (**Figure 2B**). Cast-Control samples were represented by a high relative abundance of aromatic-C and carboxyl-C, while 8 week Cast-Shoot and Cast-Root samples contained higher relative abundance of O-N-alkyl-C. At 8 weeks, the Cast-Root MOM fraction remained relatively close to the Cast-Root POM fraction and the initial root chemical characteristics. In contrast, Cast-Shoot MOM fraction at 8 weeks presented similar characteristics to the samples collected after 54 weeks. After 54 weeks, the OM in Cast-Shoot and Cast-Root samples tended to evolve toward Cast-Control chemical characteristics (**Figure 2**). Compared with 8 weeks, the relative abundance of O-N-alkyl-C decreased, while that of alkyl-C and aromatic-C increased (**Figure 2B**), resulting in a higher alkyl-C/O-N-alkyl-C ratio for both Cast-Root and Cast-Shoot (**Table 2**).

Cast-Root and Cast-Shoot at the Microscale

Structures of intact cast samples were analyzed with TEM and NanoSIMS in order to obtain detailed information of the microscale spatial assembly of the biogeochemical interfaces. At 8 weeks, plant residues incorporated in earthworm casts presented similar structures compared to the initial shoots and roots (**Figures S1**, **S2**), although they showed different degradation stages (**Figure 3**). For example, parenchyma cells were partially degraded in Cast-Shoot

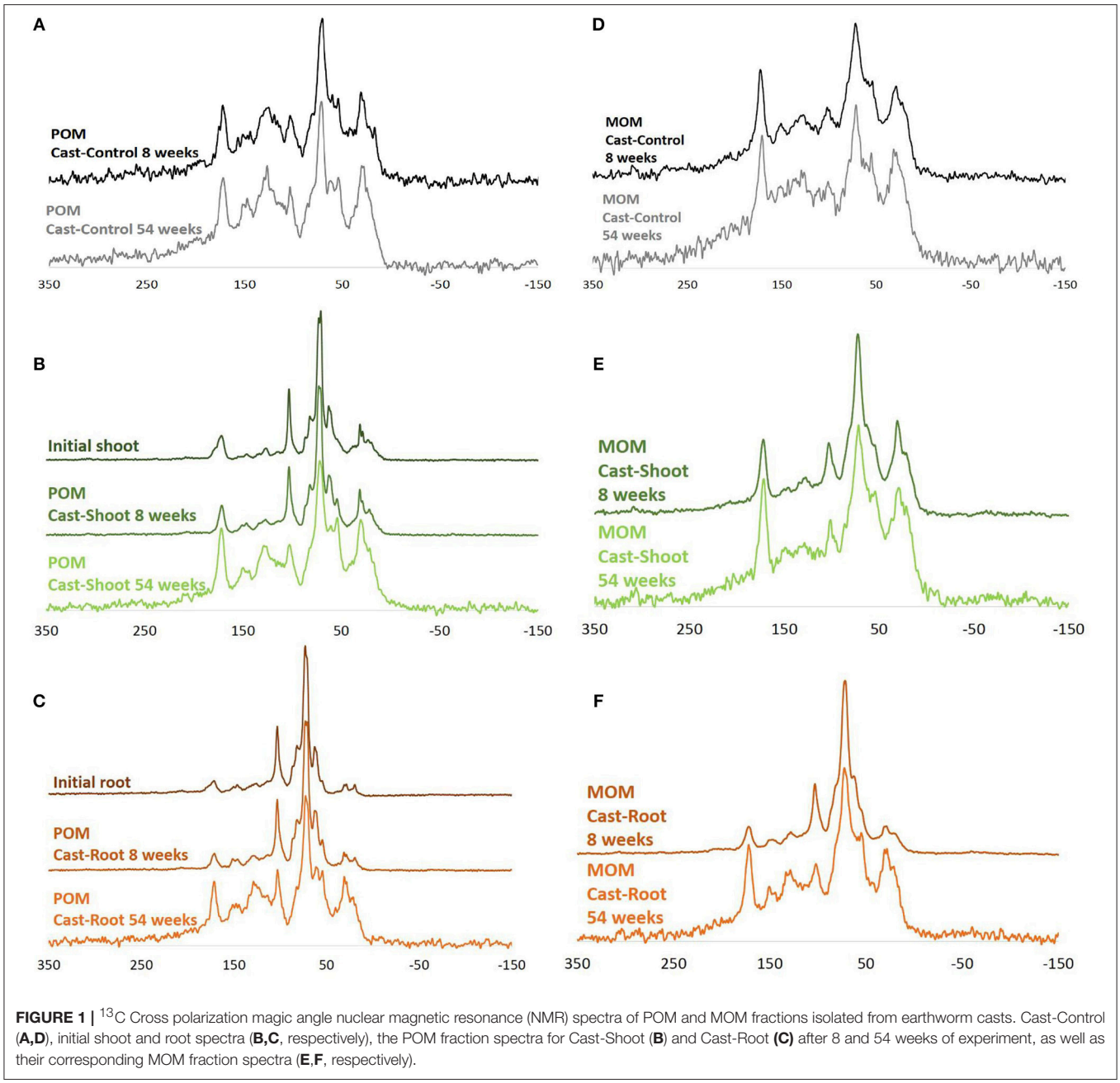

FIGURE 1 | ^{13}C Cross polarization magic angle nuclear magnetic resonance (NMR) spectra of POM and MOM fractions isolated from earthworm casts. Cast-Control (**A,D**), initial shoot and root spectra (**B,C**, respectively), the POM fraction spectra for Cast-Shoot (**B**) and Cast-Root **(C)** after 8 and 54 weeks of experiment, as well as their corresponding MOM fraction spectra (**E**,**F**, respectively).

(**Figure 3A**) compared with initial shoot tissues (**Figure S1A**), while woody tissues were well preserved (**Figure 3A**). Both Cast-Shoot and Cast-Root images highlighted some preserved plant tissues (**Figures 3A,G**), long and thin laces identified as parenchyma cell wall residues (**Figures 3B,F,H**), microaggregates (**Figures 3B,I, 4A,B**) and microorganisms (**Figures 3C–E,H**). Intact or barely degraded plant structures were prevalent in Cast-Root (**Figures 3G,H**). Various microorganisms, mainly fungi and bacteria, were depicted within Cast-Shoot: fungi attacking cell walls of woody tissues (**Figure 3D**), bacteria colonizing parenchyma cells (**Figure 3E**), or microorganisms within microaggregates (**Figure 3C**). A few microorganisms were also observed in Cast-Root (**Figures 3G,H**).

Many features identified in TEM images were recognized in NanoSIMS images. For Cast-Shoot, **Figures 4A,B** were comparable to **Figures 3C,B**, respectively, with the clear occurrence of a labeled fungus (**Figure 4A**), amorphous OM and plant cell wall (**Figure 4B**). On these images, $^{28}Si^-$ maps reflect an important proportion of small size mineral particles (i.e., mainly clay size $< 2\,\mu m$) on images and $^{12}C^{14}N^-$ maps showed organic structures among these mineral particles. Cast-Shoot images showed the presence of partially degraded plant structures derived from the labeled plants and labeled

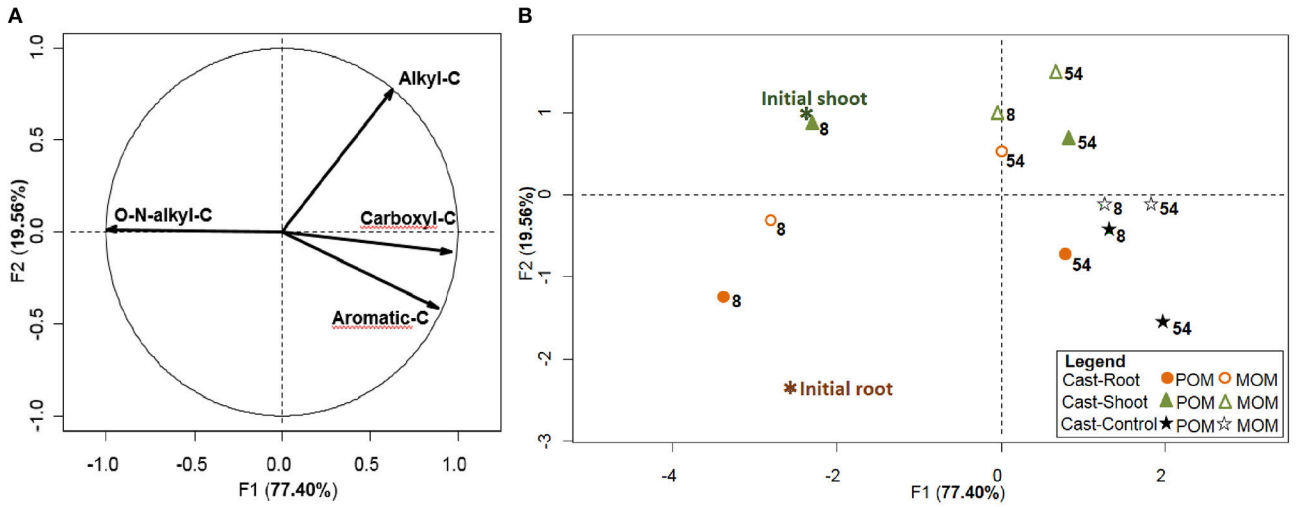

FIGURE 2 | Chemical characterization of earthworm casts. PCA carried out with the 4 variables of the 6 cast samples, fractionated into POM and MOM. The variables correspond to the 4 chemical shift regions integrated from ^{13}C CPMAS NMR. **(A)** distribution of variables on the correlation circle and **(B)** distribution of the cast samples, with root and shoot litter as illustrative samples. Weeks after the beginning of the experiment are indicated using numbers next to samples.

microorganisms involved in plant decomposition (**Figures 4A,B**, respectively). Degraded plant structures and microorganisms were both integrated into organo-mineral aggregates. For Cast-Root, **Figures 4C,D** were comparable to **Figures 3G,H**. Labeled plant structures observed on NanoSIMS Cast-Root images presented a lower degree of degradation and reduced associations with mineral particles (**Figures 4C,D**), compared with Cast-Shoot images.

At 54 weeks, microaggregates (from 20 to 30 μm) with complex organo-mineral composition, were frequently observed on Cast-Shoot (**Figures 5A–C**) and Cast-Root (**Figure 5G**) images. Highly degraded plant tissues, cell walls or amorphous organic residues were prevalent in Cast-Shoot. Residues of woody tissues were still recognizable and colonized by bacteria (**Figure 5D**). On Cast-Root images, some cell wall residues surrounded by mineral particles were identified (**Figure 5E**) and some cell intersections were still recognizable (**Figure 5F**). Microorganisms were prevalent on both Cast-Shoot and Cast-Root images. Bacteria were either intact, present under residual form (dead microorganisms leaving cell wall residues) (**Figures 5B,D**; **Figures 5E,H**) or spores (presenting dark core and coat) (**Figures 5B,H**).

On NanoSIMS images, labeled areas were scarce (**Figure 6**) compared with samples observed after 8 weeks of experiment (**Figure 4**). Three types of labeled structures are identified on Cast-Shoot and Cast-Root images: (1) well-defined spots corresponding to bacteria (**Figure 6C**) or fungi (**Figure 6D**), (2) organic structures similar to cell wall residues (**Figure 6B**) and (3) "diffused" labeling probably corresponding to highly degraded organic structures (**Figure 6A**). δ^{13}C values are highly variable on all images, with 165‰ < δ^{13}C <1131‰ in structures identified as microorganisms on Cast-Root (7 images observed, data not shown). No clear differences in δ^{13}C values were observed between Cast-Root and Cast-Shoot.

DISCUSSION

Litter Type has a Short-Term Impact on Cast Composition Which Is Smoothed on the Longer Term

As mentioned above, root and shoot materials were both deposited onto the soil surface in the same conditions to focus on the impact of the sole chemical composition of litter type, without considering physical interactions between roots and soil. Even though other soil biota and physico-chemical processes may play a role in litter degradation, the action of earthworms is major in temperate soils. The present study focuses on this action through the analysis of root and shoot incorporation within casts. Although it is difficult to generalize the obtained results to soils, it must be noted that casts might account for half of the soil surface layer in natural systems containing earthworms (Ponomareva, 1950; Six et al., 2004).

At 8 weeks, the high percentage of litter-derived carbon in Cast-Root and Cast-Shoot (**Table 2**), their proximity in chemical composition with the corresponding initial plant residues revealed by the PCA (**Figure 2**), as well as the observation of plant structures within casts (**Figures 3**, **4**), highlighted the capacity of earthworms to incorporate both shoots and roots in casts. However, some differences could be evidenced between Cast-Root and Cast-Shoot at 8 weeks, which reflect different incorporation and/or decomposition extents for the two types of plant residues. Indeed, Cast-Root can be distinguished from Cast-Shoot by a higher quantity of plant residues isolated as POM, a higher labeling and C/N ratio (**Table 2**) a lower level of degradation of the observed plant structures (**Figures 3**, **4**), as well as a higher relative abundance of O-N-alkyl-C (**Figure 2**), as typically observed for fresh residues (Lorenz et al., 2007). These results suggested either a delayed incorporation of roots

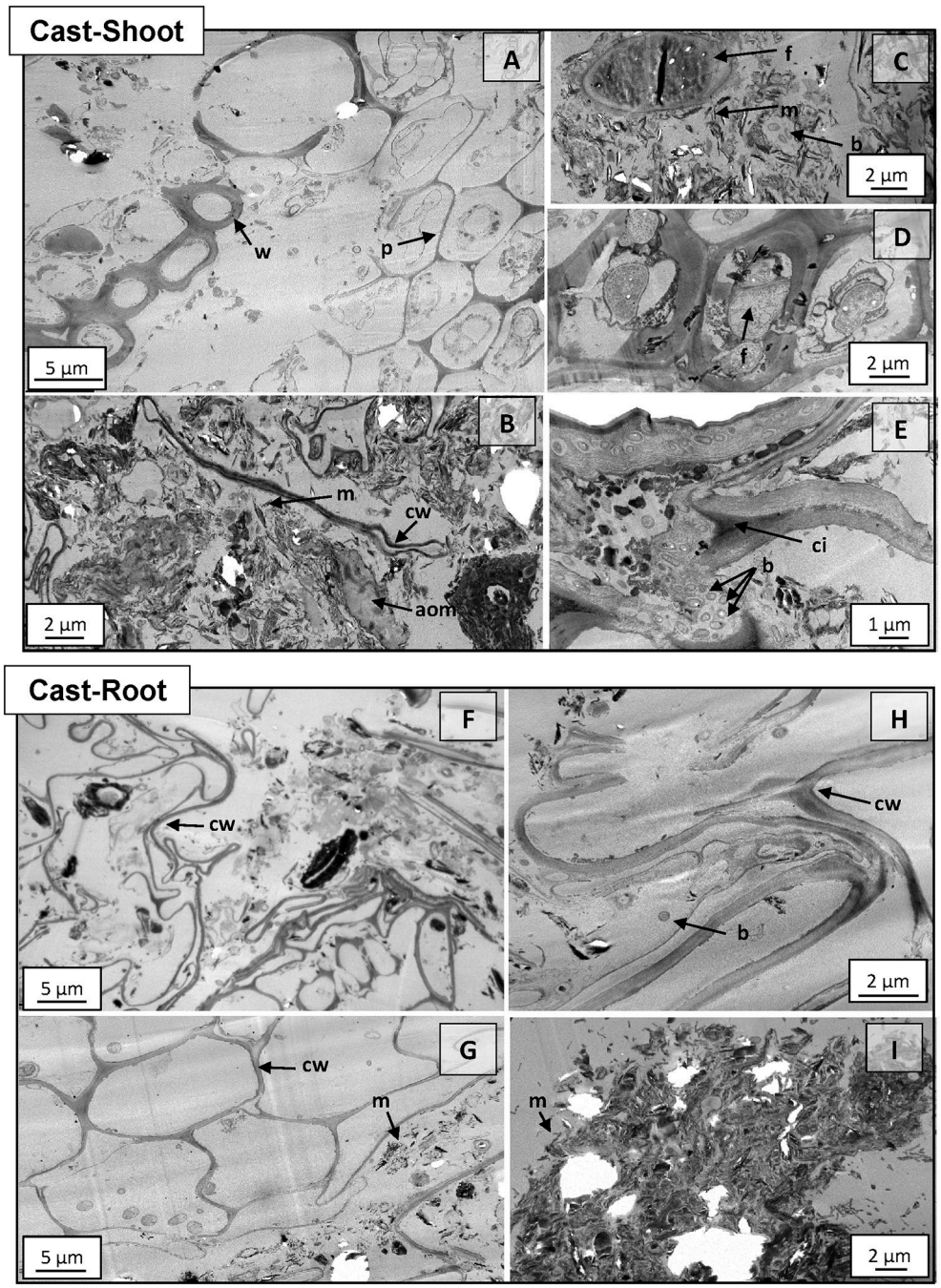

FIGURE 3 | TEM micrographs of Cast-Shoot and Cast-Root samples after 8 weeks of experiment. **(A)** Partially degraded parenchyma cells and well-preserved woody tissues; **(B)** organo-mineral aggregate; **(C)** fungus in an organo-mineral aggregate; **(D)** fungi attacking woody tissues; **(E)** bacteria inside cell walls; **(F,H)** compacted cell walls; **(G)** intact cell walls and **(I)** organo-mineral aggregate dominated by mineral particles. aom, amorphous organic matter; b, bacteria; cw, cell wall; ci, cell intersection; f, fungus; m, mineral particle; p, parenchyma cell; w, woody tissue.

by earthworms and/or a slower decomposition of roots in casts. Both hypotheses are plausible, as the root lignin content (aromatic-C relative abundance, **Table S1**) makes roots less palatable for earthworms (delayed incorporation) (Tian et al., 1995) and slower to decompose (Balesdent and Balabane, 1996; Puget and Drinkwater, 2001; Lu et al., 2003). The difference in plant decomposition is also corroborated by the clear difference in the C/N ratio of the initial roots compared with shoots (30.7 vs. 14.8) (Vidal et al., 2017). We were thus able to demonstrate a clear relationship between the chemical composition of the type of applied plant residue and its short-term degradation as promoted by earthworm and microorganism activity.

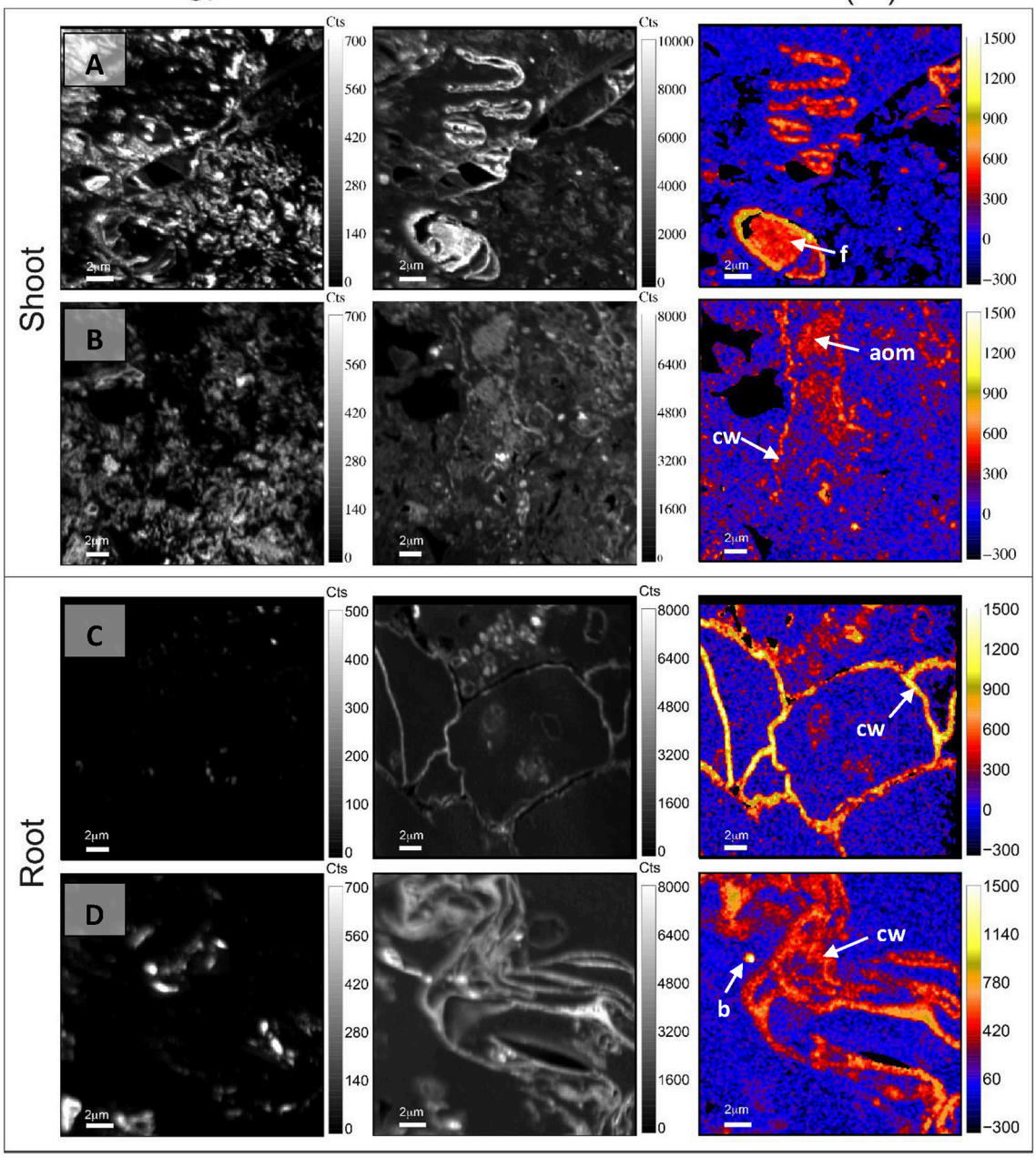

FIGURE 4 | NanoSIMS maps of $^{28}Si^-$, $^{12}C^{14}N^-$, and $\delta^{13}C$ illustrating contrasted degradation stages of labeled plant tissues and microorganisms implied in their decomposition, after 8 weeks of experiment, for both Cast-Shoot **(A,B)** and Cast-Root **(C,D)**. aom, amorphous organic matter; b, bacteria; cw, cell wall; f, fungus.

Studies depicting the impact of earthworms on soil carbon cycle are mainly restricted to short-term experiments (<200 days) (Lubbers et al., 2013). The present study tracked litter-derived C for more than 1 year in earthworm casts. The aforementioned structural and chemical differences between Cast-Root and Cast-Shoot were reduced after 54 weeks, highlighting the capacity of earthworms to efficiently degrade both shoots and roots. The chemical changes in casts between 8 and 54 weeks reflected a commonly illustrated litter decay process (Baldock and Skjemstad, 2000; Lorenz et al., 2007; Mueller et al., 2009, 2014; Preston et al., 2009; Cepáková and Frouz, 2015). The decrease in POM quantity and plant-derived-C (**Table 2**) highlighted the degradation of plant residues, via the loss of labile carbon and mineralization (Cotrufo et al., 2015). This was corroborated by the alkyl-C/O-N-alkyl-C ratio increase in POM and MOM fractions (**Table 2**) which reflected a relative decrease

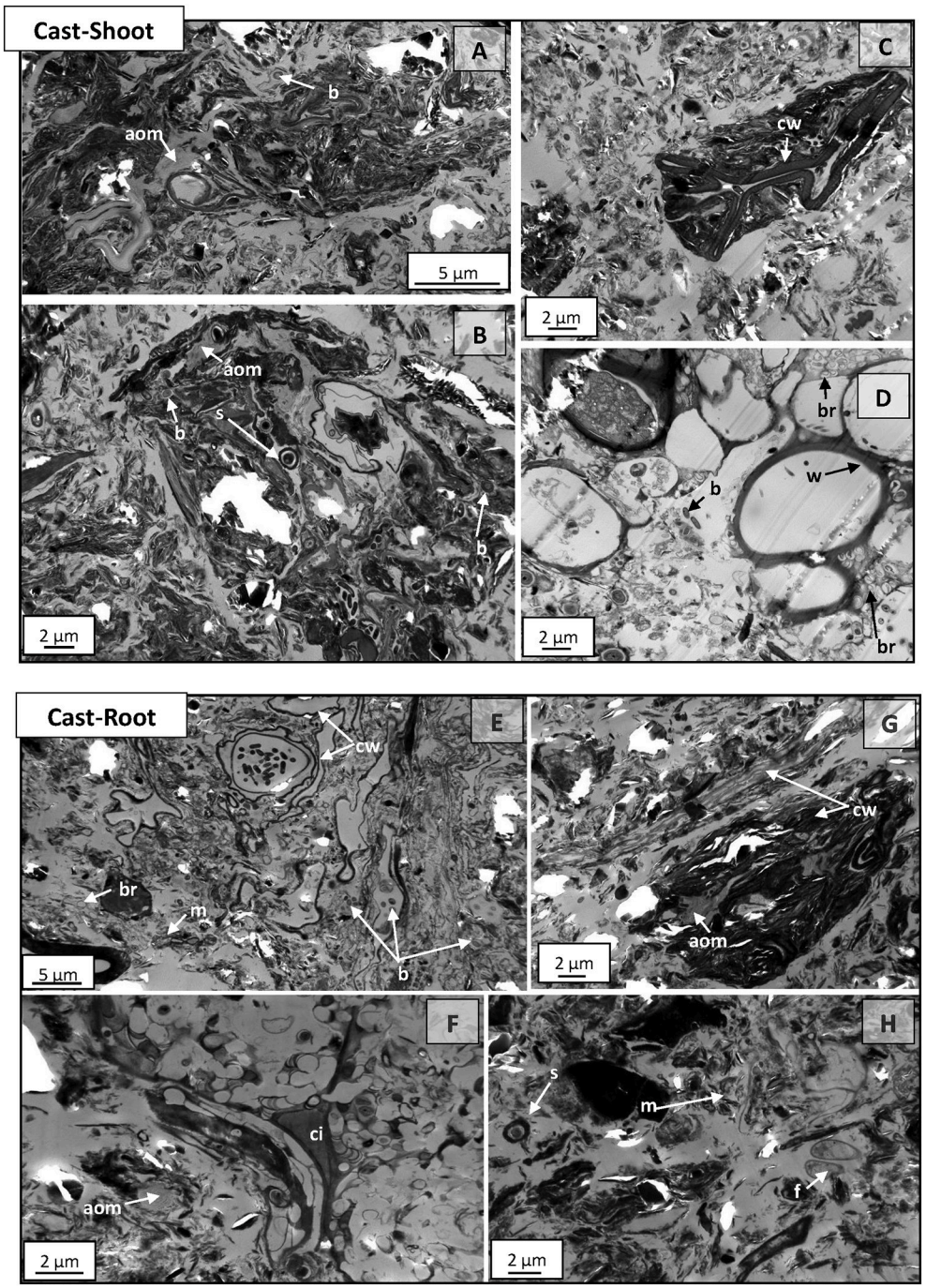

FIGURE 5 | TEM micrographs of Cast-Shoot and Cast-Root samples after 54 weeks of experiment. **(A–C,G)** organo-mineral aggregates; **(D)** partially degraded woody tissues; **(E)** cell walls residues; **(F)** highly degraded cells with the cell intersection left; **(A,B,D,E,H)** microorganisms. aom, amorphous organic matter; b, bacteria; br, bacterial residue; cw, cell wall; ci, cell intersection; f, fungus; m, mineral particle; s, bacterial spore; w, woody tissue.

in easily degradable compounds such as carbohydrates (O-N-alkyl-C) and/or a relative accumulation of biologically stable polymethylenic compounds (alkyl-C) including hydrophobic by-products of decomposition (Bonanomi et al., 2013). The relative increase in aromatic-C (**Figure 2** and **Table S2**) indicated a higher contribution of lignin-derived compounds in plant residues (Angst et al., 2016). These chemical changes were correlated to microscopic observations, as remaining plant structures were represented by structural plant parts (e.g., cell walls, cell intersections and woody tissues) (**Figure 5**) which

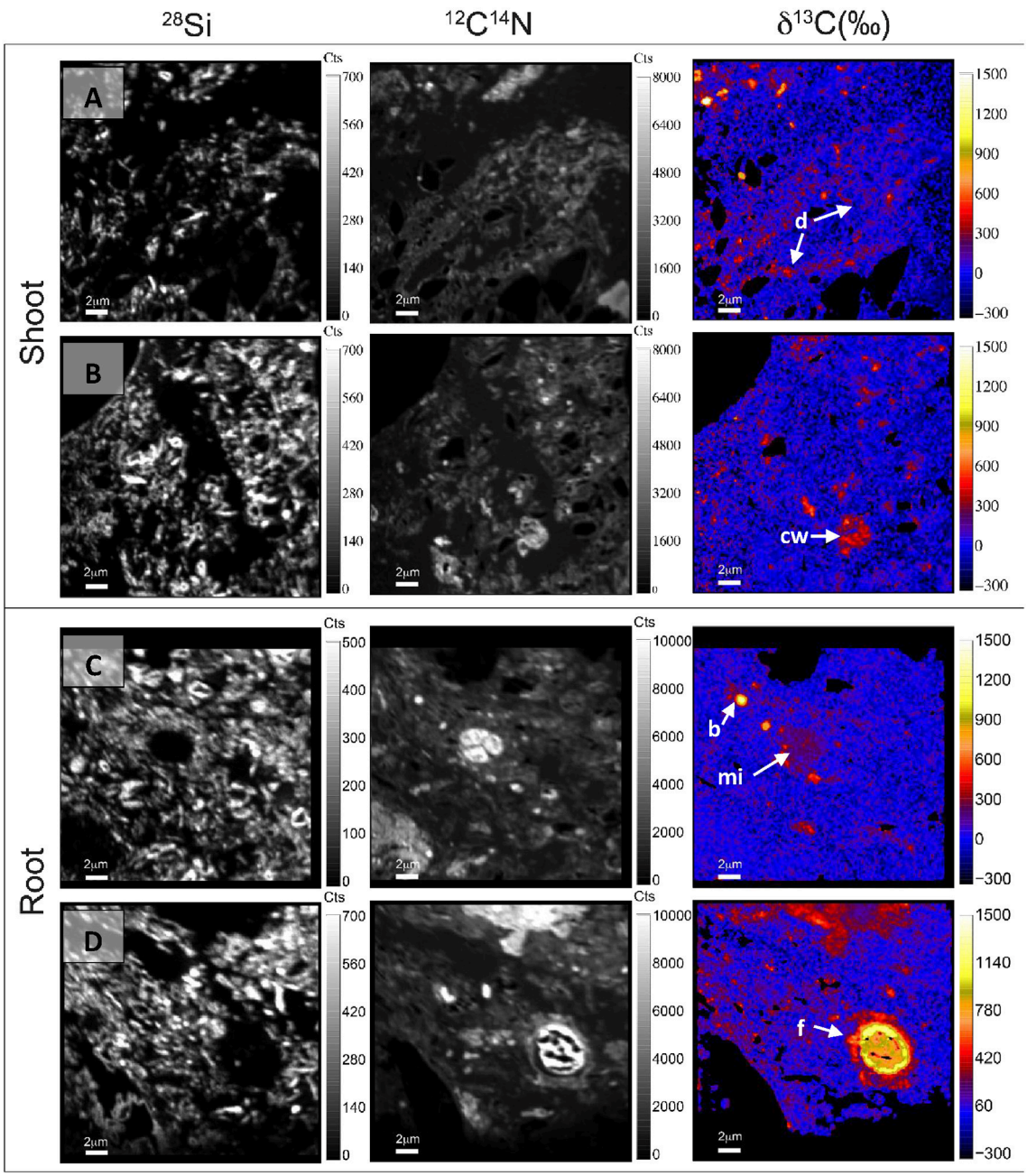

FIGURE 6 | NanoSIMS maps of $^{28}Si^-$, $^{12}C^{14}N^-$, and $\delta^{13}C$ illustrating the integration of labeled organic structures (both plant tissues and microorganisms) inside organo-mineral aggregates, after 54 weeks of experiment, for both Cast-Shoot **(A,B)** and Cast-Root **(C,D)**. b, bacteria; cw, cell wall; d, "diffused" labeling; f, fungus; mi, microorganism (bacteria or fungi).

show higher resistance to degradation and generally present higher lignin concentrations compared to non-structural parts (Fahey et al., 2011; Jouanin and Lapierre, 2012; Cotrufo et al., 2015). The bacteria or bacterial residues associated to woody tissues (**Figure 5D**) were also a sign for advanced degradation.

These results are consistent with recent findings highlighting that although earthworms avoid phenolic-rich substrates, such as roots, they tend to accelerate their degradation with time (Bi et al., 2016; Angst et al., 2017). The short-term impact of the litter type was smoothed at the longer term, which strengthened the need to consider the long-term (> 200 days) role of earthworms on litter decomposition and carbon cycling (Lubbers et al., 2013; Angst et al., 2017).

Connecting Litter Decomposition Features With Microorganisms

Thanks to NMR spectroscopy and EA-IRMS analyses on fractionated cast material, we were able to depict the plant degradation stages and the contribution of mineral-associated organic matter along the year of experiment. In intact samples, i.e., considering the complex microscale architecture of casts, TEM and NanoSIMS revealed information on the location and structural evolution of plant residues along degradation, as well as on the microorganisms feeding on these residues.

The lower amount of intact shoot structures (vs. root) in casts at 8 weeks (**Figures 3**, **4**) can be related to the abundance and diversity of the observed microorganisms. Indeed, a clear contribution of specific microorganisms to shoot degradation is evidenced by their colonization of partly degraded shoot structures (**Figures 3D,E**) associated with their high isotopic enrichment (**Figure 4A**). Abundant microbial communities have frequently been observed in fresh earthworm casts due to the high amount of available substrate (i.e., mucus, plant structures) (Parle, 1963; Drake and Horn, 2007; Frouz et al., 2011), partly released during the digestion activities of the earthworms (Brown et al., 2000). These microorganisms tend to use the more labile and easily available content first (e.g., cell contents) (Fahey et al., 2011), the latter representing their main source of energy and carbon (Cotrufo et al., 2015). This was reflected by the extensive biodegradation of parenchyma cells, while woody tissues often remained intact (**Figure 3A**) and corroborated by the slight relative increase in lignin-derived signals in the NMR spectra of the POM fractions (vs. initial shoots) (**Tables S1, S2**). Moreover, the diversity of microorganism metabolic capacities in casts (Brown, 1995) is illustrated by the occurrence of some bacterial clusters associated to plant cell walls (**Figure 3E**) and of fungi, attacking cell walls (**Figure 3D**). As earthworms are not able to decompose lignin without the participation of microorganisms (Neuhauser et al., 1978; Curry and Schmidt, 2007), the degradation of woody tissues was initiated by fungi, which are able to degrade more resistant tissues compared to bacteria (Bossuyt et al., 2001) and are particularly implied in lignin decomposition (Tuor et al., 1995; Filley et al., 2002; Dignac et al., 2005). The action of fungi provided bacteria with intermediate decomposition products and enable their colonization of woody tissues (Roman et al., 2006).

While most microorganism cells identified at 8 weeks were in an intact form, they exhibited various stages of structural degradation at 54 weeks (**Figures 5**, **6**). In parallel, the OC content decreased in casts (**Table 1**), the substrates started to become less decomposable (e.g., increase in lignin-derived compounds), entrapped within microaggregates (**Table 2** and **Figure 2**) and thus limiting for microorganisms, which could progressively starve (Miltner et al., 2012). Indeed, organo-mineral interactions have been previously reported as one of the main drivers for SOM stabilization, leading to a restriction in substrate bioavailability and diffusion (Mueller et al., 2014; Angst G. et al., 2017). With a decrease in substrate bioavailability some bacteria formed spores (**Figures 5B,H**). The sporulation might either be caused by the anoxic conditions created during the earthworm gut transit or by the lack of substrate (Brown, 1995; Drake and Horn, 2007). The exhaustion of easily available substrate can also lead to the starvation of microorganisms and the autolysis of bacterial cells (van Veen et al., 1997; Zelenev et al., 2000; Ekschmitt et al., 2005). Thus, other bacteria which were not able to overcome this lack of energy and carbon died and were left as bacterial residues (**Figures 5B–E**) (Miltner et al., 2012).

Thus, with the decay of the litter-derived OM, we could show the parallel buildup of microbial dominated OM. This is also supported by the decrease in C/N ratios in Cast-Root POM and MOM fractions (**Table 2**) that reflect the decay of litter and the relatively higher microbial contribution (Mueller et al., 2014; Cepáková and Frouz, 2015). The presence of labeled microorganisms enclosed within microaggregates (**Figures 6C,D**) pointed to the limitation of the accessibility of microbial-derived OC for degradation. The spatial inaccessibility created by this process could lead to a protection of this microbial-derived OC from degradation (Lützow et al., 2006; Shan et al., 2013) and could represent a source of carbon at the longer term (Miltner et al., 2012).

Organo-Mineral Associations Prevailed After 1 Year

At 54 weeks, there was a relative shift from a POM-dominated to a MOM-dominated system. Organo-mineral associations prevailed in casts, as supported by the high percentage of OC of bulk in MOM fractions (**Table 2**) and the abundance of microaggregates with high mineral contribution on TEM and NanoSIMS images (**Figures 5**, **6**). Thus, after the destruction of existing microstructure during the gut transit (Shipitalo and Protz, 1989; Six et al., 2004), new microaggregates developed in casts under the combined effect of mineral properties (e.g., adsorption capacities) and microorganism activity. Interactions between minerals and OM are partly controlled by mineral features, such as mineralogy and chemical compositions influencing their capacity to adsorb organic material (Baldock and Skjemstad, 2000; Eusterhues et al., 2003, 2005; Sollins et al., 2009; Kaiser et al., 2015). For example, the clay size particles ($< 2\,\mu m$), as those observed in casts (**Figures 5**, **6**), are known to have high surface areas and adsorption capacities (Kögel-Knabner et al., 2008). In addition to mineral properties, living microorganisms produce polysaccharides during OM decomposition processes that favor adsorption of minerals and increase inter-particle cohesion (Chenu et al., 2002), leading to a strengthening of organo-mineral bonds in casts (Shipitalo and Protz, 1989).

In a unique way, the association of quantitative biogeochemical information and fine scale elemental and isotopic information led to depict the fate of shoot and root litter in earthworm casts. The chemical composition appeared as a driving parameter for litter degradation at the early stage

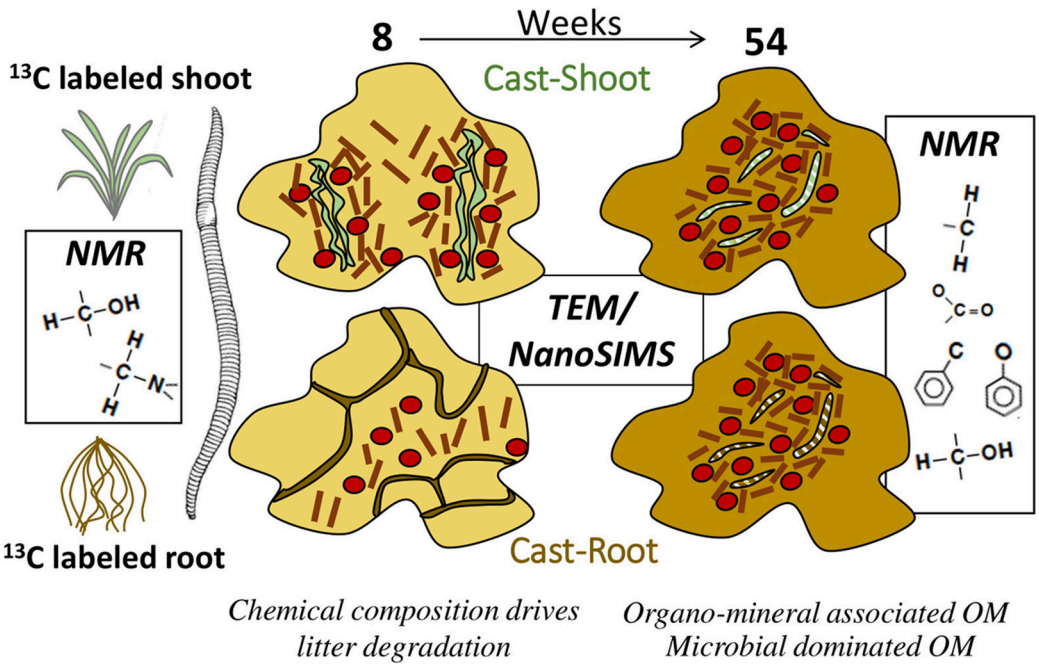

FIGURE 7 | Simplified illustration of the main findings in the present study.

of decomposition in earthworm casts. A clear difference in the short-term (8 weeks) fate of shoot- and root- derived OC could be evidenced with a higher abundance of less degraded root residues recovered as particulate organic matter in the casts. After 1 year, the structural and chemical differences between shoots and roots ceased and the system dominated by plant-derived OM shifted toward a system where mineral-associated OM prevailed. Along with this shift, we demonstrate the buildup of microbial-dominated OM, both as living microbial biomass and dead microbial residues. Thus, microorganisms played a key role in litter degradation, producing binding agents for microaggregate formation and as an important carbon source in casts. These main findings are summarized in **Figure 7**. We emphasized the complex and dynamic role of earthworm casts as hot spots for OC inputs and microbial activity at the short term and potential stable carbon source at the longer term in soils. We were able to demonstrate the role of earthworms for the formation of presumably stable organo-mineral associations sequestering litter-derived carbon on longer timescales.

AUTHOR CONTRIBUTIONS

AV collected and analyzed the data, wrote the manuscript, and ensured the exchange between all co-authors. FW performed TEM analyses and gave technical and scientific support. LR performed nanoSIMS analyses and, gave technical and scientific support. CM supervised the sample fractionation and ^{13}C-NMR analyses and, gave technical and scientific support. T-TN participated in discussing the results obtained for the NanoSIMS and TEM analyses. FB realized the EA-IRMS analyses of the fractionated samples. SD and KQ participated to design the experiment and supervised the project. All authors discussed the results and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2019.00055/full#supplementary-material

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frontiers in Environmental Science

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Functional Assemblages of Collembola Determine Soil Microbial Communities and Associated Functions

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Soil processes such as decomposition are mainly performed by soil biota. Although soils worldwide are extremely biodiverse, the relationship between decomposers (fauna and microorganisms), and ecosystem function is poorly understood. Collembola are abundant and ubiquitous microarthropods that are found in terrestrial ecosystems. They can affect the amount of biomass and the activity of microbial communities, either directly through selectively feeding on fungi and bacteria, or indirectly by dissemination of microbial propagules, and the alteration of nutrient availability. However, despite the functional role they play in belowground food webs, the interactions between natural assemblages of soil microbes and Collembola receive little attention. This study, conducted in microcosm conditions, examines the effects of two distinct natural assemblages of functional groups of Collembola (ep- and euedaphic) upon microbial communities using PLFA markers and their associated soil functions (e.g., enzymatic activities and C mineralization rate) over a 2-month period. Our principal objective was to determine whether different functional groups of Collembola had varying effects on microbial soil community abundance, structure and activity, resulting in potentially important effects on ecosystem processes. Our findings show that the interactions of the functional groups of Collembola with microbial communities vary significantly whether they are alone or combined. A distinct response in the composition of the microbial communities was found at the end of the 2-month period. The communities were significantly different from each other in terms of PLFA marker composition. We found that the epedaphic species were related to and promoted Gram+ bacteria whereas euedaphic species were related to Gram- bacterial markers. This had further repercussions on soil function, such as nutrient recycling. Combining both functional groups did not lead to a complementary effect on soil microbial properties, with a drastically different outcome between the first and the second month of the experiment. Additional research dealing with the interactions between decomposers using natural assemblages will help to predict the functional outcomes of soil biota structure and composition.

Keywords: PLFAs, enzymatic activities, springtails, life-forms, belowground interactions

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HIGHLIGHTS

- Functional groups of Collembola shape soil microbial communities.
- Each functional group has a specific effect on microbial communities and associated roles.
- No complementarity was observed between epedaphic and euedaphic collembolan species
- Response of microbial communities to the presence of Collembola is time dependent.

INTRODUCTION

Decomposition, with primary production, is one of the most important ecosystem functions found in soil. Indeed, ∼90% of the terrestrial net primary production (NPP) enters the soil food web to be consumed and then decomposed into mineral forms and eventually reabsorbed by plants. Decomposition processes are mainly regulated by environmental drivers including abiotic factors (e.g., water content, temperature), litter quantity and quality, and by the activity of the decomposer biota (Seastedt, 1984; Anderson, 1991; Wardle et al., 2004).

Decomposition in terrestrial ecosystems and nutrient cycling are primarily regulated by soil micro-organisms (fungi and bacteria). Although, most nutrient mineralization is governed directly by the activities of bacteria and fungi, this mineralization process is regulated by interactions with soil fauna (Bardgett and Cook, 1998; Kaneko et al., 1998; Bardgett and Shine, 1999; Mikola et al., 2002; Tiunov and Scheu, 2005; Lenoir et al., 2007; Chauvat et al., 2014). For example, several studies showed that soil food web properties strongly impact organic matter recycling and, thus, affect the quality and quantity of the nutrients available to plants (Heemsbergen et al., 2004), see Whalen et al. (2013) for a complete review on N cycling. Other studies also highlighted links between the structure and composition of soil fauna and several enzymatic activities (Sauvadet et al., 2017).

Within terrestrial soil fauna, Collembola are abundant and ubiquitous microarthropods, which feed predominantly on fungi, but also bacteria, actinomycetes, and algae (Chen, 1995). Soil Collembola can affect the biomass and activity of the microbial community, either directly through selectively feeding on fungi and bacteria, or indirectly by comminution of organic matter, dissemination of microbial propagules, and the alteration of nutrient availability (Moore et al., 1988; Verhoef and Brussaard, 1990; Lussenhop, 1992; Griffiths and Bardgett, 1997). Grazing pressure exerted by Collembola depends on invertebrate body size, population density as well as feeding preferences (Hedlund and Augustsson, 1995; Kaneko et al., 1998; Crowther and A'Bear, 2012). The interaction between soil microbes and Collembola is important because of their trophic and functional significance within belowground food webs. However, within this framework, most mechanistic studies have focused on a limited number of Collembola species, while none to our knowledge have considered natural assemblages of Collembola. Though, these microarthropods belong to a very heterogeneous group with contrasting life-forms, e.g., litter-dwelling or soil-dwelling, they occupy different soil sub-horizons. Basically, epedaphic collembolan species are large-bodied, have a high metabolic activity, consume a food substrate of high quality and are surface-dwellers. Conversely, euedaphic species are small deep-living species that consume low-quality food and have low metabolic activity. Euedaphic species are colorless with reduced appendices (e.g., furca, antennae, leg). Finally, the hemiedaphic group includes species sharing intermediate attributes (Petersen, 2002). Considering their functional traits, it is common to consider these groups as functional groups, even if we lack knowledge on how they perform or drive different functions. For example, Caravaca and Ruess (2014) showed that varied and specific grazing intensities were associated with each life-form (one species per life-form) of arbuscular mycorrhizal fungi. Other studies highlighted clear positive relationships between euedaphic collembolans and microbial biomass (Perez et al., 2013), while less obvious relationships were depicted for epedaphic ones. Furthermore, different feeding preferences of epedaphic and euedaphic species upon fungi have been highlighted (Thimm and Larink, 1995; Ponge, 2000; Nakano et al., 2017). Finally, these functional groups have been shown to express different foraging patterns toward microbial food resources (Chauvat et al., 2014). For example, intermediate levels of fungal grazing by Collembola can stimulate fungal growth and promote soil respiration, whereas overgrazing can depress fungal populations, causing a decline in rates of carbon mineralization (Anderson et al., 1981; Hedlund and Öhrn, 2000; Cortet et al., 2003; Cole et al., 2004).

To gain further insights on how different functional groups within a single decomposer taxa (i.e., Collembola) impact soil processes, we performed a microcosm experiment investigating the response of microbial communities (structurally and functionally) to different Collembola functional groups, alone or in combination. We hypothesized that (i) each Collembola functional group, due to the differences in life-history traits, has a specific impact on soil microflora, (ii) the presence of euedaphic species will generate a stronger response in soil microflora (biomass or activities) than would the presence of epedaphic species compared to a control without Collembola. Furthermore, Eisenhauer et al. (2010) showed that functionally dissimilar decomposer groups could synergistically impact soil processes. We, thus, hypothesized that (iii) complementarity may occur between different Collembola functional groups using different resources along the soil profile. Finally, as time is a determinant aspect of the outcome of biotic interactions, we investigated the response of microflora to the different Collembola treatments over a 2-month period.

MATERIALS AND METHODS

Experimental Setup

The experiment was conducted under microcosm conditions in closed glass jars (9 cm diameter and 9.5 cm height). The soil, microorganisms, and Collembola come from a low intensity cow-grazing area established since 1968 and managed by the Lycée Agricole d'Enseignement Générale et Technique Agricole of Yvetot (north-west France, 49°37′04.00″N, 0°45′18.76″E). The climate of the region is temperate oceanic, with an average annual

temperature and rainfall of 10°C and 800 mm, respectively. The original soil was classified as Neoluvisol-Luvisol (pH water = 6.1, clay = 15%, silt = 65%, sand = 20%, total carbon = 10.40 g kg^{-1}, total nitrogen = 1.04 g kg^{-1}; IUSS, 2006) and supported a vegetation dominated by *Agrostis capillaris* (L.), *Lolium perenne* (L.), and *Ranunculus acris* (L.).

Substrate

The substrate in the microcosm consisted of a mixture of 1 part sand to 5 parts soil. The soil was collected at a depth of 0–15 cm and sieved through a 5 mm mesh. To eliminate the original fauna and microflora, the substrate was autoclaved with two cycles of 105°C at 48 h intervals. Subsequently, the substrate was dried at 105°C and aliquots of the substrate were sampled to determine the soil water holding capacity. Finally, each microcosm was filled with 150 g of dry soil and soil suspension was used to adjust the soil moisture to 70% of the soil water-holding capacity.

Extraction and Inoculation of Microorganisms

Microorganisms were extracted from soil broth filtrates, prepared by weighing 500 g of sieved fresh soil and dissolved with 2 L of physiological water (0.85% NaCl), according to the protocol of Eisenhauer et al. (2009). A volume of 37 ml of microbial filtrate (i.e., microbial suspension) was inoculated into each microcosm. In order to establish the microbial community, the microcosms were then incubated at a temperature of 25°C for 10 days.

Collembola Extraction and Composition of Treatments

Collembola were extracted from soil monoliths using the Berlese-Tullgren device (Tullgren, 1918). In order to select and sample the two functional groups of Collembola (epedaphic or euedaphic), either the top 2 cm or the bottom 4 cm of the monoliths (10 cm depth) were placed in the Berlese-Tullgren device. The Collembola were placed into pots filled with moist plaster, and were then transferred 10 days after microbial inoculation with pooters into the microcosms to establish four different treatments: a control without Collembola, a treatment with only epedaphic Collembola ("Ep"), a treatment with only eudedaphic species ("Eu"), and a treatment with both epedaphic and euedaphic species ("Ep + Eu"). To efficiently set up the different treatments, Collembola were sorted out under binocular before being transferred with the pooters into the different microcosms. During this phase, Collembola were believed to be epedaphic based on three morphological criteria (Petersen, 2000): presence of pigmentation, presence of a large patch of ocelli, and presence of a well-developed jump organ: the furca (i.e., the mucro of the furca ending beyond the end of the abdomen). Alternatively, individuals were considered as euedaphic if they were not pigmented, had no ocelli and no furca observable at the binocular. Each treatment was replicated 16 times resulting in a total of 64 microcosms. Collembola addition to the microcosms resulted in different numbers of epedaphic or euedaphic individuals, reflecting differences occurring in natural conditions. We were more interested in the functional importance of each group (relying on natural abundances or either epedaphic or euedaphic collembolans) rather than the functional identity of each group of Collembola. The microcosms were closed and stored in a climate chamber at 18°C, with a daily light/dark cycle of 12/12 h for 60 days. The microcosms were opened every week to aerate and adjust soil moisture. The influence of Collembola on microbial variables was assessed 1 and 2 months after Collembola inoculation. At the end of the experiment, Collembola were extracted from 4 of the microcoms to check functional collembolan assemblages in each treatment (see **Table 1**).

At each sampling period of 1 and 2 months, 32 microcosms (eight replicates per treatment) were dismantled.

Microbial Community Structure

At each sampling period, the microbial community structure was determined by Phospholipid fatty acid analysis (PFLA) and microbial activity by potential C mineralization rate and by enzymatic activities.

PLFA extraction and analysis was performed using a modified protocol from Frostegård and Bååth (1996) (see details in **Supplementary Material**). The results were expressed as nmol PLFA g^{-1} dry soil. We used the bacterial acid methyl ester (BAME) 26 Mix of Sigma-Aldritch as a reference and further in the analyses only considered those identified markers and did not take into account un-recognized peaks, as they accounted for <1% in area in our chromatograms compared to the whole set of the BAME 26 mix reference. We retained the following 16 PLFAs as indicators of the microbial community structure: branched and saturated PLFAs i15:0, a15:0, i16:0, and i17:0 (Gram+ positive bacteria); mono-unsaturated and cyclopropyl PLFAs 16:1ω7c (16:1ω9), cy17:0, (Gram-negative bacteria) 18:1ω9c, 18:1ω9t, 18:2ω6,9 (fungi), 10me-16:0 and 10me-18:0 (Actinobacteria), and, lastly, 14:0, 15:0, 16:0, 17:0, 18:0 (general indicators).

We assessed the potential Carbon mineralization rate of the microorganisms by measuring CO_2 evolution at both 30 and 60 days after the introduction of Collembola (Anderson and Domsch, 1978). Twenty grams of soil under controlled conditions (28°C and initial sample humidity) for a period of 10 days in hermetic pots. CO_2 released was captured by NaOH (0.2 M) and measured using a conductivity meter (ThermoScientific, Orion 011007; see Perez et al., 2013).

TABLE 1 | Mean (and standard deviation) densities of Collembola (number of individuals) in each treatment after 2 months.

	Density	
Treatment	**Ep**	**Eu**
Control	0.25 (0.5)	0 (0)
Ep	21.0 (10.1)	2.0 (4.0)
Eu	1.5 (1.3)	38.5 (11.9)
Ep + Eu	16.2 (8.0)	50.2 (17.1)

The most dominant species per life form were: Ep (Isotoma anglicana, Lepidocyrtus violaceus, Isotomurus palustris gr); Eu (Folsomia fimetaria, Protaphorura armata gr., Mesaphorura sp.). Ep, Epedaphic; Eu, Euedaphic.

We also measured three enzymatic activities related to C and N cycles. Beta-glucosidase activity (C cycle), Urease (N cycle), and Fluorescein DiAcetate (FDA) hydrolysis (measures a wide spectrum of enzymes, such as proteases, lipases, and esterases). FDA is a method of measuring overall activity potential. Beta-glucosidase was evaluated by Eivazi and Tabatabai method 1988, using the *p*-nitrophenyl-beta-glucopyranoside (*p*NPG) as a substrate (see **Supplementary Material** for details). Urease activity, an enzyme linked to the conversion of the amine (NH2) to ammonium (NH4+), was measured by determining the amount of ammonium released during incubation (2 h at 37°C) following Kandeler and Gerber (1988) (see **Supplementary Material** for details). The FDA activity was determined according to the method of Schnürer and Rosswall (1982) (see **Supplementary Material**).

Statistical Analyses

Statistical analyses were performed on the data collected for each time period (Month 1 and 2). Normality and homoscedasticity of the data were tested (Shapiro-Wilk and Bartlett, $\alpha = 0.05$) to decide whether to use parametric tests (the data met both assumptions) or non-parametric tests (at least one of the assumptions was not met). Analysis of Variance (ANOVA) was used to examine the effect of treatments on measured variables over the whole course of the experiment. Single groups ("Ep" & "Eu"), their combination ("Ep + Eu"), and the control treatment were taken as single treatments in these analyses, as performed in Cragg and Bardgett (2001). Therefore, we used the following model y∼Treatment and not y∼EP*EU. Regarding biological factors, in our case Collembola introduced in microcosms, it is almost impossible to account for initial numbers and trace their survival during the experiment. Therefore, the combination of the treatments could have not been properly controlled in order to assess interactive effects. Tukey's HSD (honestly significant difference) was used to identify means that were significantly different at the 5% level. We performed analyses to quantify changes between the two sampling periods for a single treatment. Means between the 2 months were compared with Pairwise *t*-tests or Wilcoxon. Significance was tested with $\alpha = 0.05$. Furthermore, to evaluate how the temporal aspect might or might not influence our results, we performed a second analysis with "treatments" as a fixed factor and "time" as a random factor. However, as trajectories of biological communities in closed and simplified environment are surely biased compared to natural system, we interpret them with caution, and rather focused on differences between treatments and the control at each sampling period.

In order to summarize/visualize the effect of Collembola life forms on the 20 PLFA markers, we performed a principal component analysis (PCA) for each sampling period (1 and 2 months). Prior to analysis data were z-transformed and submitted to Hellinger transformation (Legendre and Gallagher, 2001). We added microbial activity (CO_2) and the three enzymatic activities as supplementary passive variables.

Finally, the hypothesis of no difference in PLFA marker assemblages between treatments was tested using one-way ANOSIM based on the Bray Curtis dissimilarity distance configuration (Clarke et al., 2006). If two groups of treatments are different in their PLFA marker assemblages, then compositional dissimilarities between the groups ought to be greater than those within the groups. ANOSIM was performed with 10,000 permutations and Bonferroni's correction was applied a posteriori. In the case of significant results, SIMPER analysis based on the Bray-Curtis dissimilarity distance was run to determine the PLFAs that contribute most in differentiating the two groups tested by ANOSIM.

Except ANOSIM and SIMPER analyses performed with the free PAST 3.14 software, all statistics were performed in R 3.1.2 (R Core Team, 2013) using the Rstudio (RStudio Team, 2015). The following libraries were used: ade4, lme4, MuMIn (Barton, 2013), multcomp (Hothorn et al., 2008), car (Fox and Weisberg, 2011), and vegan (Oksanen et al., 2013).

RESULTS

PLFA

Total PLFAs differed significantly between treatments at each sampling period (**Figure 1**). After the first month, "Ep" treatment positively influenced total PLFA with higher values (1,240 nmol g^{-1} of dry soil, on average) compared to the control (1,052 nmol of g^{-1} of dry soil, on average). In contrast, we found lower values of total PLFAs in the "Ep + Eu" treatment (350 nmol g^{-1} dry soil, on average) compared to all other treatments. Finally, the "Eu" treatment had intermediate values of total PLFAs between the "Ep" and the control treatments. After 2 months, all treatments with Collembola ("Ep," "Eu," and "Ep + Eu") significantly and positively influenced total PLFAs (mean value of 892, 874, and 988 nmol g^{-1} dry soil, respectively) compared to the control treatment without Collembola (627 nmol g^{-1} dry soil, on average). No difference was noticed between the different functional group treatments. Finally, among all the experimental treatments, only the "Ep + Eu" treatment showed higher values of total PLFA at the second sampling period compared to the first one, with an almost 3-fold increase (**Figure 1**).

Regarding the ratio of bacterial to fungal PLFA markers, no difference was observed between all the treatments at both sampling periods, except for "Ep + Eu" that significantly increased the bacterial/fungal ratio during the 2 months of experimentation (+23.5%; **Table 2**).

The Gram+ and Gram– bacterial PLFA markers and their ratios (Gram+/Gram–) differed significantly between treatments on the two sampling periods (**Table 3**). During the first month, only the "Ep + Eu" treatment had a negative influence on the PLFA markers. After 2 months, the pattern changed, the "Ep" and "Ep + Eu" treatments had higher concentrations of Gram+ bacteria compared to both the "Eu" and control treatments. In parallel, after 2 months, "Eu" had a higher concentration of Gram- bacteria and a higher ratio of Gram+/Gram– compared to both "Ep" and "Ep + Eu," the control being intermediate. Between the two sampling periods, the amount of PLFA Gram+ significantly decreased in all treatments (by 1.2 to 1.6 times) except in "Ep + Eu" where it increased by 3.2 times. The amount of PLFA Gram– significantly decreased by more than 2-fold in the control and in "Ep" between the first and second month. Finally,

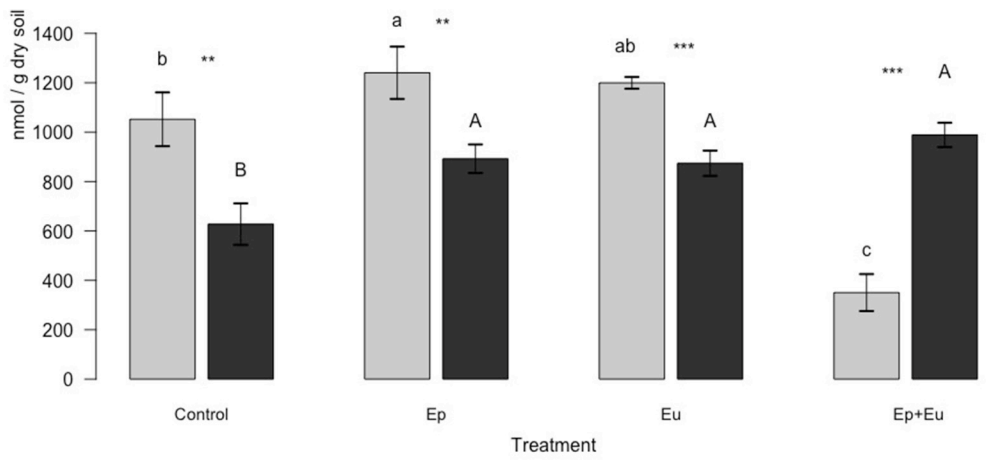

FIGURE 1 | Total PLFA (mean ± standard deviation) of four experimental treatments 1 and 2 months after Collembola re-inoculation. Gray bar-plot = first month, black bar-plot = second month. Different minuscule letters (i.e., "a", "b", "c") indicate significant differences ($\alpha = 0.05$) between treatments for first month. Different capital letters (i.e., "A," "B") indicate significant differences ($\alpha = 0.05$) between treatments for second month. The asterisks indicate significant differences between the months for a given treatment (***$p < 0.001$, **$p < 0.01$). Control, without Collembola; Ep, Epedaphic Collembola; Eu, Euedaphic Collembola; Ep+Eu, mixed functional groups ($n = 4$).

TABLE 2 | Ratio of bacterial to fungal PLFA (mean ± standard deviation) of four experimental treatments 1 and 2 months after Collembola re-inoculation.

	Bacteria/Fungi		*t*-test	
	Month 1	**Month 2**	***t***	***p***
Control	1.76 ± 0.75	1.29 ± 0.12	1.24	ns
Ep	1.38 ± 0.06	1.41 ± 0.06	−0.93	ns
Eu	1.50 ± 0.09	1.33 ± 0.13	2.10	ns
Ep+Eu	1.15 ± 0.13	1.42 ± 0.07	−3.60	*
LMM output				
F	1.73	1.70		
p	ns	ns		
R^2 (%)	25.71	25.43		

*t-test was provided to evaluate the significant differences between the 2 sampling periods for a given treatment. LMM (linear mixed effect model) output was provided to evaluate significant differences between treatments for a sampling period (*p < 0.05, ns p > 0.05). Control, without Collembola; Ep, Epedaphic Collembola; Eu, Euedaphic Collembola; Ep+Eu, mixed functional groups (n = 4).*

the ratio Gram+/Gram– decreased significantly in "Ep" and in "Ep + Eu" between the two dates.

When considering the temporal aspect as a random factor, besides Gram+ bacterial PLFA markers and the ratio of bacterial to fungal PLFA markers, all other PLFA variables significantly differed between the treatments (**Appendix A Table A.1**). The total PLFA, bacterial PLFA, fungal PLFA, the Gram+ bacterial PLFA markers were all significantly higher in the "Ep" and the "Eu" treatments compared to the "Ep + Eu" treatment; the control showing intermediates values. The amount of PLFA Gram– had almost the same pattern being significantly higher in "Eu" compared to "Ep + Eu," the two other treatments showing intermediates values. Including time as a random factor drastically increased the explained variance compared to a model only including the fixed factor "treatment" for two variables: PLFA gram– and the ratio of bacterial to fungal PLFA markers (**Appendix A Table A.1**).

Microbial Activities

Significant differences in released CO_2 were observed between treatments for both sampling periods. During the first sampling period, almost twice the amount of CO_2 was released in the control and "Ep" treatments compared to the "Eu" and "Ep + Eu" treatments. During the second sampling period, released CO_2 was much lower for all treatments. However, it was still significantly higher in all treatments containing Collembola than in the control (**Figure 2**).

In terms of enzymatic activity, for the first sampling period, the FDA activity was significantly higher (55–92.5%) in all treatments containing Collembola, either alone or in combination, than in the control (**Figure 3**). This pattern changed radically after 2 months. Significantly higher FDA activity was found in the control than in the "Eu" and the "Ep + Eu" treatments (**Figure 3**). The "Ep + Eu" treatment was also significantly different from single functional group treatments with a lower value of FDA. Overall, the FDA activity decreased from −14% in "Ep" to −70% in "Ep + Eu" during the course of the experiment except for the control treatment where it increased by 55%.

The urease activity only differed between treatments during the first sampling period, with a higher mean value in the "Ep" treatment compared to all other treatments (**Table 4**). In opposite, the beta-glucosidase activity only differed between treatments during the second sampling period, with a 2-fold higher activity in the control and the "Ep" treatments compared to the "Eu" and the "Ep + Eu" treatments (**Table 4**).

TABLE 3 | Gram$^+$ and Gram$^-$ bacterial PLFA and their ratios (mean ± standard deviation) for four experimental treatments 1 and 2 months after Collembola re-inoculation.

	Gram$^+$		t-test		Gram$^-$		t-test		Gram$^+$/Gram$^-$		t-test	
	Month 1	Month 2	*t*	*p*	Month 1	Month 2	*t*	*p*	Month 1	Month 2	*t*	*p*
Control	473.88 ± 60.67^{b}	285.41 ± 52.67^{C}	4.69	**	48.89 ± 9.69^{a}	23.29 ± 3.41^{AB}	4.98	**	9.79 ± 0.76^{bc}	12.50 ± 3.11^{AB}	−1.69	ns
Ep	505.56 ± 45.07^{ab}	408.93 ± 33.63^{AB}	3.44	*	46.61 ± 7.72^{a}	18.49 ± 4.88^{B}	6.15	**	11.03 ± 1.84^{ab}	23.66 ± 8.41^{A}	−2.93	*
Eu	585.71 ± 32.87^{a}	356.48 ± 21.29^{BC}	11.71	***	39.73 ± 7.52^{a}	44.56 ± 20.74^{A}	−0.44	ns	15.25 ± 3.59^{a}	9.42 ± 4.12^{B}	2.13	ns
Ep+Eu	135.08 ± 33.98^{c}	442.45 ± 26.75^{A}	−14.21	***	23.13 ± 5.98^{b}	20.96 ± 5.15^{B}	0.55	ns	6.23 ± 2.78^{c}	22.11 ± 5.93^{A}	−4.85	**
LMM output												
F	79.71	14.78			8.82	4.67			9.04	5.95		
p	***	***			**	*			**	**		
R^2 (%)	94.10	74.72			63.82	48.29			64.38	54.36		

*Different minuscule letters (i.e., "a", "b", "c") indicate significant differences (α = 0.05) between treatments for first month. Different capital letters (i.e., "A", "B", "C") indicate significant differences (α = 0.05) between treatments for second month. t-test was provided to evaluate the significant differences between the 2 sampling periods for a given treatment. LMM (linear mixed effect model) output was provided to evaluate significant differences between treatments for a sampling period. (***p < 0.001, **p < 0.01, *p < 0.05, ns p > 0.05). Control, without Collembola; Ep, Epedaphic Collembola; Eu, Euedaphic Collembola; Ep+Eu, mixed functional groups (n = 4).*

When considering the temporal aspect as a random factor, all variables but FDA activity significantly differed between the treatments (**Appendix A Table A.1**). The released CO_2 was higher in "Ep" compared to both "Eu" and "Ep + Eu." The beta-glucosidase activities were higher in the Control and in "Ep" compared to "Eu" and "Ep + Eu." Finally, Urease activity was higher in "Ep" compared to "Eu." Including the temporal aspect as a random factor led to obtain a much higher explained variance for all the variables related to microbial activity (**Appendix A Table A.1**).

Interdependence of Collembola and Microbial Community

For the first sampling period, the proportion of the total variance in the PCA (**Figure 4**), which is explained by all PLFA markers, is 60%. The first-third axes accounted for 49, 18, and 10% of the variance, respectively. The first axis clearly separated "Ep + Eu" from all other treatments, while the second axis separated the two single functional groups "Ep" and "Eu." "Ep + Eu" was related to higher values of C18.2, C11, C17D, and high values of FDA activity. "Ep" was related to higher values of CO_2 and urease activity and high concentrations of C16.1.9, C18. Lastly, "Eu" was related to C15a and C15i.

For the first sampling period, the ANOSIM analyses (**Table 5**) showed, that PLFA assemblages found in the "Ep +Eu" were significantly different from the communities found in the control or "Ep," or "Eu" treatments. Furthermore, "Eu" and "Ep" were also significantly different from each other. This pattern was due to the presence of five PLFA markers, C16, C15a, C16i, C15i, C18.1.c (cf. **Appendix A Table A.2**).

For the second sampling period, the total variance in the PCA (**Figure 5**), which is explained by all PLFA markers, is 75%. The first-third axes accounted for 35, 24, and 12% of the variance, respectively. The first axis separated the control from all other treatments. The second axis separated the "Eu" treatment from the treatments containing the epedaphic species "Ep" and "Ep + Eu." "Eu" was related to higher values of C18 and C16.1.9 (General indicators and bacterial Gram–, rather r-strategists). "Ep" and "Ep + Eu" were correlated, with higher values of C15i, C17i (bacterial Gram+ that are rather k-strategists). The control was variable and was correlated to higher values of C17D (bacterial Gram-), C15a, C16i (bacterial Gram+), C18.1.c, C18.2, and C18.1.t (fungal indicators). Differences between assemblages of PLFA markers were stronger during the second sampling period. The assemblages in all of the experimental treatments differed significantly from each other. Lastly, a different set of PLFA markers contributed to dissimilarity (cf. **Appendix A Table A.3**).

Finally, the treatments containing Collembola ("Ep," "Eu," and "Ep + Eu") were more correlated with higher values of CO_2 and urease activity, while the control treatment was correlated with beta-glucosidase and FDA activities.

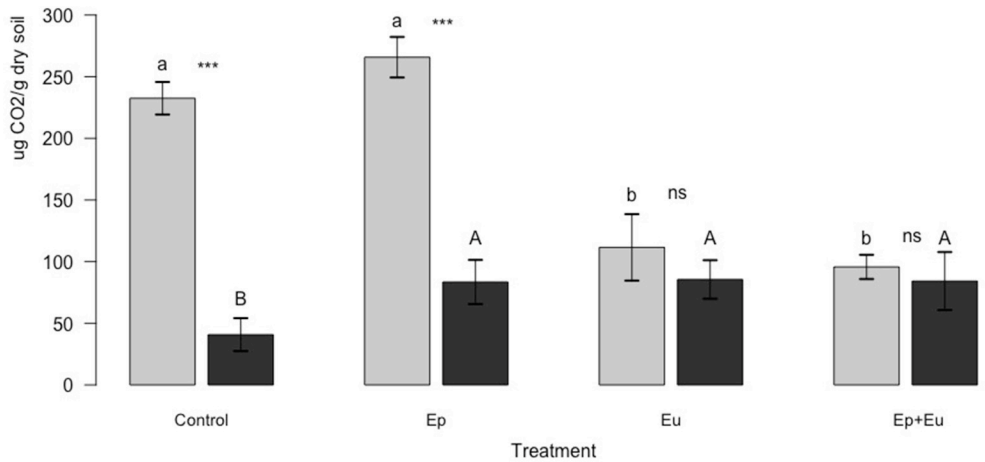

FIGURE 2 | CO_2 release (mean ± standard deviation) of four experimental treatments 1 and 2 months after Collembola re-inoculation. Gray bar-plot = first month, black bar-plot = second month. Different minuscule letters (i.e., "a," "b") indicate significant differences ($\alpha = 0.05$) between treatments for first month; Different capital letters (i.e., "A," "B") indicate significant differences ($\alpha = 0.05$) between treatments for second month. The asterisks indicate significant differences between the months for a given treatment (***$p < 0.001$, ns $p > 0.05$). Control, without Collembola; Ep, Epedaphic Collembola; Eu, Euedaphic Collembola; Ep+Eu, mixed functional groups ($n = 4$).

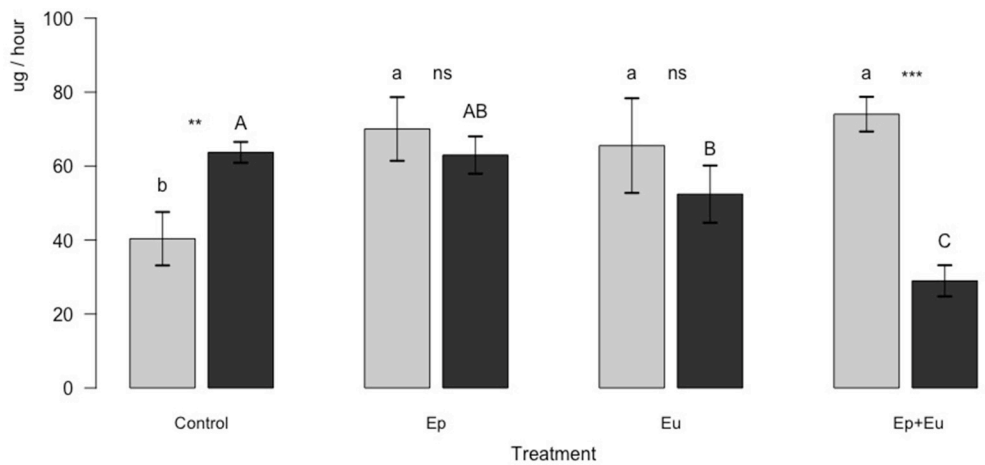

FIGURE 3 | FDA activity (mean ± standard deviation) of four experimental treatments 1 and 2 months after Collembola re-inoculation. Gray bar-plot = first month, black bar-plot = second month. Different minuscule letters (i.e., "a," "b") indicate significant differences ($\alpha = 0.05$) between treatments for first month; Different capital letters (i.e., "A", "B", "C") indicate significant differences ($\alpha = 0.05$) between treatments for second month. The asterisks indicate significant differences between the months for a given treatment (***$p < 0.001$, **$p < 0.01$, ns $p > 0.05$). Control, without Collembola; Ep, Epedaphic Collembola; Eu, Euedaphic Collembola; Ep+Eu, mixed functional groups ($n = 4$).

DISCUSSION

Our results clearly demonstrate that the presence of Collembola drives the trajectories of soil microbial communities over time. Furthermore, categorizing the functional identity of Collembola assemblages is an important key to explaining the nature and intensity of microflora responses.

At the conclusion of the experiment, all treatments with Collembola promoted microorganism biomass (all PLFAs) to the same extent as the control. However, a clear difference in abundance of PLFA markers in the various treatments with Collembola, demonstrated that each functional group of Collembola did impact the structure of the microbial assemblages, but not necessarily the biomass. Microbial communities and activities were both affected by Collembola, often in different ways. Overall a coarse value like respiration indicates overall higher metabolic activity with the presence of Collembola. Our design does not allow us to disentangle the role of the abundance and identity of each functional groups upon microbial community. However, our study dealt with natural assemblages of either epedaphic or euedaphic Collembola, and therefore gives insights on how microbial communities respond

TABLE 4 | Urease and beta-glucosidase activities (means ± standard deviation) of four experimental treatments in 1 and 2 months after Collembola re-inoculation.

	Urease (μg N-NH4 g-1 h-1)		*t*-test		B-glucosidase (μg g-1 h-1)		*t*-test	
	Month 1	**Month 2**	***t***	***p***	**Month 1**	**Month 2**	***t***	***p***
Control	30.20 ± 6.23^{b}	18.16 ± 2.92	3.50	*	6.23 ± 1.83	12.68 ± 1.09^{A}	−6.05	**
Ep	35.0 ± 5.70^{a}	20.83 ± 4.79	3.80	**	7.90 ± 2.17	11.59 ± 1.54^{A}	−2.77	*
Eu	20.20 ± 4.56^{b}	16.89 ± 1.75	1.35	ns	6.12 ± 1.12	5.98 ± 0.55^{B}	0.22	ns
Ep+Eu	22.69 ± 4.04^{b}	22.02 ± 4.19	0.23	ns	6.51 ± 1.89	6.84 ± 0.84^{B}	−0.32	ns
LMM output								
F	6.83	1.71			0.84	39.42		
p	**	ns			ns	***		
R^2 (%)	57.75	25.47			14.41	88.74		

*Different minuscule letters (i.e., "a," "b") indicate significant differences (α = 0.05) between treatments for first month; Different capital letters (i.e., "A," "B") indicate significant differences (α = 0.05) between treatments for second month. t-test was provided to evaluate the significant differences between the 2 sampling periods for a given treatment. LMM (linear mixed effect model) output was provided to evaluate significant differences between treatments for a sampling period. (***p < 0.001, **p < 0.01, *p < 0.05, ns p > 0.05). Control, without Collembola; Ep, Epedaphic Collembola; Eu, Euedaphic Collembola; Ep+Eu, mixed functional groups (n = 4).*

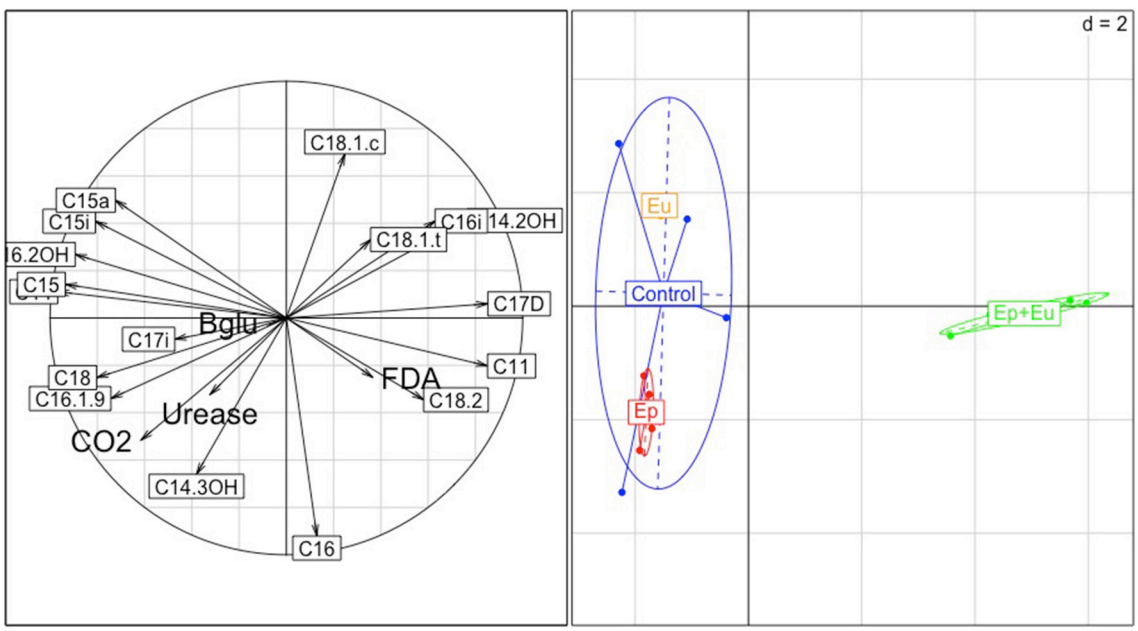

FIGURE 4 | Principal Component Analysis (PCA) of soil microbial parameters 1 month after Collembola re-inoculation. Correlation circle with the variables is on the left; and projection of the treatments on the right panel. The centroids are the barycenters of each treatment and each ellipse represents a 95% confidence interval. Control, without Collembola; Ep, Epedaphic Collembola; Eu, Euedaphic Collembola; Ep+Eu, mixed functional groups; FDA, FDA activity; Urease, Urease activity; CO2, CO_2 activity.

to these groups representatives of natural conditions in terms of both species and abundances.

Our second hypothesis was that the presence of euedaphic species would generate a stronger response in soil microflora than would the presence of epedaphic species due to their closer link to the soil microbial compartment (Perez et al., 2013). In general, our results showed that euedaphic species ("Eu") did not induce a stronger response in microbial communities, but rather a different response from the epedaphic species ("Ep") compared to the control. This differentiation of response clearly appeared in the composition of the microbial communities at the end of the 60-day experiment with a significant difference in PLFA marker composition. In a recent study, a single euedaphic Collembola species (*Protaphorura armata*) was shown to reduce the Gram+/Gram− ratio after 20 days (Maboreke et al., 2017). Although Collembola are depicted as fungivores, with many studies highlighting strong linkages between fungi and Collembola species, especially euedaphic (Gange, 2000; Jørgensen et al., 2005; A'Bear et al., 2012), in our study, the bacterial/fungal PLFA marker ratio did not vary across treatments. Using PLFA markers, Kutáková et al. (2018) found a similar pattern that showed a stronger impact of three

sympatric species of Collembola upon bacterial communities than upon fungal communities. This indicates a stronger interaction between all collembolan functional groups and soil bacteria than originally thought. The fact that epedaphic and euedaphic species led to different assemblages of PLFA markers supports the differentiation of niches between those functional groups. Furthermore, the differentiation in the composition of microbial communities observed in presence of Collembola and, also, between the "Ep" and "Eu" treatments impacted soil functions such as nutrient recycling (Kaneko et al., 1998; Chauvat et al., 2014). Unlike FDA and beta-glucosidase activities, at the end of the experiment, microbial and urease activity (linked to the N cycle) were the highest in Collembola treatments. This is consistent with the results found by Cragg and Bardgett (2001) with a positive effect of three distinct Collembola species on both microbial activity and leaching of nitrate. These authors showed that after 70 days, microbial activity and nitrate release were significantly higher in microcosms containing Collembola compared to a defaunated control. As suggested earlier, positive effects of Collembola on ecosystem processes are likely to be indirect. Though, a positive effect of their feeding on the activity of microorganisms is an increase in enzymatic activities and excretion of nutrients (Visser et al., 1981; Bardgett et al., 1993). Furthermore, our study revealed a temporal change in soil processes associated with microbial communities under the influence of Collembola. After 1 month, potential C mineralization was strongly reduced in the "Eu" treatment compared to the control or the "Ep" treatment. This fits our initial hypothesis, with a strong link between euedaphic species and microbial assemblages. We do not know the mechanism causing the difference in C mineralization. Other than urease, none of the other parameters (i.e., PLFA, enzymatic activities) showed a difference between "Ep" and "Eu" treatments after 1 month. We have shown that there is a temporal impact on community differences, demonstrating strong driving forces exerted by the different Collembola assemblages, even if the microcosms do not have resources (e.g., root exudates) coming from primary producers. As exemplified in other studies, positive effects of Collembola on belowground processes after 2 months of experimentation may have consequences on aboveground

TABLE 5 | Results of ANOSIM analyses between PLFA compositions 2 months after Collembola re-inoculation of four treatments.

	Control	Ep	Eu	Ep+Eu
First month				
Control	0.1147 (0.12)	0.0609 (0.19)	**0.029 (1)**	
Ep		**0.0303 (0.99)**	**0.031 (1)**	
Eu			**0.0255 (1)**	
Ep+Eu				
Second month				
Control	**0.0315 (0.90)**	**0.0308 (0.87)**	**0.029 (0.97)**	
Ep		**0.0303 (0.65)**	**0.031 (0.75)**	
Eu			**0.0255 (1)**	
Ep+Eu				

Control, without Collembola; Ep, Epedaphic Collembola; Eu, Euedaphic Collembola; Ep+Eu, mixed functional groups. p-value and R-values (within brackets) are given. For clarity, significant results are in bold.

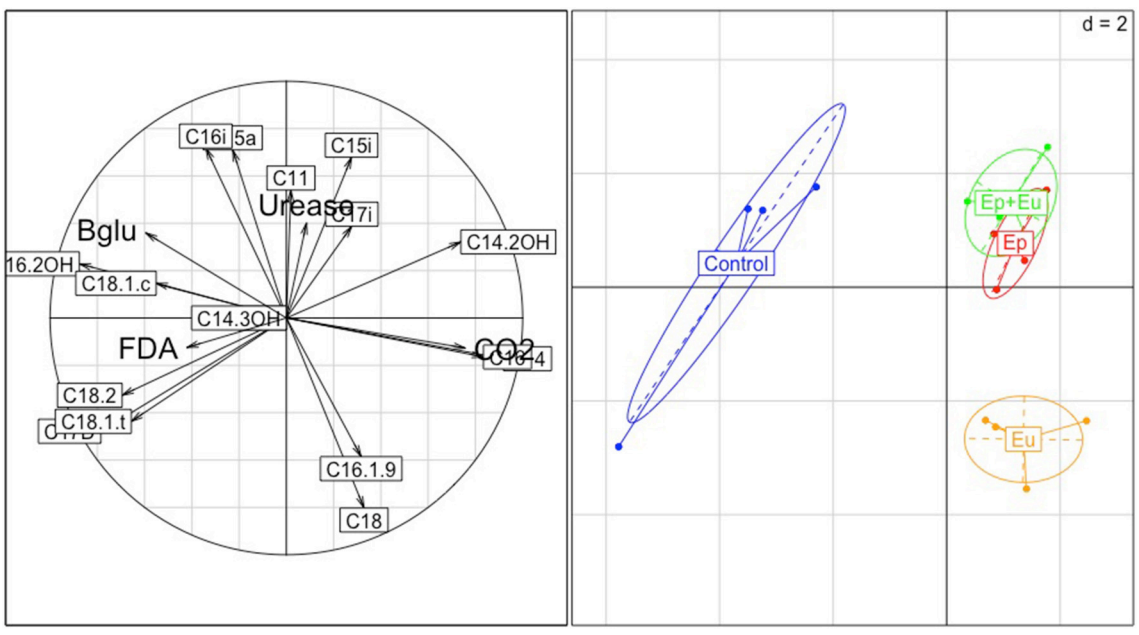

FIGURE 5 | Principal Component Analysis (PCA) of soil microbial parameters during the second month after Collembola re-inoculation. Correlation circle with the variables is on the left; and projection of the treatments on the right. Control, without Collembola; Ep, Epedaphic Collembola; Eu, Euedaphic Collembola; Ep+Eu, mixed functional groups; FDA, FDA activity; Urease, Urease activity; CO2, CO_2 activity.

systems through plant growth or phenology (Forey et al., 2015) or on plant soil feedbacks (Kutáková et al., 2018).

We also aimed to explore the functional complementarity effect by combining collembolan functional groups. Surprisingly, the combination of functional groups ("Ep + Eu") did not always further promote the microbial community structure, composition, or activities. One month after the beginning of the experiment, treatments with a single Collembola functional group significantly promoted the total PLFAs compared to the defaunated treatment, the "Ep + Eu" treatment strongly inhibited it. Previous studies demonstrated that Collembola trigger compensatory growth of the fungi on which they graze (Hanlon, 1981; Hedlund et al., 1991), but the outcome of this interaction is largely dependent on the species composition and population density of the fungivores, with high Collembola densities hampering microbial biomass (Ek et al., 1994; Mikola and Setälä, 1998). We may thus hypothesize that adding natural assemblages of both "Ep" and "Eu" together led to an important top-down regulation on microbial communities. This pauperization of the microbial communities led in parallel to a decrease of CO_2 release and urease activity. In the same way, PCA ordination of the experimental treatments over the first month on each PLFAs marker revealed a clear differentiation between Control, "Ep" and "Eu" on one side and "Ep + Eu" on the other side of the second axis.

As previously mentioned, differentiation between treatments varies according to the time elapsed since the beginning of the experiment. The "Ep + Eu" treatment was more similar to "Ep" treatment than to the "Eu" treatment, suggesting a dominant role of epedaphic species in the combined treatment. This does not support our hypothesis of a complementarity between the functional groups of Collembola. However, this study was conducted under controlled microcosm conditions with all the limitations resulting from this experimental design. for example, microcosms may artificially increase the interactions between epedaphic and euedaphic individuals, probably slightly modifying our complementarity results. However, our study still showed how both functional groups may directly or not interact to drive the microbial community. Overall, we need to be very cautious when extrapolating results from microcosm experiments to field situation, especially regarding the temporal aspect (Carpenter, 1996). Despite these limitations of not adequately reproduce environmental ecosystems, our microcosms offered the opportunity, based on a simplified system, to focus on processes or mechanisms at fine spatial and temporal scale to better understand relationships between soil organisms.

The fact that the influence of Collembola can vary depending on their ecological traits is an important finding for soil food web and interaction web research, as was the virtual lack of complementarity observed between the two functional groups investigated. Furthermore, although Collembola are thought to be primarily fungivores, they largely influenced composition of bacterial-related PLFAs, supporting the emerging view of strong indirect or non-trophic interactions between Collembola and soil bacterial communities.

AUTHOR CONTRIBUTIONS

MC and SC formulated the initial idea. SC, BW, MC, and EF designed the experiment. SC and BW conceived and performed the experiment. SC and BW collected the data including microbial analyzes. SC, MA-V, and LM conducted the PLFA analyzes. SC, BW, and ML conducted the enzymatic activities analyzes. SC performed the statistical analyzes. SC, MC, BW, and EF wrote the manuscript. Additionally, MC supervised the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2019.00052/full#supplementary-material

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Earthworms Building Up Soil Microbiota, a Review

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The positive effect of earthworms on soil processes and plant growth has been extensively documented. The capacity of earthworms to decompose organic matter has been attributed to the microbial communities that inhabit their digestive track or the structures they build, which in turn contribute to make up the drilosphere, a hotspot for microbial activity. However, how earthworms modify the structure of soil microbial communities and how these changes affect soil microbial processes is still unclear. Do earthworms reduce microbial abundance and activity because they feed on microorganisms or do they select and stimulate specific microbial groups? We hypothesise that "the effect of earthworms on nutrient cycling and plant growth is not only a direct effect but is mainly mediated indirectly, via modifications of the microbial community." The objective of this review is to synthesize the existing literature concerning the influence of earthworms on the structure and function of soil microbial communities, as well as to understand how earthworm-induced changes in the soil microbiota would in turn impact soil processes, particularly those occurring in the rhizosphere and involved in plant growth and health. Recent reports have shown that specific bacterial groups consistently increase in soils where earthworms are present, regardless of the earthworm functional group. The extent of this increase seems to be dependent upon the type of substrate under study. Our synthesis also reveals that endogeic and anecic earthworms regularly induce an increase in soil nutrients, whilst this positive effect is not as evident in the presence of epigeic earthworms. The effect of earthworms on nutrient cycling has been further investigated with microbial functional genes, although existing reports largely focus on nitrogen cycling. Earthworms seem to enhance denitrification, most likely through the increase in organic compounds due to organic matter decomposition. By enhancing soil nutrient availability, earthworms indirectly promote plant growth, which has also been attributed to the induction of signal molecules. However, no experiment to date has been able to prove a direct causal relationship between specific signal molecules, earthworms and plant growth promotion. Finally, we propose a framework for earthworm-microbiota interactions and recommend further research.

Keywords: soil nutrient hotspots, interactions, soil biotransformation, signal molecules, drilosphere, microbiome

INTRODUCTION

Earthworms are considered as ecosystem engineers that play an important role in shaping soil structure and cycling nutrients (Blouin et al., 2013). Earthworms promote litter decomposition, nitrogen (N) mineralisation and water infiltration, as a result of their feeding and burrowing habits (Baker, 2007), and therefore deeply affect soil properties (Hättenschwiler and Gasser, 2005). They also play a crucial role in the provision of soil ecosystem services (Lavelle et al., 2016). The soil volume directly influenced by earthworms, known as the drilosphere (Bouché, 1977; Lavelle, 2002), is an important functional region of the soil, made by the earthworm community and the structures it creates: middens, burrows, tunnels, and casts. Earthworms are thus builders of habitats for other organisms, which establishes them as physical or allogenic engineers (Jones et al., 1994; Lavelle et al., 1997, 2016). Besides, these building activities constitute an input of organic matter to the soil and a pathway for the stabilization of soil organic carbon (Corg) through the formation of organo-mineral aggregates (Deeb et al., 2017). This enrichment in organic matter mainly results from earthworm food choice (Curry and Schmidt, 2007), its digestion and excretion of intestinal or cutaneous mucus that can be cementing (Shipitalo and Le Bayon, 2004) or used as an energy source (Lavelle et al., 1995). Therefore, in addition to shaping soil structure, earthworms also have an important impact on soil organic matter dynamics and microorganisms in their gut, casts and drilosphere (Andriuzzi et al., 2016) and are also identified as biochemical (Lavelle et al., 2016) or autogenic ecosystem engineers (Lawton and Jones, 1995).

Earthworms are divided into three main functional groups or ecological categories, which determines how they influence the soil compartment and its microbial communities (Thakuria et al., 2010): (1) epigeic earthworms live on the soil surface and feed from the litter; (2) endogeic earthworms live in the soil and produce horizontal tunnels, while feeding on mineral soil and partially decomposed material, being then geophagous; (3) anecic earthworms produce permanent vertical burrows and feed on the litter that they drag into their burrows to be pre-decomposed by microorganisms, while depositing their casts at the burrow entrance (Bouché, 1977; Lavelle, 1981; Lee, 1985).

Earthworms are considered as key ecological mediators that have the capacity to affect soil functions and microbial activities (Binet et al., 1998; Lavelle et al., 2016), by producing an energy-rich mucus that activates microorganisms through a priming effect (Jenkinson, 1966) and signal molecules that have hormone-like effects and influence plant gene expression (Puga-Freitas and Blouin, 2015). The mutualistic interaction existing between earthworms and the soil microbiota has been named the "Sleeping Beauty Paradox" (Lavelle et al., 1995; Brown et al., 2000), where *dormant* soil microorganisms, awaiting suitable environmental conditions are activated by the *kiss* of the earthworm made of easily assimilable glycoproteins present in the drilosphere in the form of intestinal or cutaneous mucus as already mentioned. This triggers the acceleration of microbial processes for a short period of time ("hot moment") and in a limited soil space ("hot spot"), at the microscale of a biopore or aggregate (Kuzyakov and Blagodatskaya, 2015) which reverberates on a larger scale, at the drilosphere and soil levels (Brown et al., 2000; Hoang et al., 2016; Lipiec et al., 2016).

Earthworms have a direct and important effect on the soil microbiota through their nutrition. This effect may depend on their food preference, selection, food ingestion rate, digestion and assimilation, as mentioned by Curry and Schmidt (2007). Earthworms can digest microorganisms (Brown, 1995; Chapuis-Lardy et al., 2010) thereby decreasing microbial biomass, especially that of fungi (Shan et al., 2013). They may also select or stimulate soil microbes (Khomyakov et al., 2007; Nechitaylo et al., 2010) which help them digest the soil organic matter, since the earthworm gut often lacks the sufficient enzymes to do so (Lattaud et al., 1997, 1998; Fujii et al., 2012). This process may enrich the soil in certain bacterial taxa, for example in bacteria able to decompose the organic matter that earthworms feed on or in denitrifying bacteria able to survive in the reduced oxygen conditions of the earthworm gut (Drake and Horn, 2007; Hong et al., 2011).

The physiology, morphology and behaviour of earthworms is essential to understand their effect on soil functions (**Figure 1**, arrow 1). However, there is increasing evidence that the effect of earthworms on soil functions may be mediated through soil microbial communities (**Figure 1**, arrow 2). It is yet not clear how the different ecological groups may promote or select soil microorganisms and there are many contradictory results concerning the effect of earthworms on soil microbial communities (Byzov et al., 2015). However, the drilosphere is generally acknowledged as being a soil hotspot with a positive effect on ecosystem functions such as nutrient cycling and plant growth (Brown et al., 1999; Scheu, 2003; Van Groenigen et al., 2014).

Considering that the involvement of microorganisms in these functions is fundamental, it is therefore necessary to consider microbial communities and how they are influenced by earthworms in order to understand and predict the effect of earthworms on ecosystem functions. It is our hypothesis that "the effect of earthworms on nutrient cycling and plant growth is not only a direct effect but is mainly mediated indirectly, via modifications of the microbial community" (**Figure 1**). Thus, the objectives of this review are two. The first is to determine whether some patterns can be drawn from the existing literature regarding the effect of the different earthworm functional groups (epigeic, endogeic, and anecic) on the abundance, structure and diversity of soil microorganisms (bacteria, archae, and fungi) at the different sites (earthworm gut, casts, burrows, bulk soil, rhizophere, others). The analytical methods used are also considered [Gram+/–, fingerprinting, phospholipid fatty acids (PLFA), sequencing]. The second is to establish the impact of earthworms on microbial processes involved in nutrient cycling, on the production of signal molecules and as a consequence, on plant growth promotion. The selected literature was mainly chosen from studies that deal with earthworms and microbial (microorganisms, microbiome) interactions and nutrient cycling (Nitrogen, Phosphorus) between 1980 and 2018; however, for

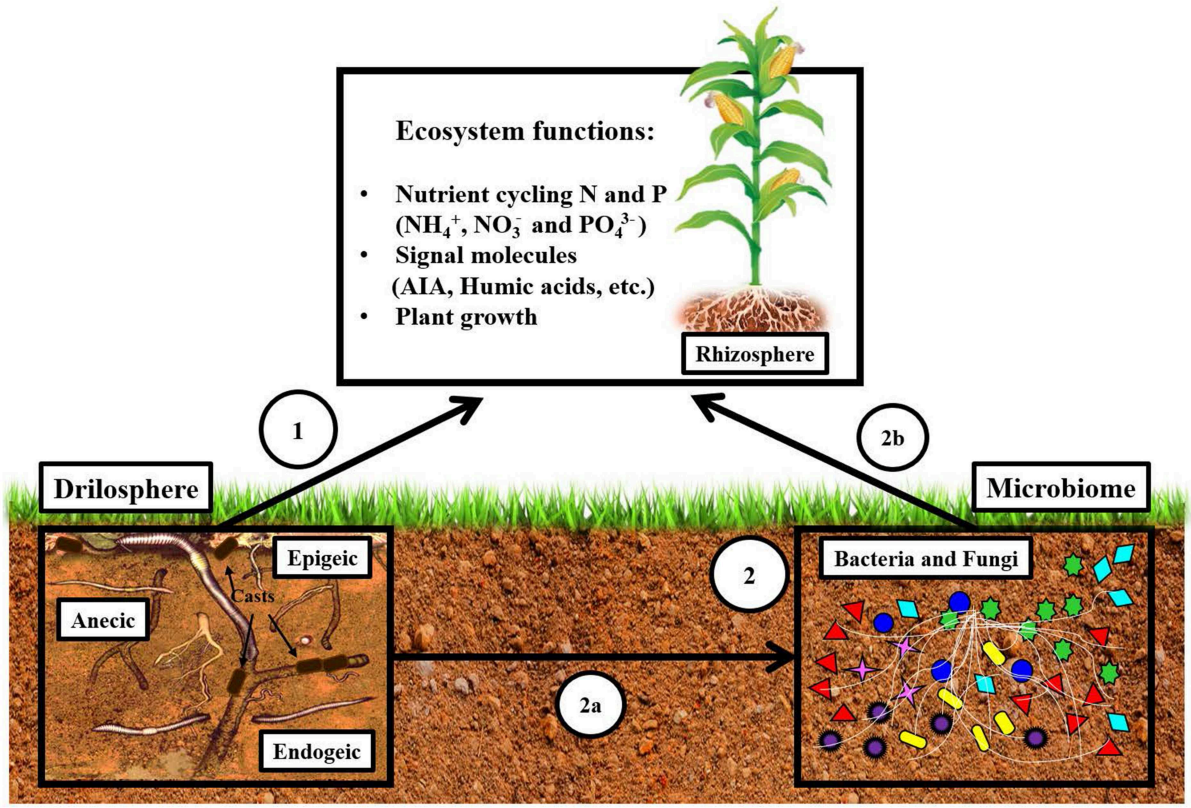

FIGURE 1 | Hypothesis: effect of earthworms on nutrient cycling and plant growth is not only a direct effect but it is mainly mediated indirectly by microorganisms. The impact of earthworms is direct (1), or indirect, through the stimulation of microorganisms (2). By modifying microbial communities (2a), earthworms impact the way bacteria are influencing ecosystem functions (2b). This figure aims at illustrating the fact that indirect effects are as important as direct ones.

specific topics we used the Web of Science (WOS) with precise keywords (see **Figures 4–6** legend).

Finally, we propose a framework for earthworm—soil microbiome interactions and recommend further research to be directed towards elucidating the microbial processes occurring in the drilosphere.

THE IMPACT OF EARTHWORMS ON THE ABUNDANCE AND ACTIVITY OF SOIL MICROORGANISMS

The effect of earthworms on soil microbial communities is critical as they are one of the most important fauna group in soils, in terms of number and biomass (Blouin et al., 2013). Besides, earthworms can have a very high rate of substrate or soil ingestion. Epigeic earthworms can ingest 3–50 mg (dry matter) of dung or any other kind of litter per gram of earthworm per day and the geophagous worms 200–6,700 mg (dry matter) of soil per gram of earthworm per day (Curry and Schmidt, 2007). In this section, we will synthesize the available information regarding how earthworms influence the abundance or activity of soil microorganisms, depending on their functional groups.

The Epigeics

The consequences of the presence of epigeic earthworms on soil microbial abundance are variable (**Figure 2**). The literature shows that they can provoke either a decrease or an increase in microbial biomass. Less frequently, reports show that the number of microorganisms remains unaffected by their action. Most studies found in the literature are performed under artificial laboratory conditions and use epigeic earthworm species *Eisenia andrei* and *E. fetida*, grown in different feedstocks (dungs, agriculture by-products and mixtures of organic matter and soil). These studies report that these species induce an increase of the microbial biomass in the transformed substrate which is made up mainly of casts, although the magnitude of this effect varies through different time scales. The activity and the numbers of microorganisms have a peak at the beginning of the digestion which lasts at the most a few hours in the gut (Brown et al., 2000) and a bit longer in the fresh casts, these sites being "hot spots and moments." After some months (3–4) there is a decrease in microbial activity and numbers in the casts or vermicompost and then there is a stabilization of both (Yakushev et al., 2009) Koubová et al. (2015) indicated that microbial biomass measured by PLFA was 2-fold greater in the earthworm gut than in the non-ingested substrate and that biomass was also higher in casts than in the surrounding substrate, although this increase was

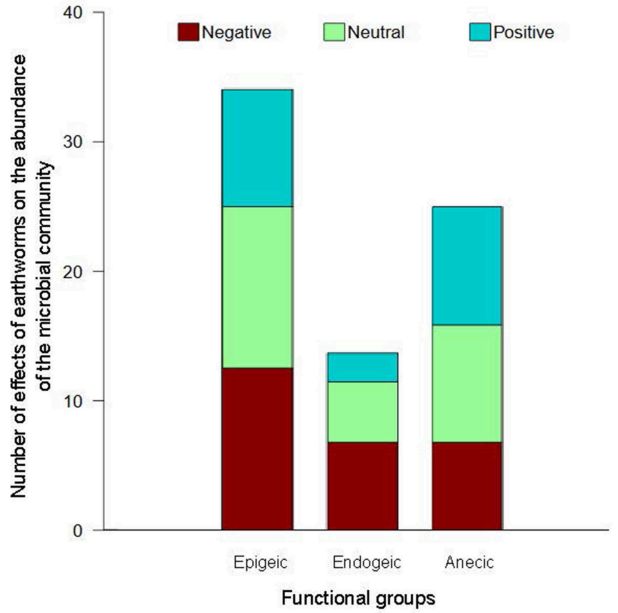

FIGURE 2 | The effect of earthworms on microbial abundance depending on their functional group. Thirty-one studies from 1986 to 2018 were considered. All the studies were carried out in controlled conditions with the exception of one (Stromberger et al., 2012). Five articles were from tropical regions and all the others from temperate regions. The microbial abundance was measured in five gut contents, 11 casts, 12 vermicomposts, 11 soils, four burrows, and one midden from the different studies. Some studies presented results from more than one earthworm species, ecological category and site measurement.

less important than in the gut. Yakushev et al. (2009) showed an increase in microbial biomass of 2.7 times in the casts of *E. fetida* and evidenced that microbial growth in a period of 9 h was 124 times higher in vermicompost than in compost. In a mixture of pig manure and soil, the microbial biomass was 1.3 times higher in the presence of earthworms (Aira et al., 2007). Toyota and Kimura (2000) found that bacterial biomass, evaluated by counting colony forming units (CFU), increased from 3.2×10^4 CFU g^{-1} in composted farmyard manure to 1.3×10^7 CFU g^{-1} in vermicompost with *E. fetida*.

Although other studies show that epigeic earthworms do not have any impact on microbial abundance in the soil, this effect seems to be dependent upon the kind of feedstock assessed. On leaf compost from alder, willow and birch with a $C/N = 19.2$, the presence of *E. fetida/E. andrei* induced a great increase in the number of microorganisms whereas vermicompost made from cattle manure ($C/N = 15.4$) did not present any differences with its respective control compost without earthworms ($C/N = 16.5$) (Yakushev et al., 2009). Sheehan et al. (2008) using mesocosms showed that, in addition to the influence of the food supply, the effect of epigeic earthworms on the abundance of microorganisms also depends on the soil layer under study and reported a larger increase in microbial biomass in the upper layers (0–7 cm) than in the deeper ones (7–14 cm).

On the other hand, several studies demonstrated that epigeic worms can decrease microbial biomass in their casts or in the substrate they live on (**Figure 2**). Through measurements of total PLFA, Gómez-Brandón et al. (2012) and Aira et al. (2002, 2006) found less microbial biomass in *E. andrei* vermicompost from grape bagasse and in *E. fetida* and *Eudrilus eugeniae* vermicompost from pig manure than in substrates without earthworms, although this effect seemed to depend upon the earthworm density (Aira et al., 2002). Overall, literature findings show that no clear effect of epigeic earthworms can be detected in microbial biomass, nor on the growth rate of microbial populations. Discrepancies may be attributed to the different species or substrates under study, as well as the different analytical methods implemented (Yakushev et al., 2009; Gómez-Brandón et al., 2012; Koubová et al., 2015).

The Endogeics

Although endogeic earthworms have been less studied, results show contrasting effects on microbial abundance, compared with the epigeics (**Figure 2**). For endogeics, the majority of the works that observe their impact on the microbiome are made comparing soils with and without earthworms. There are less studies on the "hotspots" (gut and fresh casts). In mesocosms with soil from arable fields (3.8% of organic matter) and potato residues on the surface, the endogeic *Aporrectodea caliginosa* did not impact soil microbial biomass which was obtained from the biovolume of bacterial numbers measured by confocal laser scanning microscopy (Postma-Blaauw et al., 2006). In contrast, Chang et al. (2016) found an increase in the bacterial biomass of a forest soil mixed with litter, measured through PLFA, in the presence of *Octolasion lacteum*. Similar findings were reported for a pot experiment by Braga et al. (2016), who detected increases in the bacterial 16S rRNA gene abundance in soils with the tropical earthworm *Pontoscolex corethrurus,* compared with soils without earthworms. In another study, microbial activity increased 6-fold in the gut of *P. corethrurus* and 1.6-fold in its casts, compared with the surrounding vertisol; more CFU were also observed in the casts by plate counting (Barois and Lavelle, 1986; Barois, 1987). Contrastingly, soil microbial biomass has also been shown to decrease with increasing endogeic earthworm biomass (Scheu et al., 2002). Chapuis-Lardy et al. (2010) found that fungal and bacterial biomass significantly decreased (∼2-fold) in casts from *P. corethrurus* when compared to the parent soil, although the fungal-to-bacterial ratio was not modified by the earthworm casting activity. The effect of endogeic earthworms on soil microbial biomass may also depend on the soil depth: it decreases with soil depth (Sheehan et al., 2008).

The Anecics

The anecic worms feed on the surface and build permanent tunnels that allow them to mix the different soil horizons. They form middens on the soil surface at the entrance of their burrows that contain visible pieces of organic matter and casts, these as well are excreted into the soil profile.

The impact of anecics on the soil may reach the whole soil profile and this is not the case with the other functional groups. Most reports show that anecic earthworms have a positive or neutral impact on microbial abundance in the soil (**Figure 2**),

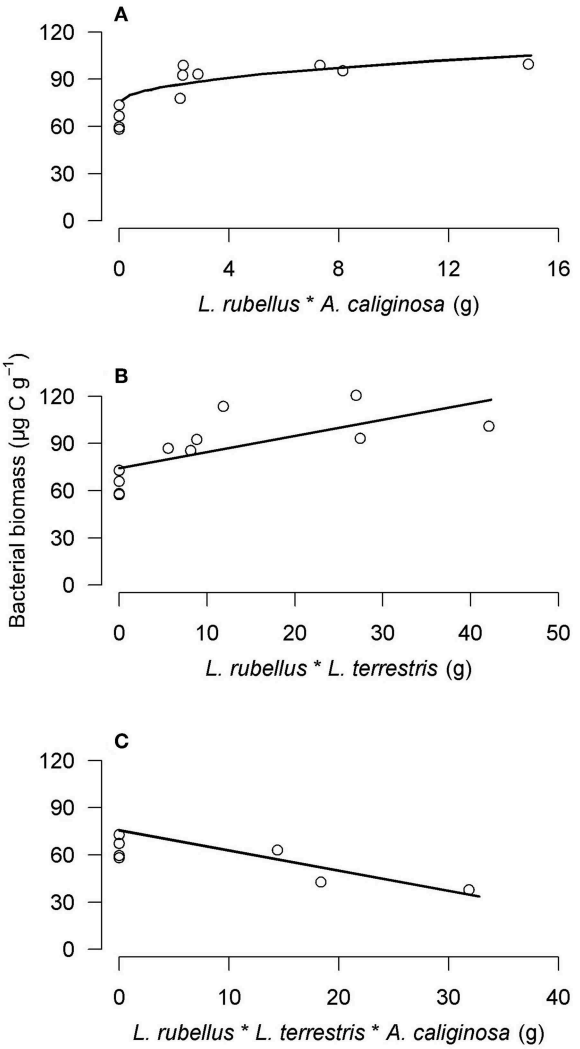

FIGURE 3 | Partial residual plots of the interaction effects (*) between **(A)** *L. rubellus* and *A. caliginosa* (g), **(B)** *L. rubellus* and *L. terrestris* (g) and **(C)** *L. rubellus, A. caliginosa* and *L. terrestris* (g) on the bacterial biomass (µg C g^{-1}), as determined by backward regression analysis (modified from Postma-Blaauw et al., 2006).

although this may be biased by the fact that most studies are carried out in temperate regions, using *Lumbricus terrestris* as a model species. As shown in **Figure 2**, very few reports are available for anecics in tropical regions.

Devliegher and Verstraete (1997) showed that the number of CFU in the soil surface layer (0–5 cm) was 60–320 times larger in the casts of *L. terrestris,* fed with lettuce, than in the surrounding soil. In a deeper layer (5–22 cm), the magnitude of the increase was lower but still significant, with 6–32 times more bacterial CFU in the drilosphere than in the soil. The influence of soil depth was further corroborated by Sheehan et al. (2008), who observed a differential impact of the anecic worms *Lumbricus friendi* and *Aporrectodea longa* on microbial biomass depending on the soil horizon under study. In a microcosm experiment using soil and litter from lime (*Tilia cordia*), the anecic *L. terrestris* induced more microbial biomass in soil retrieved from its burrow walls, compared with the surrounding soil (Tiunov et al., 2001). However, these results seem to depend on the litter source and the soil Corg and N content, as the same experiment repeated with beech litter soil (which has three times more Corg and N than the lime litter soil) showed a smaller increase in burrow microbial biomass. Stromberger et al. (2012) also evidenced a larger abundance of microorganisms measured by PLFA in the burrow walls of *L. terrestris* when compared with the bulk soil (89.4 and 56.7 nmol g^{-1} soil respectively). Similar results measured also with microbial PLFA were found by Sampedro and Whalen (2007) in the gut of *L. terrestris* and by Aira et al. (2009) in middens. Overall, most of the literature points towards a positive effect of anecics on microbial biomass in their burrows, middens, or casts, although a few reports observed a neutral effect (Postma-Blaauw et al., 2006) or negative effect (Zhang et al., 2000; Yu et al., 2008).

Although most studies focus on the effect of one earthworm species or species from the same ecological category on microbial biomass, it is important to remember that all three functional groups coexist in natural conditions. Investigating the earthworm impact on soil microorganisms should therefore integrate the whole earthworm community. Few studies have taken this point into account, with the exception of Postma-Blaauw et al. (2006) who showed that, although *L. terrestris* (anecic) did not have any effect on soil microorganisms, the combined presence of *L. rubellus* (epigeic) and *L. terrestris* induced an increase in microbial biomass. These authors also observed a smaller increase in the microbial biomass when *L. rubellus* and *A. caliginosa* (endogeic) were tested together, and a decrease of the microbial biomass when all three functional groups were combined (**Figure 3**). The importance of investigating the combined effect from distinct earthworm functional groups was further confirmed by Scheu et al. (2002) who determined, in a mesocosm experiment, that when epigeics (three species) and endogeics (three species) were put together, soil microbial biomass was larger than that observed when each earthworm group was studied independently. Finally, it must be considered that assessing soil microbial biomass as a whole may hide the spatial heterogeneity of the effect of earthworms. This could increase microbial abundance only in hot spots and decrease it in the bulk soil, with the resulting effect depending on the rate of soil ingestion, earthworm density, and presence of the functional groups (Sheehan et al., 2008). Considering microbial abundance as a whole can also prevent the detection of the fact that some specific microbial taxa may have been promoted while others hindered. Therefore, it is important to look at how earthworms modify microbial community structure.

IMPACT OF EARTHWORMS ON THE STRUCTURE OF SOIL MICROBIAL COMMUNITIES

By feeding on soil and influencing soil factors such as porosity, water content, mineral N (NO_3^-, NH_4^+) or organic matter

content, earthworms modify soil habitats and their resident microbial communities. In this section, we will make available information regarding the impact of earthworms on the structure and diversity of soil microbial communities and determine whether the resulting changes are consistent among functional groups of earthworms.

Earthworms Modify the Diversity of Soil Microbial Communities

The effect of earthworms on the richness and diversity of microbial communities can be neutral, negative or positive, depending on the earthworm species and on the "micro-habitat" considered, i.e., whether the study focuses on the earthworm gut, casts, or on the surrounding soil. Neutral effects of earthworms on soil bacterial communities have been reported by de Menezes et al. (2018), who showed that the introduction of the endogeic *Aporrectodea trapezoides* did not influence the number of bacterial OTUs (Operational Taxonomical Units) nor the Chao1 richness estimator of the whole soil. On the other hand, positive effects on bacterial richness and diversity were observed by Hoeffner et al. (2018) in the burrows created by four epi-anecic species from the *Lumbricus* genus, compared to the bulk soil. These authors, however, showed that fungal diversity remained unaffected by the earthworms. The impact that earthworms may have on soil microbial diversity was also investigated through the study of the vermicomposting process. The epigeic earthworms *Eudrilus* sp. or *E. fetida* increased bacterial diversity in the substrate, at least during the first stages of their vermicomposting (Vivas et al., 2009; Gopal et al., 2017), which showed the importance of considering different time scales in the study of bacterial diversity enhancement.

Contrary results were observed when considering earthworm gut and casts. Negative effects of earthworms on bacterial richness were found in earthworm gut and casts by Koubová et al. (2015), who showed that bacterial species richness (estimated from culturable bacteria) decreased during the passage through the epigeic *Eisenia's gut*. Soil ingestion by epigeic earthworms was also reported to decrease microbial diversity, as observed in the gut of *Eudrilus* sp. (Gopal et al., 2017) and in casts of *L. rubellus* (Furlong et al., 2002). This decrease in microbial diversity after soil ingestion has been attributed to the increased dominance of several bacterial groups in the earthworm casts, more specifically to an enrichment in bacterial taxa able to degrade benzoic and aromatic compounds (Furlong et al., 2002; Gopal et al., 2017). Further studies evidenced that the type of food that earthworms ingest seems to have little influence on the diversity of bacterial communities in casts, as shown by Aira et al. (2016) in the epigeic *E andrei*.

Overall, these studies show that the influence of earthworms on microbial communities varies between micro-habitats, although Egert et al. (2004) only found slight differences between the community structure of bacteria and archea in the gut, the casts and the surrounding soil in the case of the anecic earthworm *L. terrestris*. On the other hand, for the same species, Sampedro and Whalen (2007) found that the microbiome of its gut was different from the bulk soil. The contrasting findings highlighted here may be partly explained by the different methods that were employed in the study of microbial diversity. Whilst several results were obtained by using Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Egert et al., 2004; Hoeffner et al., 2018) or clone libraries of the bacterial 16S rRNA gene (Furlong et al., 2002), other studies have used next generation sequencing (NGS) to increase the resolution of diversity estimates (Gopal et al., 2017). The overall effect of earthworms on the soil microbial community also depends on soil conditions, particularly nutrient content. Koubová et al. (2015) showed that the effect of the epigeic earthworm *E. fetida* on soil microbial community biomass and composition, assessed through PLFA and culturable bacterial counts, was stronger in nutrient-poor habitats, where the stimulation of bacterial growth in the earthworm intestine was more noticeable.

Earthworms Modify the Abundance of Specific Taxa Within the Microbial Community

Soil passage through earthworm gut has been reported to consistently increase the abundance of specific bacterial groups within the microbial community, such as that of Flavobacterium (Schönholzer et al., 2002), Actinobacteria (Furlong et al., 2002; Rattray et al., 2010; Aira et al., 2016; Gopal et al., 2017; Ma et al., 2017), Firmicutes (Furlong et al., 2002; Rattray et al., 2010; Singh et al., 2015; Gopal et al., 2017; Ma et al., 2017) and γ-Proteobacteria, in particular members of the Pseudomonas genus; (Furlong et al., 2002; Aira et al., 2016; Ma et al., 2017). Earthworms generally promote the growth of fast-growing bacteria such as γ-Proteobacteria due to the labile carbon substrates they produce (Braga et al., 2016) in their gut or from their skin, which leads to increases in the Proteobacteria:Acidobacteria ratio (Gong et al., 2018). Specific functional groups have also been shown to be enhanced by the presence of earthworms, such as denitrifiers (Ihssen et al., 2003) or cellobiose utilizers (Karsten and Drake, 1995). Sampedro and Whalen (2007) also found significant changes in microbial-derived PLFA profiles of soil and gut and described that gut passage significantly increased the concentration of biomarkers indicative of aerobic bacteria, microeukaryotes, and fungi.

The advent of high-throughput sequencing of 16S rRNA gene amplicons has allowed us to confirm and refine these results. The presence of endogeic earthworms (*A. trapezoides, Metaphire guillelmi,* or *P. corethrurus*) is associated with increases in Bacteroidetes (especially in Flavobacteriaceae and Sphingobacteriales), β-Proteobacteria (especially in Rhodocyclaceae), Firmicutes (especially in Paenibacillaceae), Verrucomicrobia and ammonia-oxidizing *Nitrosovibrio* in the soil (Bernard et al., 2012; de Menezes et al., 2018; Gong et al., 2018). The observed enrichment in these bacterial taxa is usually attributed to an increase in the mineralisation of organic residues (Bernard et al., 2012). Bernard et al. (2012) and de Menezes et al. (2018) also found a promotion of chitinolytic bacterial taxa by *P. corethrurus* and *A. trapezoides,* respectively, such as Chitinophagaceae, Cytophagaceae, Neisseriaceae, and Microbacteriaceae. The release of chitin in the soil, either

through the production of chitinase by earthworms or through the degradation of fungal hyphae during gut passage, may be responsible for this increase in chitinolytic bacteria. In general, gut bacteria of anecic and endogeic earthworms seemed to be determined, in descending order of importance, by earthworm ecological group, habitat, and species (Thakuria et al., 2010).

In the epigeic earthworms *E. fetida* and *Perionyx excavatus*, gut bacterial communities were shown to be dominated by Proteobacteria, Actinobacteria, and Firmicutes, with several differences according to the species. Verrucomicrobia and Chloroflexi were abundant in the gut of *E. fetida* whilst they were absent in that of *P. excavatus*. On the contrary, Spirochaetes were abundant in *P. excavatus* but not in *E. fetida* (Singh et al., 2015). The earthworm intestinal tract constitutes an environment that is enriched in C, N and water content and impoverished in oxygen when compared with the surrounding soil (Barois and Lavelle, 1986). It has therefore been consistently shown to favour the occurrence of anaerobic or facultatively anaerobic bacteria and archea (Barois et al., 1987; Horn et al., 2003; Koubová et al., 2015). Bacterial genera such as *Aeromonas, Bacillus, Clostridium, Paenibacillus, Propionibacterium,* or *Staphylococcus* were shown to be abundant in the guts of epigeic *Eisenia* earthworms (Toyota and Kimura, 2000; Shin et al., 2004; Koubová et al., 2015). König (2006) reported that *Bacillus* and *Paenibacillus*, in particular, were commonly detected in the gut of earthworms and were especially relevant since they were able to degrade aromatic compounds under oxygen limiting conditions.

Although consistent patterns could be observed, the effect of earthworms on soil bacterial community composition seems to be mostly dependent upon the type of substrate under study (de Menezes et al., 2018). This was demonstrated by Gopal et al. (2017) who showed that bacterial community structure changed throughout the vermicomposting process, as nutrient dynamics were modified. Gong et al. (2018) reported a decrease in the relative abundance of Cloroflexi and Fibrobacteres by the anecic *M. guillelmi* in rice fields where mulch was applied, whereas their dominance increased in rice fields where straw was incorporated. These authors also reported a shift in keystone taxa within the soil microbial community, which was dependent upon the applied organic amendment. These findings were consistent with those described by Koubová et al. (2015), who recorded distinct shifts in microbial taxa depending on the environment under study. Earthworm (*Eisenia* spp.) excreta were enriched in Actinobacteria in compost pile (plant remains) whereas they were enriched in Firmicutes in large scale vermiculture plant (cattle manure and agricultural waste) and forest soil. On the other hand, increases in Gammaproteobacteria were detected in the gut of earthworms. Increases in Gammaproteobacteria were also found by Fjøsne et al. (2018) in the soil when the epigeic earthworm *Dendrobaena veneta* was present. These authors consistently observed increases in *Kluyvera cryocrescens* and *Pseudomonas putida*, independently from the initial composition of the soil microbial community.

To synthesize the available information, we looked at how often microbial phyla were found in soils or substrates influenced by earthworms belonging to different ecological groups. **Figure 4** shows that Proteobacteria, Actinobacteria, Firmicutes, Acidobacteria, Planctomycetes, Bacteroidetes, Nitrospirae, and Chloroflexi have the highest relative abundance in soils where earthworms are present, regardless of the ecological category they belong to. Although epigeic earthworms seem to induce a higher microbial diversity than endogeic and anecic earthworms, these latter may impact the soil bacterial community in a more consistent manner, as shown by a lesser proportion of rare phyla (**Figure 4**). A network analysis (**Figure 5**) confirmed that the above referred eight phyla form the core of the network while interacting or being promoted by most earthworm species. It also revealed that epigeic earthworms promote more rare phyla of bacteria (seven phyla) than do endogeic earthworms (two phyla). Altogether, these findings suggest that some bacterial taxa respond in a consistent manner to the presence of earthworms and could constitute good indicators for predicting the impact of earthworms on soil ecosystems.

THE IMPACT OF EARTHWORMS ON NUTRIENT CYCLING THROUGH THE MODIFICATION OF SOIL MICROBIAL COMMUNITIES

Earthworms are decomposers feeding on organic matter, thereby releasing nutrients through digestion and excretion with direct consequences on plant growth (**Figure 1**, arrow 1). They also have an important impact on microbial communities which in turn affects nutrient cycling and plant development through their interactions (**Figure 1**, arrows 2a and 2b). In a recent study, Braga et al. (2016) showed that the introduction of the endogeic *P. corethrurus* in the soil significantly changed around 70 microbial functions in the bulk soil and in the rhizosphere, which were mainly related to biosynthesis and plant-microbe symbiosis. The presence of earthworms also modified the ecological interactions among microbial functions. As shown in the previous section, earthworms stimulate certain microbial taxa, and by doing so increase the importance of keystone functions (Braga et al., 2016). In this section, we will summarise the main findings concerning the impact of earthworms on microbial functions, emphasising how information about earthworm-associated microbial communities needs to be integrated in order to improve knowledge of the influence of earthworms on nutrient cycling.

Earthworms Increase Nutrient Mineralisation in the Soil

Earthworms, in particular endogeic geophagous earthworms, are known to promote C and N mineralisation in the soil (Lavelle et al., 1998; Araujo et al., 2004; Coq et al., 2007; Gopal et al., 2017), most likely through a priming effect affecting decomposition rates of the soil organic matter (SOM) (Barois et al., 1987; Bernard et al., 2012). This positive priming effect is expected to promote the recycling of nutrients, especially of organic N and P, in the SOM (Kuzyakov et al., 2000; Bertrand et al., 2015). This has been shown for *P. corethrurus* in several studies, summarised in the recent review by Taheri et al. (2018).

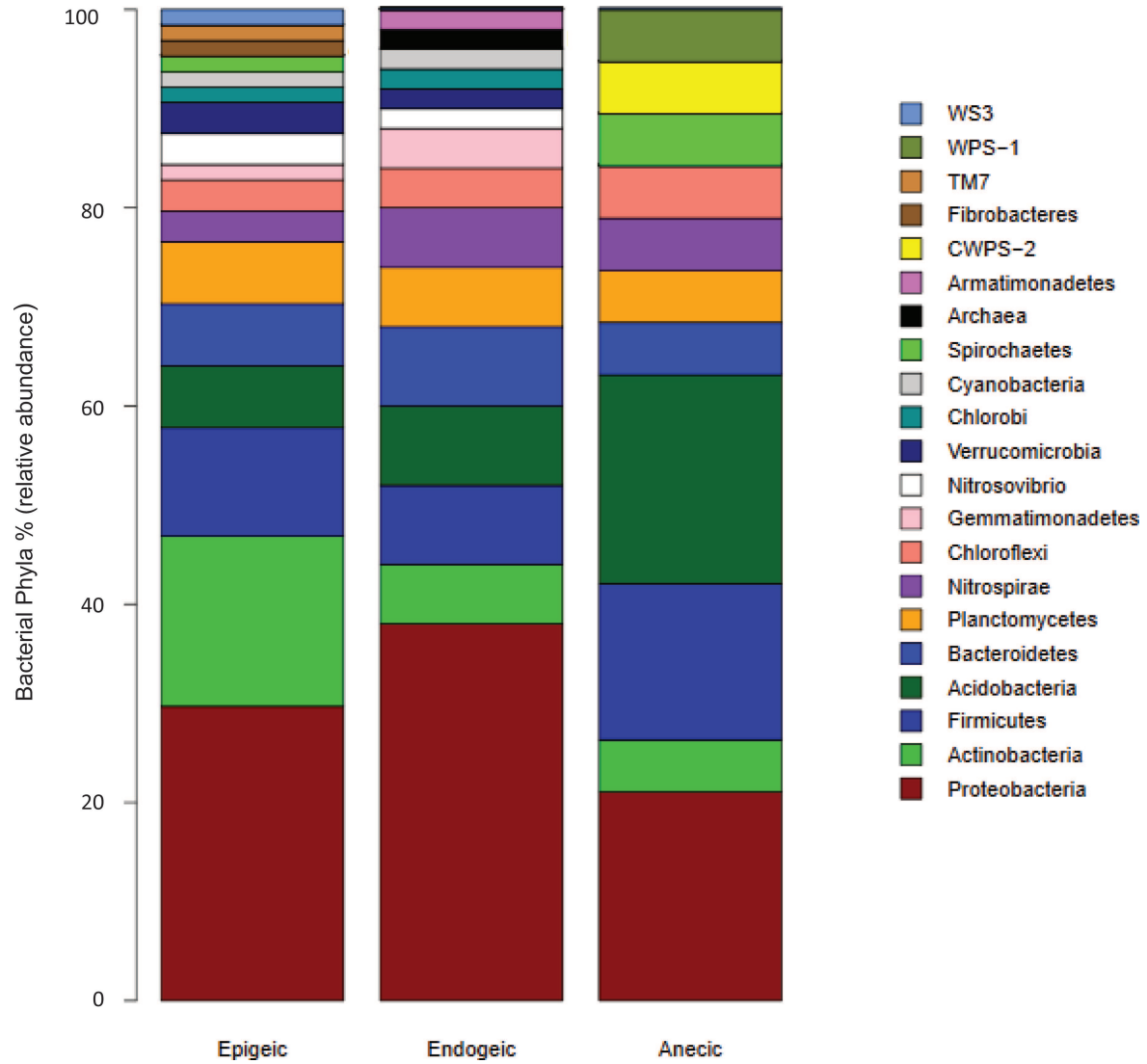

FIGURE 4 | Relative abundance of microbial phyla reported in soils or substrates processed by earthworms of different functional groups. Data was obtained from 11 peer-reviewed publications retrieved after a search made using the words: "earthworms," "soil microbial communities," and "phyla" in the Web of Science from 2009 to 2018 (before that, no information was found with the keywords "microbial phyla").

Two to three-fold increases in mineralised C have also been observed in casts of the endogeic *A. caliginosa*, compared with the surrounding soil, which is attributed to the priming effect caused by earthworm ingestion and digestion (Abail et al., 2017). Epigeic earthworms such as *E. fetida* and *P. excavatus* have also been reported to enhance the decomposition rates of organic matter (Singh et al., 2015).

The increase in SOM mineralisation in earthworm casts, compared with the surrounding soil, is associated with an enrichment in labile compounds and with a subsequent increase in microbial activity (Barois and Lavelle, 1986; Coq et al., 2007; Abail et al., 2017), which could be attributed to the earthworm digestion itself and to the influence of the gut microbiome. The enhancement of r-strategist bacteria with fast growth rates and specialised catabolic capabilities (Bernard et al., 2012), which are thought to be responsible for the observed increase in SOM mineralisation by earthworms, was defined by Lavelle et al. (1995) as the "Sleeping Beauty" paradox. As described previously, the promotion of fast-growing bacteria (γ-Proteobacteria for example) may be driven by the N-rich gut mucus, changes in soil physico-chemical characteristics or to the degradation of fungal biomass during gut transit, through which earthworms can produce labile C substrates (Brown, 1995; Brown et al., 2000; Braga et al., 2016). The induction of a priming effect by earthworm gut transit is further confirmed by observations showing that SOM mineralisation rates are lower in old casts than in recent ones (Pulleman et al., 2005; Bertrand et al., 2015).

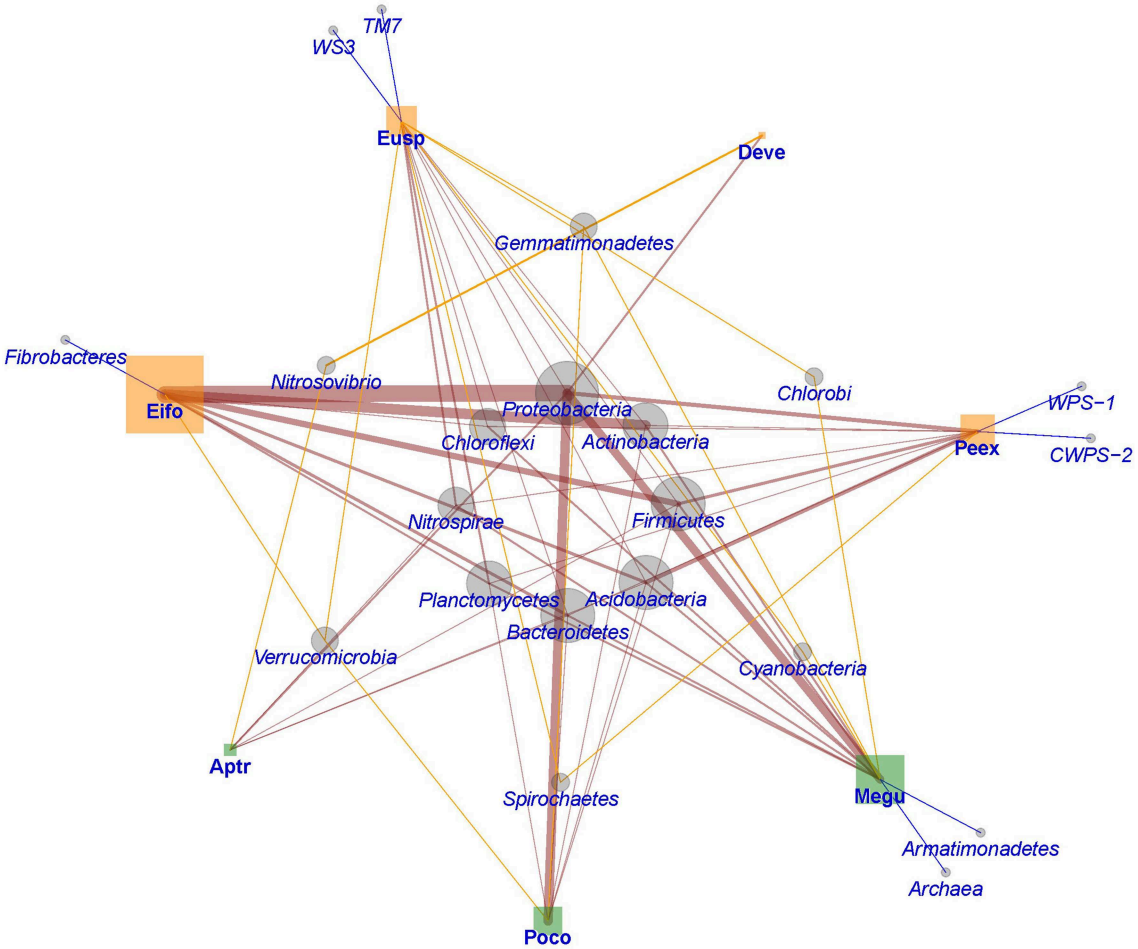

FIGURE 5 | Network representation of microbial phyla (circles) in soils or substrates processed by earthworms (squares) of different functional groups (green = endogeic and orange = epigeic). The size of the figures represents the relative frequency of reports for each taxon whilst the width of the links is the relative frequency of each pair of interactions ($n = 11$). Aptr, *Aporrectodea trapezoides*; Deve, *Dendrobaena veneta*; Eifo, *Eisenia fetida*; Eusp, *Eudrilus* sp.; Megu, *Metaphire guillelmi*; Peex, *Perionyx excavatus*; Poco, *Pontoscolex corethrurus.* The data is the same as that used in **Figure 4**.

Although the effect of earthworms on soil N dynamics may vary depending upon the species considered (Clause et al., 2014; Groffman et al., 2015), increases in mineral N in earthworm casts from the different functional groups have been consistently observed (Decaëns et al., 1999; Aira et al., 2005; Clause et al., 2014). Mineral N concentrations have been measured as 5-folds in casts of *P. corethrurus* when compared with those of the surrounding soil (Lavelle et al., 1992). Increases of 31 and 4% in soil NO_3^--N and NH_4^+-N, respectively, have also been observed in soils with the presence of *A. caliginosa* (McDaniel et al., 2013). The epigeic *E. fetida* also enhanced organic N mineralisation in the rhizosphere of *Phormium tenax*, a New Zealand lilaceous perennial (Zhong et al., 2017). The overall positive effect of earthworms on C and N mineralisation in the rhizosphere was shown by Wu et al. (2017) who demonstrated that *P. corethrurus* affected C and N processes and the soil microbial community in plots where living plants were present, in contrast to plots where artificial plants were used as controls. This was further confirmed by Athmann et al. (2017) who evidenced a positive effect of root and earthworm (*L. terrestris*) biopores, compared with the bulk soil, on the activity of several enzymes involved in the C and N cycle, resulting in an increase in nutrient mobilisation. These findings point out a positive interaction effect on nutrient mineralisation at the drilosphere and rhizosphere level, two hotspots of microbial activity in the soil. As recently highlighted by Bray et al. (2019), there is a stimulatory effect of earthworms and other soil macrofauna on rhizosphere microbial communities and on the microbially-mediated processes, particularly on N mineralisation and SOM formation.

The enhancement of C and N mineralisation by the earthworm-associated microbiota is mediated by an increasing enzyme activity. Some of the bacterial taxa that may be promoted by earthworms, such as *Pseudomonas* spp., have been associated with the production of enzymes involved in the degradation of complex organic molecules, which could favour

BOX 1 | Earthworms interact with mycorrhizal fungi

Beside soil bacteria, fungi are key organisms in the dynamics of soil biogeochemistry and its ultimate effect on plant growth. In contrast to with bacteria, much less information is available regarding their interactions with earthworms and the outcome of these interactions. Most of the attention, if not all in the fungi-earthworm interactions have been focused on arbuscular mycorrhizal fungi. In a similar way to the interaction with bacteria, the interactions between earthworms and mycorrhizal fungi, particularly arbuscular mycorrhizal fungi, have been found to modify the soil chemistry (Zhang et al., 2016, 2018) and soil nutrient availability (Milleret et al., 2009; Xiang and Li, 2014) and, critically important, the uptake of nutrients by plants (Milleret et al., 2009; Li et al., 2012, 2013a,b; Aghababaei et al., 2014) and the composition and abundance of the fungal community (Gormsen et al., 2004; Dempsey et al., 2013; Cao et al., 2015a,b,c, 2016, 2018; Zhang et al., 2016). Although the understanding of the interactions between earthworms and mycorrhizal fungi has not been the primary focus of most published works, there is a considerable amount of data that permits us to gain some insights on these interactions and their synergistic effects on plant performance (Wurst et al., 2004; Yu et al., 2005; Zaller et al., 2011; Li et al., 2013b).

The scientific interest in the interactions between earthworms and mycorrhizal fungi dates back almost 30 years and tackled the fundamental question of how the trophic activity of earthworms affects the availability of infective units of mycorrhizal fungi. While all studies focused on the abundance of spores of mycorrhizal fungi in earthworms' casts found a concentration effect, the density of spores in the casts was on average 66% higher than in surrounding non-earthworm processed soil (Gange, 1993; Harinikumar and Bagyaraj, 1994; Lee et al., 1996) and remained viable for up to a year (Reddell and Spain, 1991). Another investigation found no effect of earthworms in dispersing effectively the infective units of mycorrhizal fungi (Pattinson et al., 1997). However, it must be noted that the only investigation on the dispersion of mycorrhizal infective units by earthworms used a different species of earthworm (the endogeic *A. trapezoides*) compared to the many studies where concentration of the spores in the casts from different species were measured (the anecic *L. terrestris* and the endogeic *P. corethrurus*, *Ochochaetona phillotti,* and *Lampito mauritii*). As considerable variation has been reported among species of earthworms in their ability to concentrate infective units of mycorrhizal fungi, the lack of support for the capacity of earthworms to disperse the infective units of mycorrhizal fungi must be taken with caution until further investigation including the three functional groups of earthworms (anecic, endogeic, and epigeic) is carried out.

Over 60% of the reviewed cases reported that earthworm activities enhanced root colonization by mycorrhizal fungi while 25% reported a reduction in root colonization. It is worth noting that while for anecic and epigeic earthworms the ratio of positive to negative and neutral effects on root colonization by mycorrhizal fungi worked out to 3:1 and 4:1, respectively, for endogeic species this was inverted to a 1:2 ratio. The mechanisms that may differentially affect root colonization by mycorrhizal fungi when interacting with anecic/epigeic, and endogeic earthworms are poorly understood. Drilling by earthworms may damage the hyphal networks and fine roots of plants (Gange and Brown, 2002; McLean et al., 2006). Horizontal drilling of endogeic species may cause a more extensive disruption of the extraradical mycelium compared to the vertical burrowing of anecic species and this may affect the capacity of the fungi to colonize the roots. Cast deposits on the surface of the soil carried out by epigeic and anecic species may favour the dispersion of mycorrhizal infective units and this in turn may favour the colonization of roots. The unique investigation tackling the question of earthworms as dispersion agents of mycorrhizal infective units showed negative results and concordantly was carried out with endogeic species, which are frequently reported to decrease root colonization by mycorrhizal fungi. A likely explanation is that fungi are often considered as earthworm food (Curry and Schmidt, 2007; Shan et al., 2013). No data exists regarding the effectiveness of anecic and epigeic species in dispersing infective propagules of mycorrhizal fungi. Therefore, a comparative study of earthworms with different ecologies as dispersing agents of mycorrhizal infective units is needed. Additionally, earthworms may favour root colonization by mycorrhizal fungi indirectly by promoting particular groups of soil microorganisms that may cooperate with mycorrhizal fungi (Zhang et al., 2016). Whether earthworms of different behaviours could favour the proliferation of particular microbial groups that in turn facilitate the interaction between mycorrhizal fungi and plant roots is a totally unexplored area, although some efforts report correlative changes of Gram positive bacteria together with mycorrhizal fungi (Dempsey et al., 2013).

A major drawback in the investigation of the interaction between earthworms and mycorrhizal fungi is that only a handful of species of fungi have been used in the experimentation (*Rhizophagus intraradices, Rhizophagus irregularis, Funneliformis mosseae, Glomus geosporum, Glomus caledoniun, Glomus etunicatum, Claroideoglomus claroideum,* and *Acaulospora* sp.), all from the Glomeraceae. This means that our understanding of these interactions is rather partial and efforts need to be made to understand the dynamics of these interactions with a wider range of species of fungi from different families as it is known that there are marked differences in colonization rates, growth of extra-radical mycelium and capabilities to move soil nutrients to their host plants. Surprisingly, we are aware of no study documenting the role of earthworms as potential drivers of the composition and structure of mycorrhizal communities. Simple pot and mesocosm experiments with known initial composition of added mycorrhizal communities with and without earthworms will help to advance this field. This is important as a great deal of efforts are being made to include earthworms and mycorrhizal fungi in sustainable agricultural practices and we need to understand their fundamental interactions and outcomes.

SOM decomposition (Bertrand et al., 2015; Fjøsne et al., 2018). Enzymes produced by the earthworm-associated microbiota are also responsible for the reported increases in soil NO_3^--N and NH_4^+-N in the presence of earthworms. For example, the activity of the soil enzyme β-N-acetylglucosaminidase has been shown to be promoted in presence of *P. corethrurus,* which resulted in "NH_4^+-N hotspots" that might be accessed by arbuscular mycorrhizal fungi (**Box 1**), hence providing benefits for plant growth (He et al., 2018). Increases in phenol oxidase and glucosidase activity by earthworms and other macrovertebrates were also observed in the rhizosphere of *Festuca arundinacea* (Bray et al., 2019), and were attributed to the ingestion of fine roots and the stimulation of microbial activity during gut passage.

The reduction of microbial immobilisation has been suggested as another driver of enhanced N mineralisation by earthworms, which may ultimately lead to an increase in NO_3^--N leaching (Domínguez et al., 2004). Some authors, however, did not detect any earthworm effect on potentially mineralisable N (Fonte and Six, 2010) or, on the contrary, evidenced a decrease in N mineralisation by earthworms (Groffman et al., 2015), most likely due to an increase in microbial immobilisation that caused total soil N to decrease by 90 g N m^{-2} in presence of the epigeic *L. rubellus*. A possible explanation which has been proposed by several authors is that N mineralised by earthworms and their associated microorganisms might be used more readily by plants, thereby masking an increase in soil available N concentrations (Pashanasi et al., 1996; González and Zou, 1999; Wu et al., 2017).

Similarly, the amount of readily available phosphorus (P) has been shown to be affected by earthworms, levels of available P being higher in casts (Jiménez et al., 2003; Kuczak et al., 2006; Vos et al., 2014; Ros et al., 2017) or in biopores formed by *L. terrestris* (Athmann et al., 2017) than in the bulk soil.

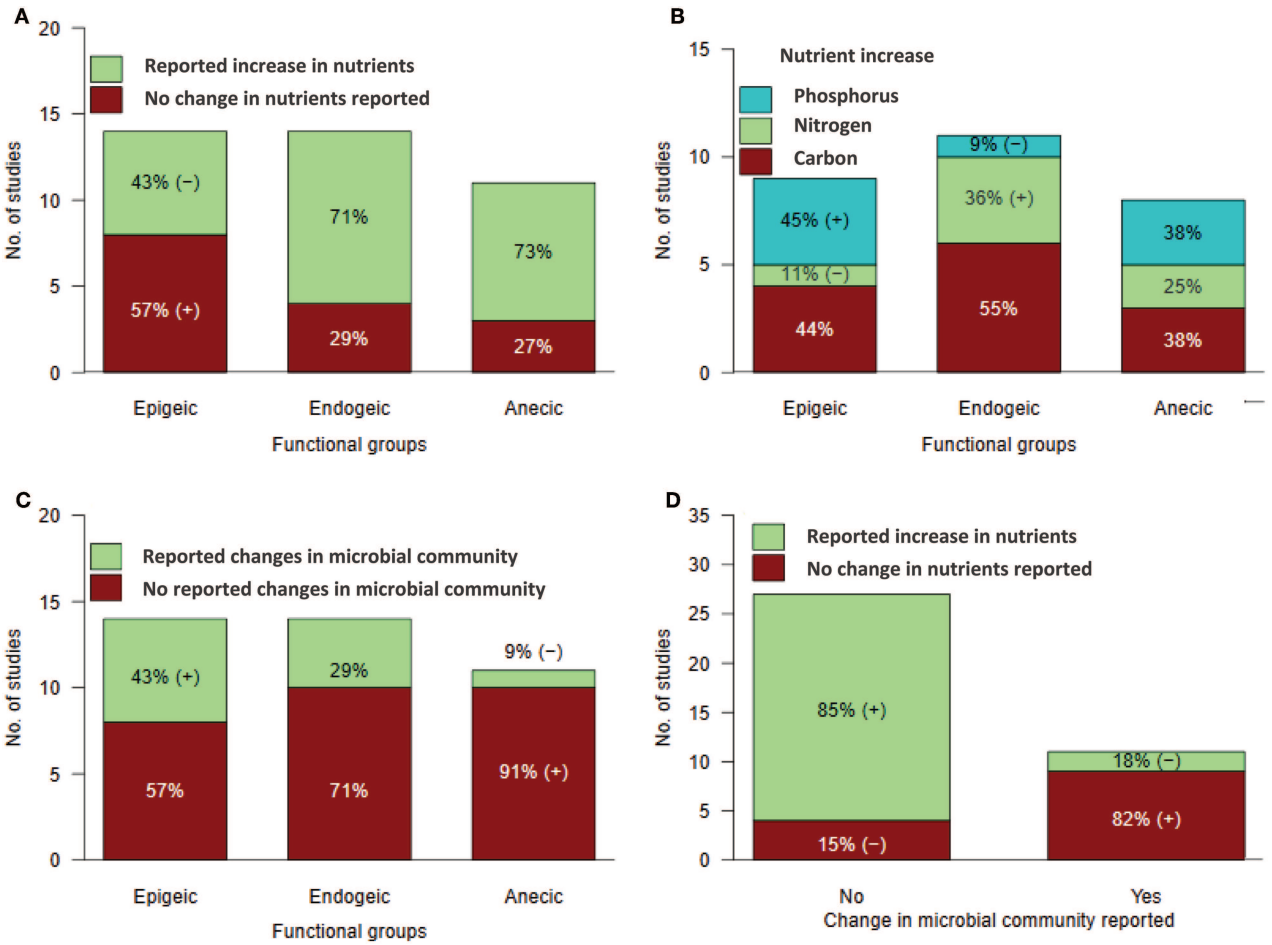

FIGURE 6 | Frequency of studies reporting: **(A)** overall changes in soil nutrients when processed by earthworms of different functional groups, **(B)** increments in carbon, nitrogen and phosphorus in the soil or substrates when processed by earthworms of different functional groups, **(C)** overall changes in the soil microbial community when the soil was processed by earthworms of different functional groups and **(D)** concurrence of data revealing changes in the soil microbial community and in the soil nutrients. Percentage values are relative to each bar. (+) and (–) indicate when the frequency of studies is higher or lower respectively than that expected under the null hypothesis: the chances of detecting nutrient increments are independent of the functional group of the earthworms. Data was obtained from 40 peer-reviewed publications retrieved after a search made using the words: "earthworms," "soil microbial communities," "nitrogen," "phosphorus," and "nutrient cycling" in the Web of Science from 2003 to 2018. In **Figure 6B** only 70% of the studies were taken into account as only increases in nutrients were considered.

Concentrations of water-extractable P in casts of the anecic earthworm *L. terrestris* have been reported to be 30–1000 times larger than those found in bulk soil (Ros et al., 2017). These earthworm-induced "P hotspots" depend upon the earthworm species and have been shown to be larger for the epigeic *L. rubellus* than for the anecic *L. terrestris* or the endogeic *A. caliginosa* (Vos et al., 2014). The influence of earthworms on available P is particularly relevant in the rhizosphere, where earthworms can interact with arbuscular mycorrhizal fungi to enhance P solubility and transfer to the plant (Milleret et al., 2009; Cao et al., 2015a) (**Box 1**). Soil available P has been reported to increase in the presence of the endogeic earthworm *P. corethrurus* (Lopez-Hernandez et al., 1993; Chapuis-Lardy et al., 1998; Patron et al., 1999), or of epigeic *E. fetida* (Cao et al., 2015a), which has been linked to the enhanced microbial activity during soil ingestion or in earthworm casts, although the magnitude of the increase in available P may differ depending on earthworm functional groups (Wan and Wong, 2004; Bernard et al., 2012; Vos et al., 2014).

Our synthesis of literature over the past 15 years revealed that endogeic and anecic earthworms induced an increase in soil nutrients in around 70% of the consulted studies; epigeic earthworms, however, only induced an increase in soil nutrients in 43% of the reported studies (**Figure 6A**). When analysing the effects of the different earthworm functional groups on particular nutrients (C, N, and P), more differences emerged. Epigeic earthworms were reported to increase P levels in the soil or substrate under study more frequently than expected under the null hypothesis, whilst endogeics were associated with N increases in the soil more frequently

than the other two groups of earthworms (**Figure 6B**). It is noteworthy that the reported increases in soil nutrients by earthworms were rarely related to changes in the soil microbial community (**Figures 6C,D**); moreover, when changes in microbial communities induced by earthworms were investigated, most studies (82%) did not report associated changes in soil nutrient contents (**Figure 6D**). Considering the functional groups of the bacterial phyla promoted by earthworms (mainly Proteobacteria, Actinobacteria, Firmicutes, and Acidobacteria) it is expected that the observed changes in nutrient availability associated to earthworms are at least in part caused by the metabolic activity of bacteria rather than by direct effects of the earthworms. This calls for more studies integrating earthworm effects on soil microbial communities at a taxonomic and functional level, to unravel the link between microbial diversity and ecosystem functions.

Earthworms Affect Microbial Functional Genes Involved in Nutrient Cycling

The influence of earthworms on nutrient cycling is not restricted to their impact on SOM mineralisation through an induced priming effect. Several studies have also demonstrated a direct effect on the expression of bacterial genes involved in the N cycle. Soil N transformations, and thus soil fertility, have often been investigated through the study of microbial functional genes, which emphasise their importance as functional genetic markers (Hosseini Bai et al., 2015; Ribbons et al., 2018).

Generally, the presence of earthworms has been associated with an increase in denitrification. The presence of the endogeic *P. corethrurus* was shown to increase the abundance of bacterial functional genes related to denitrification (*nirK* and *nosZ*) in the soil and in the rhizosphere (Chapuis-Lardy et al., 2010; Braga et al., 2016). Similar findings were found by Nebert et al. (2011) for the epigeic *L. rubellus*. The expression of the *nosZ* gene, which encodes for the nitrous oxide (N_2O) reductase, is directly linked with the amount of N_2O, an important greenhouse gas (GHG) of which earthworms are thought to be promotors (de Menezes et al., 2018). An increase in the abundance of the *nosZ* gene in the presence of earthworms may indicate the presence of larger denitrifying bacterial communities (Reverchon et al., 2015), which are known to be influenced by the quantity and composition of organic compounds resulting from the decomposition of organic residues (Kandeler et al., 2006). Earthworms and their associated microbiota, by promoting the decomposition of SOM, could therefore create soil conditions that are able to sustain more abundant denitrifier communities. Horn et al. (2003, 2006) indicated that the earthworm gut is a microenvironment ideal for N_2O-producing bacteria and that gut denitrifiers are probably soil-derived. However, increases in N_2O emissions and in the abundance of the gene *nosZ* seem to be species-dependent, as no effect of the endogeic *A. caliginosa* was detected on denitrification genes (Nebert et al., 2011). This is also consistent with results by Depkat-Jakob et al. (2010) who found that *nosZ*-containing taxa were not uniformly stimulated in the guts of worms from different feeding guilds. On the other hand, the anecic earthworm *Maoridrilus transalpinus* was shown to reduce N_2O emissions when associated with rhizobial bacteria, most likely due to the aerobic conditions created by burrowing, which are detrimental to denitrification (Kim et al., 2017). These contrasting findings may be due to the different experimental settings that were implemented to study the effect of earthworms on N_2O emissions. Lubbers et al. (2013) for example, conducted a meta-analysis showing that earthworms increase GHG emissions, in which most referenced studies are based on very short and simplified experimental set ups, in which there are no plants to uptake the mineralised N, which could indeed favour the emission of N_2O. Complexification of experimental set ups towards an integration of complex interactions between plants, macrofauna and microorganisms is therefore required in order to elucidate whether the presence of earthworms increase or decrease GHG emission in the long term.

Other microbial processes have also been reported to be positively affected by earthworms. Functional genes associated with carbohydrate and lipid metabolisms, biosynthetical pathways, translation, reduction-oxidation and cell proliferation processes were more abundant in the soil when *P. corethrurus* was present (Braga et al., 2016). The introduction of *P. corethrurus* also promoted microbial functions associated with plant-microbe symbiosis in the rhizosphere of sugarcane, such as plant cell colonization by N-fixing bacteria or plant growth regulation (Braga et al., 2016). Finally, despite the reported effect of earthworms on P mobilisation, no studies have yet investigated, to the best of our knowledge, how earthworms may alter microbial functional genes associated with the P cycle.

THE IMPACT OF EARTHWORMS ON SIGNAL MOLECULES PROMOTING PLANT GROWTH

The positive effects of earthworms on plant growth and yield are known to be related to improved soil physico-chemical variables, as earthworms facilitate the penetration of roots in the soil, the absorption of nutrients and the exchange of gases (**Figure 1**, arrow 1). Recently, these positive effects have also been attributed to the soil microbiota (**Figure 1**, arrows 2a and 2b), through the activation of microorganisms producing signal molecules.

Despite all the literature documenting the co-occurrence between changes in the N cycle by earthworms and their positive effect on plant growth (Van Groenigen et al., 2014), some studies suggest that an increased nutrient mineralisation is not sufficient to explain the effect of earthworms on plant growth by itself (Blouin et al., 2006; Laossi et al., 2010). There are other concomitant mechanisms, especially the emission of signal molecules (SM) in the presence of earthworms, which are involved in the effect of earthworms on plant growth (Puga-Freitas et al., 2012b) and help explaining the earthworms positive effects. SM are molecules with strong effects on plant physiology despite their presence at low concentration and are generally associated with qualitative changes. For example, SM are the main factors driving plant development and immunity (Taiz and Zeiger, 2010). In turn, these qualitative changes can induce

quantitative changes (e.g., growth). It is important to notice, that SM differ from nutrients which are constitutive of biomass, generally present at relatively high concentration and mainly responsible for quantitative changes.

It is widely accepted that SM are not exclusively produced by plants. They are also produced by almost all soil organisms, including soil fauna and microorganisms (Brito-Vega and Espinosa-Victoria, 2009; Puga-Freitas and Blouin, 2015). Multiple organic compounds are included in SM, such as sugars, organic acids and vitamins; these compounds are often involved in the initiation of signalling pathways leading to the production of phytohormones (auxins, gibberellins, cytokinins, ethylene, and abscisic acid), as well as secondary metabolites or volatile compounds that activate the plant's immune system or regulate its growth and development. Up to date, it is unclear if soil fauna is able to produce these SM by itself, or if it activates microorganisms that produce them. However, Puga-Freitas et al. (2012a) revealed that culturable microorganisms extracted from earthworm-worked soils where producing more indole acetic acid (IAA) (+46%) as compared with a control soil without earthworms, which supports the second hypothesis of a stimulation of bacteria (probably Plant Growth Promoting Bacteria, or PGPB) by earthworms.

Humic acids, IAA, aminocyclopropane-1-carboxylate (ACC), as well as molecules tentatively identified as auxins and ethylene have been reported as SM produced in the presence of earthworms, using indirect methods such as colourimetry. In many cases, their presence has been deduced from observations on plants that are similar to results observed in the presence of exogenous SM application. However, recent evidence shows that SM have been unequivocally identified by a reverse phase ultra-high-resolution liquid chromatography (UPLC) system coupled to a triple quadrupole mass spectrometry analyser, which allowed to determine the presence of jasmonic (JA), salicylic (SA), and abscisic acid (ABA) in vermicompost of *E. fetida* (Hernández, 2019). Nevertheless, the involvement of microorganisms in the secretion of these molecules was not elucidated with the exception of Pathma and Sakthivel (2013) who identified bacteria from *E. fetida* casts. Most of the studies investigating the chemical composition of SM in earthworm casts were conducted on epigeic earthworms, particularly on *E. fetida*, while one single paper studied the endogeic species *Aporrectodea caliginosa* (synonym *Nicodrilus caliginosus*) and *Aporrectodea rosea* (synonym *Allolobophora rosea*). Finally, all studies have solely been related to the casts of earthworms (**Table 1**). So far, there are no publications that confirm the isolation and unequivocal quantification of compounds such as auxins or gibberellins and the identity of the microorganisms associated with these molecules. Only two studies, at a 17-year interval (Canellas et al., 2002 and Hernández, 2019), provided an unambiguous identification of SM (humic acids with ABA, SA, and JA).

In the light of the new era of technology for the analysis and quantification of organic molecules, a new panorama opens to understand "the universe of molecules' diversity" of soil. More research is required to elucidate the most efficient extraction methods and identification of these molecules on earthworms or their casts. Transcriptomic approaches could also help unravel the microbially-mediated impact of earthworms on plant growth (Puga-Freitas et al., 2012b). Furthermore, considering the growing information available regarding earthworm-associated microbial communities, it is necessary to carry out more systematic research on the SM produced by microorganisms that are detected in earthworm digestive tracts, casts, and tunnels. *Pseudomonas* spp., for example, have been detected in the gut of *E. fetida* (Pathma and Sakthivel, 2013). Since *Pseudomonas* spp. have frequently been shown to emit SM that may promote plant growth, for instance through an induction of plant resistance to pathogens (Bloemberg and Lugtenberg, 2001; Pieterse et al., 2009; González et al., 2017); the combining next-generation sequencing with state of the art metabolomic tools may help understanding the joint effect of earthworm and PGPB on plant growth.

Regarding plant response to SM in the presence of earthworms, many observations of the "hormone-like effect" have been made with reference to vermicompost, for example increased growth and yield, development of flowers and fruits, and other processes related to tolerance to biotic and abiotic stresses (**Table 1**). In general, *E. fetida, A. caliginosa* and *A. rosea* are the earthworm species that have presented greater positive effects in plants, which has been attributed to the presence of IAA, ACC, and humic acids produced by their associated bacteria. Humic acids are SM extracted from vermicompost produced from cattle manure that also enhanced root growth and the number of sites of lateral root emergence in maize seedlings (*Zea mays*); these molecules were also shown to be responsible for a stimulation of the plasma membrane H+-ATPase activity (Canellas et al., 2002). Quaggiotti et al. (2004) reported an accumulation of H+-ATPase gene transcripts in the roots and an increase of nitrate transporter gene transcripts in the shoots of plants exposed to earthworm-producing humic substances. Using a transcriptomic approach for the screening of gene expression in *Arabidopsis thaliana*, Puga-Freitas et al. (2012b) found an accumulation of transcripts of 57 genes, most of which are known to be induced by exogenous hormone application or microbial elicitors. They also showed the reversion of the dwarf phenotype of an *A. thaliana* mutant for IAA transport in the presence of earthworms, suggesting that earthworms were compensating the low auxin level in root cells by producing auxin-like compounds in the soil, which were able to penetrate plant roots (Puga-Freitas et al., 2012b). Transcriptomic studies and exploration of plant signalling pathways using mutants could be developed for different stages of plant development to better characterize plant response to the presence of earthworms.

A hypothesis has been put forward that the activity of earthworms has a positive impact on plant growth through SM released in the soil. However, the literature is composed of many "chapters" (reported in **Table 1**) relying either on changes in the soil microbial community, an increase or decrease of SM or modifications in plant development or immunity. This is mainly due to the numerous scientific expertise required in soil chemistry, microbiology, plant physiology, and soil ecology. Therefore, nowadays there is no single study integrating all the chapters in a complete story.

TABLE 1 | Studies showing the effects of earthworms on plants under controlled conditions.

Earthworm species	Functional group	Area of influence	Microorganism species	Signal molecule released or related	Identification method	Effect of the molecule on plants	References
Eisenia fetida	Epigeic	Casts	Not reported	IAA and humic acids	Gas chromatography coupled to masses	Growth of corn seedling root (*Zea mays*)	Canellas et al., 2002
				IAA	Not reported	Growth regulator	Arancon et al., 2006
				Humic acids		Increased the development of flowers and fruits in peppers	
		Casts	*Pseudomonas aeruginosa, P. monteilii, P. fluorescens, Bacillus pumilis, B. subtilis, B. flexus, Microbacterium schleiferi, Acinetobacter calcoaceticus, A. baumannii, A. junii, A. schindleri, Stenotrophomonas maltophilia* and *Enterobacter cloacae*	IAA	Colorimetric method	Growth regulator	Pathma and Sakthivel, 2013
			Bacillus megaterium, B. thuringiensis, B. amyloliquefaciens, B. tequilensis, B. licheniformis, B. cereus, B. pumilus, B. aryabhattai, B. subtilis, B. flexus, B. aquimaris, B. marisflavi, Microbacterium takaoensis, Acinetobacter calcoaceticus, A. baumannii, A. junii, A. schindleri, Arthrobacter nicotianae, Rhodococcus ruber and *Enterobacter cloacae*	Aminocyclopropeno-1-carboxilate (ACC ethylene precursor)		Regulator of ethylene level in plants for optimal growth	
		Casts	Not reported	ABA	Identified unequivocally	Not reported	Hernández, 2019
				JA	through		
				SA	UPLC in a directed study		
*Nicodrilus caliginosus** and *Allolobophora rosea***	Endogeic	Casts	Not reported	Auxin-like	Root growth inhibition test	Root growth in *Daucus carota*	Muscolo et al., 1999
*Nicodrilus caliginosus** and *Allolobophora rosea***				IAA and low molecular size humic substances	Enzyme linked immuno-sorbent assay (ELISA)	Stimulates the uptake of nitrate by roots and the accumulation of the anion at the leaf level in *Zea mays*	Quaggiotti et al., 2004
Aporrectodea caliginosa				Similar to phytohormone, such as auxin and ethylene	Not reported	Increased total biomass and biomass production of *Lolium perennial* meristem	Puga-Freitas et al., 2012b

We highlight the compounds or signal molecules (SM) that, are responsible for the reported effects on plants.
**Synonym: Aporrectodea caliginosa. **Synonym: Aporrectodea rosea.*

CONCLUSIONS

Earthworms are known to play a critical role in ecological processes, through their improvement of soil structure, nutrient cycling and plant growth. Evidence also shows that earthworms contribute towards the structuring of soil microbial communities, either directly through their ingestion or indirectly through a priming effect resulting from an increase of available labile substances. However, few investigations have combined data on earthworm –microorganism interactions with studies on soil nutrient cycling, especially on P cycling, or the production of signal molecules, which prevents us to fully understand the mechanisms underlying the effect of microbial hotspots in the drilosphere on soil functioning. Our hypothesis "the effect of earthworms on nutrient cycling and plant growth is not only a direct effect but it is mainly mediated indirectly, via modifications of the microbial community" is largely verified at the small spatial and short temporal scale (gut, casts, burrows and tunnels).

Earthworms influence microbial biomass and activity in the soil but contrasting results can be found in the literature regarding the direction of this effect. This could be due to the nature of the organic matter earthworms feed on, particularly in the case of epigeic species or the substrate they live in. This could also be linked with experimental conditions, since the most variable effects on microbial abundance were observed in laboratory studies and consistent increases of microbial abundance by endogeic species were observed in the field (**Figure 2**). However, the effect on microbial communities is less or neutral when the feedstock or the soil they feed on is rich in assimilable organic matter independently of the functional group. Nonetheless, a recurrent result of our review is the relevance of considering earthworm ecological category (epigeic, anecic, or endogeic) to highlight some trends in the effect of earthworms on the structure and function of microbial communities. Complexification of experimental design, with interactions between earthworm ecological groups and the presence of plants should therefore be considered in mesocosm studies in order to better mimic natural conditions and avoid experimental artifacts.

Although there is still no clarity in understanding if earthworms have their own intestinal microbiome or it comes from the soil, most of the information says it comes from the soil. We can however stress on some general patterns: taxa such as Flavobacterium, Actinobacteria, Firmicutes, and γ-Proteobacteria are consistently reported to be promoted by gut transit and could therefore constitute good indicators for predicting the impact of earthworms on soil processes. The increasing use of Next Generation Sequencing (NGS) technologies in the study of soil microbial communities and their diversity will help to refine our understanding of how earthworms may shape them. Information is also critically needed regarding the role of earthworms as potential drivers of the composition and structure of fungal communities, particularly mycorrhizal fungi because of their direct interaction with plants (**Box 1**). On the other hand, saprotrophic fungi, like many bacteria, are key drivers of soil biogeochemistry but their roles have been largely neglected especially in their synergistic or antagonist interactions with earthworms.

Earthworms promote the mineralisation of N and P and alter microbial functional genes which modifies soil functions. More information is needed to understand which microorganisms and microbial genes are activated by earthworms, especially on the P cycle.

Finally, the consequences of these earthworm-induced changes in soil functioning on plant growth cannot be fully understood without the study of SM, produced either by the earthworms or most likely by microorganisms created by earthworm activity. The involvement of specific microbial taxa in the secretion of these molecules needs to be elucidated and this requires a collaborative effort from disciplines such a metabolomics, microbiology, transcriptomics, and biochemistry in order to unequivocally identify SM in earthworms or in their casts.

To plagiarize Aristotle, earthworms are indeed the intestine of the Earth, with their specific microbiota, which brings us to a large spatial and temporal scale. This intestine is complex to understand because of its dynamics associated with the activity of earthworms, other soil organisms and plant roots. Nevertheless, we have to consider these complex effects of earthworms on microbial communities in order to understand the effect of earthworms on nutrient cycling and plant growth promoting SM and ultimately to predict plant-soil interactions, especially if earthworms ingest hundreds or thousands of tons of substrates or soil per hectare and per year.

AUTHOR CONTRIBUTIONS

RM-S and RG did the bibliometric data analysis. FR, AD, JG-A, MÁ-J, MB, RM-S, RG, and IB contributed with the writing of different sections. LV and CC gave ideas for the structure and illustrations of the article and reviewed the manuscript. MÁ-J and MB designed and produced the illustrations. MB gave constructive suggestions throughout the work to organise the manuscript. FR and IB coordinated the work and integrated and edited the different sections of this article.

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Soil Health Changes Over a 25-Year Chronosequence From Forest to Plantations in Rubber Tree (*Hevea brasiliensis*) Landscapes in Southern Côte d'Ivoire: Do Earthworms Play a Role?

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The agro-ecological drawbacks of the spread of rubber tree plantations in Côte d'Ivoire since the 1990's are obvious even though they have not been properly investigated. They consist of biodiversity loss, land degradation and food insecurity, which have extended into the existing cocoa-led degraded areas whose rehabilitation have unfortunately not started. This situation increases not only the threat on soil health status but also undermines the capability of soils to deliver ecosystem services that are key to sustainable agricultural production. The current study took advantage of a chronosequence in rubber tree landscapes to assess soil health deterioration in general and possibly earthworm-mediated role in soil health changes. The hypothesis underpinning this study was that earthworms contribute to mitigate soil health deterioration in rubber-dominated landscapes due to their key role in soil functioning. This study confirmed that the conversion of forest to rubber tree plantations significantly impaired all soil biological, physical, and chemical parameters at the beginning (7 years) of the chronosequence; followed further by a restorative trend taking place beneath the plantations from 12 years. However, this study failed to find evidence of a direct role of earthworms in soil health rehabilitation over time. Mesoscale studies along with the use of appropriate models could help unravel this "black box" and shed some light on the contribution of earthworms as key soil ecosystem engineers.

Keywords: biodiversity, earthworms, functional groups, land use change, soil degradation, soil threats, rubber tree plantations

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INTRODUCTION

In the last two decades, replacement of degraded lands by rubber tree plantations has become common in the humid and sub-humid areas of Côte d'Ivoire. The overwhelming presence of these new tree plantations in the agro-ecological landscapes is due to its huge economic returns in lieu of the low profitability of cocoa due to falling world market prices (Ruf, 2012). As a result, smallholder farmers seeking livelihood improvement have entered the scene by (i) replacing their old cocoa or coffee plantations with rubber trees, and (ii) converting the remaining portions of secondary forests and fallow lands into rubber stands. This has significantly contributed to an over 99% rise in the area and production of rubber tree plantations at the national level since 1960 (FAOSTAT, 2016). The immediate outcome is the rise of Côte d'Ivoire to the top as Africa's leading rubber producer with a total production share of 45.9% (FAOSTAT, 2016) The total area and production are estimated at 189,937 ha and 310,655 tons, respectively. It is well-documented that the conversion of natural ecosystems to rubber tree stands resulted in agro-ecological and environmental drawbacks in areas of the world where the cultivation is possible. They include loss of

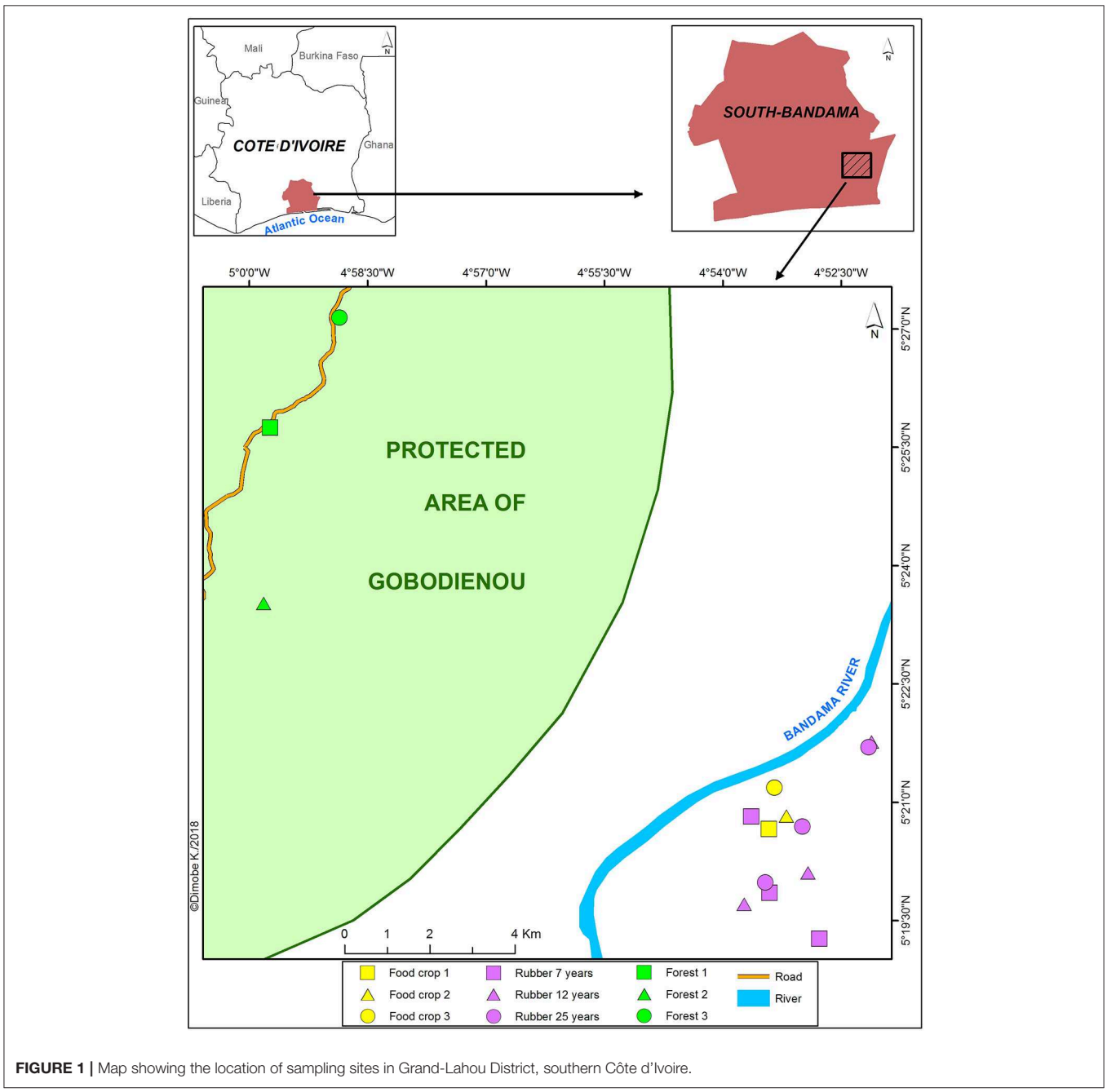

FIGURE 1 | Map showing the location of sampling sites in Grand-Lahou District, southern Côte d'Ivoire.

biodiversity (Pia and Konrad, 2015), deterioration of soil quality and reduction of soil organic carbon stocks (Oku et al., 2012; de Blécourt et al., 2013), acute soil erosion, disruption of streams, and risk of landslides (Fox et al., 2014). In other words, as a land-use system characterized by sequential changes in space and time, monoculture rubber tree farming has the potential to increase the magnitude of threats to soil over time (Günal et al., 2015). Soil threats due to land use change and exploitation are summarized in 10 main groups out of which soil erosion, soil organic carbon (SOC) deterioration, nutrient imbalance, loss of soil biodiversity, and soil compaction are the most important (FAO and ITPS, 2015).

It is now well-acknowledged that soils are self-organized ecological systems within which organisms (microorganisms, predators organized in micro food webs, and ecosystems engineers) interact in a nested suite of discrete scales (Lavelle, 1997; Lavelle et al., 2016). Soil ecosystem engineers composed mostly of earthworms, termites, and ants play key roles in creating habitats for other organisms and controlling their activities through physical and biochemical processes (Lavelle, 1997; Jouquet et al., 2006). Furthermore, they contribute to deliver ecosystem services through three different processes (Puga-Freitas et al., 2012; Puga-Freitas and Blouin, 2015; Lavelle et al., 2016): (i) the organization of soil physical structure and associated ecosystem services, (ii) the selection and activation of plant, microbial and smaller invertebrate communities that determine decomposition and nutrient cycling processes, and (iii) the release of hormones that regulate primary production.

All terrestrial ecosystems consist of aboveground and belowground components that interact to influence community and ecosystem-level processes and properties (Wardle et al., 2004). This is particularly true in rubber tree landscapes presumably characterized by the successional replacement of land use and land cover that drive above and belowground interactions. This change also undermines the deliverance of soil-based ecosystem services which are driven by soil organisms among which soil macroinvertebrates play a critical role like any other land uses around the world (Spurgeon et al., 2013; Franco et al., 2016; de Valença et al., 2017).

One way of assessing the reaction of soil systems to the perturbations brought about by rubber tree plantations is to assess the health of soil in these derived landscapes. Such an assessment should be integrated and holistic including physical, chemical, and biological components. The latter comprise key organisms and community structure with a special reference to their interactive feedback with abiotic parameters. Earthworms are key soil macroinvertebrates due to their contribution to the functioning of ecosystems (Blanchart et al., 1999, 2004; Guéi et al., 2012; Blouin et al., 2013; Fischer et al., 2014) and plant productivity (van Groenigen et al., 2014; Xiao et al., 2018) which are very well-documented. These findings mostly from studies carried out in controlled conditions (laboratory, mesocosms, etc.) are indicative of the potential interactive feedback between earthworms and soil processes. Furthermore, semi-natural studies in mesocosms have revealed that tropical earthworms are organized in ecological groups composed of detritivores and geophageous polyhumics, mesohumics and oligohumics according to their feeding behavior (Lavelle, 1981). There are also two main groups, compacting, and decompacting, based on their physical impact on soils (Blanchart et al., 1999, 2004; Guéi et al., 2012). To date, studies have rarely attempted to investigate the extent to which interactive feedbacks between earthworms and soil processes influence soil health in field conditions. The successional stages of different land use along a chronosequence in rubber tree landscapes offer the framework to such a study. This study aims to investigate earthworm-mediated role in soil health changes beneath rubber tree plantations as compared to baseline forests. The hypothesis underpinning the current study is that earthworms help mitigate soil health deterioration in rubber-dominated landscapes through their functional impact on soil chemical and physical characteristics.

METHODS

Study Site

The study was conducted in the village Tiéviessou (Latitude: 5°08′13″N; longitude: 5°01′26″W; elevation: 6 m) located in Grand-Lahou District, southwestern Côte d'Ivoire. This region is characterized by a bimodal humid tropical climate marked by two rainy seasons and two dry seasons with steady significant seasonal variation in the past two decades. The total annual rainfall and average temperature of the study year (2013) were 1085.35 mm and 26.9°C, respectively. The study site is in the Guinean domain and belongs to the ombrophilous area characterized by dense evergreen forests (Guillaumet and Adjanohoun, 1971). The area has experienced tremendous human pressure leading to human-derived landscapes made of degraded secondary forests, plantations of oil palm, rubber tree and cocoa along with food crops. However, a portion of the landscape has been reserved as a protected area known as the Classified Forest of Gobodienou (**Figure 1**). This landscape is highly irrigated due to the presence

TABLE 1 | Characteristics of sampling sites.

Land use type	Age (year)	Surface (ha)	Land-use history	Fertilization status
Secondary forest	-	2	Primary forest	NA
Secondary forest	-	2	Primary forest	NA
Secondary forest	-	2	Primary forest	NA
Food crops	1	0.5	Fallow	No
Food crops	2	0.5	Fallow	No
Food crops	3	0.33	Fallow	No
Rubber tree	7	1.5	Palm tree plantation	Yes
Rubber tree	7	1.5	Coffee plantation	Yes
Rubber tree	7	1	Coffee, palm tree	Yes
Rubber tree	12	0.5	Palm tree plantation	Yes
Rubber tree	12	1	Secondary forest	Yes
Rubber tree	12	1	Cocoa plantation	Yes
Rubber tree	25	2	Palm tree plantation	Yes
Rubber tree	25	0.7	Secondary forest	Yes
Rubber tree	25	1	Secondary forest	Yes

NA, not applicable.

of the Bandama river, and lagoons with mangrove vegetation at the edges. The vegetation is mostly composed of *Diospyros* spp. (Ebenaceae) and *Mapania* spp. (Cyperaceae) (Eldin, 1971). The main soil type are Ferralsols (World Resource Base, 2006) with a sandy loam texture.

Sampling Design

To meet the objective of the study, sampling plots were selected to capture the most representative features of the rubber tree (*Hevea brasiliensis*) landscapes together with portions of the baseline ecosystem along a chronosequence: food crops (cassava) of 1 to 3-year-old; 7, 12, and 25-year-old smallholder plantations around Tiéviéssou village and the two settlements, Agnouanssou and Betesso (**Table 1**). Smallholder famers who are owners of these plantations use very few inorganic inputs in general. A survey conducted at the onset of this study revealed that they only used inorganic fertilizers including urea and NPK as inputs in the early stages of the plantations to help trees grow smoothly. The chronosequence that is a set of rubber tree plantations in the landscape that share similar attributes but with different ages, was found to be most relevant approach in line with the objective of this study as the initial date of the disturbance and subsequent history of the sites were known (Walker et al., 2010; Zhou et al., 2017). The general assumption supporting chronosequence-based study uses natural forests as baseline ecosystems from which all man-made systems were at first derived. In most cases, the cycle of smallholder plantations starts with food crops that are grown in association with planted rubber trees which will form the basis of monospecific plantations 3 years later. The 7-year-old plantations represent the initial stage of production of the plantation, the 12-year-old ones are halfway of their productive life and the 25-year-old plantations are considered as fully mature because the complete production cycle of a plantation can reach 40 years. In addition to plantations, secondary forests selected from the protected area were considered as baseline ecosystems.

By a stratified sampling approach, three sampling plots of size 10 m × 50 m each, were used as replicates and selected afterwards in each land use type (LUT) such that the total number of plots amounted to 15. In each plot, five sampling points of which one was placed at the center point and the remaining at the four corners, were established. Hence, in total, 15 plots were selected along the chronosequence (forest, food crops, rubber 7, rubber 12, and 25 years) and geo-referenced using GPS.

TABLE 2 | Occurrence (1, presence; 0, absence) of earthworm species and ecological categories in the rubber tree plantation landscapes.

Family	Species	Functional group	Forest	Food crops	Rubber 7 y	Rubber 12 y	Rubber 25 y
Glossoscolecidae	*Pontoscolex corethrurus* (Bather, 1920)	Geophageous polyhumic	0	0	0	1	0
Acanthodrilidae	*Millsonia Omodeoi* (Sims, 1986)	Geophageous mesohumic	1	1	1	1	1
	Millsonia sp.		0	1	0	0	1
	Dichogaster baeri (Sciacchitano, 1952)	Detritivore	1	0	1	1	1
	D. terrae-nigrae (Omodeo and Vaillaud, 1967)	Geophageous oligohumic	0	0	1	0	0
	D. saliens (Beddard, 1893)	Geophageous mesohumic	1	1	0	1	1
	D. erhrhardti (Michaelsen, 1898)	Detritivore	1	0	0	0	1
	D. papillosa (Omodeo, 1958)	Detritivore	1	0	1	1	1
	D. eburnea (Csuzdi and Tondoh, 2007)	Detritivore	1	0	1	1	1
	D. mamillata (Csuzdi and Tondoh, 2007)	Detritivore	0	0	1	0	0
	D. leroyi (Omodeo, 1958)	Detritivore	0	0	0	1	1
	Dichogaster sp1	Detritivore	1	1	0	1	1
	Dichogaster sp2	Detritivore	1	0	0	1	1
	Dichogaster sp3	Detritivore	0	0	1	1	0
	Agastrodrilus multivesiculatus (Omodeo and Vaillaud, 1967)	Geophageous oligohumic	0	1	1	1	0
	Agastrodrilus opisthogynus (Omodeo and Vaillaud, 1967)	Geophageous oligohumic	0	0	0	0	1
Eudrilidae	*Scolecillus compositus* (Omodeo, 1958)	Geophageous polyhumic	1	1	0	0	0
	Stuhlmannia zielae (Omodeo, 1963)	Geophageous polyhumic	1	1	1	1	1
	S. palustris (Omodeo and Vaillaud, 1967)	Geophageous polyhumic	1	0	0	0	0
	Eudrilus eugeniae (Kinberg, 1867)	Detritivore	0	0	1	0	0
	Hyperiodrilus africanus (Beddard, 1891)	Geophageous polyhumic	1	0	1	1	1
	Hyperiodrilus sp.	Geophageous polyhumic	1	0	0	0	1
	Gordiodrilus paski (Stephenson, 1928)	Geophageous polyhumic	0	1	1	1	1
Ocnerodrilidae	*Ocnerodriliae* sp.	Geophageous polyhumic	1	0	1	1	1
Total species			14	8	13	15	16

Rubber 7 y, 12 y, 25 y stand for 7, 12, 25-year-old rubber tree plantations.

Soil health indicators that are sensitive to land use changes and related to some key soil functions such as nutrient cycling, soil structure regulation, soil biodiversity conservation (Schulte et al., 2015) were measured. Chemical parameters namely pH-H_2O (pH), soil organic carbon (SOC) and macronutrients including total nitrogen (N), total phosphorus (P) and exchangeable potassium (K) were measured. Physical parameters, namely bulk density, aggregate size distribution, and stability were measured. Ecological metrics (density, biomass, species richness, and diversity) and community structure of earthworms were considered as proxies of soil organisms.

Field campaigns to collect data pertaining to soil health indicators were conducted from mid-September to mid-November 2013, corresponding to the short rainy season, which is the most suitable period for earthworm collection.

Soil Sampling and Chemical Analyses

In each plot, soil samples were collected from five distinct points: one at the center, four points at 2 m and arranged in the 4 cardinal points. The samples were collected from topsoil (0–20 cm) using an auger. They were thereafter pooled and thoroughly mixed as a single composite sample. Each composite sample was used as replicates and thus totaling 15 samples per LUT, giving 75 (5 × 15, as we have 5 LUT) in the study area. Samples were air-dried for a week and homogenized using a 2-mm mesh sieve. An aliquot of 100 g of the fine fraction was used and analyzed for pH-H_2O (pH), soil organic carbon (SOC) and nutrients including total nitrogen (N), total phosphorus (P), exchangeable potassium (K) determination. SOC concentration was measured using the modified method of Anne (Nelson and Sommers, 1982) while N content was extracted by the method of Nelson and Sommers (1980) and determined using the Technicon autoanalyzer (Technicon Industrial Systems, 1977). Phosphorus (P) was measured by colorimetry following nitriperchloric acid digestion and subsequent molybdenum-blue color development (Olsen and Sommers, 1982). Potassium (K) was extracted using ammonium acetate buffer (pH 7) and determined by means of atomic absorption spectrophotometry techniques (Anderson and Ingram, 1993).

FIGURE 2 | CA plot showing the distribution patterns of earthworm species according to the forest (blue curve), rubber 25 years (green curve), rubber 12 years (red curve), and rubber 7 years and food crop (yellow curve). *Stuh_palu, Stuhlmannia palustri; Dich_erhr, Dichogaster erhrhardti; Dich_sp2, Dichogaster sp2; Dich_papi, D. papillosa; Dicho_baeri, D. baeri; Scol_comp, Scolecillus compositus; Agas_ops, Agastrodrilus opisthogynus; Dicho_Sal, Dichogaster saliens; Dicho_sp1, Dichogaster sp1; Ocne_sp, Ocnerodriliae* sp., *Agas_mult, Agastrodrilus multivesiculatus; Stuh_ziel, Stuhlmannia zielae; Dicho_ebur, Dichogater eburnea; Dicho_lero, D. leroyi; Dicho_sp3, Dichogaster sp3; Pont_core, Pontoscolex corethrurus; Mill_sp, Millsonia sp; Mill omod, Millsonia omodoei; Dicho_terr, Dichogaster terraenigrae; Hyper_afri, Hyperiodrilus africanus; Eudr_euge, Eudrilus eugeniae; Gord_paski, Gordiodrilus paski*.

Soil Physical Property Measurement

Bulk Density

Soil samples were collected using the cylinder in layers 0–10 cm and 10–20 cm. The bulk density (BD) was calculated at laboratory depending on the inner diameter of the core sampler, sampling depth and the oven dried weight at 105°C. Soil water content was measured gravimetrically and expressed as a percentage of soil water to dry soil weight.

Aggregate Size Distribution and Mean Weight Diameter (MWD)

Soil aggregate distribution was determined using the dry-sieving method (Gilot, 1994) consisting of soil cores collection from each LUT using a cylinder in 0–10 cm and 10–20 cm layers and air-dried to a moisture content of about 5% of the dry weight. The total mass was weighed and identified aggregates further broken by dropping the dry soil blocks from a constant height (1 m) onto a hard surface. Subsequently, the samples were successively placed on a set of six stacking sieves of different meshes (50, 100, 200, 500, 1,000 and 2,000 mμ in ascending order resulting in six aggregate size fractions: <50 mμ, 50–100 mμ 100–200 mμ, 200–500 mμ, 500–1,000 mμ, 1,000–2,000 mμ and >2,000 mμ). All fractions were weighed, and data analyzed to compute the proportion of aggregate distribution and the mean weight diameter (MWD). MWD is used to measure soil structural stability (Ge et al., 2018). This index is calculated as follows:

$$\mathrm{MWD} = \sum_{i=0}^{n} \frac{w_i}{100} \bar{x}_i \text{ where,}$$

- MWD is the mean weight diameter (mm),
- n is the number of aggregate fractions (five),
- x_i is the mean diameter of the ith fraction
- w_i is the weight of soil in the fraction i expressed as a percentage of the dry soil mass.

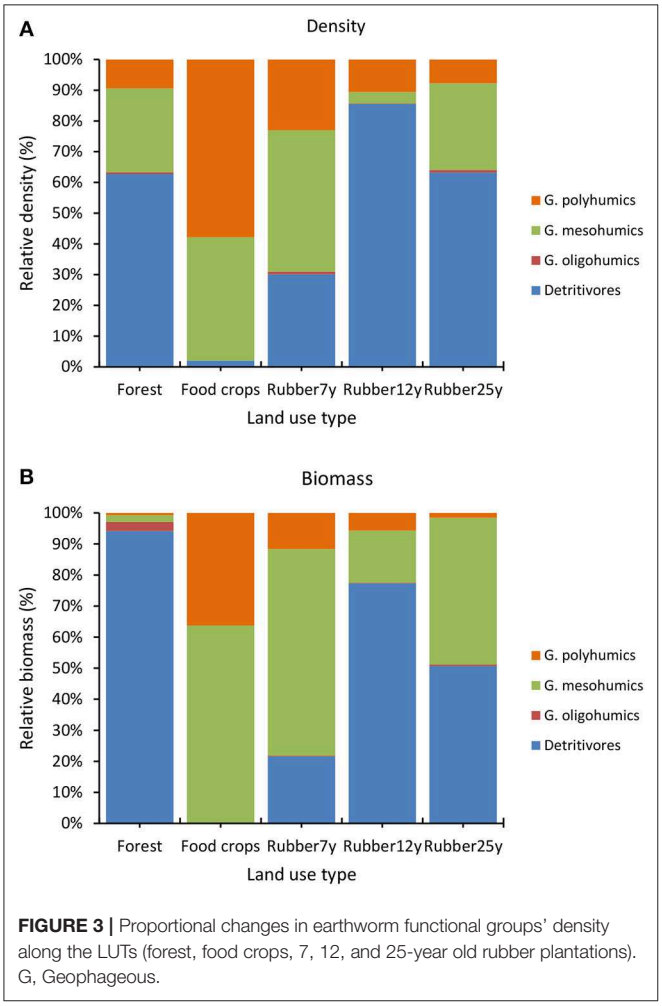

FIGURE 3 | Proportional changes in earthworm functional groups' density along the LUTs (forest, food crops, 7, 12, and 25-year old rubber plantations). G, Geophageous.

Sampling and Identification of Earthworms

Earthworms were sampled using a modified method recommended for tropical soils (Anderson and Ingram, 1993), which involves digging of 5 monoliths (25 × 25 × 30 cm) along a transect stretching across the sampling plot. Since we were not concerned with the vertical distribution of earthworms, the modified size and depth of the monolith of this sampling scheme was used in this study. A soil monolith (50 × 50 × 20 cm) was dug out at each sampling point and used as replicates in each plot with 5 replicates in total per plot and thus 15 per LUT. The monolith was surrounded by a trench of 20 cm depth preventing earthworms from escaping. The sampled soil blocks were deposited in a bucket and specimen were collected by hand-sorting in trays according to the layers (0–10 cm and 10–20 cm) and were preserved in 4% formaldehyde solution. Earthworm specimen were identified to species level (Tondoh and Lavelle, 2005; Csuzdi and Tondoh, 2007), counted, weighed and further allocated into four functional groups. The most common accepted ecological classification is a division in three groups (Bouché, 1977); anecics (vertical burrowers), epigeics (litter layer/surface inhabitants), and endogeics (mineral soil inhabitants). Later on, based on their feeding behavior and ecological characteristics (Lavelle, 1981), provided a nomenclature fitting the tropical context as follows: detritivores and geophageous polyhumics, mesohumics and oligohumics. Detritivores are litter feeders, which feed at or near the soil surface on plant litter. Geophageous earthworms feed deeper in the soil and derive their nutrition from soil organic matter and dead roots ingested with mineral soil (Lee, 1985). Owing to their dependence on soil organic matter (Lavelle, 1981), geophageous earthworms were further divided into three groups: the polyhumics, which feed on decaying residues mixed with little mineral soil, the mesohumics, which feed on soil fairly rich in organic matter, and the oligohumics, which feed on organic matter-poor soil.

DATA PROCESSING AND ANALYSIS

To characterize earthworm community structure in the different LUTs, a Correspondence Analysis (CA) was performed on the matrix of species abundance per LUT using the FactoMineR and factoextra packages in R statistical software. The CA uses

a simple Chi square statistic to test for significant dependence between species and LUTs. It also provides the factor scores for species and LUTs, which we used to represent their association graphically. The distance between species is indicative of their similarity (or dissimilarity) in the LUTs space and was thus used to associate each species to each LUT. For each LUT, diversity indices (species richness, Shannon–Wiener and Evenness indices) were computed.

The significance of the effects of land use change on earthworm ecological metrics (density and biomass, species richness, diversity indexes) and soil organic carbon (SOC) was tested using a separate Generalized Linear Mixed effects models (GLMM). LUT effects were considered as fixed, and soil chemical parameters (total N, P, K, and pH) as random factors to account for unknown heterogeneity effects. SOC was analyzed as continuous variable, and thereafter applied GLMM to Gaussian distribution after log-transformation. Population density and biomass, and species richness were modeled as count data. The over-dispersion in earthworm density and biomass was tested using the *qcc* package in the R software. In cases of over-dispersion, the fits of Poisson regression and quasi-Poisson regression were compared with negative binomial regression. Parameters of the mixed-effects models were estimated using *lme4* package with the restricted maximum likelihood (REML) method (Bates et al., 2015), and *p*-values computed based on the Satterthwaite approximations to the degrees of freedom in the *lmerTest* package (Kuznetsova et al., 2016). The conditional (variance explained by fixed and random factors) and marginal (variance explained by fixed effects only) R^2-values were calculated following Nakagawa and Schielzeth (2013).

The possible sources of variation in soil chemical and physical characteristics were further investigated focusing on species richness, earthworm density, earthworm biomass and land use type. Using boxplots, we first examined the effect of LUTs on earthworm communities and on soil chemical and physical characteristics. The R package *ggpubr* was used to compare means. Correlations between earthworm density and soil variables were determined by Pearson's correlation coefficient.

A Biplot Principal Component Analyses were performed using the *FactoMineR* package to determine the relationships that could exist between earthworm species along with functional groups and soil parameters.

Soil Health Deterioration Index

Changes in soil health along the chronosequence from forest to aged plantations were assessed using degradation/deterioration indices (DIs). Soil degradation index for each soil property was calculated as the difference between mean values of individual

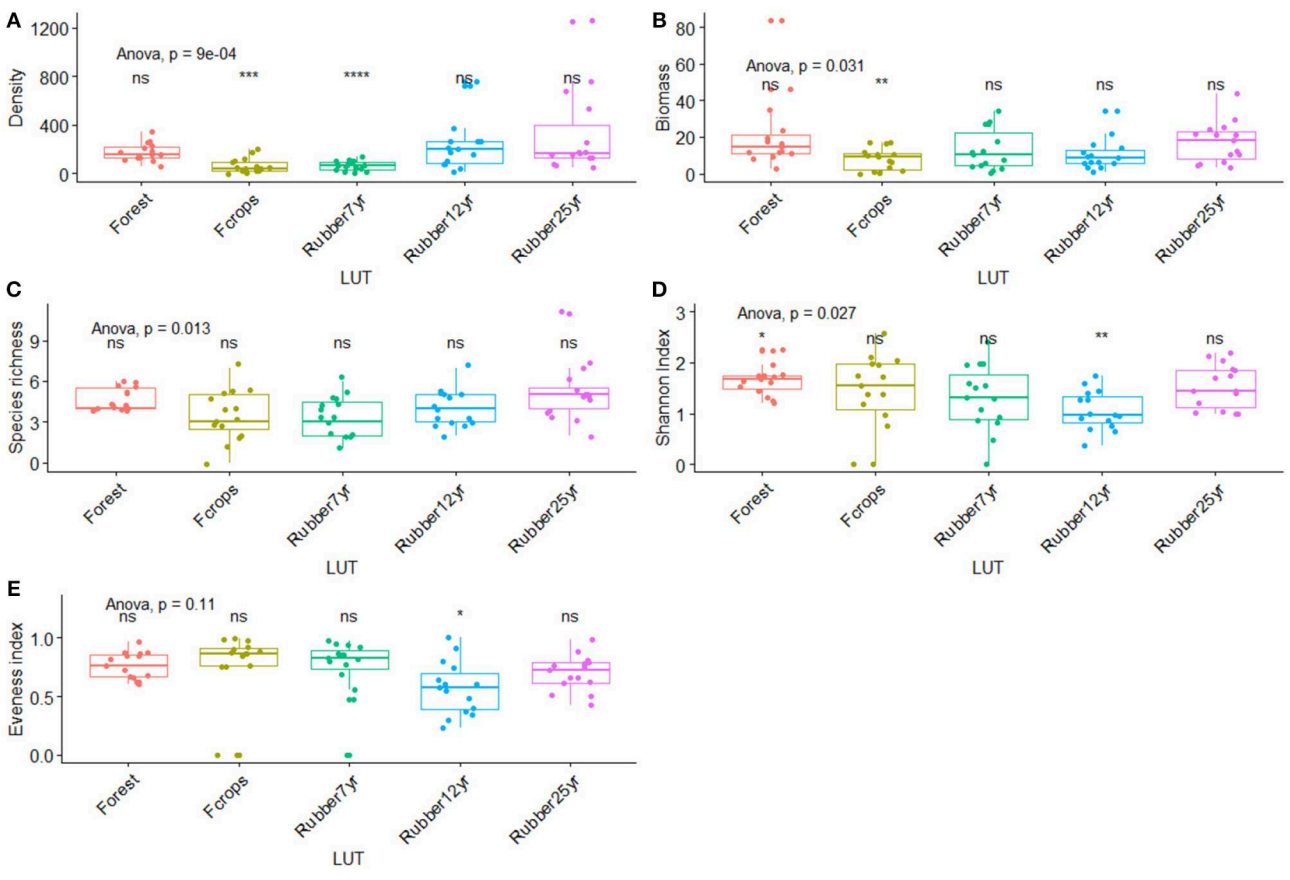

FIGURE 4 | Changes in earthworm density, biomass, species richness, Shannon, and Evenness indices along the rubber tree chronosequence. **(A)** Density (ind m^{-2}). **(B)** Biomass (g m^{-2}). **(C)** Species richness. **(D)** Shannon index. **(E)** Eveness index. $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$; $^{****}p < 0.00001$.

soil properties under each LUT and the reference values of corresponding soil parameters in the baseline secondary forest (Lemenih et al., 2005; Dawoe et al., 2014; Tondoh et al., 2015). This index is expressed as a percentage of the mean values under the natural ecosystem. Furthermore, a cumulative DI was obtained by summing up the resultant positive and negative DI's of the individual soil properties for each farm field to be used as an index of soil quality responses (either degradation or improvement) to forest clearing and subsequent cultivation. Soil pH values were not considered in this calculation because the criteria of "more is better" is not true or uncertain over the range of values found in this study (Islam and Weil, 2000).

Interactive Feedback Between Earthworms and Soil Properties

Structural equation modeling was used to investigate the relationships between the abundance of earthworm functional groups (See **Dataset S1**) and soil properties including pH, soil organic carbon, total nitrogen, total phosphorus, mean weight diameter and bulk density (See **Dataset S2, S3**). Two models were considered: the first evaluating the direct and indirect effect of soil properties (here considered as reflective indicators) on earthworm density through correlation path analysis; and the second looking at the feedback effect of earthworms on soil health (expressed as latent variable manifested by soil properties). Data were standardized to homogenize measurement scales and to keep the same magnitude order between the observed variances. SEM was carried out with the package "Lavaan" (Rosseel, 2012), and path diagrams were generated with the package "semPlot" (Epskamp, 2015). Evaluation of the model fit was based on the analysis of Chi square, Tucker-Lewis index (TLI), comparative fit index (CFI), and mean square error of approximation (RMSEA).

All statistical analyses were carried out using the R software (R Core Team, 2018).

RESULTS

Community Structure of Earthworms in the Landscape

Up to 24 earthworm species belonging to four families, namely Glossoscolocidae, Acanthodrilidae, Eudrilidae, and Ocnerodrilidae, were collected in the entire landscape (**Table 2**). The Acanthodrilidae family harbored the most important community composed of 15 native species while the Eudrilidae's

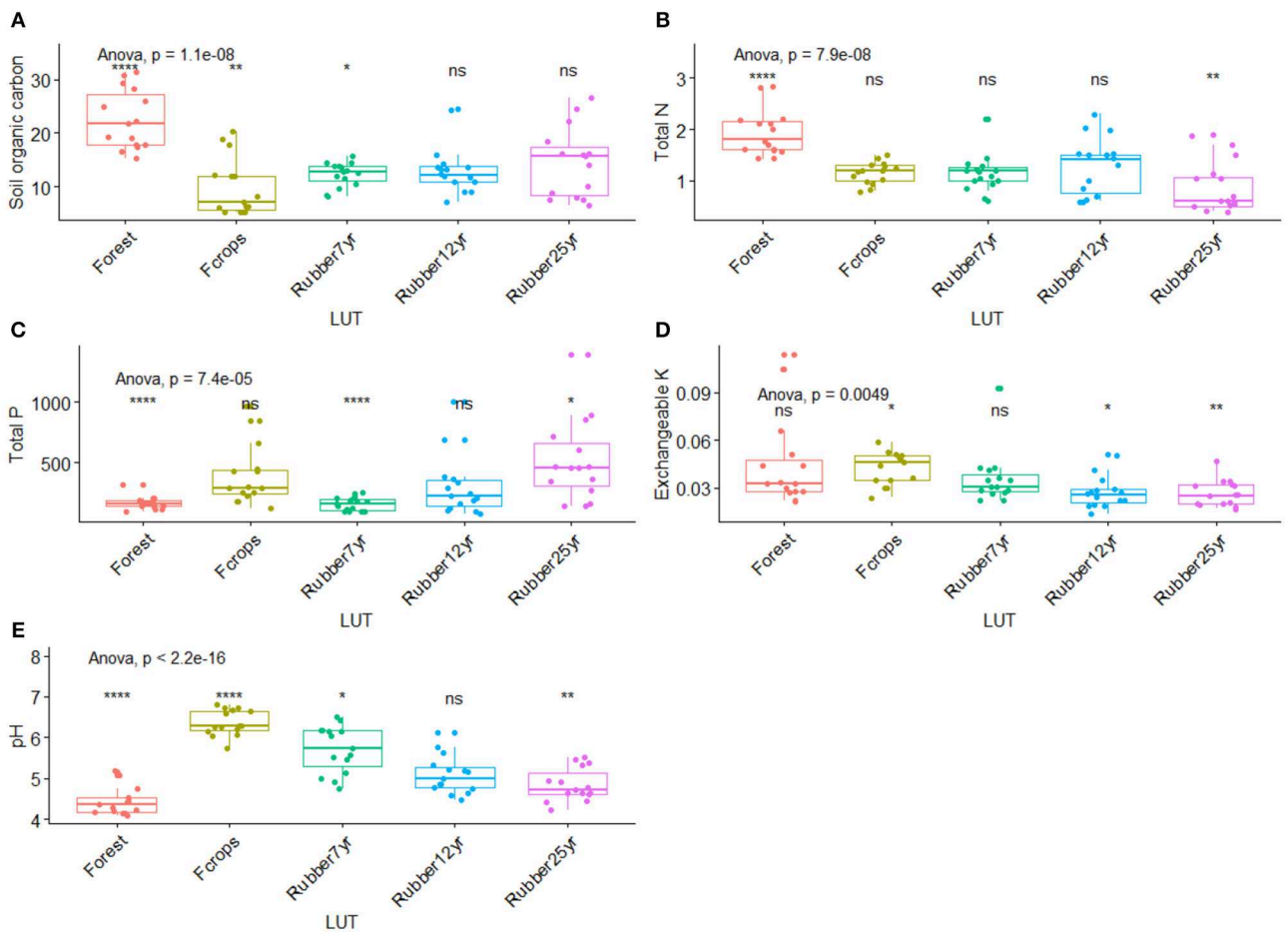

FIGURE 5 | Changes in soil chemical characteristics along the rubber tree chronosequence. **(A)** Soil organic carbon (g kg^{-1}). **(B)** Total nitrogen (g kg^{-1}). **(C)** Total phosphorus (mg g^{-1}). **(D)** Exchangeable potassium (cmolc kg^{-1}). **(E)** pH. *$p < 0.01$; **$p < 0.001$; ****$p < 0.00001$.

family is composed of 7 species in the entire landscape. It is noteworthy that the whole community is composed of the pantropical species *Pontoscolex corethrurus* of the Glossoscolocidae family and *Eudrilus eugeniae* and *Hyperiodrilus africanus* of the Eudrilidae family whose distribution is spread in degraded lands in Africa. From a functional viewpoint, earthworms are classified into detritivores, geophageous oligo, meso, and polyhumics (**Table 2**). The biplot CA performed on the 24 earthworm species (**Figure 2**) evidenced significant association between species and LUT ($\chi^2 = 946.54$, $p < 0.001$). As a matter of fact, along the first axis (47.7%, eigenvalue = 0.51), species associated with food crops and the plantations of 7 years, including *H. africanus*, *E. eugeniae*, *M. omodeoi*, *Dichogaster terraenigrae*, *Millsonia* sp, *Dichogaster mamillata*, *Gordiodrilus paski*, are opposed to those attached to forest lands and the two aged rubber tree stands. On the contrary, axis 2 (33.15%) revealed the opposition of forested areas characterized by *Stuhlmannia palustri*, *D. erhrhardti*, *D. papillosa*, *D. baeri*, *S. compositus,* and rubber tree plantations of 12 years where *S. zielae*, *D. eburnea*, *P. corethrurus,* and *D. leroyi* are common.

With regards to functional attributes, in line with density of the polyhumics, the geophageous oligohumics did not show significant changes in density (**Figure 3**) and biomass (**Figure 4**) after the conversion of forest into rubber tree plantations. Conversely, significant changes were found in the density and biomass of detritivores ($p < 0.0001$) and mesohumics ($p = 0.024$); and the biomass of polyhumics ($p = 0.0001$). As for density, detritivore earthworms represented the most important group in several land uses (62.7–63.2%) except the youngest plantations (30.1%) and food crops (2.0%), where they were less present (**Figure 3**). On the contrary, mesohumics were fairly well-represented in cultivated areas including food crops (40.2%), 7-year (46. %) 25-year-old plantations (28.3%) while polyhumics were strongly associated with food crops (57.8%) and the youngest plantations (23.0%). Similar trends were found with the biomass (**Figure 4**) as detritivores accounted for 50.7–94.2% in forest and the two last plantations in the chronosequence, mesohumics being in the range 47.3–66.6% (food crops, rubber 7 and 25 years) and the polyhumics being mostly harbored by food crops at 36.2%.

Changes in Earthworm Communities

Generally speaking, unlike the Evenness index, ecological metrics values revealed significant changes in earthworm communities over 25-years along the chronosequence in rubber-dominated landscapes (**Figure 5**). The highest average values of density were

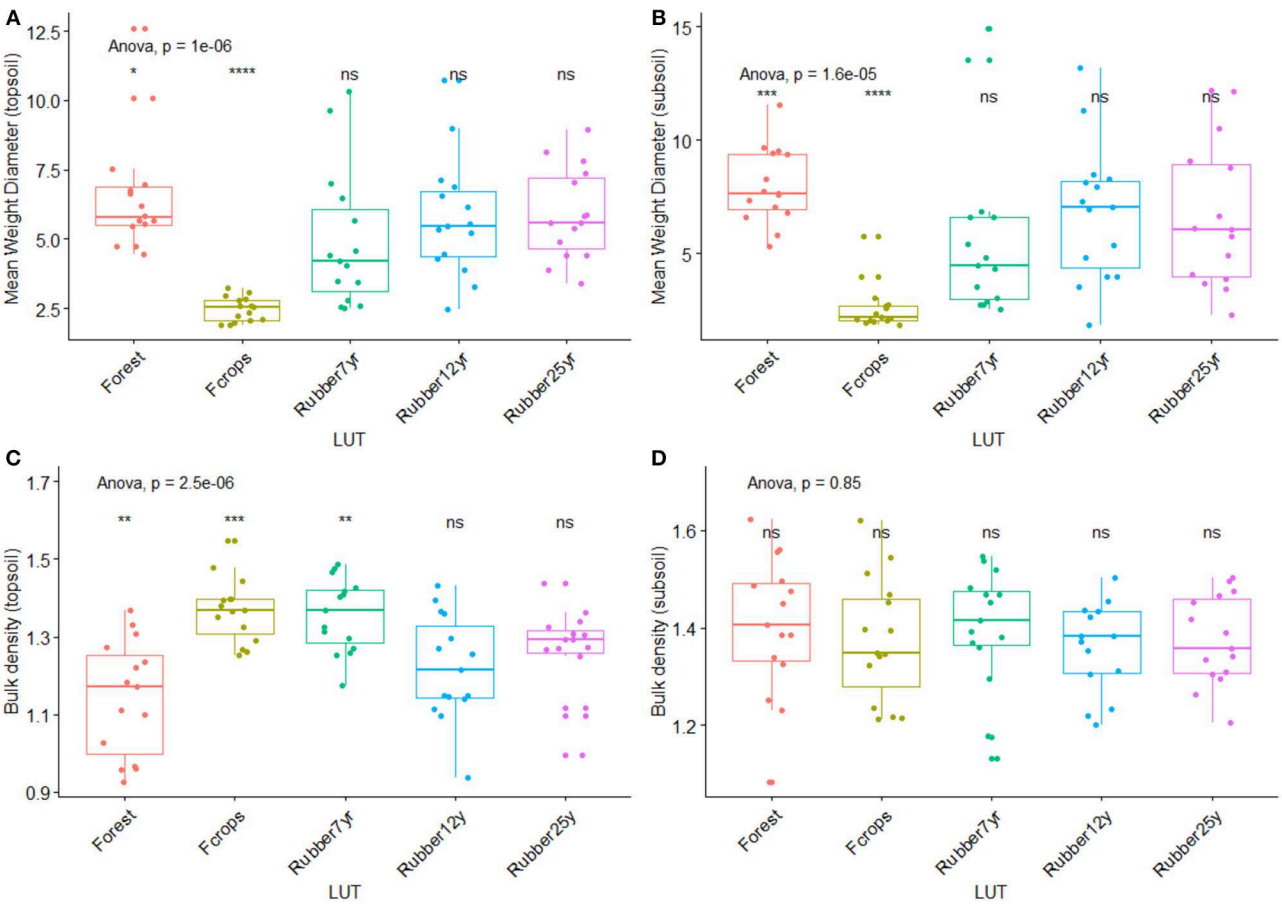

FIGURE 6 | Changes in soil physical characteristics along the rubber tree chronosequence. **(A)** Mean weight diameter (Topsoil: mm). **(B)** Mean weight diameter (Subsoil: mm). **(C)** Bulk density (Topsoil: g cm^{-3}). **(D)** Bulk density (Subsoil: g cm^{-3}). *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$; ****$p < 0.00001$.

found in the 12 and 25-year old plantations (246.9 and 318.1 ind m^{-2}). Intermediate values (176 ind m^{-2}) were found in the forest, while the lowest values were found in the 7-year-old rubber stands (63.7 ind m^{-2}) and the food crops (66.4 ind m^{-2}). The variation in biomass values showed a reverse trend with the highest value in the forests (21.5 g m^{-2}), intermediate values in the 7- and 25-year-old plantations (13 g m^{-2} and 17.3 g m^{-2}) and the lowest values in food crops (8 g m^{-2}) and the 12-year-old plantations (10.5 g m^{-2}). Subsequently, earthworm density, biomass, species richness, and Shannon index (**Figure 5**) varied significantly along the chronosequence. Species richness values were significantly higher in the oldest plantation (25 years) along with the forest, while Shannon (H) and evenness (E) indices were highest in forest and food crops, respectively (**Figure 5**). Moreover, H significantly had the lowest values in forest and in rubber tree plantations of 12 years.

Changes in Soil Chemical Characteristics

The conversion of forest into rubber-dominated landscapes resulted in significant shifts ($p < 0.001$) in the average values of soil chemical characteristics as portrayed in **Figure 6**. The greatest value of SOC (22.5 g kg^{-1}) was recorded in forest soils, followed by intermediate values in 7-year (12.8 g kg^{-1}), 12-year-old plantations (14.5 g kg^{-1}), and food crops (12.3 g kg^{-1}) and the lowest values were recorded in the 25-year old plantations (9.9). Total N showed a similar trend with the highest value beneath forest (1.93 g kg^{-1}) and the lowest in the 25-year old plantations. The value of P significantly increased along the chronosequence with greater ones found in the 25-year old plantations (515.9 mg g^{-1}), food crops (391.6 mg g^{-1}), and the 12-year old plantations (299.6 mg g^{-1}). On the contrary, K did not show a decreasing trend, recording the highest values in the forest and food crops and lowest values over ages of plantations. After an initial low average value in forest (4.43), pH values increased up to the top level in food crops (6.36) before undergoing a steady drop in the plantations (5.71, 5.08, 4.84).

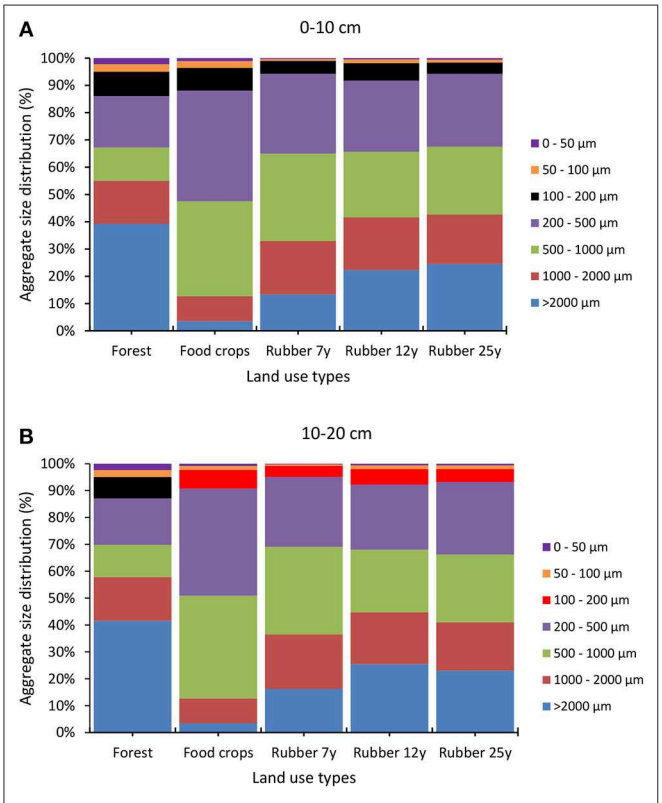

FIGURE 7 | Change in aggregate size distribution across land use types in the rubber tree landscapes.

Changes in Soil Physical Characteristics

The distribution of aggregates size significantly ($p < 0.0001$) varied across LUTs in the 0–10 cm and 10–20 cm soil layers. The impact of forest conversion into rubber tree landscapes was mostly reflected in the macroaggregates (>2,000 µm) that showed a sharp drop in food crops followed by a recovering along with a steady increase in the plantations. The aggregate size classes 1,000-2,000 µm and 500–1,000 µm were the most important in the landscape, although they did not show a clear trend revealing the impact of forest conversion. Overall, the mean weight diameter (MWD) in top and subsoil varied significantly across LUTs ($p < 0.001$) as shown in **Figure 9**. The lowest values of MWD in top (2.47) and subsoil (2.62) were recorded in food crops, while the highest values were recorded in forest (6.59 and 7.98) and last plantations in the chronosequence. Intermediate values were found in the last two plantations of the chronosequence (**Figure 7**). As for bulk density, values in the 0–10 cm layer was low (1.14) in the forest before a significant rise occurred in the food crops (1.37) with inconsistent trends in plantations (1.36, 1.22, 1.26) thereafter. On the contrary, no significant changes occurred in the 10–20 cm layer.

Soil Health Degradation Indices

The analysis of soil deterioration indices depicted in **Table 3** revealed that soil health was severely impaired in food crops (DI = −173.7) and the 7-year old plantations (DI = −345.8). However, this situation reversed after 12 years (DI = −69.9) before full restoration in plantations of 25 years (+84.1). Earthworm density and species richness along with total P were instrumental in reversing the trend of soil health degradation caused by significant drops in SOC, total N and extractable K along the chronosequence (**Table 4**).

Relationship Between Earthworm Abundance and Biomass and Soil Organic Carbon

The results of GLMM showed that SOC, earthworm density and biomass varied significantly among LUTs, with 42, 13, and 19% of variance explained, respectively (**Table 5**).

For SOC, forest had a regression coefficient which was 14.06 significantly higher than that of the other LUT (**Table 3**), indicating that SOC was higher in forest followed by rubber plantations in a decreasing order and food crops. A similar trend was observed for earthworm biomass while density displayed the highest coefficient models in 25 and 12-year-old plantations,

TABLE 3 | Average density (ind m $^{-2}$± standard error) and biomass (g m $^{-2}$± standard error) of earthworm species across the rubber tree plantation landscapes.

	Secondary forest		Food crops		Rubber tree 7-year-old		Rubber tree 12-year-old		Rubber tree 25-year-old	
	Density	**Biomass**	**Density**	**Biomass**	**Density**	**Biomass**	**Density**	**Biomass**	**Density**	**Biomass**
P. corethrurus	0.00	0.00	0.00	0.00	0.00	0.00	2.67 ± 2.67	1.00 ± 1.00	0.00	0.00
M. lamtoiana	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
M. omodeoi	0.27 ± 0.27	0.1 ± 0.1	17.33 ± 4.05	4.95 ± 1.22	29.07 ± 8.07	8.66 ± 2.45	4.27 ± 1.63	0.71 ± 0.40	21.33 ± 7.82	7.26 ± 2.52
Millsonia sp.	0.00	0.00	2.93 ± 2.93	0.001 ± 0.00	0.00	0.00	0.00	0.00	3.2 ± 2.65	0.61 ± 0.40
D. baeri	14.67 ± 3.37	15.45 ± 5.23	0.00	0.00	4.53 ± 2.13	2.21 ± 1.72	2.4 ± 1.34	0.38 ± 0.21	0.8 ± 0.58	0.29 ± 0.23
D. terraenigrae	0.00	0.00	0.00	0.00	0.27 ± 0.27	0.001 ± 0.001	0.00	0.00	0.00	0.00
D. saliens	47.73 ± 13.94	0.37 ± 0.10	6.4 ± 2.73	0.15 ± 0.06	0.00	0.00	2.4 ± 1.45	0.05 ± 0.03	65.33 ± 25.02	0.31 ± 0.12
D. erhrhardti	15.47 ± 7.96	2.41 ± 1.16	0.00	0.00	0.00	0.00	0.00	0.00	1.33 ± 0.84	0.15 ± 0.10
D. lamottei	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
D. papillosa	35.73 ± 14.26	0.52 ± 0.19	0.00	0.00	2.67 ± 1.73	0.03 ± 0.02	5.87 ± 2.73	0.06 ± 0.03	10.4 ± 3.12	0.13 ± 0.04
D. eburnea	23.47 ± 8.26	1.37 ± 0.40	0.00	0.00	6.67 ± 3.59	0.18 ± 0.09	197.07 ± 50.2	6.84 ± 1.40	160 ± 60.03	7.58 ± 2.73
D. mamillata	0.00	0.00	0.00	0.00	2.4 ± 2.4	0.08 ± 0.08	0.00	0.00	0.00	0.00
D. leroyi	0.00	0.00	0.00	0.00	0.00	0.00	0.8 ± 0.8	0.03 ± 0.03	0.53 ± 0.36	0.24 ± 0.21
Dichogaster sp1	17.6 ± 7.85	0.45 ± 0.18	1.33 ± 0.93	0.02 ± 0.01	0.00	0.00	1.33 ± 1.08	0.02 ± 0.02	26.93 ± 9.68	0.36 ± 0.11
Dichogaster sp2	3.47 ± 1.70	0.04 ± 0.02	0.00	0.00	0.00	0.00	0.27 ± 0.27	0.03 ± 0.03	1.06 ± 0.83	0.01 ± 0.01
Dichogaster sp3	0.00	0.00	0.00	0.00	1.33 ± 0.75	0.03 ± 0.02	3.47 ± 2.67	0.19 ± 0.13	0.00	0.00
A. multivesiculatus	0.53 ± 0.36	0.02 ± 0.02	0.00	0.00	0.53 ± 0.53	0.03 ± 0.03	0.27 ± 0.27	0.02 ± 0.02	1.87 ± 0.95	0.08 ± 0.04
A. opisthogynus	0.53 ± 0.53	0.6 ± 0.6	0.00	0.00	0.00	0.00	0.00	0.00	0.8 ± 0.8	0.001 ± 0.00
H. africanus	0.00	0.00	19.47 ± 4	2.56 ± 0.66	10.93 ± 3.59	1.48 ± 0.56	1.07 ± 0.61	0.001 ± 0.00	3.47 ± 2.43	0.001 ± 0.00
S. compositus	1.6 ± 1.60	0.03 ± 0.03	0.8 ± 0.58	0.021 ± 0.02	0.00	0.00	0.00	0.00	0.00	0.00
S. zielae	8.27 ± 4.77	0.14 ± 0.08	7.73 ± 2.12	0.27 ± 0.14	0.27 ± 0.27	0.01 ± 0.01	21.33 ± 5.72	21.33 ± 5.72	0.57 ± 0.14	0.24 ± 0.14
S. palustris	1.07 ± 1.07	0.01 ± 0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
E. eugeniae	0.00	0.00	0.00	0.00	1.6 ± 0.94	0.28 ± 0.19	0.00	0.00	0.00	0.00
G. paski	0.00	0.00	10.4 ± 6.03	0.08 ± 0.05	1.87 ± 0.77	0.02 ± 0.01	0.53 ± 0.53	0.03 ± 0.03	1.6 ± 0.94	0.02 ± 0.01
Ocnerodriliae sp.	5.6 ± 5.32	0.001 ± 0.00	0.00	0.00	1.6 ± 0.94	0.01 ± 0.00	3.2 ± 2.68	0.02 ± 0.02	5.87 ± 5.32	0.01 ± 0.00
Average	176 ± 19.2	21.49 ± 5.3	66.4 ± 15.8	8.02 ± 1.56	63.7 ± 10.1	13. ± 2.9	246.9 ± 57.5	17.3 ± 2.9	318.1 ± 88.2	17.3 ± 2.9

indicating higher values in these two plantations as opposed to lower values in 7-year-old stands and in food crops.

Relationship Between Earthworm Species and Soil Parameters

The first two axes of the biplot PCA used to test the interaction between earthworm species and soil parameters accounted for a total variance of 82.1% (**Figure 8**). Axis 1 (54.8%) represented LUTs rich in SOC, total N, P with moderate values of mean weight diameter which are significantly associated with *Dichogaster baeri*, *Millsonia omodeoi*, Eudrilus *eugeniae, Millsonia omodeoi,* and *Stuhlmannia zielae*. The second axis (27.3%) characterizes LUTs with high values of pH and bulk density along with moderate values of P which showed close association with *Pontoscolex corethrurus*, *Hyperiodrilus africanus* and *Dichogaster papillosa* (**Figure 8**).

TABLE 4 | Degradation indices (%) for 0–20 cm soil layer along a 25-year-old chronosequence in rubber tree landscapes following conversion of forest.

	Food crops	Rubber 7 y	Rubber 12 y	Rubber 25 y
Density (individual m^{-2})	−62.3	−63.8	+40.3	+80.8
Biomass (g m^{-2})	−62.7	−39.5	−51.3	−90.4
Species richness	−25.7	−27.1	−14.3	+10.0
Soil organic carbon (g kg^{-1})	−45.5	−43.4	−35.5	−35.5
Total Nitrogen (g kg^{-1})	−40.5	−39.8	−33.2	−33.2
Total Phosphorus (mg g^{-1})	+133.5	−4.8	+78.7	+207.6
K (cmolc kg^{-1})	−5.6	−100.0	−40.6	−41.0
MWD (mm)	−65	−27.4	−13.9	−14
Cumulative Degradation Index (CDI)	−173.7	−345.8	−69.9	+84.1

Rubber, rubber tree plantation; yr, years.

Interactive Feedback Between Earthworms and Soil Properties

The direct influence of soil physico-chemical parameters on earthworm functional groups, evaluated from the first path analysis, revealed that SOC ($T = 0.5$, $p = 0.0000$), MWD_top soil ($T = 0.40$, $p = 0.008$), K ($T = 0.32$, $p = 0.024$), and P ($T = 0.21$, $p = 0.04$) have a strong causal effect on geophageous mesohumic earthworms, while K and MWD_sub show negative correlations with detritivores ($T = -0.33$, $p = 0.039$) and polyhumics ($T = -0.34$, $p = 0.04$), see **Figure 9** and **Table 6**. However, there is no significant relationship between the abundance of oligohumic earthworms and the physico-chemical soil parameters considered in this study.

The second structural model, proposed for assessing feedback effects of earthworms on soil health, was characterized by a poor overall fit. The low values obtained for all evaluation indices including the Comparative Fit Index (CFI: 0.558), the Tucker-Lewis Index (TLI: −1.16), and the Root Mean Square Error (RMSEA: 0.355) indicate that the proposed model was not able

TABLE 5 | Results of generalized linear mixed effects models testing the effects of LULC categories on (a) soil organic carbon, (b) earthworm biomass and (c) earthworm density.

	Fixed effects				Random effects (variance)					R^2(%)	
	Est.	SE	*t*-value	*p*-value	*N*	*P*	*K*	pH-H_2O	Resid.	Marg	Cond.
SOC											
Intercept	9.86	1.47	6.70	<0.001	12.78	15.73	4.41	0.08	0.00	42	99
Forest	14.06	2.04	6.88	<0.001							
Rubber 25 y	6.39	1.85	3.45	0.001							
Rubber 12 y	4.41	1.36	3.24	0.002							
Rubber 7 y	1.95	1.11	1.75	0.08							
Food crops											
BIOMASS											
Intercept	8.23	3.24	2.54	0.013	0.00	0.000	6.51	0.00	147.90	13	16
Forest	12.97	4.55	2.85	0.006							
Rubber 25 y	9.04	4.58	1.97	0.052							
Rubber 12 y	2.17	4.56	0.48	0.64							
Rubber 7 y	4.47	4.53	0.98	0.33							
Food crops											
DENSITY											
Intercept	66.77	52.96	1.26	0.21	2,303	1,021	12,660	0.00	21,010	19	54
Forest	101.15	70.38	1.44	0.16							
Rubber 25 y	235.25	73.67	3.19	0.002							
Rubber 12 y	188.11	68.21	2.76	0.007							
Rubber 7 y	8.81	63.43	0.14	0.89							
Food crops											

Est. and SE represent estimates of regression coefficient and stand error, respectively.

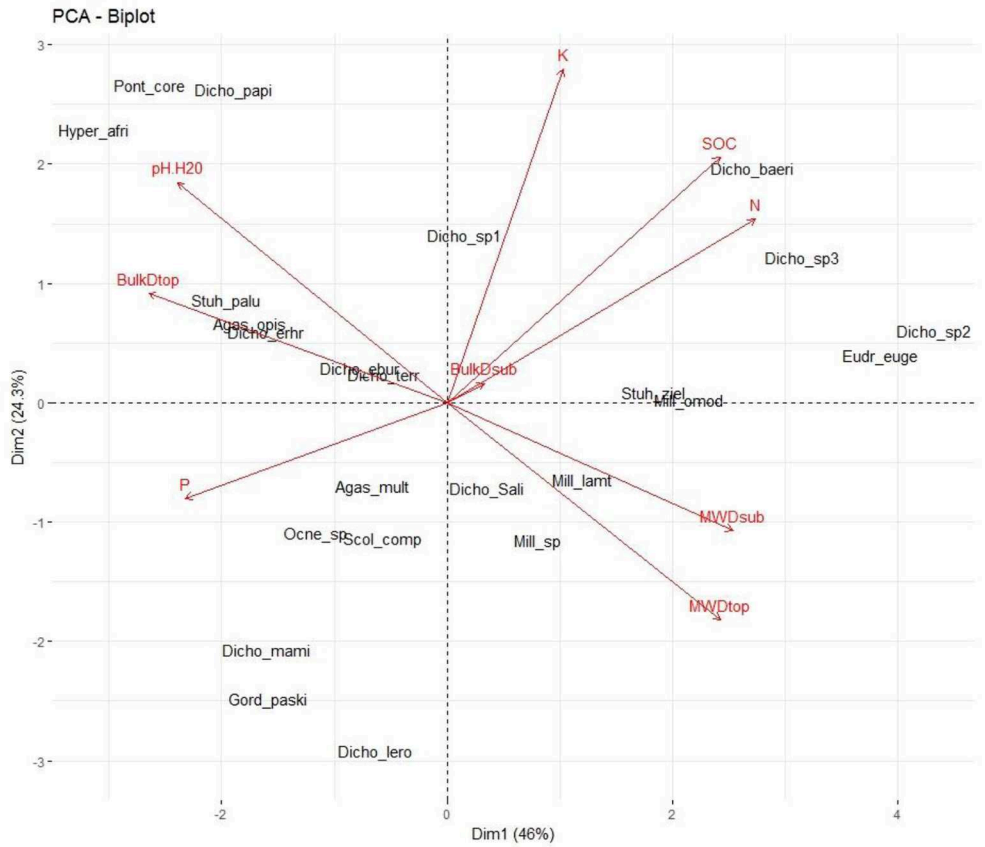

FIGURE 8 | PCA biplot showing the distribution of earthworm species along with soil properties (SOC, soil organic carbon, N, total nitrogen; MWDtop, Mean Weigh Diameter of layer 0–10cm; MWDsub, Mean Weight Diameter of layer 10–20 cm; BulkDtop, bulk density of 0–10 cm layer; BulkDsub, bulk density of 10–20 cm layer) within the factorial plane 1–2. For species abbreviations, refer to **Figure 2**.

to accurately reflects the variability observed in the data. Direct effects of earthworms on soil health were not clearly evidenced by the results (**Figure 10**).

DISCUSSION

Changes in Earthworm Communities Along the Chronosequence

Apart from the presence of *Pontoscolex corethrurus* and *Eudrilus eugeniae*, species composition of earthworm communities was similar to those collected in southern west Côte d'Ivoire (Tondoh et al., 2011, 2015; Guéi and Tondoh, 2012). The conversion of forest into rubber tree landscapes has resulted in a significant shift in earthworm communities both at taxonomical and functional levels. After a significant drop in the food crops and the 7-year-old plantations, population density of earthworms showed a significant increase in the 12-year and the 25-year-old plantations. These increases did not differ significantly from that of the forest. Similar trends were reported in previous studies in western Côte d'Ivoire in cocoa growing landscapes (Tondoh et al., 2011, 2015). This trend is likely due to the proliferation and the mixture of the pantropical earthworm *P. corethrurus*, the African-Wide earthworms *Hyperiodrilus africanus* and native species (*Dichogaster saliens* and *Dichopaster eburnean*) in the two last plantations of the sequence. Growth and expansion of these populations are most likely due to their capability of withstanding degraded agro-ecosystems with low soil organic carbon content (Marichal et al., 2010; Guéi and Tondoh, 2012). This explains the higher species richness and diversity in plantations due to gradual enrichment or increase in species composition consistently with findings in cocoa landscape in southwestern Côte d'Ivoire (Tondoh et al., 2015). Another explanation is supported by the high soil water content in the 25-year-old plantation characterized by great production of litter (Chaudhuri et al., 2013; N'Dri et al., 2018) of good quality due to its low content in polyphenol, flavonoid and lignin (Chaudhuri et al., 2013). Moreover, the same trend of increased density of earthworm populations was found along rubber chronosequence in southern Côte d'Ivoire (Gilot et al., 1995; N'Dri et al., 2018) and in Tripura, India (Chaudhuri et al., 2013). The trend in biomass was different with higher values in the forest compared to human-derived ecosystems, mostly characterized by the prevalence of small and medium-sized species. It's noteworthy that in the current study, *P. corethrurus* did not represent the dominant species of the earthworm community as it is in

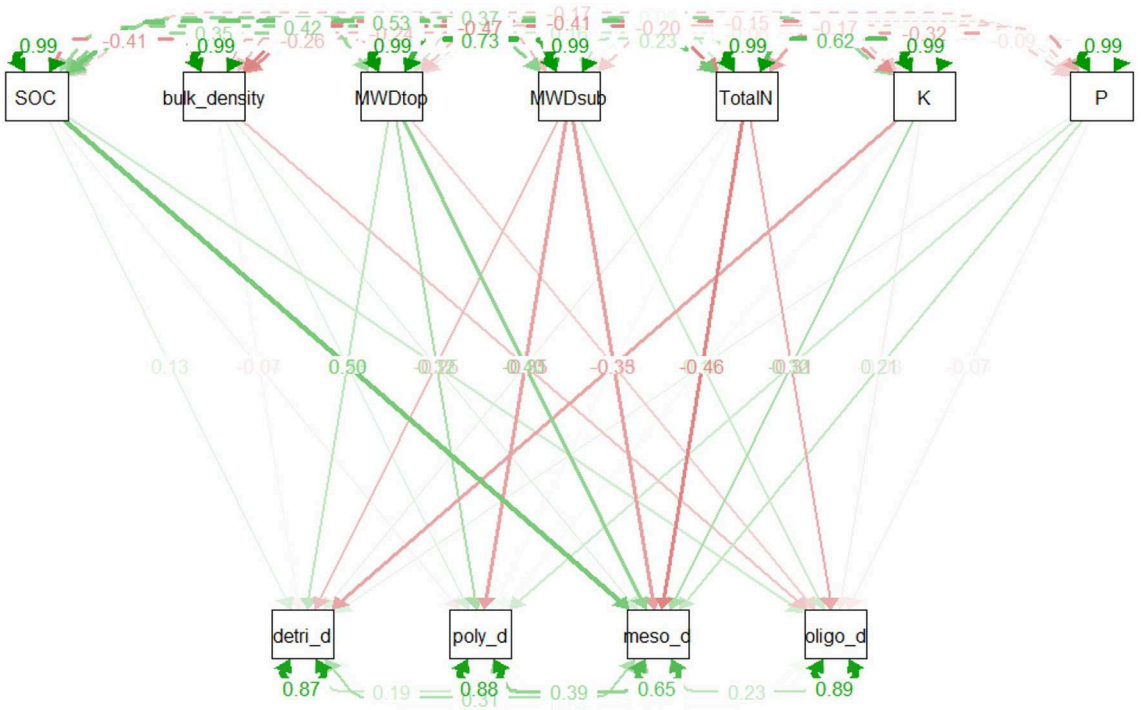

FIGURE 9 | Correlation Path diagram relating soil parameters as exogenous variables and earthworm functional groups as endogenous variables. The value indicates the path coefficients (T). Node opacity indicates their significance (p-value); the width its importance and the color its direction (green for a positive correlation, red for a negative). detri_d, meso_d, oligo_d, poly_d represents respectively density of detritivores, geophageous mesohumic, oligohumic, and polyhumic earthworms. SOC, soil organic carbon; bulk density, soil bulk density, MWDtop and MWDSub, mean weight diameter of top and sub soil; TotalN , total soil nitrogen; K, exchangeable potassium; and P, total phosphorus.

disturbed agro-ecosystems. It has been reported in rubber tree plantations in India and across the Amazonia that this species populations represented up to 70% total earthworm populations in density and biomass, respectively (Chaudhuri et al., 2008, 2013; Marichal et al., 2010). The detritivores and the geophageous mesohumic were the most important functional groups in forest-derived landscapes with a marked presence of native species that were composed of earthworms from both functional groups.

Impact of Land Use Change on Soil Properties

Soil organic carbon (SOC), pH, and total phosphorus (P) showed significant variations in their values along the chronosequence, indicating consistent changes after forest conversion into rubber tree plantations. These findings agree with previous research in southwestern Côte d'Ivoire in cocoa landscapes (Tondoh et al., 2011, 2015), the humid forest zone of Nigeria (Oku et al., 2012), in Asia (de Blécourt et al., 2013) and in the Western Kenya highlands (Nyberg et al., 2012). SOC concentration was high and above 20 g kg^{-1}, which is considered as the threshold value of a soil of good quality (Musinguzi et al., 2013; Lal, 2015), indicating deterioration of SOC by rubber farming. Conversely, the steady increase of P in rubber tree plantations over time was noteworthy and is likely to be factored into fertilizer management. Indeed, with money made out of their plantations, farmers were keen on maintaining their soil fertility status by integrating mineral and organic fertilizers in alleys as shown in legume-based cropping systems in middle Côte d'Ivoire (Koné et al., 2008). Contrasting soil pH values across the landscape revealed that soil acidity should be handled with care as it tended to be low in forest and rubber tree-derived ecosystems. Findings pertaining to SOC, pH and bulk density confirmed results by N'Dri et al. (2018), who found similar trends along another chronosequence in the same study site.

The conversion of forest into rubber tree landscapes has resulted in a significant drop in aggregate stability and large macroaggregates (>2 mm) in the food crops prior to a gradual replenishment over time in both layers (0–10 cm and 10–20 cm) of the plantations. In contrast, bulk density was low in the forest but was high under human-derived systems most likely indicating impaired soil porosity in the plantations (Kakaire et al., 2015). Furthermore, the proportion of macroaggregates was severely reduced in the food crop systems and afterwards restored in the plantations. These findings point out the beneficial role of trees through their roots in shaping up soil structure. The following explanations likely account for the following processes: (i) the huge abundance of soil organisms (macro and meso invertebrates, particularly soil mites) that increased at a rate of +121 % in the 25-year old plantations and are known to be active in the breakdown of fresh inputs of organic material abundantly produced in 12 and 25-year-old plantations (N'Dri et al. (2018); (ii) soil aggregation in aged plantations is facilitated

TABLE 6 | Summary of correlation and p-values from correlation path analysis.

Regression		Path coefficient	Std.Err	*z*-value	*P* (>|z|)
Detri_d ~					
	SOC	0.130	0.142	0.916	0.36
	Bulk_density	−0.040	0.133	−0.304	0.761
	MWDtop	0.228	0.176	1.293	0.196
	MWDsub	−0.247	0.168	−1.464	0.143
	TotalN	0.116	0.167	0.698	0.485
	K	−0.334	0.162	−2.062	0.039
	P	0.114	0.117	0.971	0.331
Poly_d ~					
	SOC	−0.069	0.143	−0.485	0.628
	Bulk_density	0.143	0.134	1.067	0.286
	MWDtop	0.316	0.177	1.782	0.075
	MWDsub	−0.345	0.170	−2.035	0.042
	Total	0.038	0.168	0.224	0.822
	K	0.048	0.163	0.30	0.768
	P	0.162	0.118	1.377	0.169
Meso_d ~					
	SOC	0.502	0.123	4.062	0
	Bulk_density	0.155	0.115	1.341	0.18
	MWDtop	0.402	0.153	2.633	0.008
	MWDsub	−0.353	0.146	−2.418	0.016
	TotalN	−0.461	0.145	−3.187	0.001
	K	0.316	0.140	2.253	0.024
	P	0.208	0.101	2.054	0.04
Oligo_d ~					
	SOC	0.167	0.144	1.158	0.247
	Bulk_density	−0.207	0.135	−1.539	0.124
	MWDtop	−0.164	0.178	−0.92	0.358
	MWDsub	0.183	0.171	1.072	0.284
	TotalN	−0.314	0.169	−1.86	0.063
	K	−0.075	0.164	−0.46	0.645
	P	−0.067	0.118	−0.565	0.572

(Value in red represents significant correlation at 0.05 threshold). Detri_d, Poly_d, Meso_d and Oligo_d stand respectively for detritivores, polyhumic, mesohumic, and oligohumic earthworms' densities. SOC, soil organic carbon; bulk_density, soil bulk density; MWDtop and MWDSub, mean weight diameter for top and subsoil; TotalN, total nitrogen; K, exchangeable potassium; and P, total phosphorus.

by vegetation restoration that caused huge fresh organic matter returns amounting to 3.9 ± 0.1 – 5.1 ± 0.6 t ha^{-1} year^{-1} (N'Dri et al., 2018) to enhance the aggregation of soil particles with plantation stand age (Bronick and Lal, 2005). In contrast, soil exposure along with lack of residue inputs in the food crops caused declines in aggregation and organic carbon, both of which make soil susceptible to erosion (Pinheiro et al., 2004). Furthermore, the increased fresh organic C in 12 and 25-year old plantation might have enhanced the stability of the aggregates through the binding of mineral particles and the formation of stable aggregates as reported by Demenois et al. (2018) in New Caledonia.

Drivers of Soil Health Change in Rubber Tree Landscapes

The conversion of forest into rubber tree plantations significantly impaired all soil biological, physical and chemical parameters in the first place. As a result, soil health was heavily degraded in food crops and 7-year old plantations. Similar results were reported in cocoa landscapes after 7 years of cocoa cropping in center-west Côte d'Ivoire (Tondoh et al., 2015) and after 3 years in the Ashanti Region of Ghana (Dawoe et al., 2014). The improvement of soil health from 12 years in the rubber tree landscapes is consecutive to the increase in earthworm abundance and P concentration. To some extent, the gentle increase in SOC, the significant rise in large soil aggregate proportion and aggregate stability under the aged plantations contributed to improve soil health status. These findings revealed the beneficial impact of rubber stands on soil health improvement in the long run as the lifespan of the cropping cycle is estimated at 40 years. Indeed, Dawoe et al. (2014) reported an improved soil quality after 30 years of shaded-cocoa farming in the Ashanti region of Ghana owing to the re-accumulation of 85% of the initial forest soil organic carbon stock. Similarly, it was revealed that up to five decades was necessary to significantly improve SOC beneath tree plantations derived from croplands (Sauer et al., 2012). It is therefore not surprising that alternative cropping options, such as rubber-based agroforestry systems (*Hevea brasiliensis—Theobroma cacao* and *H. brasiliensis—Dalbergia cochinchinensis*), have the ability to improve soil quality and ecosystem resilience (Chen et al., 2017).

Can Earthworms Play a Troubleshooter's Role in Mitigating Soil Health Deterioration Issues in Rubber Tree Landscapes?

The Biplot-PCA revealed that in the rubber tree landscapes, earthworms' assemblage was featured by land use change, which in turn strongly impacted the soil chemical and physical characteristics. As a result, two functional groups of earthworms were found to be involved. The first group was made up of *Dichogaster baeri*, *Millsonia omodeoi* and *Stuhlmannia zielae*, which are associated either with LUTs rich in SOC, total N and P (i.e., forest and 25-year old rubber tree plantations). The second group consisted of *Pontoscolex corethrurus*, *Hyperiodrilus africanus* and *Dichogaster papillosa*, commonly found in degraded LUTs—food crops and 7-year old plantation—with high pH and bulk density along with moderate total P. Unlike *M. omodeoi* that was found in forested areas, a similar association was reported in the center west cocoa landscapes of Côte d'Ivoire (Guéi and Tondoh, 2012). Indeed, as highlighted by Koné et al. (2012), *M. omodeoi* can be viewed as a persistent species due to its ability to adapt to contrasted environments. Furthermore, the strong relationship between earthworms and SOC agrees with previous findings (Tondoh et al., 2011; Guéi and Tondoh, 2012) and confirms the role of SOC as a key driver of earthworms' abundance and community structure in agro-ecological landscapes. Although these findings were expected, they don't provide much clarity on the direction of the interaction between both abiotic and biological components.

The structural equation modeling was used to shed light on the relationship between earthworms and soil physico-chemical parameters by testing two hypotheses: the first highlighting the strong influence of soil physico-chemical parameters on earthworm communities, and the second addressing the potential

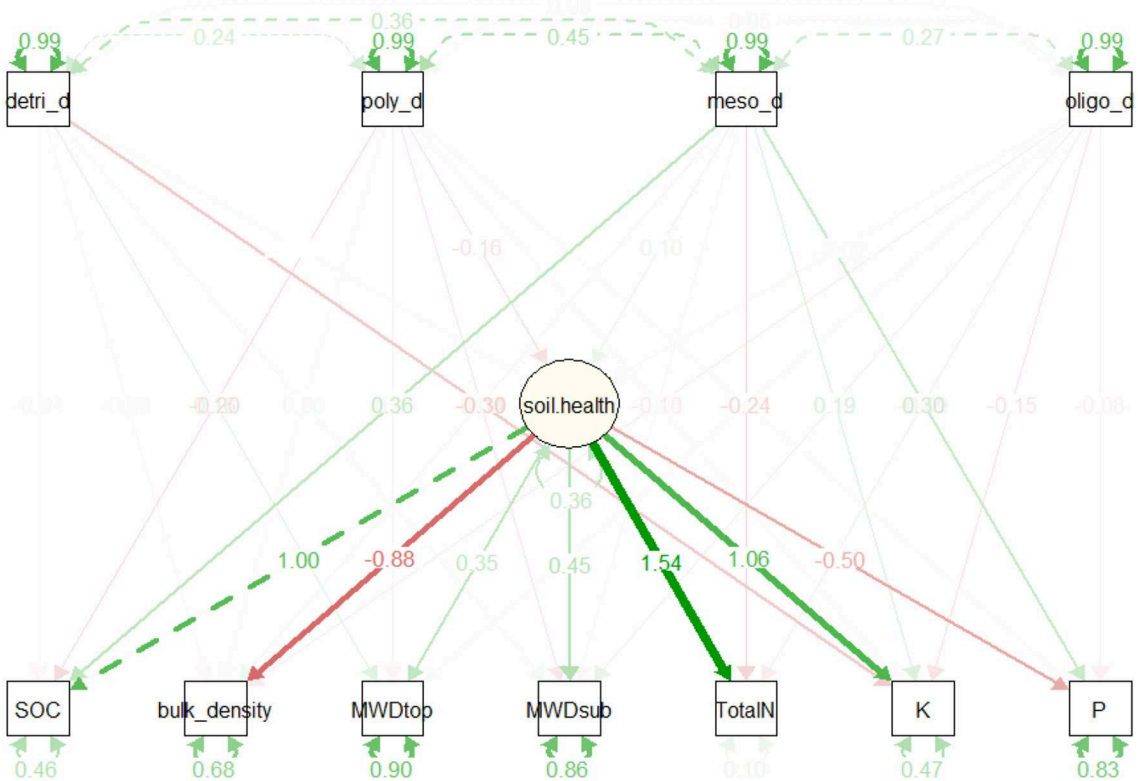

FIGURE 10 | Path diagram relating earthworm functional groups as exogenous variables and soil health as endogenous variables. The value indicates the path coefficient (T). Node opacity indicates their significance (p-value); the width its importance and the color its direction (green for a positive correlation, red for a negative). See **Figure 9** for the explanation of abbreviations.

feedback effects on these same soil properties, caused by earthworm activities. The results of the correlation path analysis derived from the first hypothesis evidenced the well-documented direct dependency between soil parameters, notably soil organic carbon on abundance of earthworm functional groups. Unfortunately, the poor overall fit of the measurement model, proposed to test feedback effect of earthworms on soil health, as assessed by the low values obtained for all evaluation indices (0.558, −1.16, and 0.355, respectively for CFI, TLI, and RMSEA), indicated that the suggested model did not properly depict the network of relationships between earthworms and soil health. However, it is now widely recognized that as ecosystem engineers, earthworms modulate the availability of soil nutrients, including organic carbon (Lavelle et al., 2006, 2016). This pattern appears to be reflected in the model results, which indicate a greater influence of mesohumic earthworms on soil organic carbon and total phosphorus than other functional groups. But, the poor fit of the suggested measurement model indicates that the right paths of these interactions are still not well-identified. The findings suggest a more complex network of relationships than the one proposed in this work. Indeed, one of the weaknesses of this study lies in its observational nature that could not provide insights into causal inference that is mostly driven by land use change to the detriment of soil organisms. Future research should focus on adjusting the model structure by identifying relevant interaction paths that explain the effect of these macro-invertebrates on the soil.

CONCLUSION

The study confirmed the detrimental impact of forest conversion into rubber tree landscapes in southern Côte d'Ivoire on soil health within the first 7 years and the restorative trend taking place beneath the plantations in year 12 and above. This was due to an enabling microenvironment (improved SOC, aggregate stability, exchangeable K, total phosphorus) taking place in rubber tree plantations, which was key for the development of geophageous mesohumic earthworms. In turn, they might have improved soil health through their interaction with soil organic carbon and total phosphorus. However, there were no evidence of direct effect of earthworms on soil heath, suggesting that more investigations at mesoscale is worth being undertaken.

AUTHOR CONTRIBUTIONS

JT conceived the study, designed, and coordinated the write-up of this manuscript. KD and YB contributed to statistical analyses and the review of the manuscript. AG, LA, and JN were

involved in data collection and preprocessing and the review of the manuscript. GF was tasked with the proofreading and review of the manuscript.

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SUPPLEMENTARY MATERIAL

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Conservation Agriculture Enhances Soil Fauna Richness and Abundance in Low Input Systems: Examples From Kenya

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Conservation agriculture (CA) (zero tillage + organic inputs as surface residue) is believed to improve soil nutrient status, soil structure, control soil erosion, and also enhance soil fauna diversity. Despite the widespread interest in CA, empirical evidence of the benefits of CA on soil fauna diversity is limited, especially in low-input systems of sub-Saharan Africa (SSA). Consequently, the magnitude and effect by CA on soil fauna remains unquantified. The aim of this study was to evaluate the effect of CA and associated management practices on soil fauna richness and abundance. We hypothesized that CA and mixed cropping would positively influence soil fauna richness and abundance. We compared CA with conventional till (CT; with or without residues) in sole maize and maize-bean cropping systems. Soil macrofauna and mesofauna were sampled across the treatments in medium-term (6 years) trials in Embu, Central Kenya, and Kakamega (6 years) and a long-term trial in Nyabeda (15 years) using soil monoliths and core samplers, respectively. In agreement with our hypothesis, higher macrofauna taxonomic richness and mesofauna was recorded in CA than in CT without residues. This study demonstrated that: (1) medium to long-term addition of organic residues enhances soil fauna richness and abundance, (2) CA increases soil fauna taxonomic richness and abundance compared with CT, and (3) CA under maize-bean intercropping, rotation and sole maize cropping systems promote soil fauna richness and abundance compared with sole legume (common beans). We conclude that adoption of CA is important in enhancing richness of soil fauna. Given the numerous challenges faced by smallholder farmers of SSA in the adoption of CA, who in most cases rarely practice all the three CA principles simultaneously, we propose a further study that will determine the effects and interactions between each of the CA components on soil fauna richness and abundance.

Keywords: tillage, organic resources, soil fauna, richness, abundance

INTRODUCTION

Conservation agriculture (CA), which encompasses minimum soil disturbance, soil cover, and crop diversification as its three main principles [with a fourth principle on fertilizer application being recently suggested by Vanlauwe et al. (2014)], has been successfully promoted in different parts of the world such as the temperate zone and parts of Latin America (Lal, 2007; Wall, 2007; Derpsch et al., 2010). Despite its low adoption in sub-Saharan Africa (SSA), the rapidly changing demographics in the region including a transformation of its economies to middle class, and the ongoing large initiatives to promote CA present opportunities of increasing its adoption. As such, evidence is needed around the various sustainability indicators of CA including soil biological indices (Ayuke et al., 2011a,b; Paul et al., 2015). Indeed, CA has been shown to stimulate soil fauna that play important role in soil aggregation, soil C sequestration, soil nutrient and water use efficiencies, and influence crop yields (Nhamo, 2007; Castellanos-Navarrete et al., 2012; Paul et al., 2015).

In many parts of the world, especially areas of SSA, the challenge of feeding an ever increasing population is persistent, due to prevalent land degradation and low soil productivity. Farmers' efforts to restore and maintain soil fertility are constrained by various challenges, the major ones being accessibility, affordability and knowledge on input management (Karanja et al., 2006; Vanlauwe et al., 2015). Historically, increasing food production in Africa has largely been attributed to land intensification and there has been efforts to intensify by increasing productivity per unit area. Sustainable intensification (SI) attempts to promote increased crop productivity by small-holder farmers in SSA (Garnett et al., 2013). Although SI has addressed the yield component, other aspects of sustainability such as soil biological functions have not been sufficiently addressed. SI advocates for management practices that reconcile environmental conservation and sustainability, and food security (Brussaard et al., 2010).

Despite the widespread interest in CA, empirical evidence of the benefits of CA in SSA is limited (Paul et al., 2013). For instance, the magnitude and direction of effect by CA on soil fauna, however, remains unquantified, especially in low-input systems of Sub-Saharan Africa. Response of soil fauna to soil tillage, available crop residues, and cropping practices in the region is largely unclear yet such knowledge is imperative for environmental conservation, sustainability and improved ecosystem services. Therefore, the aim of this study was to evaluate the effect of CA and associated management practices on soil fauna richness and abundance. Specifically, the study was to assess how conservation agriculture and its principal elements that encompass zero tillage, application of organic or inorganic inputs and cropping system, affect soil fauna richness and abundance. It is hypothesized that CA, organic or inorganic inputs and crop rotation and or mixed cropping would positively influence soil fauna richness and abundance.

MATERIALS AND METHODS

Study Sites

The studies were conducted at three sites (**Plate 1**). The first included a medium-term (6 years) trial, based at the Kenya Agricultural and Livestock Research Institute (KALRO) in Embu County, about 130 km north of Nairobi, Kenya. The trial is located in the sub-humid Central highlands of Kenya on the southern slopes of Mt Kenya at: latitude 0° 32′ S, longitude 37° 37′ E and an altitude of 1,480 m (**Table 1**). The average temperature is 19.5°C. The area receives bimodal rains with a mean total of 1,450 mm in two distinct seasons: long rains (March to August) and short rains (October to January). Agroecological zone is Upper mid land (UM3) (Jaetzold et al., 2007). The Soils are mainly Humic Nitisols (FAO, 1989) derived from basic volcanic rocks (FAO-UNESCO, 1997). They are deep, well-weathered with a clay texture (% sand, clay, silt: 3, 22, and 75) with moderate to high inherent fertility. The second is a medium-term trial (6 years) based at the Kenya Agricultural and Livestock Research Institute (KALRO) Kakamega (also in sub-humid western Kenya, Kakamega County), while the third is Nyabeda, a sub-humid site in western Kenya, within Siaya County where a long-term trial (15 years) is located. The Kakamega and Nyabeda sites are characterized by two rainy seasons: a long rainy season between March and August and short rainy season between September and January (Jaetzold et al., 2007). In all the sites, maize is the main staple crop and is normally grown either as a monocrop or in association with legumes, mainly common beans and groundnuts. Soybean, a cash crop, is also grown by farmers in the western Kenya region. All sites have predominantly smallholder settlements, with land sizes ranging from 0.3 to 3 ha per household. The study sites represent different soil types with varied physical and chemical characteristics. The study was conducted in December 2015 for the sites in eastern Kenya and June–July 2016 for the two trials in western Kenya.

Experimental Design

Embu Trial

The medium-term (6 years) field trial was established in 2010. Treatments tested for soil fauna include tillage (conventional till: CT, zero till: CA), organic residues (maize or bean residues: CR) and cropping systems (sole maize: SM, sole bean: SB and maize-bean intercrop: MBi) (**Table 2**). During the cropping seasons, plots are planted with maize (*Zea mays* Hybrid 512) and beans (*Phaseolus vulgaris*, variety Rose Coco) as per treatments. Mineral N fertilizer is applied targeting 60 kg N ha^{-1} for sole maize (SM) and 20 kg N ha^{-1} for sole beans (SB), applied as calcium ammonium nitrate (CAN), one-third at planting, and two-third at stage V6 of maize. Maize-bean intercrop (MBi) received 80 kg N ha^{-1}. Irrespective of tillage methods, SM, SB, and MBi received 60, 51, and 111 kg P_2O_5 ha^{-1} from [N23P23], [N18P46], and combination of [N23P23] + [N18P46] fertilizer materials, respectively (Micheni et al., 2016). For the treatments receiving organic inputs, all residues from the preceding crop were incorporated into the soil, thus providing about 39.2 t C

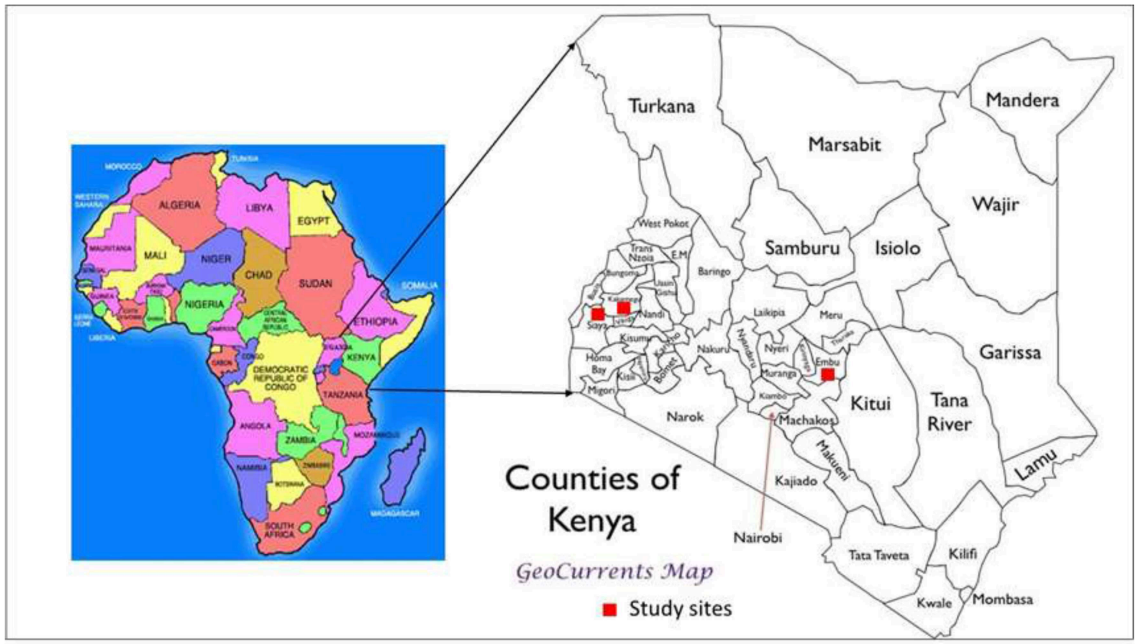

PLATE 1 | Map showing location of the study sites.

ha^{-1} and 67.4 t of SOM, respectively. Residues were removed from those treatment not receiving residues. The treatments were replicated three times in a randomized complete block design with plots of 10 m long by 8 m wide. Conventional till was done by hand-hoeing to a maximum depth of 15 cm, whereas no tillage was conducted in the zero till plots. Sole maize (SM): Maize spacing was 75 cm between rows and 50 cm within. Three seeds were sown per hill and thinned 1 week after crop emergence to two plants per hill, to give a plant population of 53,333 plants ha^{-1}. Sole bean (SB): Sole bean were spaced at 50 cm between rows and 15 cm within rows while maintaining one plant per hill giving plant population of 133,330 plants ha^{-1}. Maize bean intercrop (MBi): Maize spacing was maintained like in maize sole crop but bean spacing was slightly adjusted to 50 cm between rows and 20 cm within rows and two plants per hill. This gave plant population of 133,330 plants ha^{-1}, which was the same as in SB configuration. This was done to minimize confounding effects due to plant population differences (Micheni et al., 2016). Under CT, two weed control events were conducted manually using hand tools (machete and hoes) within a given season. The first weeding was done 1–2 weeks after crop emergence and the second was conducted in approximately one and half months after the first weeding. In the CA treatments, weeds were controlled using pre and post-emergence herbicides. Roundup (Glyphosate), a post-emergence herbicide, was applied at the rate of 3.0 liters (L) ha^{-1} to kill weeds at the beginning of the seasons. Dual Gold (960 g L^{-1} Metolachlor), a pre-emergence herbicide, was applied at the rate of 2.0 L ha^{-1} on relatively moist soil surface after planting but before emergence of crops and weeds. One month after the crop emergence, selective Basagran post-emergence herbicide was applied at the rate of 2.0 Lha^{-1} to manage actively growing grass and broad-leafed weeds in maize-bean intercrop.

The herbicide is effective mainly through contact action and therefore care was taken to have all weeds thoroughly covered with the herbicide sprays while avoiding maize and bean leaves.

Kakamega

The experimental set up, design and management of this trial was the same as that of Embu medium-term. The trial tested the effect of different treatments on arable crop production and these include: tillage (conventional till, zero till), organic (maize residues) and cropping systems (continuous maize, maize-bean rotation and intercrop; **Table 2**). However, fertilizer was applied at a rate of 50 kg N ha^{-1} and 25 kg P ha^{-1}. The organic inputs are applied onto the soil surface every season at 2 t ha^{-1}. The residues, however, were removed from those treatment not receiving residues. The treatments were replicated three times in a randomized complete block design with plots of 10 m long by 8 m wide. Treatments selected for soil fauna studies included: maize-bean intercrop (MBi) under conventional (CT) and zero (CA) till, but all with crop residues (CR) applied. A no input treatment that typically represented farmer practice (or control) was also sampled (**Table 2**).

Nyabeda Trial

The field experiment was established in March 2003, and has been managed by the International Center for Tropical Agriculture (CIAT). The details of the trial set-up and management are documented in Kihara et al. (2012). The treatments used to study soil fauna diversity include a farmer practice under sole maize, CT with residues added, CA under maize-soybean

TABLE 1 | Location, climatic, and soil characteristics of the study sites.

Parameter	Embu	Kakamega	Nyabeda
Year established	MT-2010	MT-2010	LT-2003
Agro-climatic zone	Humid	Sub-humid	Sub-humid
Agro-ecological zone	Upper midland 3	Upper midland 1	lower midland 2
Latitude	00° 33.18′ S	0° 16.96′ N	0° 07′ N
Longitude	037° 53.27′ E	34 46.07′ E	34° 24′ E
Altitude (m.a.s.l.)	1,420	1,534	1,420
Total annual rainfall (mm)	1,250	1,978	1,800[a]
Daily temperatures (°C):			
Mean	20	21	23.2
Minimum	16–21	11	14
Maximum	21–28	26	31
Soil type	Humic Nitisols[b]	Eutric Nitisol[c]	Ferralsol[c]
Sand:silt:clay ratio	3:22:75	13:34:53	15:21:64
pH (water)	3.88	5.40	5.08
Extractable K (me 100 g^{-1})	0.27	0.70	0.10
P (mg P kg^{-1})	16.13	3.40	2.99
Ca (cmolc kg^{-1})	2.15	0.93	4.69*
Mg (cmolc kg^{-1})	0.45	0.05	1.68*
Total SOC (%)	3.70	4.10	1.35
Total Nitrogen (%)	0.37	0.30	0.15

**Value obtained in meq 100 g^{-1} of soil.*
[a]2002–2008 period.
[b]See Jaetzold et al. (2007) for details.
[c]See Jaetzold et al. (2006) for details.
MT, medium-term; LT, long-term.

rotation and intercropping. There were 3 replicates included and individual plots measured 7 × 4.5 m. The crop rotation since trial establishment consisted of soybean (*Glycine max* L.) during short rains and maize (*Zea mays* L.) during long rains. All plots were fertilized with 60 kg ha^{-1} N (urea), 60 kg ha^{-1} P (Triple Super Phosphate), and 60 kg ha^{-1} K (Muriate of Potash) per season (**Table 2**). To control stem borer, 5 kg ha^{-1} of granulated Bulldock (beta-cyfluthrin) was applied in the funnel of the maize plants during the 5th week after planting in all treatments. Under CT, the seedbed was prepared by hand-hoeing to 15 cm soil depth. Weeding was performed three times per season using hand hoe. Under CA, a 3 cm deep seedbed was prepared with the hand hoe. Weeding was performed three times per season by hand pulling until the long rainy season of 2009. Thereafter, herbicides (glyphosate and 2, 4-dichlorophenoxyacetic acid) have been applied to all CA treatments before planting and subsequent weeding done by hand pulling. Maize residues were collected after crop harvest, dried, chopped and stored during the dry season. At the time of soybean planting, the residues were reapplied at a rate of 2 h ha^{-1} for those treatments receiving crop residues. Since soybeans drop leaves prior to grain maturity, soybean residues (leaves and stems) always remained in the field after harvesting, irrespective of treatment. These soybean residues were then either incorporated in CT or remained at the soil surface in CA. In our fauna sampling regime, only a few of the trial treatments were selected, and a no input treatment was also sampled as in Kakamega (**Table 2**).

Faunal Sampling Techniques

Two techniques were employed to sample fauna for richness and abundance.

Macrofauna

Using a monolith of size 25 cm × 25 cm × 30 cm, samples were taken 8 weeks (in December 2015 for the sites in eastern Kenya and June–July 2016 two experimental trials in western Kenya) after planting crops in the season (Swift and Bignell, 2001; Bignell et al., 2008): At each observation, one sample was taken randomly from each plot. The monolith was situated over a randomly selected spot and dug with a spade and hoe to a 30 cm soil depth (**Plate 2**). The soil from the monolith was removed by hand depth-wise (0–15 and 15–30 cm) into plastic buckets. The soil sample from each depth was placed in different plastic trays (20 cm by 30 cm) and gently sorted out to locate the animals. The animals were separated into major taxonomic groups, recorded and then collected in plastic bottles. The soil fauna collected were preserved in 75% alcohol for subsequent identification at the Soil microbiology laboratory of CIAT, ICIPE Duduville Campus, Nairobi, Kenya. Earthworms were killed in 75% alcohol and fixed in 4% formaldehyde. In the laboratory, counting was done. Species richness, and number of different categories of animals were expressed per meter square.

Mesofauna

At the same time of sampling for macrofauna soil samples were collected for mesofauna observations using a metallic core of 10 cm diameter up to 30 cm depth (and at same 0–15 or 15–30 cm depths as macrofauna). One sample was taken in each plot at each sampling. The samples were taken to the CIAT laboratory where mesofauna groups were extracted using the behavioral or dynamic method with Berlese-Tullgren as the basic apparatus (**Plate 3**) (Southwood, 1995). This apparatus was originally designed by an Italian entomologist, Berlese, A. and later modified by a Swede, Tullgren, A. who used a light bulb as the source of heat. The apparatus has since been modified by many workers (Southwood, 1995). For this study the apparatus was designed and constructed locally.

Basically, the collected soil was poured into the perforated soil sample containers of the "Berlese-Tullgren" funnel apparatus over a funnel. Heat was applied to the soil using a 75 watts bulb placed above the sample container. Heat supplied was regulated by upward adjustments of the sample containers. Thus, the animals were exposed to a controlled gradient of high to low temperature and light, and low to high humidity from top to bottom, so that the animals are driven gradually downwards and out into the collection jars filled with 75% alcohol. The set up was left for 24 h. After this period, the animals collected in the jars were sorted out and counted under a light microscope. The preserved animals were also stored and later taken to the laboratory for taxonomic analyses.

Soil Sampling and Nutrient Analysis

Immediately after handpicking the soil macrofauna, soils from each monolith was mixed thoroughly to make

TABLE 2 | Treatment selected and descriptions.

Treatment	Tillage	Cropping	Organic input	Inorganic input
Embu medium-term trial				
1. CTMBi−CR	Conventional	Maize-beans intercrop	None	80 kg N, 111 P_2O_5
2. CASB+CR	Zero	Sole beans	Beans residues	20 kg N, 51 kg P_2O_5
3. CASM+CR	Zero	Sole maize	Maize residues	60 kg N, 60 kg P_2O_5
4. CAMBi+CR	Zero	Maize-beans intercrop	Maize and beans residues	80 kg N, 111 P_2O_5
Kakamega medium-term trial				
1. FP (Farmer practice)	Conventional	Sole maize	None	None
2. CTMBi+CR	Conventional	Maize-bean intercrop	2 t/ha maize residues	50N, 25P
3. CAMBi+CR	Zero	Maize-bean intercrop	2 t/ha maize residues	50N, 25P
Nyabeda long-term trial				
1. FP (Farmer practice)	Conventional	Sole maize	None	None
2. CTMSr+CR	Conventional	Maize-soybean rotation	2 t/ha maize residues	60 kg N/ha-Urea
3. CAMSr+CR	Zero	Maize-soybean rotation	2 t/ha maize residues	60 kg N/ha-Urea
4. CAMSi+CR	Zero	Maize-soybean intercrop	2 t/ha maize residues	60 kg P/ha-TSP

CT, Conventional till; CA, Conservation agriculture (Zero till); FP, Farmer practice; SM, Sole maize; SB, Sole beans; MBi, Maize bean intercrop; MSi, Maize-soybean intercrop; MSr, Maize-soybean rotation CR, Crop residue; N, Nitrogen; +/– denotes with or without crop residues and with or without nitrogen.

PLATE 2 | Soil monolith excavation and macrofauna sampling.

a composite sample of about 500 g for analysis. Soil parameters measured included: soil pH total organic C and N, available P, and exchangeable bases (Na, K, Ca, and Mg). Soil pH was determined using a pH meter with soil-water ratio of 1:2.5 (Anderson and Ingram, 1993). Total organic C and N were determined using a CN-analyser, while P and the bases were extracted by the Mehlich-3 procedure (Mehlich, 1984) and measured through inductively coupled plasma atomic emission spectroscopy (Isaac and Johnson, 1998).

Statistical Analyses

The data obtained on soil fauna richness and abundance and soil chemical properties were subjected to analysis of variance (ANOVA) with Genstat 17.1 (2015). Levene's test was used to test for homogeneity of variances (Field, 2005). In case of non-homogeneity of variances, data were square root $(x + 0.5)^{1/2}$ transformed before further analysis. Fauna data were analyzed separately for each depth (0–15 and 15–30 cm). Linear Mixed Model was fitted by Restricted Maximum Likelihood (RELM) procedure using the Genstat package. This procedure allows for inclusion of both fixed- and random-effects terms in the model such that profiled deviance of RELM criterion is optimized for the parameter estimates (Kuznetsova et al., 2014; Bates et al., 2015). Treatments were included in the model as fixed factors, whereas block was defined as a random factor. The statistical significance was determined at $p \leq 0.05$ and levels of significance among the different treatments were evaluated using Fischer's least significance difference (LSD). Correlation analysis (Pearson correlations), was conducted to establish the significance of the relationships between soil fauna and soil factors. Because fauna and soil variables had different units of measurement, they were standardized first so that each variable received equal weight in the analysis and also to make the coefficient (r) values comparable (Cao et al., 1999; Jongman et al., 2005).

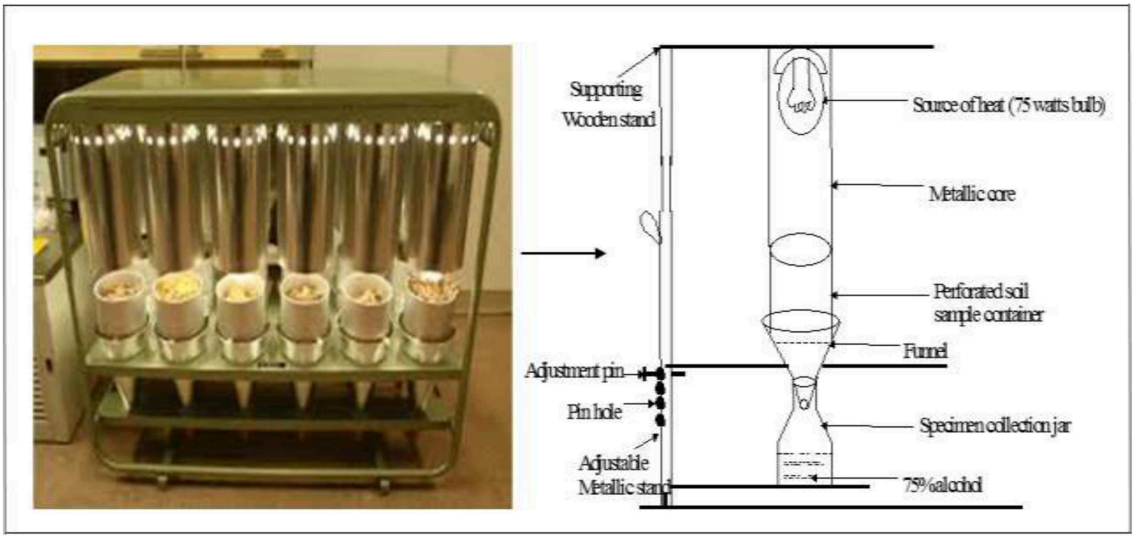

PLATE 3 | Photo and sketch diagram showing the Berlese-Tullgren apparatus.

RESULTS

Soil Fauna Composition

Overall, a total of 58 macrofauna species classified into 14 major groups were sampled across the three trials in eastern and western Kenya. However, it should be noted that, the numbers given are estimations because it is difficult (or even impossible) to be sure that these additional categories consist of just one species (already listed or not) or more species. The medium trial of Embu had relatively higher species richness compared to the medium-term trial of Kakamega and long-term trial of Nyabeda, and the highest number of species (37) was recorded in the Embu medium-term trial followed by Nyabeda (32) > Kakamega (27) (Data is available as **Supplementary Tables**). The macrofauna groups were dominated by Oligochaeta (earthworms), Isoptera (termites), Hymenoptera (ants), and Coleoptera (beetles), with these groups constituting over 80% of mean totals across the different trials (**Table 3**). In Embu medium-term trial, Hymenoptera were the most abundant of these faunal groups, constituting about 39% of the mean total followed by Isoptera (28%), Coleoptera (12%), and Oligochaeta (10%) (**Table 3**). In Kakamega trial, Oligochaeta (64%) was the most dominant of all the macrofauna groups followed by Isoptera (7%) > Hymenoptera (6%) > Coleoptera (5%), whereas in Nyabeda, Isoptera was dominant (55%) followed by Oligochaeta (21%) > Hymenoptera > Coleoptera (6%) in that order. In all the trials, however, the other macrofauna groups were observed in very low numbers, with each group constituting ≤ 5% (**Table 3**). Generally, macrofauna were more abundant in the eastern Kenya than in western Kenya sites, with the former recording a mean total between 875 and 1,386 numbers per m^{-2} compared to that between 565 and 784 numbers per m^{-2} in the latter (**Table 3**).

For mesofauna groups, 18 species classified into seven major groups were observed across the four trials, and the western Kenya sites (Nyabeda and Kakamega) had relatively higher mesofauna richness compared to the eastern Kenya (Embu) sites (Data is available as a **Supplementary Tables**). Mesofauna was equally relatively more abundant in the western Kenya sites compared to the eastern Kenya site (**Table 3**). In the eastern Kenya (Embu) trial, mesofauna groups were dominated by Acarina, which constituted > 50% followed by Collembolla (16–17%) and Enchytraeids (10–15%), with each of the other groups constituting < 6% (**Table 3**). Western Kenya trials were also dominated by Acarina (40–59%) and Collembolla (35–46%) and the other groups each constituted ≤ 5% (**Table 3**).

Soil Fauna Taxonomic Richness

The combinations of tillage practice, organic residues and cropping system had significant effect on macrofauna taxonomic richness ($p < 0.05$) largely at the top 0–15 cm soil depth than at the lower 15–30 cm soil depth in most of the study sites (**Figure 1**). At 0–15 cm soil depth of the Embu medium-term trial, mean macrofauna taxonomic richness was significantly higher in both conservation agriculture under sole maize (CASM+CR) cropping and conservation agriculture under maize-bean intercrop (CAMBi+CR) treatments than in either conventional till minus crop residues under maize-bean intercrop (CTMBi-CR) or conservation agriculture under sole beans (CASB+CR) (**Figure 1A**). At 15–30 cm, mean macrofauna taxonomic richness did not differ among the treatments ($p = 0.503$) (**Figure 1B**). At the Kakamega, no significant differences were noted for macrofauna mean richness among the treatments at both 0–15 and 15–30 cm soil depths (**Figures 1C,D**). At Nyabeda, macrofauna richness was significantly lower in conventional (typical farmer's practice without residues) till without inputs than in the other treatments (**Figure 1E**). At 15–30 cm, mean macrofauna taxonomic richness did not differ among the treatments ($p = 0.370$) (**Figure 1F**).

In addition, no significant differences were noted for mesofauna mean richness among the treatments at both 0–15 cm and 15–30 cm soil depths in all study sites (**Figure 2**). As

TABLE 3 | Mean total population and percent composition of macrofauna and mesofauna within each group across medium-term (MT) and long-term (LT) trials in Embu, Kakamega, and Nyabeda, Kenya.

	Embu-MT		Kakamega-MT		Nyabeda-LT	
Macrofauna group	**Mean total**	**% of total**	**Mean total**	**% of total**	**Mean total**	**% of total**
Oligochaeta	136	9.8	499	63.6	120	21.2
Isoptera	381	27.5	58	7.4	312	55.2
Hymenoptera	545	39.3	46	5.9	56	9.9
Coleoptera	171	12.3	39	5.0	36	6.4
Diptera	15	1.1	4	0.5	4	0.7
Lepidoptera	33	2.4	16	2.0	4	0.7
Diplopoda	24	1.7	38	4.8	1	0.2
Chilopoda	34	2.4	39	5.0	7	1.2
Orthoptera	21	1.5	4	0.5	3	0.5
Araneae	11	0.8	20	2.5	17	3.1
Odonata	3	0.2	–	–	4	0.7
Hemiptera	4	0.3	22	2.8	1	0.2
Blattoidea	5	0.4	–	–	–	–
Isopoda	3	0.2	–	–	–	–
Total	**1,386**	**100**	**784**	**100**	**565**	**100**
Mesofauna group						
Acarina	592	57.9	1,196	39.5	2,900	58.9
Arachnida	3	0.2	–	–	–	–
Collembolla	172	16.9	1,392	46.0	1,741	35.3
Diplura	50	4.9	155	5.1	32	0.6
Enchytraeidae	150	14.7	14	0.5	11	0.2
Protura	55	5.4	–	–	–	–
Symphyla	–	–	268	8.9	243	4.9
Total	**1,022**	**100**	**3,025**	**100**	**4,927**	**100**

expected, soil fauna richness reduced with depth where these were nearly ≤50% that of top soil for each of the treatments.

Soil Fauna Abundance

Across all sites, and in both (0–15 and 15–30 cm) soil depths, no significant effect of tillage, cropping and organic inputs on soil macrofauna abundance were observed (**Figure 3**). Equally no significant treatment effects on soil mesofauna abundance were observed in Embu medium-term (**Figures 4A,B**) and Nyabeda long-term trials for both soil depths (**Figures 4E,F**). Although a similar observation was made on mesofauna abundance at the 15–30 cm soil depth of Kakamega trial (**Figure 4D**), at 0–15 cm soil depth, maize-bean intercrop system under conservation agriculture (CAMBi+CR) had significantly higher mesofauna abundance than the convention till with similar management (CTMBi+CR) practices or conventional till (farmers practice) without any inputs (**Figure 4C**).

Soil Fauna Group Abundance Across Treatments

Faunal abundance were analyzed and assessment made depth-wise across treatments for the various groups.

Macrofauna

In the long-term trial, tillage, residue application and cropping system affected only a few of the macrofauna groups such as Oligochaeta, Chilopoda, and Araneae, but only at the top soil depth. This is unlike medium-term trials where significant effects were noted in either depths (**Table 4**).

At 0–15 cm soil depth, in the Embu medium-term trial, Oligochaete, and Araneae were significantly more abundant in both conservation agriculture, maize-bean intercropping system with residues (CAMB+CR) and conservation agriculture, sole maize with residues (CASM+CR) treatments than zero till, sole beans with residues (CASB+CR) and conventional till, maize bean system without residues treatments (CTMB-CR) (**Table 4**). Chilopoda, however, were significantly more abundant in CASM+CR than in the other treatments. The other macrofauna groups did not differ among the treatments, and at 15–30 cm, all the macrofauna groups did not differ among the treatments as well.

In Kakamega, management practices affected only Oligochaeta and Chilopoda groups but only at 0–15 and 15–30 cm soil depths, respectively. At 0–15 cm depth, maize-bean intercrop system under conservation agriculture with residue applied (CAMBi+CR) had significantly higher Oligochaete abundance than the convention till with similar management (CTMBi+CR) practices or conventional till (farmers practice: FP) without any inputs. At 0–15 cm, the predacious Chilopod group, was on the other hand, significantly higher in famers practice (FP-conventional till without any inputs) than in the other treatments.

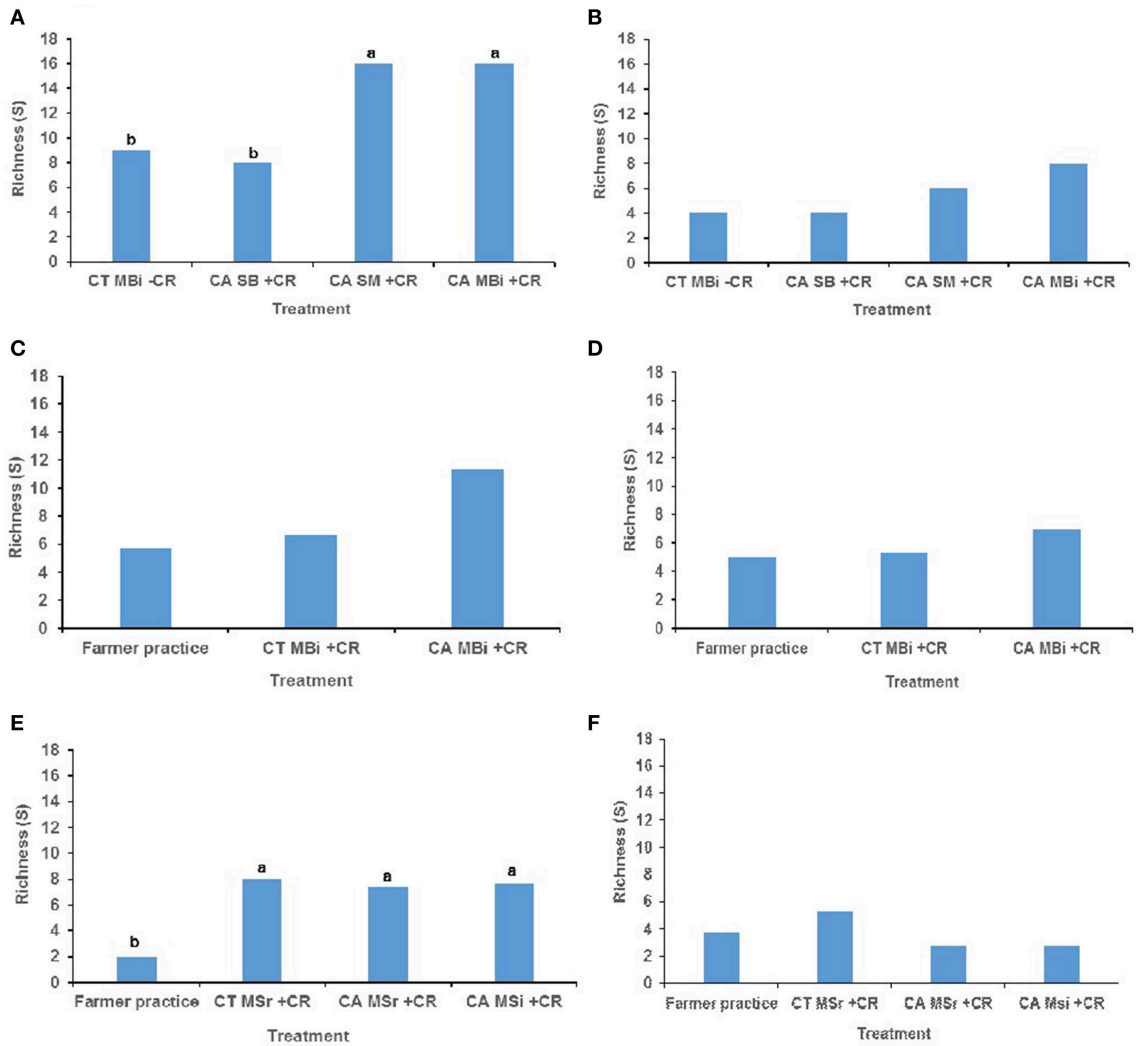

FIGURE 1 | Soil macrofauna diversity (richness) across trials of Embu, Kakamega, and Nyabeda. MT, Medium-term; LT, Long-term; CT, Conventional till; CA, Conservation agriculture (Zero till); SM, Sole maize; SB, Sole beans; MBi, Maize bean intercrop; MSi, Maize-soybean intercrop; MSr, Maize-soybean rotation; CR, Crop residue. **(A)** Embu MT (0-15 cm, p = 0.027*); **(B)** Embu MT (15-30 cm, p = 0.503); **(C)** Kakamega MT (0-15 cm, p = 0.329); **(D)** Kakamega MT (15-30 cm, p = 0.414); **(E)** Nyabeda LT (0-15 cm, p = 0.029*); **(F)** Nyabeda LT (15-30 cm, p = 0.370). Bars with different lower case letters are statistically significantly different at $p < 0.05$.

At 0–15 cm soil depth of Nyabeda, cropping system and residue addition seem to be the influencing factors for Hymenoptera abundance as this group was significantly higher under maize-soybean rotation system for both conventional (CTMSr+CR) and conservation agriculture systems with crop residue applied (CAMSr+CR) than in the conventional till under continuous maize without inputs (FP) and conservation agriculture under maize soybean intercrop but with crop residues (CAMSr+CR). No significant treatment effects were observed for the other macrofauna groups at this top soil depth and for all the macrofauna groups at 15–30 cm soil depth.

Mesofauna

In the medium-term trial of Embu, and in both soil depths, all the mesofauna groups did not differ among the treatments indicating lack of significant influence of tillage, organic residue application and crop management (**Table 5**). However, significant effect of tillage on mesofauna abundance were noted at top 0–15 cm soil depths for both Kakamega and Nyabeda trials. At Kakamega, Collembolan group was significantly higher in the conservation agriculture practices than in the conventional till practices, and the same pattern was observed for Symphyla group in the Nyabeda trial. As expected, mesofauna groups were less abundant in the lower soil depths compared to the upper depths.

Management Practices and Effect on Soil Chemical Properties

Soil management practices had significant effects on soil chemical properties (**Table 6**). In Embu site, soil TOC was significantly lower in conservation tillage under sole maize (CASM+CR) than in all the other treatments. Total organic N, on the other hand, was significantly higher in conventional tillage under

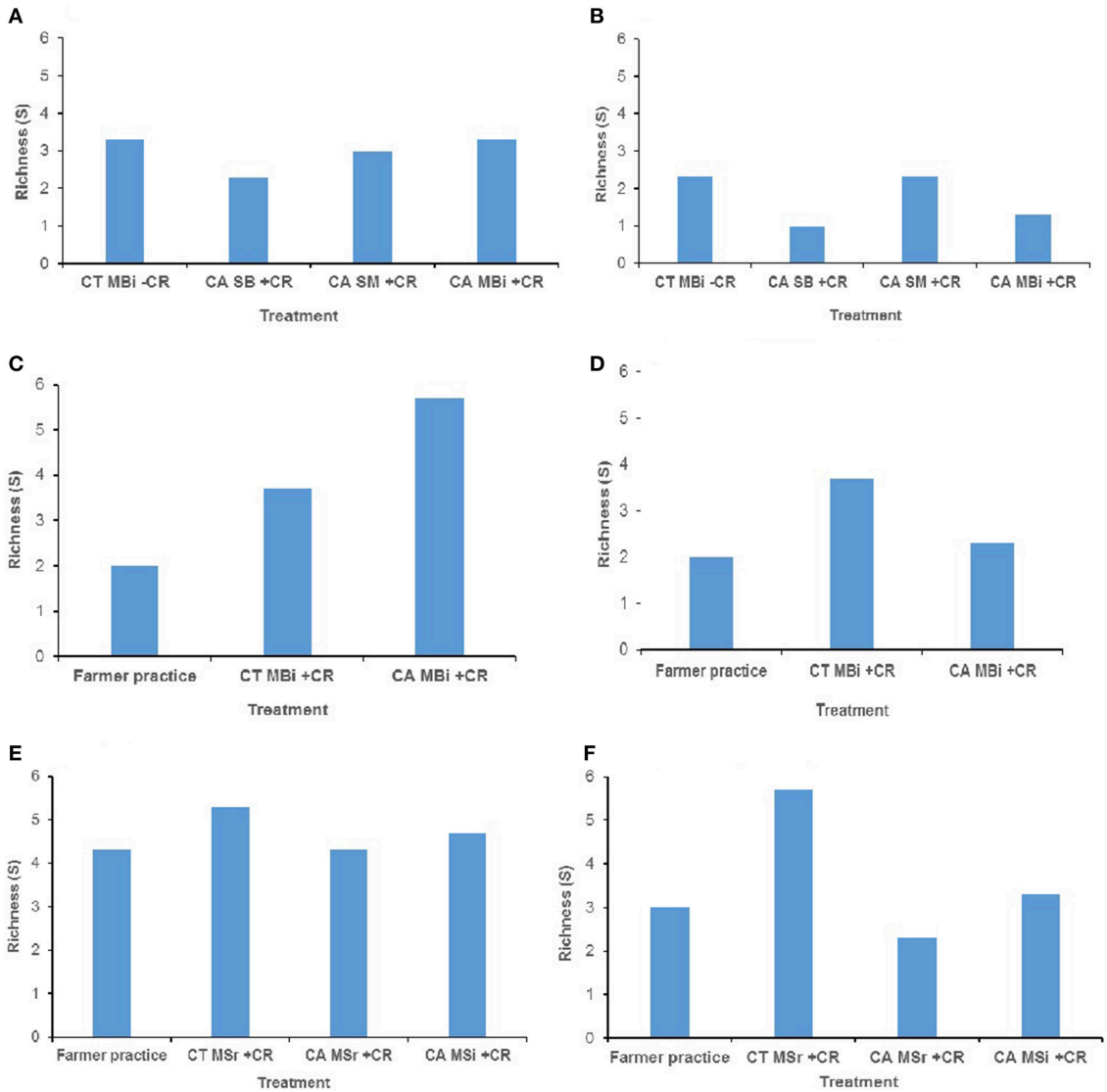

FIGURE 2 | Soil mesofauna diversity (richness) across trials of Embu, Kakamega, and Nyabeda. MT, Medium-term; LT, Long-term; CT, Conventional till; CA, Conservation agriculture (Zero till); SM, Sole maize; SB, Sole beans; MBi, Maize bean intercrop; MSi, Maize-soybean intercrop; MSr, Maize-soybean rotation; CR, Crop residue. **(A)** Embu MT (0-15 cm, p = 0.864); **(B)** Embu MT (15-30 cm, p = 0.515); **(C)** Kakamega MT (0-15 cm, p = 0.058); **(D)** Kakamega MT (15-30 cm, p = 0.502); **(E)** Nyabeda LT (0-15 cm, p = 0.405); **(F)** Nyabeda LT (15-30 cm, p = 0.125).

maize-bean intercrop without crop residues (CTMBi-CR) than in the conservation tillage practices under sole maize and maize-bean intercrop (**Table 6**). It however, did not differ from conservation tillage under sole beans. In Kakamega site, soil pH was significantly higher in conventional tillage under maize-bean intercrop with crop residues than in conservation tillage under intercrop (CAMBi+CR) and farmer practice (FP), and a trend similar to that of soil pH was observed for Ca and Mg (**Table 6**). Tillage influenced TOC and P contents, and were significantly higher in conservation tillage under intercrop (CAMBi+CR) than in the conventional tillage under intercrop (CA MBi+CR) and farmer practice (FP). Although TON was significantly higher in CAMBi+CR than FP, it did not differ from CTMBi+CR (**Table 6**). At Nyabeda, treatment effect was observed only for soil pH, Ca, and Mg. Soil pH was higher in CTMSr+CR than either CAMSi+CR or FP although it did not differ from CAMSr+CR. Calcium content was highest in CAMSr+CR > CTMSr+CR > FP, but lowest in CAMSi +CR. Magnesium content on the other hand was highest in CTMSr+CR > CAMSr+CR > FP, but again lowest in CAMSi+CR (**Table 6**).

Correlation Between Soil Fauna and Soil Chemical Properties

Over 60% of the macrofauna, and over 70% of mesofauna showed significant correlation with soil parameters (**Table 7**). Oligochaeta, Diplopoda, and Hemiptera significantly positively

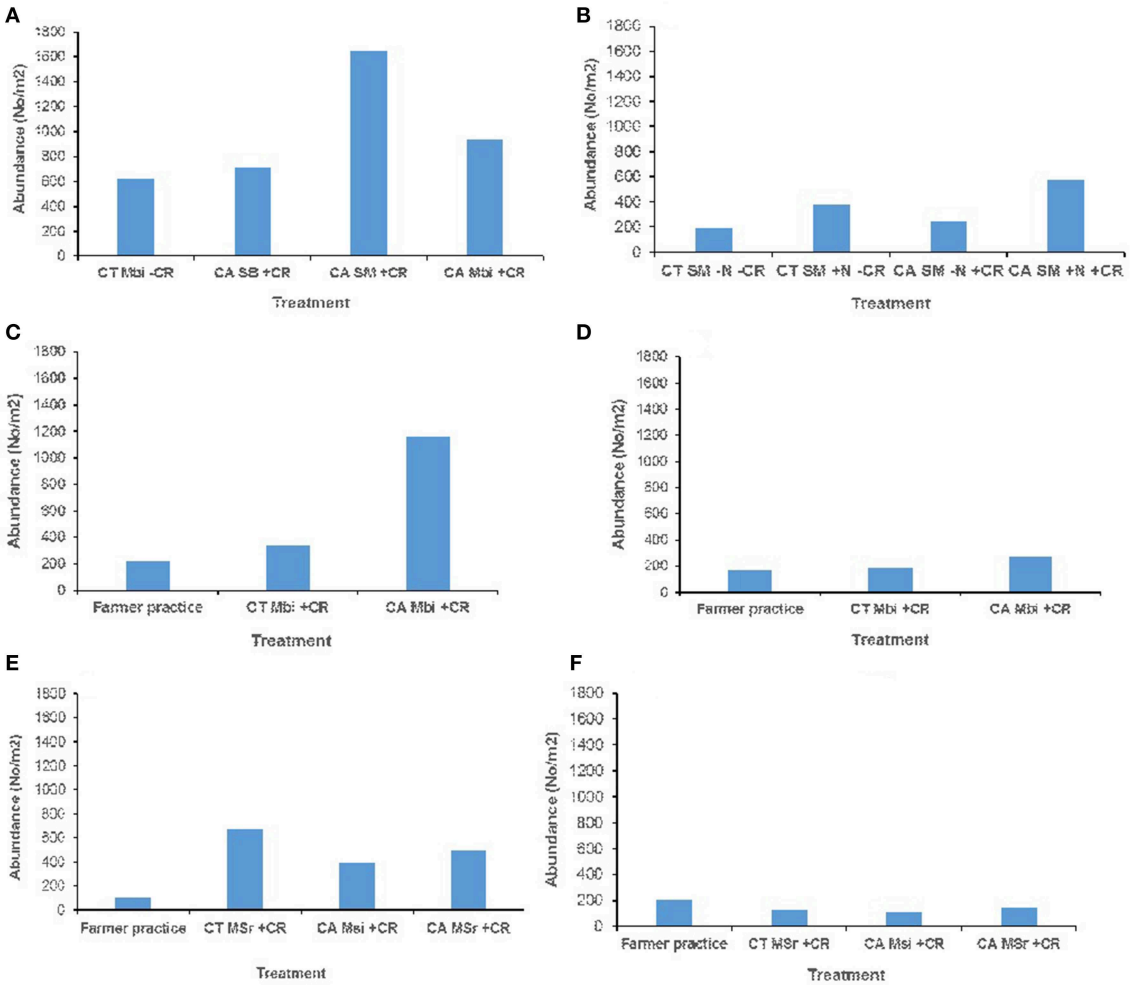

FIGURE 3 | Soil macrofauna abundance across trials of Embu, Kakamega, and Nyabeda. MT, Medium-term; LT, Long-term; CT, Conventional till; CA, Conservation agriculture (Zero till); SM, Sole maize; SB, Sole beans; MBi, Maize bean intercrop; MSi, Maize-soybean intercrop; MSr, Maize-soybean rotation; CR, Crop residue. **(A)** Embu MT (0-15 cm, p = 0.316); **(B)** Embu ST (15-30 cm, p = 0.075); **(C)** Kakamega MT (0-15 cm, p = 0.128); **(D)** Kakamega MT (15-30 cm, p = 0.383); **(E)** Nyabeda LT (0-15 cm, p = 0.285); **(F)** Nyabeda LT (15-30 cm, p = 0.843).

correlated with soil organic matter (TOC, TON) and P, whereas Hymenoptera, Coleoptera and Blattodea positively correlated with nearly all the exchangeable bases (Na, K, Ca, and Mg). Diptera, Lepidoptera, and Odonata on the other hand significantly correlated with all exchangeable bases measured (except K and Mg). Whereas, Orthoptera positively correlated with Mg, and Isopoda positively with K, Chilopoda negatively correlated with soil pH (**Table 7**). Mesofauna groups on the other hand showed significant correlations with exchangeable bases (Na, K, Ca, and Mg) only. Whereas, Enchytraeid and Protura positively correlated with all the exchangeable bases and Arachnida with Mg, Acarina, Collembolla, and Symphila negatively correlated with all the bases.

DISCUSSION

The total number of macrofauna taxa of between 25 and 37 recorded in our study sites conforms with the range of 35–38 taxa recorded by Ayuke (2000) and Ayuke et al. (2009) across arable fields within western and eastern Kenya, respectively. However, these taxa were much lower than 75 taxa recorded by Karanja et al. (2009) across arable sites of a different ecozone, the Coastal region of Kenya. Mesofauna total taxa were, however, three times higher than that recorded under biomass transfer agroforestry technology at Maseno, Western Kenya (Ayuke, 2000).

Results of this study have demonstrated the benefits of conservation agriculture in enhancing soil fauna richness and abundance. Higher macrofauna taxonomic richness and abundance of mesofauna in CA treatments than in CT without residue application are related to an improved microclimate and access to food in the CA system. Disturbances caused by tillage operations and residue removal (CT) are known to negatively affect sensitive fauna (Ayuke et al., 2011a,b), except for the predating and foraging groups like Hymenoptera (ants), Chilopoda (centipedes) and some species of Isoptera (termites) e.g., *Microtermes* sp. Earthworm species, among them, the

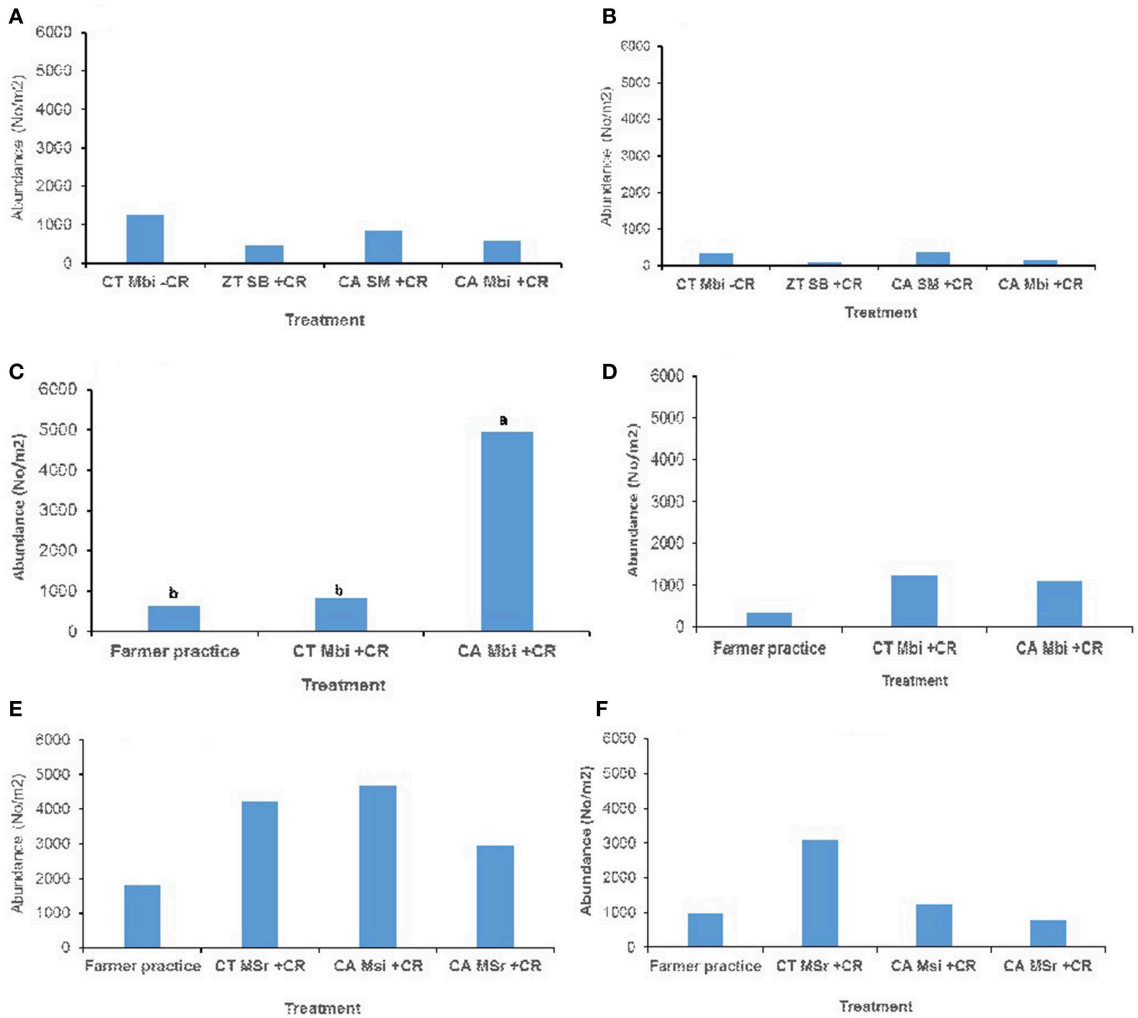

FIGURE 4 | Soil mesofauna abundance across trials of Embu, Kakamega, and Nyabeda. MT, Medium-term; LT, Long-term; CT, Conventional till; CA, Conservation agriculture (Zero till); SM, Sole maize; SB, Sole beans; MBi, Maize bean intercrop; MSi, Maize-soybean intercrop; MSr, Maize-soybean rotation; CR, Crop residue. **(A)** Embu MT (0-15 cm, p = 0.664); **(B)** Embu MT (15-30 cm, p = 0.233); **(C)** Kakamega MT (0-15 cm, p = 0.036*); **(D)** Kakamega MT (15-30 cm, p = 0.347); **(E)** Nyabeda LT (0-15 cm, p = 0.425); **(F)** Nyabeda LT (15-30 cm, p = 0.256). Bars with different lower case letters are statistically significantly different at $p < 0.05$.

Dichogaster sp. are also sensitive to disturbance (Ayuke et al., 2011a,b), and so were lacking under conventional till as opposed to conservation agriculture system.

Microclimate modification, food resource availability, and land Management practices (e.g., tillage, organic resource use, crop rotation, and application of agrochemicals such as pesticides, herbicides, and inorganic fertilizers) are known to either positively or negatively influence the diversity and abundance of soil fauna communities (Dangerfield, 1993; Beare et al., 1997; Nhamo, 2007; Gianessi, 2010; Isenring, 2010). Tillage influences soil fauna in several ways: the mechanical and physical disturbance due to tillage can cause habitat destruction for some of the macrofauna groups. When vegetation are removed, the resultant change in habitat structure, the reduced range and abundance of food resources and the extreme climate at the soil surface, most likely combine to create harsh environment that may be intolerable to most soil fauna groups among them earthworms. Consequently, the population of these soil fauna groups could be suppressed, possibly explaining why some species of earthworms, springtails (Collembolla) and Symphyla were absent from the conventional till systems, hence the lower diversity and abundances observed. However, predacious fauna groups such as ants (Hymenoptera) were favored by tillage as this exposed their likely prey among them termites, on to the soil surface hence abundant food.

Conservation agriculture favored those fauna groups e.g., earthworms (Oligochaeta) and predacious spiders (Araneae) that are sensitive to disturbance caused by tillage. Many known Araneae (spider) species live on the soil surface or in soil crevices, invade the natural pore system of soils or are in some way closely associated with the soil systems. Higher Araneae recorded under CA practices could be attributed to less disturbance

TABLE 4 | Mean abundance of soil macrofauna groups across treatments in medium-term and long term trials of Embu, Kakamega, and Nyabeda, Kenya.

Group	0–15 cm					15–30 cm				
	Treatment				*P*-value	Treatment				*P*-value
Group	**CTMBi-CR**	**CASB+CR**	**CASM+CR**	**CAMBi+CR**	***P*-value**	**CTMBi-CR**	**CAMBi+CR**	**CASB+CR**	**CASM+CR**	***P*-value**
Embu medium-term trial										
Oligochaeta	80b	0b	192a	197a	**0.029***	11	48	0	16	0.225
Isoptera	272	43	283	187	0.810	165	37	107	432	0.420
Hymenoptera	16	501	901	251	0.283	21	91	144	256	0.388
Coleoptera	139	59	139	160	0.215	85	21	43	37	0.064
Diptera	27	43	0	5	0.091	11	0	0	0	0.070
Lepidoptera	59	32	0	37	0.412	5	0	0	0	0.455
Diplopoda	11	0	27	37	0.070	0	16	0	5	0.572
Chilopoda	0b	0b	64a	5b	**0.031***	21	5	5	21	0.349
Orthoptera	0	48	21	5	0.566	0	11	0	0	0.455
Araneae	0b	0b	16a	21a	**0.035***	5	5	5	5	0.455
Odonata	5	0	0	5	0.654	0	0	0	0	–
Hemiptera	11	5	0	0	0.189	0	0	0	0	–
Blattoidea	0	5	0	16	0.216	0	0	0	0	–
Isopoda	5	0	5	0	0.455	0	0	0	0	–
Group	**FP**	**CTMBi+CR**	**CAMBi+CR**		***P*-value**	**FP**	**CTMBi+CR**	**CAMBi+CR**		***P*-value**
Kakamega medium-term trial										
Oligochaeta	112b	213b	869a		**0.016***	64	91	149		0.493
Isoptera	27	5	5		0.444	69	16	53		0.476
Hymenoptera	16	48	32		0.444	16	16	11		0.790
Coleoptera	27	16	32		0.815	5	21	16		0.444
Diptera	0	5	0		0.444	0	0	5		0.444
Lepidoptera	0	0	43		0.218	0	0	5		0.444
Diplopoda	0	5	101		0.444	0	0	5		0.444
Chilopoda	27	16	32		0.744	37a	0b	5b		**0.048***
Orthoptera	0	0	0		–	0	11	0		0.111
Araneae	11	16	0		0.373	0	16	16		0.617
Hemiptera	0	11	48		0.444	0	0	5		0.444
Group	**FP**	**CTMSr+CR**	**CAMSi+CR**	**CAMSr+CR**	***P*-value**	**FP**	**CTMSr+CR**	**CAMSi+CR**	**CAMSr+CR**	***P*-value**
Nyabeda long-term trial										
Oligochaeta	91	27	17	112	0.736	32	64	21	75	0.628
Isoptera	5b	0b	224a	5b	**0.005****	139	11	43	64	0.667
Hymenoptera	5	85	37	69	0.161	16	43	16	0	0.175
Coleoptera	5	80	11	21	0.495	0	11	5	5	0.572
Diptera	5	405	0	261	0.542	5	0	0	0	0.455
Lepidoptera	0	11	5	0	0.455	5	0	0	0	0.455
Diplopoda	0	0	0	0	–	5	0	0	0	0.455
Chilopoda	0	11	0	0	0.591	0	0	0	0	–
Orthoptera	0	5	0	0	0.455	0	0	0	0	–
Araneae	0	43	21	5	0.392	0	0	5	5	0.654
Hemiptera	0	5	0	0	0.455	0	5	5	0	0.654
Blattoidea	0	0	0	5	0.455	0	0	0	0	–

*Means followed by the same lowercase letters across rows are not significantly different at $p < 0.05$. Significant p-values are indicated in bold. For some of the groups with zeros at lower depths, ANOVA did not calculate the p-value. For treatment abbreviations (see **Table 2**). *Significant; **Highly significant; ***Very highly significant.*

that might have affected the populations of their likely prey. It has been shown that, reduced- or no-tillage agriculture is beneficial for soil conservation in that, soil-surface accumulation of crop residues as a result of minimal soil disturbance protects soil from water and wind erosion (Stinner and House, 1990). Unlike in conventional tillage, litter and soil organic matter

TABLE 5 | Mean abundance of soil mesofauna groups across treatments in long- and short-term trials of Embu, Nyabeda, and Kakamega.

	0–15 cm Treatment					15–30 cm Treatment				
Group	**CTMBi-CR**	**CASB+CR**	**CASM+CR**	**CAMBi+CR**	***P*-value**	**CTMBi-CR**	**CASB+CR**	**CASM+CR**	**CAMBi+CR**	***P*-value**
Embu medium-term trial										
Acarina	1,100	233	533	233	0.415	33	33	133	67	0.142
Arachnida	0	167	0	167	0.654	0	0	0	0	–
Collembola	67	100	167	133	0.931	67	0	133	33	0.514
Diplura	67	33	33	0	0.714	0	0	33	33	0.654
Enchytraeidae	0	67	100	167	0.136	133	67	33	33	0.285
Protura	33	33	0	67	0.572	100	0	33	0	0.285
Group	**FP**	**CTMBi+CR**	**CAMBi+CR**		***P*-value**	**FP**	**CTMBi+CR**	**CAMBi+CR**		***P*-value**
Kakamega medium-term trial										
Acarina	464	506	1,477		0.122	84	295	759		0.322
Collembola	127b	42b	3,249a		**0.012***	42	422	295		0.391
Diplura	0	84	42		0.444	84	211	42		0.432
Enchytraeidae	42	0	0		0.444	0	0	0		–
Symphyla	0	211	169		0.132	127	295	0		0.273
Group	**FP**	**CTMSr+CR**	**CAMSi+CR**	**CAMSr+CR**	***P*-value**	**FP**	**CTMSr+CR**	**CAMSi+CR**	**CAMSr+CR**	***P*-value**
Nyabeda long-term trial										
Acarina	1,350	2,110	2,363	2,363	0.538	464	1,772	676	506	0.199
Collembola	338	2,110	2,012	295	0.224	380	1,224	338	253	0.441
Diplura	0	0	0	0	–	84	42	0	0	0.613
Enchytraeidae	42	0	0	0	0.455	0	0	0	0	–
Symphyla	84b	0b	295a	295a	**0.025***	42	42	211	0	0.150

*Means followed by the same lowercase letters across rows are not significantly different at $p < 0.05$. Significant p values are indicated in bold. For treatment abbreviations (see **Table 2**). *Significant; **Highly significant; ***Very highly significant.*

(and also nutrients) tend to concentrate near the soil surface of conservation-tillage systems, and these condition are likely to favor soil fauna within the CA. Although crop or organic residues get incorporated into the soil, albeit slowly, through invertebrate activity (Ayuke et al., 2009; Kihara et al., 2015), the litter layer is a very important factor in ameliorating soil temperature and water content of soil, thus providing a more stable environment for soil- and litter-dwelling invertebrates. This may explain the expected higher macrofauna richness and mesofauna abundance observed in top-soils than sub-soils.

The important role of organic amendments in influencing presence and abundance of specific faunal groups have been discussed (Ayuke, 2000) and their role explains why, *P. annulatus* earthworm species that thrive best under high organic matter environments were only recorded under sole maize and maize-bean CA systems where crop residues were retained, and detritus groups such as Collembolla and Symplyla were few under conventional till without residues. Equally no *P. annulatus* worms were recorded under CA with sole bean system where highly transient bean residues had been applied. Indeed, we observed during fauna sampling (eight weeks after cropping), that none of the trash remained at the soil surface, probably due to fast disappearance and rapid decomposition of bean trash. Soil fauna were deprived of food resources as a result hence were low or absent in such systems. Oligochaeta, Araneae, and Chilopoda were favored with long-term addition of organic residues while Isoptera were favored in the medium-term. In our study sites, earthworms were dominated by the epigeics (those living on top soil layers but forage primarily on plant residues—e.g., *Dichogaster* sp. and *P. annulatus*) and the endogeics (those living and feeding in the soil but foraging on soil organic matter). The main source of organic matter as food for earthworms include litter from aboveground plant parts, but dead roots and rhizodeposition can also be important food sources (Curry, 2004). Because earthworms are often food limited, their population can increase following organic amendments (Lowe and Butt, 2002; Ayuke et al., 2011b). Higher numbers of Oligochaeta (earthworms) in treatments with organic residues could be attributed to abundant food resources due to crop residues. Importance of soil organic matter as food and energy sources is reinforced by the positive correlations observed between TOC and TON as well as P with some of the macrofauna groups such as Oligochaeta, Diplopoda, and Hemiptera. Exchangeable bases (Na, K, Ca, and Mg) are equally important for some of the fauna groups where they are used in the formation of body parts such as the exoskeletons.

In natural ecosystems, spiders constitute the main invertebrate predatory group hence play an important ecological function in pest control. Araneae being mostly polyphagous predators, can significantly affect the population dynamics of many

TABLE 6 | Soil chemical characteristics (0–30 cm soil depth) across treatments of Embu, Nyabeda, and Kakamega.

Site	Treatment	Soil pH	TOC	TON	P	Na	K	Ca	Mg
Embu-MT	CT MBi -CR	4.75	2.43a	0.23a	15.87	0.27	0.58	2.23	4.30
	CA SB +CR	4.83	2.45a	0.22ab	14.20	0.24	0.67	2.27	5.25
	CA SM +CR	4.56	2.20b	0.20b	16.67	0.16	0.41	1.77	5.49
	CA MBi +CR	4.54	2.47a	0.18b	16.67	0.17	0.50	1.83	5.46
	***P*-value**	0.428	**0.040***	**0.016***	0.946	0.491	0.377	0.515	0.362
Kakamega-MT	FP	4.63b	3.26b	0.26b	36.10b	0.02	0.07	0.68b	0.08b
	CT MBi +CR	5.22a	3.57b	0.29ab	4.74b	0.02	0.16	1.34a	0.18a
	CA MBi +CR	4.59b	4.07a	0.32a	93.30a	0.02	0.11	0.59b	0.08b
	***P*-value**	**0.023***	**0.012***	**0.020***	**0.011***	0.791	0.162	**0.018***	**0.014***
Nyabeda-LT	FP	4.95b	2.39	0.18	36.57	0.03	0.09	0.73c	0.10c
	CT MSr +CR	5.39a	2.36	0.18	30.10	0.03	0.05	0.85b	0.20a
	CA MSr +CR	5.18ab	2.40	0.19	27.07	0.02	0.11	0.93a	0.14b
	CA MSi +CR	4.67b	2.35	0.18	30.69	0.03	0.07	0.57d	0.08d
	***P*-value**	**0.011***	0.954	0.856	0.181	0.735	0.114	**0.004****	**<0.001*****

*For treatment abbreviations (see **Table 2**). TOC, total organic carbon; TON, total organic nitrogen; P, phosphorus; N, sodium; K, potassium; Ca, calcium; Mg, magnesium. Significant p-values are in bold. *Significant; **Highly significant; ***Very highly significant.*

TABLE 7 | Pearson correlation matrix of soil fauna and soil parameters.

Fauna	Soil pH	TOC	TON	P	Na	K	Ca	Mg
Macrofauna								
Oligochaeta	−0.20	**0.69*****	**0.62*****	**0.66*****	−0.23	−0.15	−0.28	−0.23
Isoptera	−0.06	−0.24	−0.30	−0.07	0.11	−0.01	0.04	0.16
Hymenoptera	−0.23	−0.22	−0.26	−0.19	**0.58*****	**0.39***	**0.50****	**0.59*****
Coleoptera	−0.17	−0.25	−0.05	−0.10	**0.60*****	**0.56*****	**0.57*****	**0.59*****
Diptera	−0.17	−0.19	0.10	−0.12	**0.41***	0.26	**0.42***	0.31
Lepidoptera	−0.14	0.08	0.33	0.24	**0.47*****	0.30	**0.38***	0.32
Diplopoda	−0.18	**0.39***	**0.38***	**0.41***	0.04	0.14	0.03	0.06
Chilopoda	**–0.37***	0.32	0.27	0.06	0.02	0.25	0.11	0.17
Orthoptera	−0.21	−0.22	−0.13	−0.13	0.16	0.11	0.20	**0.44***
Areneae	−0.09	0.09	−0.07	0.26	−0.18	−0.05	−0.23	−0.16
Odonata	−0.04	−0.11	0.00	−0.08	**0.38***	0.32	**0.38***	0.23
Hemiptera	−0.13	**0.44***	**0.39***	**0.45****	−0.13	−0.04	−0.18	−0.14
Blattoidea	0.03	−0.19	−0.11	−0.22	**0.44***	**0.39***	0.34	**0.40***
Isopoda	−0.21	−0.11	−0.05	−0.11	0.22	**0.64*****	0.28	0.34
Mesofauna								
Acarina	0.25	−0.14	−0.23	0.12	**–0.43***	**–0.46****	**–0.48****	**–0.49****
Arachnida	−0.07	−0.19	−0.05	−0.12	0.25	0.16	0.23	**0.42***
Collembolla	0.10	0.21	0.08	0.34	**–0.40***	**–0.37***	**–0.41***	**–0.42***
Diplura	−0.06	0.24	0.29	−0.01	−0.10	−0.15	−0.07	−0.14
Enchytraeidae	−0.20	−0.32	−0.14	−0.31	**0.73*****	**0.48****	**0.71*****	**0.72*****
Protura	−0.13	0.23	0.04	0.19	**0.68*****	**0.61*****	**0.65*****	**0.55****
Symphyla	0.06	0.18	0.09	0.19	**–0.49****	**–0.47****	**–0.50****	**–0.53****

*For abbreviations (see **Table 6**). Significant p-values are in bold. *Significant; **Highly significant; ***Very highly significant.*

phytophagous and saprophagous invertebrates (Ekschmitt et al., 1997; Ziesche and Roth, 2008). In this study, CA most likely provided a conducive and prey-rich environment for Araneae. Chilopods (Centipedes) are among the oldest extant terrestrial arthropods and are an ecologically important group of soil and leaf litter predators (Undheim and King, 2011). In our study, higher Chilopod numbers was observed where maize stover residues had been applied in medium and long-term. It is possible that maize stover residues, apart from providing a moist conducive environment, might have favored availability of prey for Chilopods. Isoptera (termites) on the other hand, were favored by medium-term addition of organic residues and were therefore more abundant under CA than under CT without residues. Most of the isopteran species sampled in our study sites were all group II trophic members that are fungal growers, and typical feeders of wood, litter and grass. These species build subterranean nests and are sensitive to tillage disturbances (Ayuke et al., 2011b). Being foragers of wood, litter and grass, they were likely favored by addition of crop residues, and to avoid disturbance, they moved to deeper soil depths hence the significant differences observed at 15–30 cm soil depth.

Applications of plant residues have been shown to increase the population of earthworms (Ayuke et al., 2003; Fonte et al., 2009). Although other studies have demonstrated that short-term additions of organic residues increase macrofauna populations, but have little effect on their diversity (Mando, 1998; Ayuke et al., 2003; Ou'edraogo et al., 2006), our study has demonstrated that conservation agriculture and medium to long-term application of organic residues can enhance the richness and abundance of soil fauna, which can in turn promote their activity, hence important soil functions like organic matter retention, stable aggregation and water infiltration (Castellanos-Navarrete et al., 2012; Paul et al., 2015). We speculate that higher soil fauna taxonomic richness in the medium and long-term trials could be due to long-term build-up of soil organic matter as food for the soil fauna groups. Studies conducted by Ayuke et al. (2009, 2011a) showed that earthworm taxonomic richness was higher in high carbon soils than in low carbon soils and this was attributed to long-term application of the various organic amendments across the long-term trials that resulted in corresponding build-up of soil organic matter. The relatively lower macrofauna abundance in the long-term trial of Nyabeda could be attributed to removal of

crop residues during soybean planting, which possibly reversed the gains of CA that could otherwise promoted soil organic matter build-up thus food source for the soil fauna. Application of agrochemicals (e.g., pesticides and herbicides) could have also affected the soil organisms as these have been shown to directly affect the organisms through toxicity or indirectly by altering habitat structure and food chain (Gianessi, 2010; Isenring, 2010).

The soil fauna invertebrate community responses to the environmental (soil parameters) changes induced by land management practices and associated ecosystem disturbance reinforce the benefits of conservation agriculture and associated practices (e.g., the use of organic inputs). Soil invertebrates are important determinants of soil chemical and physical characteristics. As such, their potential beneficial role in biodegradation and humification of organic residues, SOM incorporation and soil aggregation in agricultural soils is well-established (Bossuyt et al., 2005; Pulleman et al., 2005a,b; Coq et al., 2007; Fonte et al., 2009; Ayuke et al., 2011b; Brussaard, 2012; Kihara et al., 2015). In view of this, we reiterate that knowledge such as of this study, that demonstrate impact of management practices among them tillage, organic resource use, crop rotation and mixed cropping, and application of inorganic fertilizers on soil fauna is important in enhancing ecosystem functioning and environmental sustainability.

CONCLUSIONS

This study assessed how conservation agriculture, and application of organic or inorganic inputs and cropping system, affected soil fauna richness and abundance. Results showed that: (1) Conservation agriculture enhances soil macrofauna taxonomic richness and mesofauna abundance than conventional agriculture, and that with addition of residues, both diversity and abundance are enhanced, both under CA and CT, (2) Rotation and mixed cropping (intercropping) such as maize legume systems, and sole maize systems coupled with organic residue addition are best bet practices that promote soil fauna diversity and abundance, and (3) Long-term addition of organic residues also enhances soil fauna diversity and abundance more than medium-term addition of organic residues.

Given the numerous challenges faced by smallholder farmers of SSA in the adoption of CA, who in most cases rarely practice all the three CA principles simultaneously, we propose a further study that will determine how each of the CA components (tillage, organic inputs and cropping systems and their interactions affect soil fauna diversity and abundance.

DATA AVAILABILITY

The datasets generated for this study can be found in **Supplementary Tables** uploaded in the site.

AUTHOR CONTRIBUTIONS

FA carried out the field activities, data analysis, and write up of the manuscript. JK financed the research activities through SIMLESA II project. He is the manager of the Nyabeda trial and also participated in the write up of the manuscript. GA managed the Kakamega medium-term trial and also contributed in the data generation and write up of the manuscript. AM managed the Embu medium-term trials also contributed in the data generation and write up of the manuscript.

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Food Choice and Fitness of *Folsomia candida* (Collembola, Isotomidae) Fed on Twelve Species of Truffle

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Fungi are a significant food resource for soil fauna, whose grazing behavior can have a significant impact on their development. This relationship is an important aspect in soil functioning, with soil fungi acting as primary agents in decomposition processes. Being one of the most abundant groups among soil fauna, springtails can play a leading role in this context. Despite several previous studies on their epigeous fungal grazing behavior, data regarding the relationship between springtails and truffles are scarce. This study aimed to investigate food preferences of the springtail *Folsomia candida* for grazing on 12 different species of truffles, 11 belonging to *Tuber* genus, and 1 to *Balsamia* genus. We also evaluated how strongly this diet influences survival and reproduction of *F. candida*. In the first experiment, *F. candida* were allowed to choose freely between a cereal mixture (choice test) and 12 different species of truffle. In the second experiment, they were fed on the truffles only (no-choice test) for 28 days. Twelve truffle species were analyzed for survival and reproduction of *F. candida*. *F. candida*'s feeding preference evolved over 72 h, beginning with a strong preference for the control and finally a general preference for truffles. Moreover, Collembola that fed on some *Tuber* species had a lower survival rate and fewer juveniles per adult compared to the control. Compared to other species, *Tuber aestivum* and *Tuber melanosporum*, which are well-known for their ability to produce brûlés, had a positive impact on collembolan fitness, whereas their palatability was not particularly prominent. Hence there was a relationship between diet and fitness in *F. candida*, whilst hardly any relationship was observed between fitness and feeding preference.

Keywords: springtail, *Tuber*, *Balsamia*, food quality, preference test, soil fauna, ectomycorrhizal fungi

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INTRODUCTION

The interaction between fungi and soil fauna is a key aspect in soil functioning, since both groups play an important role in the soil food web. Soil fauna has a significant impact on fungi, being the latter an important food resource for it (Hanlon, 1981; Jørgensen et al., 2003; Harold et al., 2005; Rotheray et al., 2011). Interactions between fungi and fungivores influence terrestrial biogeochemical cycles and can also induce fundamental changes in the performance of the plant–fungus association, through which fungal grazers affect mineralization, decomposition rates, and energy transport in soils (Ruess and Lussenhop, 2005; Fernandez et al., 2016).

The grazing of many groups of microarthropods can have either positive or negative effects on fungal communities, depending on the taxon and the abundance of animals (Bengtsson and Rundgren, 1983; Bretherton et al., 2006; Tordoff et al., 2008; Crowther et al., 2012). To prevent detrimental grazing some fungi have evolved defensive strategies, including the presence of crystal structures and other deposits on their hyphal surface, and the production of toxic or distasteful secondary metabolites (Böllmann et al., 2010).

Fungi are an important target in the feeding of springtails, one of the most abundant groups among soil fauna, and grazing by Collembola has produced evident effects on fungal development (Hopkin, 1997; Böllmann et al., 2010), either enhancing or decreasing mycorrhizal spore number (Bakonyi et al., 2002). Thus, their food choice could play a key role in this context. Despite springtails being generalists feeding on a wide range of foods, fungi are one of the main sources for most of them (Parkinson et al., 1979; Jørgensen et al., 2003; Chahartaghi et al., 2005). This group displays marked feeding preference behavior. Moreover, collembolan grazing seems to be species-specific, with selectivity depending on many fungal parameters, such as growing substrate, life stage, mycelium vitality and metabolic activity (Sabatini and Innocenti, 2000; Kaneda and Kaneko, 2004; Heděnec et al., 2013).

Whether the springtails' feeding preference is related to their development and fitness is not clearly known. Some authors (Pfeffer et al., 2010) suggest that *Folsomia candida* (Collembola, Isotomidae) is able to follow an optimal diet for its growth and reproduction rate by choosing to feed on what seems to be its favorite type of fungi. Furthermore, Heděnec et al. (2013) found that the fitness of *F. candida* strongly depended on the litter type rather than on fungal species. Truffles could play a crucial and interesting role here, since their presence influences the biochemical and physical composition of soil, especially in the rhizosphere, where they interact with soil fauna (Callot, 1999; Ricard, 2003; Granetti et al., 2005; García-Montero et al., 2012; Mello et al., 2013; Menta and Pinto, 2016). The genus *Tuber*, representing ectomycorrhizal fungi, produces hypogeous fruiting bodies that release secondary aromatic metabolites as adaptive strategy to attract feeders. This phenomenon is typical of subterranean organisms that strictly depend on animal activity for spore dispersion (Reyna Domenech, 2007). *Tuber* fungi also modify soil biogeochemistry (García-Montero et al., 2009; Trappe and Claridge, 2010) to such an extent that volatile compounds can inhibit the germination and growth of other plants around the host plant (Splivallo, 2008; Menta and Pinto, 2016), generating a burnt area called "brûlé," which could affect soil fauna in many ways. Menta et al. (2014) tried to highlight the differences between soil fauna inside and outside the brûlé resulting from the peculiar environment created by *Tuber aestivum* (Vittadini). The authors showed that some collembolan families were more present in terms of abundance and frequency outside the brûlé, while a species *Folsomia* was abundant inside the brûlé. Some authors (Menta et al., 2014; Pinto et al., 2017) suggested that the conditions created by *T. aestivum* do not have a negative impact on *Folsomia*, which is known to graze on fungi and hyphae (Moore et al., 1985; Thimm and Larink, 1995; Fountain and Hopkin, 2005). Despite previous studies on the epigeous fungal grazing behavior of Collembola, data about the feeding interaction between springtails and truffles are still scarce (Parkinson et al., 1979; Chen et al., 1995; A'Bear et al., 2012; Heděnec et al., 2013).

In this study, we focused on the preference of *F. candida* for grazing on 12 different species of hypogeous truffles, 11 of which belonged to *Tuber*, and 1 to *Balsamia*. We aimed to improve our understanding of the strict relationship between soil fauna and hypogeous fungi by investigating whether the feeding preference influences survival and reproductive performance of *F. candida*. We expect that the fungi that show the greatest palatability influence *F. candida* fitness positively.

MATERIALS AND METHODS

Hypogeous Fungi

We tested 12 species of hypogeous fungi, 11 belonging to *Tuber* and 1 belonging to *Balsamia* genus (**Table 1**). Soil fungi were collected in several municipalities located in Northern and Central Italy, in a period comprised between February 2015 and April 2016. All fungi were provided by ISPRA, the National System for Environmental Protection in Rome and classified and photographed according to their "Mycological biodiversity information system." **Table 1** reports the characteristics of the sites where the fungi were collected, the number of fungi used in the experiments for each species, and the vegetation cover of the areas. The number of truffles for each species varied between three and seven, depending on the availability of truffles during the study period. After collection, the truffle samples were gently brushed, washed with running water to remove soil residues, successively dried at room temperature for 2 h, and classified at species level using a microscope. They were then sliced (3 mm thickness) and dried at 27°C in airflow for 24 h (this treatment may have removed some VOCs but prevented proliferation of molds). The dried samples were placed into separate vacuum bags and sent to Parma University, where they were pulverized using a small grinder and immediately used for the test.

F. candida Cultures

The springtail *F. candida* Willem (Collembola: Isotomidae) is among the most intensively studied of all species of Collembola (Hopkin, 1997). This parthenogenetic species is widely distributed in many environments (Fountain and Hopkin, 2005). Cultures of this species are very easy to maintain and they are excellent for laboratory experiments due to their short reproductive cycle.

The *F. candida* came from 15 laboratory cultures at Parma University. They were reared according to ISO guidelines (ISO 11267, 1999), maintained at 20 (±2°C with 50–55% RH, and fed weekly on a pulverized mixture of dried organic cereals (20% wheat, 20% oats, 20% rye, 20% spelt, and 20% rice). The animals used for egg deposition (aimed to obtain the age-synchronized juveniles used in the test) were collected from all 15 breeding containers and mixed to prevent them originating from a single breeding line.

TABLE 1 | Species of hypogeous fungi used in the study, number of fungi per species, municipality, altitude of the area, and plant species present in the area.

Fungi species	*n*	Municipality	Altitude (m a.s.l.)	Plant species
Balsamia vulgaris (Vittadini, 1831)	3	Roma	60	*Cedrus atlantica*
Tuber aestivum (Vittadini) Var. *uncinatum* (Chatin) (Montecchi et Borelli, 1995)	7	Piacenza Rieti Roma Viterbo	55–860	*Acer campestre*, *Acer opalus*, *Carpinus betulus*, *Cornus sanguinea*, *Corylus avellana*, *Ostrya carpinifolia*, *Populus alba*, *Quercus cerris*, *Quercus ilex*, *Quercus pubescens*, *Ulmus minor*, *Prunus* spp., *Betula* spp., *Prunus laurocerasus*
Tuber borchii (Vittadini, 1831)	7	Roma Viterbo	3–670	*C. atlantica*, *Fagus sylvatica*, *Pinus halepensis*, *Pinus pinea*, *Q. pubescens*
Tuber brumale (Vittadini, 1831)	5	Rieti Roma Viterbo	55–960	*A. opalus*, *Juniperus communis*, *Prunus spinosa*, *Q. cerris*, *Q. ilex*, *Q. pubescens*
Tuber excavatum (Vittadini, 1831)	5	Frosinone Rieti Roma Viterbo	220–750	*A. campestre*, *C. sanguinea*, *C. avellana*, *Fraxinus ornus*, *O. carpinifolia*, *Populus alba*, *Pinus nigra*, *Q. cerris*, *Q. pubescens*, *U. minor*
Tuber fulgens (Quèlet, 1880)	3	Frosinone	750	*P. nigra*
Tuber macrosporum (Vittadini, 1831)	3	Rieti	170–960	*A. campestre*, *A. opalus*, *C. avellana*, *F. ornus*, *J. communis*, *O. carpinifolia*, *P. alba*, *P. spinosa*, *Q. cerris*, *Q. pubescens*
Tuber magnatum (Picco, 1788)	3	Rieti Roma	170–420	*A. campestre*, *C. sanguinea*, *C. avellana*, *F. ornus*, *O. carpinifolia*, *P. alba*, *Q. cerris*, *Q. pubescens*, *U. minor*
Tuber melanosporum (Vittadini, 1831)	3	Roma	55	*Q. pubescens*
Tuber mesentericum (Vittadini, 1831)	3	Viterbo	560	*F. sylvatica*
Tuber puberulum (Berkeley and Broome, 1846)	4	Roma Viterbo	60–380	*C. atlantica*, *Q. cerris*, *Q. ilex*, *Q. pubescens*
Tuber rufum (Picco) Var. *rufum* (Montecchi et Lazzari, 1993)	3	Frosinone Roma	60–750	*Q. ilex*, *P. nigra*

Fungi collected from February 2015 to April 2016. N, number of truffles.

All animals used in the tests were age-synchronized to 10 days by removing eggs from the deposition cultures and, once hatched, inserting juveniles into Petri dishes, with moistened breeding substrate 8:1 (*w/w*) plaster of Paris: activated carbon powder.

Feeding Preference Test

In this experiment *F. candida*'s feeding preference was tested for each fungi species separately with a binary option method consisting in allowing them to choose between a fungus species and the cereal mixture. Cereal mixture was the food used for cultures and during the synchronization phase.

Petri dishes filled with a 0.5 cm plaster layer of Paris mixed with charcoal (8:1) were used for the experiments. Two small hollows (5 × 5 × 3 mm and distant 5 cm one to each other) were made at opposite sides of the Petri dish; one hollow contained 1 gr of a pulverized fungi sample and the other one contained the same quantity of pulverized cereal mixture. There were five replicates for each truffle-control. Ten same age individuals of *F. candida* were transferred from the breeding substrate to the test substrate via an exhauster. No mortality was observed during the process. All the experiments were conducted at 20°C in dark/light 12:12 h conditions. Using a stereomicroscope, the number of *F. candida* feeding on either the truffle or the cereal mixture was checked after 24, 48, and 72 h. The count was made without removing the lid from Petri dishes to avoid Collembola displacement as result of the disturbance.

Survival and Reproduction Test

In this second experiment, survival and reproduction of *F. candida* were tested following the ISO guidelines 11267 (1999). Petri dishes were filled with a 0.5 cm plaster layer of Paris mixed with charcoal (8:1), and one hollow (5 × 5 × 3 mm) in the center was filled with one pulverized fungi sample. Five replicates were prepared for each fungi sample and for the cereal mixture. Ten *F. candida* individuals aged 10–12 days were added to each Petri dish using an exhauster. No mortality events occurred during the process. All experiments were as in Feeding Preference Test, and all fungi were tested at the same time (July, 2016). The Petri dishes were incubated for 28 days and aerated once a week. At the end of this period, the number of surviving adults and the juveniles were recorded using a stereomicroscope with floatation technique (ISO 11267/99).

Statistical Analysis

The Friedman test, followed by the Wilcoxon test for *post-hoc* comparisons, is the non-parametric alternative to the one-way ANOVA with repeated measures which was applied here to evaluate differences in collembolan feeding preference behavior among checks at 24, 48, and 72 h. Interspecific fungi differences on the number of *F. candida* were analyzed using the Kruskal-Wallis test, a non-parametric approach used to compare multiple independent samples, and the Mann-Whitney test with the Bonferroni correction for *post-hoc* analysis. The Wilcoxon test

was used to highlight differences between the *F. candida* grazing on cereal mixture vs. truffle to analyze feeding preference. To evaluate the differences on survival and reproduction between truffles and with cereal mixture, the Dunn test and Bonferroni correction were applied. The Kendall's tau was calculated to investigate the correlation between diet and fitness, in order to test the relationship between n. of *F. candida* grazing on truffles and: (i) n. of juveniles, (ii) n. of juveniles per adults introduced at the beginning, (iii) n. of juveniles per adults survived at the end of the experiment. A p-value ≤ 0.05 was considered significant. All statistical analyses were performed using R 3.6.0 software (R Core Team, 2017).

RESULTS

Feeding Preference

The number of *F. candida* feeding on truffles differed between checking hours ($p < 0.001$), with an increasing at 48 h ($p < 0.001$) and 72 h ($p < 0.001$) compared to 24 h (**Figure 1**). *F. candida* showed different responses with different truffle species ($p < 0.001$), and when compared to the cereal mixture. In general, *F. candida* showed a preference for most truffle species except for *T. excavatum* and *T. fulgens* compared to the cereal mixture (**Figure 1**). *T. macrosporum*, *T. magnatum*, and *T. mesentericum* were preferred significantly to the cereal mixture at the end of the experiment (**Figure 1**). In particular, the preference percentage of grazing *F. candida* obtained by *T. macrosporum* was higher than 85%.

Comparing the 12 truffles at 24 h, *T. magnatum* showed a higher number of *F. candida* feeders than *T. aestivum* ($p < 0.01$), *T borchii* ($p < 0.01$), *T. brumale* ($p < 0.001$), *T. puberulum* ($p < 0.05$), and *T. excavatum* ($p < 0.001$); this last truffle also differed from *T. melanosporum* ($p < 0.05$). At 48 h only *T. aestivum* differed significantly from *T. magnatum* ($p < 0.05$), and *T. excavatum* from *T. borchii* ($p < 0.05$). At the end of the experiment (72 h), only *T. macrosporum* showed higher *F. candida* feeders than *T. aestivum, T. borchii, T. excavatum* ($p < 0.01$ for all), and *T. rufum* ($p < 0.05$).

Survival and Reproduction

Results showed differences between truffles as regards collembolan survival ($p < 0.001$, **Figure 2**). *B. vulgaris, T. aestivum, T. borchii, T. melanosporum, T. mesenterivum*, and *T. puberulum* highlighted a survival percentage close to the cereal mixture (**Figure 2**). *T. excavatum, T. macrosporum* and *T. magnatum* did not differ from control. Differently, *T. rufum* determined significant lower survival when compared with the cereal mixture, but still higher than 50%. On the other hand, *T. brumale* and *T. fulgens* determined survival percentages lower than 50%, and significantly lower than the cereal mixture.

When comparing the truffles, *T. aestivum*, *T. borchii, T. melanosporum, T. mesentericum*, and *T. puberulum,* led to higher survival when compared with *T. brumale, T. fulgens*, *T. magnatum*, and *T. rufum*. *T. aestivum* and *T. puberulum* showed higher survival when compared to *T. macrosporum* (**Figure 2**; see **Supplementary Table 1** for statistical significances).

Results on reproduction of *F. candida* highlighted differences in the number of juveniles per adult depending on the species of truffle ($p < 0.001$, **Figure 3**). All truffle species, except *T. borchii, T. melanosporum*, and *T. mesentericum,* led to a lower number of juveniles when compared to the cereal mixture, but the differences resulted significant for *T. fulgens, T. brumale*, and *T. magnatum* only ($p \leq 0.001$ for all).

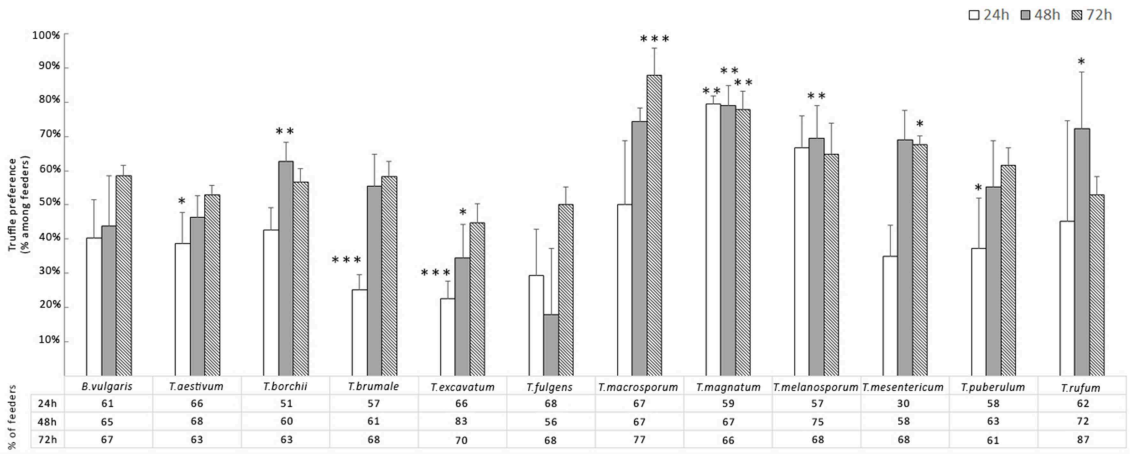
Comparing truffles, the highest reproduction rate was supported by *T. melanosporum*, significantly different from *T. aestivum*, *T. brumale*, *T. fulgens, T. macrosporum, T. magnatum, T. puberulum*, and *T. rufum* (**Figure 2**; see **Supplementary Table 1** for statistical significances).

Instead, *T. fulgens* determined the lowest number of juveniles, differing from all the other species of truffles except for *T. brumale* and *T. magnatum.* Furthermore, although *T. brumale* proved better compared to *T. magnatum*, it caused a significantly lower reproduction rate than the other species of truffle, except for *T. excavatum*, *T. fulgens*, *T. macrosporum*, and *T. rufum* (**Figure 2**; see **Supplementary Table 1** for statistical significances).

No correlation was highlighted between diet, in term of n. of *F. candida* grazing on truffles, and fitness (n. of juveniles: tau: −0.039, p: not significant; n. of juvenile per adults introduced at the beginning, tau: −0.039, p: not significant; n. of juveniles per adults survived at the end of the experiment tau: −0.045, p: not significant).

DISCUSSION

The aim of this study was to observe the grazing behavior of *F. candida* fed on twelve species of hypogeous fungi, and to evaluate if different fungi could affect survival and reproduction of this species. Consequently the aim was then to understand how much feeding preference was related to fitness of this collembolan species. It is a well-known fact that *F. candida* can exhibit distinct feeding preferences depending on the fungal species (Tordoff et al., 2008), and this study confirmed both this aspect and the important role of this species in spore dispersion and regulation of fungal community. The interesting addition is that not only do different fungal species have various palatability for this collembolan, but also that fungi were preferred as food resource when the springtails could choose between fungi and the usual food (cereal mixture in this study) that these animals had been used to feeding on for numerous generations. The feeding preference trend observed in this study showed an increment of truffle palatability already 48 h after the beginning of the experiment. This suggests that springtails need a short time to modify their feeding habits. Truffles exhibit their maximum sensorial properties when fresh. With a shelf-life of 7–10 days, truffles quickly lose their flavor intensity and start to spoil (Campo et al., 2017). Recent studies by Splivallo et al. (2015) and Splivallo and Ebeler (2015) show that bacteria associated with truffle-ascocarps and sulfur-containing volatiles, such as thiophene derivates, contribute to truffle aroma. Nevertheless, classical preservation method, like hot air drying (HAD) or dehydration of truffles, reducing



% of feeders	*B.vulgaris*	*T.aestivum*	*T.borchii*	*T.brumale*	*T.excavatum*	*T.fulgens*	*T.macrosporum*	*T.magnatum*	*T.melanosporum*	*T.mesentericum*	*T.puberulum*	*T.rufum*
24h	61	66	51	57	66	68	67	59	57	30	58	62
48h	65	68	60	61	83	56	67	67	75	58	63	72
72h	67	63	63	68	70	68	77	66	68	68	61	87

FIGURE 1 | Average percentage ± Standard Error of *F. candida* that expressed truffle preference 24, 48, and 72 h after being introduced to feeding arenas. Asterisks correspond to significant differences between truffle and cereal mixture (control): $^{*}p < 0.05$, $^{**}0.05 > p > 0.01$, $^{***}p < 0.01$.

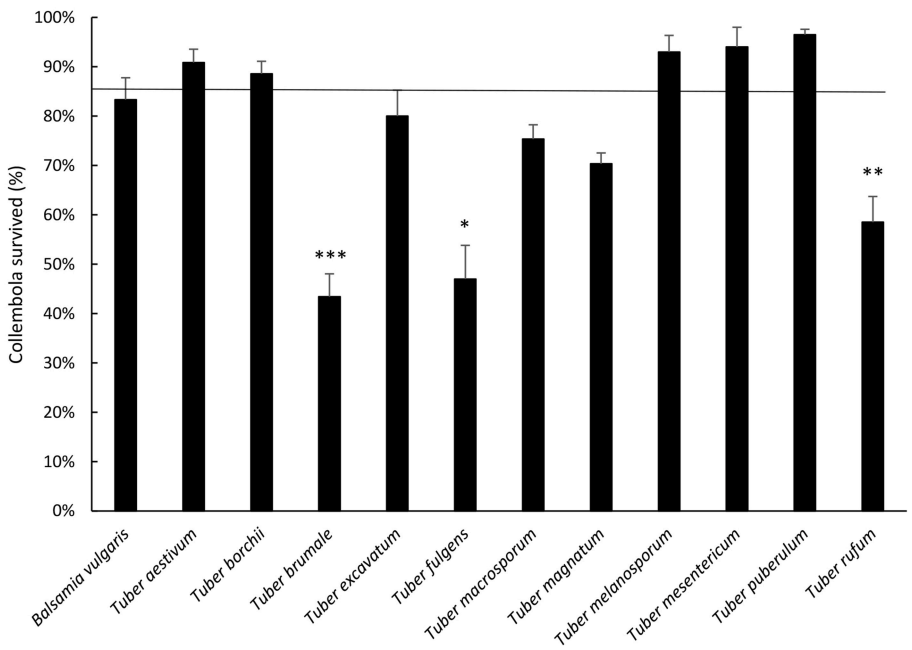


FIGURE 2 | Average percentage ± Standard Error of *F. candida* surviving adults after 28 days' feeding on truffles. The horizontal line represents cereal mixture (control). Asterisks correspond to significant differences between truffle and control: $^{*}p < 0.05$, $^{**}0.05 > p > 0.01$, $^{***}p < 0.01$. For the differences between truffles, see **Supplementary Table 1**.

water content and microbial growth, slow down enzymatic and chemical activities. The resulting microbial inhibition could partially explain the variations in truffle species preferences often observed throughout the first experiment. Moreover, the rehydration of the dried and pulverized truffle samples could have reactivated the microbiome, which could have served as food resource, being *F. candida* a fungivorous species.

We must however consider that *F. candida* showed variability between fungi. *T. macrosporum* and *T. magnatum* were the two more palatable species, while *T. excavatum* was the least favorite. Truffles attract arthropods with volatile compounds to facilitate spore dispersion through the grazers' digestive tract and, in this way, compensate their hypogeum condition (Reyna Domenech, 2007). This strategy could be particularly effective on those blind or reduced-eyesight soil-dwelling species that use odors as clues like *F. candida*. The VOCs profiles of *Tuber* spp. are highly complex and are far from being fully described (Vita et al., 2015). Moreover, the geographical origin contributes to the specific variation in VOC profiles, as reported by Üstün et al. (2018) for the white truffle *T. magnatum*. Besides, bacteria associated with truffle-fruiting bodies contribute to truffle aroma, making the system even more complex. In this study, the

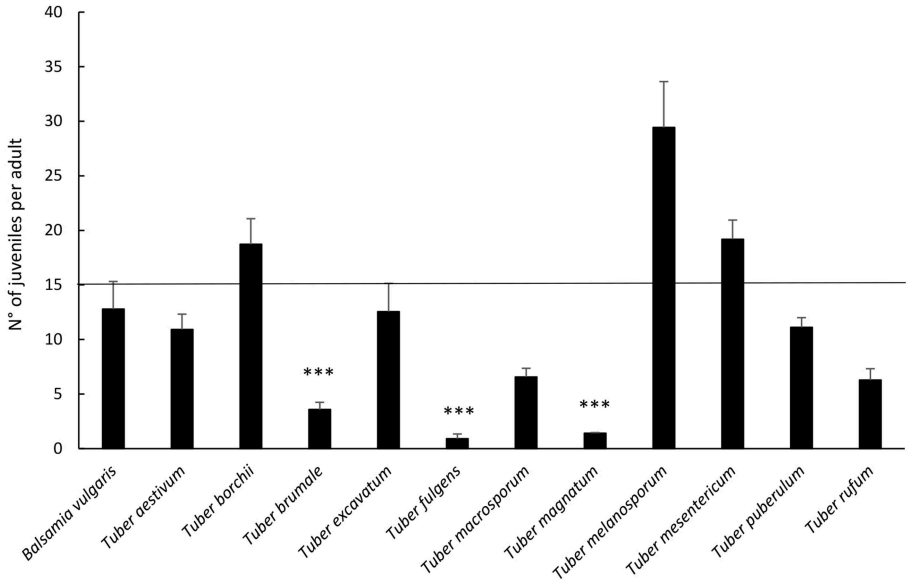


FIGURE 3 | Average percentage ± Standard Error of *F. candida* juveniles (numbers of juveniles/surviving adults) after 28 days' feeding on truffles. The horizontal line represents cereal mixture (control). Asterisks correspond to significant differences between truffle and control: $*p < 0.05$, $**0.05 > p > 0.01$, $***p < 0.01$. For the differences between truffles, see **Supplementary Table 1**.

food source was not characterized in terms of biochemical composition because the focus is on the capacity shown by *F. candida* to discriminate among different truffle species and on the effects of these species on survival and reproduction of *F. candida*. Our results show that there is a substantial variation between species of truffle not only in their palatability but also in the effects on survival and reproduction rate of *F. candida*, in agreement with previous studies (Heděnec et al., 2013). Hubert et al. (2004) suggested the existence of four fungal groups in terms of attractiveness and suitability for grazers' development: (i) preferred and suitable for growth; (ii) preferred, but unsuitable; (iii) avoided, but suitable; and (iv) avoided and unsuitable. Our results fall into the first two categories, since we found that compared with the food provided during breeding, truffle was preferred in many cases but not always suitable for collembolan development. Several authors concluded that collembolans are able to select an optimal diet in order to maximize their fitness (Sabatini and Innocenti, 2000; Jørgensen et al., 2008). However, the current study suggests an inconsistent link, if not a discrepancy, between *F. candida* grazing preference and reproduction, in accord with other experiments, such as Heděnec et al. (2013), where discrepancies between food choice and food suitability emerged. Böllmann et al. (2010) suggested that this repellent characteristic has more influence on feeding preference than fungi palatability. Indeed, *T. magnatum* showed high palatability but a low reproduction rate for *F. candida*, hence easily unsuitable in terms of quality. Furthermore, a link between attractiveness and unsuitability could constitute a mechanism of counterbalance to prevent the damages of overgrazing, since by reducing the reproduction the number of collembola that graze on truffle will be smaller. Considering this hypothesis, truffles, acting on *F. candida* fitness and collembolan population, can indirectly modify soil biochemical and physical composition and, consequently, change soil microbial community in terms of bacteria and other microorganisms that this species uses as food source. Therefore, the effects of some truffle species are direct, modifying soil biogeochemical properties, and indirect, acting on soil living community and, consequently, on soil food web, organic matter decomposition rate and biogeochemical processes that take place in the soil. The two species of truffles *T. aestivum* and *T. melanosporum* are known for their ability to create brûlés, affecting soil biogeochemistry (García-Montero et al., 2009) and soil fauna community (Menta et al., 2014; Pinto et al., 2017). Our results showed high collembolan survival and reproduction rate for both these species, even if their palatability did not differ significantly from other food resources. In particular, feeding on *T. melanosporum* resulted in the highest reproduction rate observed in this study, supporting Scheu and Simmerling hypothesis 2004 that compounds judged tasteful by Collembola may differ from those useful to enhance their fitness. These results suggest that truffle species able to create brûlés could be potentially valuable resources in terms of fitness for *F. candida*. Menta et al. (2014) proposed that *T. aestivum* metabolites could attract *Folsomia* genus unlike other soil microarthropod taxa that were more abundant outside the brûlés.

In conclusion, our data show that truffle species differed not only in their palatability but also in their effects on fitness of *F. candida*, highlighting an inconsistency between preference and suitability. Other studies should be conducted to understand if the difference in response of *F. candida* was caused by the identity of the metabolites produced by truffle species, and if similar effects are induced on other

fungal grazers. This study aims to stimulate studies for better understand the extremely complex relationship between truffles, microbiome, and soil fauna, not only for extending the scientific knowledges but also for increasing the success in consistent truffle yield. Considering latter aspect, *F. candida* can play an important role in the truffle cultivation, at least for the truffles species that showed both high palatability and fitness for this Collembola.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the ISO 11267:1999.

AUTHOR CONTRIBUTIONS

CM formulated the idea, directed the study, conducted the experiments, and wrote the manuscript. BB helped in the statistical analysis and in the writing of the manuscript. SR conducted the experiments and the statistical analysis, and contributed to writing the manuscript. CS provided and classified all the truffles used in the experiments and participated in the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2019.00114/full#supplementary-material

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