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Expanding and intensifying agriculture has led to a loss of soil carbon. As agroecosystems cover over 40% of Earth's land surface, they must be part of the solution put in action to mitigate climate change. Development of efficient management practices to maximize soil carbon retention is currently limited, in part, by a poor understanding of how plants, which input carbon to soil, and microbes, which determine its fate there, interact. Here we implement a diversity gradient by intercropping undersown species with barley in a large field trial, ranging from one to eight undersown species. We find that increasing plant diversity strengthens positive associations within the rhizosphere soil microbial community in relation to negative associations. These associations, in turn, enhance community carbon use efficiency. Jointly, our results highlight how increasing plant diversity in agriculture can be used as a management strategy to enhance carbon retention potential in agricultural soils.

Biologists have empirically tested how diversity loss can impact ecosystem processes due to shifts in energy fluxes and matter that are underlying ecosystem functioning[1,2]. Long-term ecological experiments have been crucial for increasing our understanding on how biodiversity enhances the provision of ecosystem productivity[2,3], stability[4,5] and resilience to climate extremes[6]. While the relationship between plant diversity and above ground plant productivity is to date the best studied ecosystem function[7,8]. More recently, it has been recognized that many of the mechanisms that promote positive biodiversity-ecosystem functioning relationships take place belowground[9–11].

The potential of plant diversity to influence soil carbon (C) cycling has been recognized[12,13], as soils are the biggest reservoir of terrestrial carbon and soil-atmosphere C feedbacks plays an important role in defining the world's climate evolution in the next decades[14]. While plant biomass and root exudates are the primary source of C into soils, ultimately it is the microbial activity influenced by the biodiversity of

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microorganisms living in the soil that will decompose (i.e. mineralize) plant compounds into more recalcitrant, less available soil C pools[15–19]. While the abiotic controls of C retention in soil are better understood[20,21], the importance of fungi and bacteria-derived C for soil carbon formation has been recently recognized[16,18,22]. It has been recently hypothesized that higher complexity of microbial-derived soil organic matter (SOM) compounds might translate into higher metabolic costs for decomposition and consequentially longer residence times in soil[23]. Recent findings support this hypothesis as microbial community composition explained the SOM chemical signature in a study that manipulated microbial diversity[16]. This same study showed that community composition influenced the thermal-stability of SOM and more thermal-stable SOM is less available to decomposition and might persist longer in soils[16]. When microbes metabolize plant C, a fraction of this C is allocated to growth and the resulting microbial biomass can ultimately contribute to soil C pools through exudation and cell death[16–19]. Growth efficiency or carbon use efficiency (CUE) represents the fraction of C taken up by microbial cells and retained in biomass as opposed to being respired. Predictions of soil carbon stocks are sensitive to the assumptions made about microbial CUE[24]. Diverse microbial communities allocate more C to growth in relation to respiration than species-poor communities[25], which could be explained in part by higher levels of complementarity between community members under high diversity[26]. Complementarity effects characterize processes such as niche differentiation and facilitation that arise from species interactions, and enhance resource use efficiency and productivity in more diverse communities[27]. If plant diversity or specific plant root traits fosters complementarity effects within the belowground microbial community[27], it can enhance microbial abundance[12], growth[13] and consequently microbial turnover would increase[15], promoting C retention in soils via greater microbial community CUE[16–18].

These processes have been previously integrated in the conceptual framework of the soil "microbial carbon pump" (MCP). The MCP framework captures the long-term cumulative effect of microbial catabolism and anabolism on SOM formation[17,18]. Two decades ago, a seminal plant diversity experiment was established, the Jena experiment, which has provided evidence that increasing plant diversity is followed by an increase in soil carbon content[12,13,28]. The results of this experiment also helped to further elucidate the importance of the rhizosphere microbial community in understanding the role of plant-microbe interactions for soil functioning[29–31]. For example, more diverse plant communities increase accessibility of root exudates for the rhizosphere microbial community[30], which may have consequences for community CUE and C cycling dynamics. As agricultural land represents almost half of Earth's land surface today[32], it becomes crucial to elucidate if findings observed within biodiversity experiments can be reproduced within an agricultural context.

Reproducing the diversity effects observed within biodiversity experiments within an agricultural context faces various challenges[11,33]. For example, most diversity experiment results were obtained starting with even abundances of different plant species. However, in an agricultural context, one or a few crops purposely dominate and the influence of intercropping with other species (i.e. diversity effect) might be different and/or reduced due to their limited abundances. Another challenge of diversification in an agricultural context is the implementation of high diversity treatments which is accompanied by increasing complexity for the farmers regarding sowing, harvesting and other management practices during plants' growing season. For example, the highest diversity treatment in Cedar Creek experiment was 16 species[4], while in the Jena experiment, it consisted of 60 different grassland species[13], which would represent a substantial effort considering agricultural management practices. Thus, we must investigate if we can yield positive results at lower levels of plant diversity in settings where the main crop is the dominant species within an agricultural context to facilitate their implementation by farmers. To bridge the gap between biodiversity research and agricultural sciences, the TwinWin plant diversity intercropping farming experiment was established in 2019 (see Supplementary Information; https://carbonaction.org/en/projects/). The TwinWin experiment has been designed to evaluate how a main agricultural crop grown with a gradient of undersown plant diversity (planted in intercropping) influences the provisioning of ecosystem functions compared to the monoculture of the main crop. Toward this end, barley is planted as a monoculture, as well as under increasing levels of undersown plant diversity (i.e.: barley plus 1 undersown species, barley plus 2 undersown species; barley plus 4 undersown species and barley plus 8 undersown species). The undersown species were chosen based on two root functional traits: nitrogen fixation capacity and rooting depth[34]. The species with no nitrogen fixation and shallow roots are *Lolium perenne* and *Phleum pratense*, N-fixers with shallow roots are *Trifolium hybridum* and *Trifolium repens*, N-fixers with deep roots are *Medicago sativa* and *Trifolium pratense* and deep rooters with no nitrogen fixation capacity are *Festuca arundinacea* and *Cichorium intybus*.

The overall aim of this study is to provide empirical evidence for the response of microbial community CUE in the soil rhizosphere to a plant diversity gradient in agricultural soils. A previous study showed that plant diversity enhanced C uptake within the rhizosphere microbial community[30]. Our overarching hypothesis is that undersown plant diversity influences the microbial associations in the rhizosphere of the main crop, mediating the associations within the belowground microbial community with consequences for soil C cycling dynamics (Fig. 1). Our specific hypotheses are: (1) plant diversity has a positive influence on microbial CUE in the rhizosphere; (2) an increase in plant diversity will increase soil organic carbon; and (3) plant diversity has a positive influence on microbial associations in the rhizosphere, which should influence community CUE. To test these hypotheses, we sample soil from the TwinWin experiment during the growing season and estimate CUE using the $^{18}O–H_2O$ substrate-independent method, sequence the bacterial and fungal communities and evaluate their association networks and determine the soil C quantity and quality. Using structural equation modeling to distinguish between direct and indirect drivers of CUE, our results suggest that plant diversity influences the positive associations within the microbial community, which contributes to increasing community CUE.

Results and discussion

Plant diversity drives soil carbon cycling in an agricultural soil

We observed higher soil organic carbon content in rhizospheric soils with higher diversity of undersown species accompanying barley (Fig. 2a). This is consistent with previous findings[12,13,28]. Plant diversity can increase plant biomass inputs into soil due to increased productivity[8,35], which might explain why higher total soil organic C content is observed in more diverse plant communities[12,28]. Moreover, within an agricultural context having other plants growing with barley also means that once the barley is harvested, the other plants continue to grow in the field gaining the function of"cover crops" while under barley monoculture there is no further plant growth after barley's harvest (i.e. plots are bare after harvest except for barley residues). The now "cover crops" continue to grow during autumn and spring resulting in additional plant biomass that will be incorporated within the upper soil layer before sowing the seeds in the following up season with a shallow tillage (±5 cm). We observed a positive relationship between plant biomass and plant diversity in spring (Fig. 2d) but not during the summer barley growing season (Fig. 2e, f). The spring biomass measurement captures only the undersown species and weeds biomass as the barley plants do not resprout after harvest. These results are in agreement with the growing literature showing the

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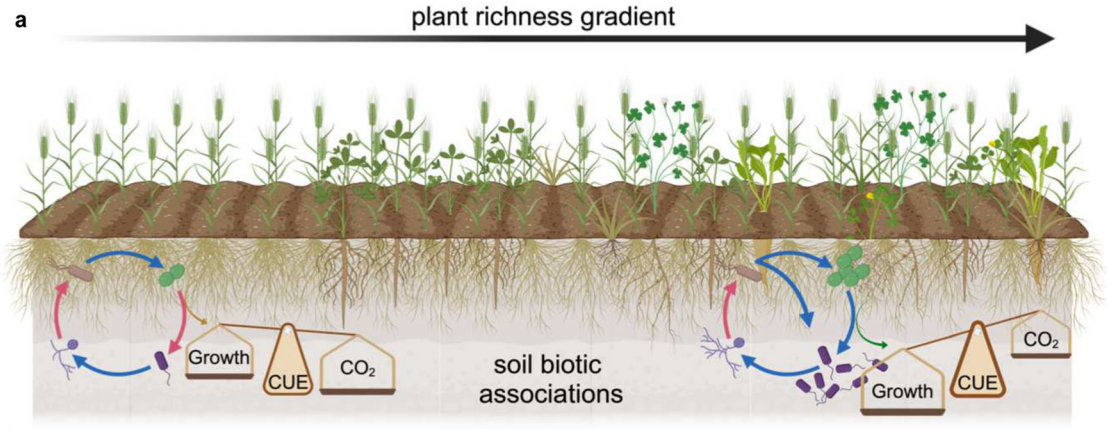
a



b

Sampling design:	Barley monoculture	Barley + 1				Barley + 4	Barley + 8
		AA	AC	IR	TG		
Plot replicates	4	3	3	3	3	4	4
Within plot level replication	6	8	8	8	8	6	6
Samples per treatment	24	24	24	24	24	24	24

Fig. 1 | TwinWin field experiment and sampling design. Graphical visualization of our hypotheses that (1) a plant diversity gradient influences soil biotic associations in the rhizosphere and that (2) such changes influence the "balance" between growth and respiration increasing microbial community CUE. Positive associations within the soil microbial community are shown in blue while negative associations are shown in red along the plant diversity gradient (**a**). Sampling design of Barley rhizosphere within the TwinWin field experiment. Number of field plot replicates sampled for each treatment and within plot replication. The number of rhizosphere samples collected within the same plot was adjusted to 6 or 8 (pseudo-replication) to yield twenty-four replicates for each specific treatment to allow the construction of association networks within each plant diversity treatment. For "Barley + 1" treatment, the sampling was focused on plots from four undersown species: *M. sativa* (AA), *T. hybridum* (AC), *L. perenne* (IR), and *F. arundinacea* (FA). The total number of rhizosphere soil samples collected was 168 (**b**). Figure 1a was created with BioRender.com released under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International license.

positive impact of cover-crops on soil C content[36,37]. This additional plant biomass observed at the higher levels of plant richness in spring likely contributes to the observed increase of SOC along this diversity gradient. Further, it has also been suggested that plant diversity per se is important for soil carbon build up, indicating that other diversity-related mechanisms are important in addition to the diversity-induced increase in biomass[35]. It is also important to consider that while the effects of plant diversity on belowground processes become progressively stronger over time[28,37], here we are studying the early responses of soil C cycling to increasing plant diversity within two years into the establishment of the TwinWin plant diversity farming experiment. The results of the Jena experiment suggests that more years into the experiment are needed to allow us to better understand SOC dynamics at different soil depths and the role of distinct plant functional groups[28].

The first results from this agricultural experiment show that manipulating undersown plant diversity in an agricultural context may have multiple positive effects as SOC increase provides various co-benefits to farmers beyond the major agricultural crop growing season[11,33]. Nevertheless, it is important to highlight the potential tradeoffs between intercropping with other plants and the main crop yield[38]. In this experiment, we observed no significant reduction in barley biomass and yield with increasing plant diversity (Supplementary Figs. 2–4). A previous study in this site observed a small negative and only marginally significant effect ($P = 0.073$) of plant diversity on yield, and this influence was driven mostly by herbicide application in barley monoculture treatment and not due to plant richness per se[39]. Moreover, we observed that the largest differences in yield were observed within the D1 treatments (Supplementary Figs. 2–4), depending on species identity rather than undersown diversification. A meta-analysis of 226 field experiments showed that while intercropping leads on average to small yield penalty for grains, a decrease in yield is not always the case specially combined with moderate N fertilizer applications[38]. However, management changes should be carefully evaluated so as not to compromise main crop yield which could lead to further conversion of non-arable land into agricultural thus exacerbating the climate crisis[14,32]. It should also be noted that the benefits of diversification schemes may vary among years, and prove particularly beneficial during drought years[40].

In addition to the soil C content, we evaluated the quality of the rhizospheric soil organic carbon (SOC) with the rock-eval ramped thermal analysis which allows the SOC to be divided into thermally labile and thermally stable fractions (I index and R index, respectively)[41]. Ramped thermal analyses of soil samples have been shown to be a promising technique to disentangle distinct organic matter (OM) compounds differing in the energy needed for thermal decomposition[42–44] and has been shown to be informative of microbial activity and functioning in soils[45]. Moreover, it was also shown that the thermally stable soil C fraction is less prone to further decomposition[16] suggesting that the thermal-stable signal captures soil C that will persist longer in soil. We observed no significant differences of thermally stable C fraction (R index) under the different plant diversity treatments (Fig. 2b). The same was observed for the thermal labile soil C fraction (Fig. 2c). While various studies have investigated the impact of plant diversity on soil C content[28,35,36,46,47], few have evaluated the plant diversity effect on C quality and its consequences for C persistence in soils[19]. More efforts are needed in this direction to evaluate changes in C quality through time since the beginning of diversification and if the higher C pools observed under higher plant diversity in other experimental sites are more resistant to decomposition. Future studies should assess whether agricultural practices can increase the residence time of soil C despite rising soil temperatures, as this may determine the fate of additional soil C on an increasingly warmer planet[45,48].

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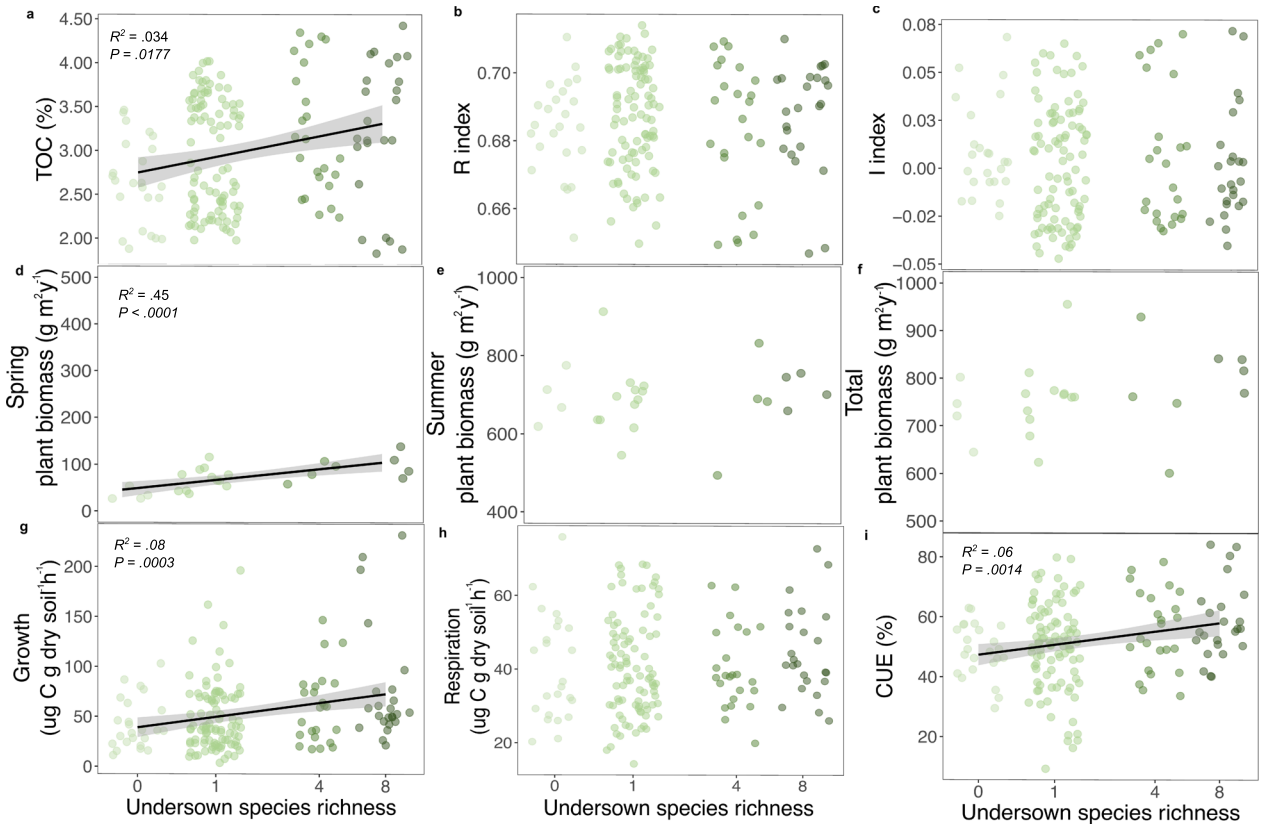

Fig. 2 | Plant diversity effects on soil carbon quantity, quality, plant biomass, and soil carbon cycling processes in the rhizosphere. Total organic carbon (%) (**a**), Thermal stability index (R index) (**b**), thermal lability index (I index) (**c**), spring above ground plant biomass (**d**), summer above ground plant biomass (**e**), total yearly above ground plant biomass (**f**) measured at the plot level within the TwinWin during two consecutive years. Growth (**g**), respiration (**h**) and CUE (**i**) observed in the rhizosphere of barley along the undersown plant diversity gradient (log). Significant relationships were evaluated with linear mixed models with location in the field (block or plot) as random effect and are presented by solid lines when significant ($p < 0.05$); exact p values are given next to the R^2 values ($n = 167$, df = 163). The shaded area denotes 95% confidence intervals around the mean values. Source data are provided as a Source Data file.

Empirical link between plant diversity and microbial CUE

Microbes are key regulators of Earth's massive soil carbon stocks, determining the partitioning of plant inputs into microbial biomass which might become part of soil organic matter[16–18] versus respiratory carbon dioxide release to the atmosphere. For this reason, understanding the influence of plant productivity, composition and diversity on microorganism physiology is of ultimate importance if we want to disentangle microbial-mediated processes relevant for C cycling[17–19]. As microbial necromass makes up to 50% of SOM[18], it is crucial to foster our understanding on the drivers of microbial growth and microbial biomass formation in soils. Carbon use efficiency (CUE) describes the proportion of a cell's resources converted into microbial biomass relative to the total resources consumed. It is thought to be an important microbial physiology parameter influencing the amount of necromass produced per unit of substrate consumed[49], and therefore, connecting microbial biomass to potential SOM formation[50]. Here we used a substrate independent method, the ^{18}O-water method[47], to evaluate microbial CUE in the rhizosphere of barley under increasing plant diversity. Interestingly, respiration and growth, the two components of CUE, responded differently to plant diversity. While growth was significantly enhanced with increasing levels of plant diversity (Fig. 2d), respiration was not (Fig. 2e). As CUE is the compilation of these two factors, CUE of the microbial community within the rhizosphere of barley increased along the undersown diversity gradient (Fig. 2f). These results can help to understand the mechanisms leading to higher SOC content in soils of more diverse plant communities, as observed here (Fig. 2a) and in previous studies[12,46]. A community that grows more efficiently should result in an increased abundance of microorganisms per gram of soil[13] and consequently, in more iterative cycles of microbial growth and death (i.e. turnover). A higher microbial turnover[15–18] under high plant diversity should accrue SOM content over time[28].

Plant diversity modulates the associations within the microbial community in the rhizosphere

There is a growing consensus that the key to understand soil functioning lies in the rhizosphere, where plants and soil meet. This is the area surrounding plant roots and is a major hotspot of soil functioning[51]. A seminal review suggested that to foster sustainable agriculture it is crucial to capitalize on the multitrophic rhizosphere-mediated interactions[52]. Here, we used network analyses to capture how a plant diversity gradient impacts the potential associations within the bacterial and fungal microbial communities in the rhizosphere of barley (Fig. 3a, b; methods; Supplementary Figs. 5–8, 11–15). To do this we built co-occurrence networks evaluating the potentially positive and negative associations within the microbial community[53] (Fig. 3a; see methods). We are using the term "potential associations" to take into consideration that networks derived from co-occurrence data are limited in their capacity to infer direct interactions[54]. Positive associations can arise both through shared responses to the environment or through co-operative interactions but it is not possible to distinguish between the two. The nodes in the networks are representing the bacterial and fungal species and the links between the nodes their potential associations. Interestingly, we observed an increase in connectivity (number of links normalized by the number of nodes present in each network) within the positive

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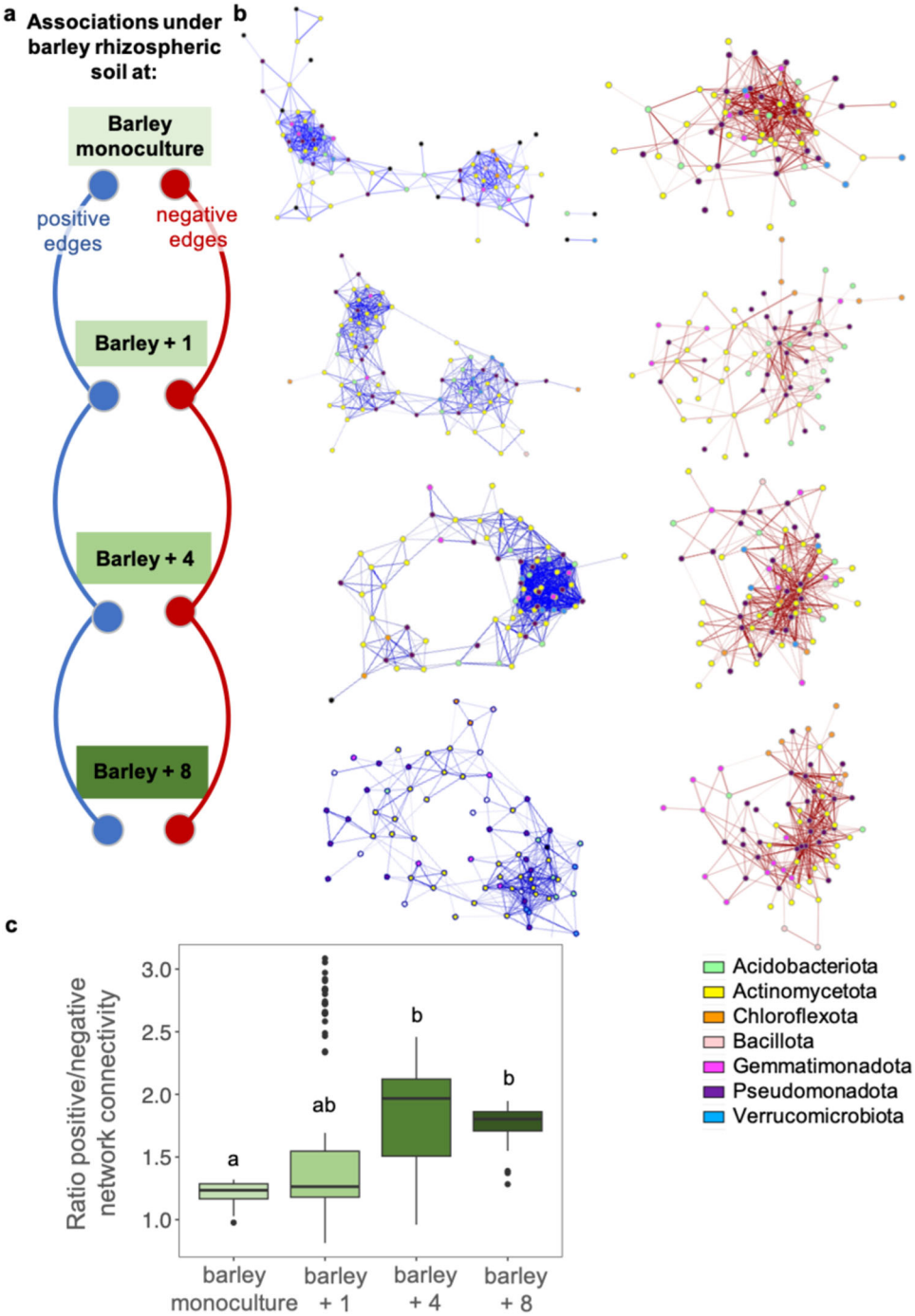

Fig. 3 | Network analysis design and findings. Network analysis approach to evaluate the impact of undersown diversity on microbial associations within the rhizosphere of barley across the diversity gradient (**a**). Bacterial networks showing edges in blue if representing positive associations or in red if representing negative associations and the colors of the nodes represent the different bacterial phylum within each plant diversity treatment from top to down: barley monoculture, barley + 1, barley + 4 and barley + 8, respectively (**b**). The Ratio of positive to negative network parameter of degree centrality which captures network connectivity at each undersown diversity treatment (**c**). Significant differences between treatments are indicated by different letters (linear mixed effects models with block as the random effect using the *nlme* package, and ANOVA type III to correct for unbalanced design, $P = 5 \times 10^{-7}$, df = 163, n = 167). In the boxplots, whiskers denote the minimum value or 1.5× interquartile range (whichever is more extreme), and box denotes interquartile range. The horizontal line denotes the median. Biological replicates: n = 24, 95, 24 and 24 for barley monoculture, barley +1, barley + 4 and barley + 8, respectively. Source data are provided as a Source Data file for Fig. 3c.

networks along the diversity gradient, while the connectivity of negative networks shows a tendency to decrease with increasing plant diversity (Fig. 3b, c, Supplementary Figs. 3–5). Co-occurrence networks have inherent method-driven biases and limitations[54–57]. Our experimental design was conceived to replicate these inherent limitations of network analysis along the diversity gradient (e.g. we used the same number of samples within each network; see methods). Therefore, captured differences in the structure of the network, are likely due to the distinct plant diversity treatments. Overall, our results suggest that plant diversity enhanced positive associations within the bacterial community compared to the negative associations (Fig. 3c, Supplementary Figs. 7–8).

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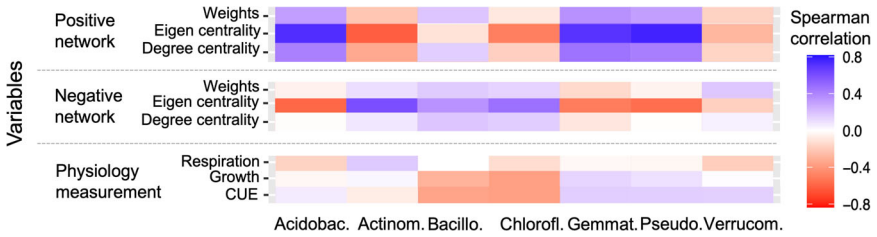

Fig. 4 | Relationship between network parameters and the response of microbial physiology to the relative abundance of different bacterial phylum across the plant diversity treatments. Heat map of positive and negative network parameters and microbial physiology measurements (respiration, growth, and CUE) in response to changes in bacterial phylum relative abundances. Weights: represent the strength of the relationship between two vertices; eigen centrality: captures the relevance of the different OTUs for the network and degree centrality is a parameter computing the connectivity among OTUs within the networks. Acidobac.: *Acidobacteriota*; Actinom.: *Actinomycetota*; Bacillo.: *Bacillota*; Chlorofl.: *Chloroflexota*; Gemmat.: *Gemmatimonadota*; Pseudo.: *Pseudomonadota* and Verrucom.: *Verrucomicrobiota*. Spearman correlation (two-tailed test) coefficients in as white where *p*-value exceeded 0.05, or in blue or red if the *p*-value is lower than 0.05 and the correlation coefficient positive or negative, respectively. Source data are provided as a Source Data file.

To evaluate how connectivity changes in positive networks in relation to negative networks, in addition to their graphical visualization (Fig. 3b), we extracted network parameters related to connectivity as the degree centrality, which captures highly connected taxa or hubs (Fig. 3c, Supplementary Figs. 7–8). When computing the ratio between the degree centrality of positive networks to the degree centrality of negative networks it corroborates our observation of enhancing connectivity in positive compared to negative networks along the diversity gradient (Fig. 3c). This relative increase in positive associations could be due in part to the enhancement of cross-feeding within the microbial community under high plant diversity compared to low plant diversity. Cross-feeding occurs when excreted by-products of metabolism of an organism or population benefits others[58–60], and is considered a key mediator of positive interactions within microbial communities[61]. Here we are hypothesizing that a more complex compilation of plant exudates and the combination of distinct litter chemistries under higher plant diversity may induce positive interactions due to cross-feeding and facilitation mechanisms (e.g. the degradation of plant by-products and its derivatives by one organism can facilitate growth of some of its neighbors who have a higher affinity to the by-product compared to the primary plant compound)[58,61]. Cross-feeding has been suggested to act as a niche construction for microorganisms[58]. Thus, if high plant diversity creates a higher niche space for the soil microbial community, this could explain why we observe higher microbial growth and growth efficiency (CUE) per gram in these soils (Fig. 2d–f). The observed increase in connectivity in the positive network could be an indicative of more cooperative associations[61] within the microbial community with increasing plant diversity. Simultaneously, the connectivity of negative networks tends to decrease with increasing plant diversity (Supplementary Fig. 8) which could mean that competition between community members decreases with plant diversity[62]. Previous studies showed that agricultural management can influence microbial association networks[63] and that network properties might be related to microbial-controlled ecosystem functions[64]. Here we show how a plant diversity gradient influences the soil bacterial network connectivity with potential consequences for soil-C cycling.

We also built the association networks using arithmetic subtraction to isolate the effect of the diversity gradient within the networks (methods; Supplementary Figs. 6 and 13)[53]. Using such an approach to compute the networks confirms our findings discussed previously of high positive network connectivity under higher plant diversity compared to low plant diversity in comparison to negative networks (Fig. 3c, Supplementary Figs. 6–8). The shift in network structure observed from barley monoculture to barley plus 1 and subsequent higher plant diversity treatments suggest changes in network organization at the community level[65]. The changes in network structure resulting in the"tightening" of the network increases the probability of species being connected to each other through direct or indirect pathways[66]. While this can be a result of enhanced cross-feeding as previously discussed, indirect effects within networks have also been acknowledged to explain the persistence of mutualism among species across time[60]. It has been shown that chemical succession in the rhizosphere of a grass during plant growth controls the microbial community assembly via microbial metabolite substrate preferences[65]. Thus, if different plant species produce a distinct assemblage of root exudates during plant growth, it should result in a differential recruitment of microbial species. The introduction of additional species can change how pre-existing species are indirectly linked to each other[66].

Our study is limited to answer the question of how distinct plant species control community assemblage as our experimental design was conceived to evaluate how undersown diversity modifies the microbial associations within the barley rhizosphere (methods). However, we observed that at the first level of diversity (barley plus 1) the undersown species identity influenced the barley rhizosphere microbial community associations (Supplementary Fig. 5). While the network structure of barley rhizosphere under *Lolium perenne*, *Trifolium hybridum* and *Festuca arundinacea* are relatively similar to each other, the network structure in plots with *Medicago sativa* shows to be very distinct from the other three species in its connectivity. Interestingly, *Medicago sativa* achieved higher plant cover in this diversity farming experiment compared to other undersown species at the same diversity level (Supplementary Fig. 16). This suggests that undersown species abundance also plays a role on influencing the major crop rhizosphere's processes. Alternatively, *Medicago sativa* is a deep rooting and nitrogen fixing specie. These changes could be in part related to the deep rooting and N fixation strategies and the recruitment of distinct microorganisms at lower depth. Previous findings suggested that changes in species associations resulted in greater changes in N- compared to C-cycling in soils[67].

We observed that changes in different bacterial phyla are responsible for the shifts in positive and negative network parameters (Fig. 4). While Acidobacteriota, Gemmatimonadota, and Pseudomonadota are more strongly related to positive changes in positive networks, Actinomycetota, Chloroflexota and Verrucomicrobiota are negatively related to changes in positive networks. This analysis also shows how the changes in the relative abundance of bacterial phylum are distinctively related to respiration, growth and CUE. In line with recent findings, we show that increases in relative abundance of Bacillota is negatively related to CUE[19]. However, it is important to consider that recent investigations have not identified any robust functional gene markers of CUE[68]. Moreover, it has been shown that inefficient taxa can increase their CUE with changes in abiotic conditions, while more efficient taxa showed a decrease in CUE with the same changes in abiotic conditions[68]. Our results suggests that CUE

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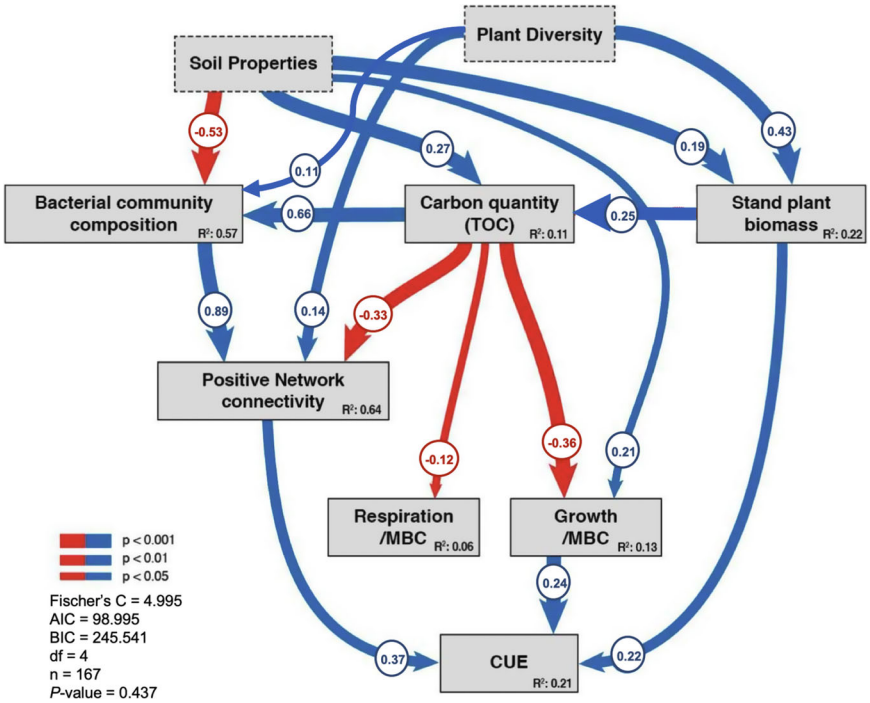

Fig. 5 | Structural equation model showing the relative importance of plant diversity, soil properties and plant biomass on CUE. Significant paths are shown in blue if positive or in red if negative. Path width corresponds to degree of significance as shown in the lower left and standard coefficient for each path is shown on a circle within each path. The amount of variance explained by the model (R^2) is shown for each response variable. Soil properties: composite variable of soil properties (i.e. pH, Calcium (g/kg soil), C/N ratio and Cation exchange capacity (cmol/kg soil)), Plant diversity: composite variable of Simpson's plant diversity index calculated based on species present in a plot and the plant cover measurements; Stand plant biomass: cumulative plant biomass measured in spring and summer from 2019 to 2021 at the plot level; Bacterial community composition: NMDS axis 1 of bacterial community structure; Positive network connectivity: positive eigen centrality from bacterial positive networks; Respiration/MBC: mass specific respiration; Growth/MBC: mass specific growth; CUE: carbon use efficiency. Global goodness-of-fit: Fisher's C. Measures of overall model fit are shown in the lower left. Source data are provided as a Source Data file.

reflect community-scale dynamics[69] and it is a flexible parameter changing in response to how abiotic and biotic conditions influence the microbial communities (Figs. 4–5). Future research should foster our understanding on how distinct plant functional groups (e.g. root depth and N-fixation) impact microbial community assembly and interactions to identify plant traits able to steer soil microbial communities to promote specific soil functions.

Bacterial networks responded to plant diversity while fungal networks did not respond to undersown plant diversity (Fig. 3, Supplementary Figs. 2–3 and 8–12). This could be due to the distinct response of fungi and bacteria to C inputs from plants[70,71]. While bacteria should respond faster to root exudate chemistry[71], fungi are known to dominate the first stages of litter decomposition[70] and fungal assembly processes can be influenced by tillage which is applied in this agricultural experiment[72]. Moreover, previous findings show that, fungal networks are more resistant to environmental stimuli[73], and that changes in fungal networks led to fewer changes in soil functioning than changes in bacterial networks[73,74]. These results could be influenced by the different ages of these experiments. The TwinWin agricultural experiment has been recently established and it is likely that changes in fungal parameters are observed once the plant treatment effects accumulate over time[75,76].

CUE as a function of interactions between biotic and abiotic drivers

We used structural equation modeling (SEM) to determine the degree to which the different components (plant diversity, plant biomass, soil properties, carbon quantity, bacterial community composition, network structure, mass specific respiration and mass specific growth) influence directly or indirectly CUE (Fig. 5 and Supplementary Figs. 17–19). The model path structure was based on the assumption that plant biomass and plant diversity drive CUE directly, but also indirectly by impacting the association between microorganisms and the soil C pool (Supplementary Fig. 17). We used the SEM to test the following hypotheses: (1) we expect plant diversity to strengthen positive associations between microbes due to cross-feeding and mutualistic interactions; (2) we expect mass specific respiration and mass specific growth to increase with increasing positive association in the microbial community, because co-existence mechanisms underlies complementary interactions that increase community efficiency; (3) we expect that increasing plant biomass will lead to higher levels of total carbon because of increased carbon inputs through dead roots and root exudation in the rhizosphere which in turn will influence mass specific respiration and mass specific growth of soil microbes due to increased substrate availability; and (4) we expect that if cross-feeding and facilitation mechanisms are captured within the positive associations these should more strongly influence growth than respiration resulting in a more efficient (less expensive) community growth, and therefore increasing CUE (Fig. 5 and Supplementary Figs. 17–19).

Although soil properties are considered a controlling variable for CUE[45,77], our structural equation model indicates that they influenced CUE only indirectly via changes in the biotic components influencing the composition of the microbial community, the mass specific growth and stand plant biomass (Fig. 5). In our SEM model soil property is a composite variable containing soil pH, C/N ratio, calcium content and the cation exchange capacity. Plant diversity positively influenced the connectivity of positive microbial association networks and the bacterial community. We cannot make conclusions from the signal of the path coefficient between bacterial community composition and network connectivity because community composition is represented by the first axis of the non-metric multidimensional scaling (NMDS) of the bacterial community, which has an arbitrary direction. Carbon

quantity had a negative effect on the positive network connectivity. Interestingly, mass specific respiration was only influenced by soil carbon quantity while mass specific growth was also impacted by soil properties.

Previous studies have shown that bacterial composition act as a direct driver of CUE in forest soils[19] and agricultural soils[78], however in our model the community composition only indirectly impacted CUE by driving associations between microorganisms. This shows that the impact of a microbial community on CUE can play out through a variety of mechanisms including associations among community members. A recent study hypothesized that biotic interactions are underestimated drivers of microbial CUE[79], we empirically showed that the degree of connectivity within positive network associations has a positive effect on CUE (Fig. 5, Supplementary Fig. 19). Because CUE is a composite variable of respiration and growth, to capture the mechanisms controlling microbial physiology we incorporated the mass specific rates of these processes in the model as previously[45]. It is important to highlight that a substantial fraction of CUE variation remains unexplained in the model, meaning that other important factors are not captured here. For example, previous studies have shown that the costs of extracellular enzyme production[80] and the availability of dissolved soil organic C[77] are factors influencing the community CUE. Moreover, while it has been shown that the presence of fungi increases community CUE[25], our results suggested that associations among fungi could have a negative influence on community CUE (Supplementary Fig. 19). It has also been shown that root biomass controls the accessibility of plant derived C to the bacterial community in the rhizosphere[30,31]. In the TwinWin experiment, it is not possible to fully disentangle plant diversity from the plant and/or root biomass and root exudation. We show that plant diversity has an impact on plant biomass enhancing plant input into the soil in spring (Fig. 2d–f) and that plant biomass has a positive influence on soil C content measured from barley's rhizospheric soil (Fig. 5). Plant biomass and the positive associations within the microbial community positively influenced CUE in our model. Altogether our results highlight how changes in plant community diversity may influence microbial communities with consequences for soil C-cycling.

Current agriculture intensification practices lead to a decrease in associated biodiversity[81]. Thus, managing agroecosystems with multiple goals and functions becomes a crucial goal for agriculture in the next decades[11,33]. The barley used in this experiment (variety Harbinger) is the most popular malt barley planted in Finland accounting for 35% of malt barley area (16000 hectares in 2017). Similarly to other major agricultural crops, this barley cultivar has been bred for maximum performance in monoculture. While barley yield has not been significantly decreased with increasing undersown diversity (Supplementary Fig. 2a) we observed potential competition with *Medicago sativa* which decreased barley yield (Supplementary Fig. 2b). Thus, it is urging to advocate breeding programmes for crop varieties to be performed using mixtures to exploit complementarity among crop species to further enhance intercropping benefits[82,83]. While in this study we showed how plant diversity drives belowground ecosystem processes relevant for soil C-cycling in agriculture, a voluminous number of studies already highlighted that plant diversity is important for other ecosystem functions[3,5], stressing the need for an "ecological intensification" within agroecosystems[11,33]. Further progress requires integrating our ecological knowledge regarding agroecosystems to the social and economic constrains of modifying management practices. Previous results suggests that small farmers play a strategic role for biodiversity conservation[84,85] and promotion of sustainability in agroecosystems[85–87]. The implementation of diversity within agroecosystems across space and time is labor intensive and is more likely to be implemented by small-scale farmers[86–89]. Policy mechanisms to promote "carbon-farming" must take into consideration the threat that small farmers will face in the next decades[90]. Comprehending the importance of public policies supporting agroecological production systems by linking the right to produce healthy food without compromising the provision of ecosystem functions and biodiversity protection is of ultimate importance for the responsible management of our agroecosystems and fostering sustainability in agriculture.

Methods

TwinWin agricultural field experiment and soil collection

Soils were collected from the TwinWin experiment located at the Helsinki University in August 2020, Finland (60°13'N/25°01'E). The TwinWin experiment was stablished in 2019 and was designed to evaluate the impact of ecological intensification within agroecosystems by evaluating the potential benefits of undersowing diverse species mixtures to promote ecosystem functioning. Here, barley (*Hordeum vulgare* L. var. Harbinger) was planted as the main crop and fertilized with 80 kg N ha^{-1} yearly. The full TwinWin experimental design consists of barley planted in monoculture with herbicide (4 plots) and without herbicide (8 plots) or with increasing levels of undersown plant diversity (i.e. barley monoculture, barley plus 1 undersown specie, barley plus 2 undersown species, barley plus 4 undersown species and barley plus 8 undersown species; Supplementary Fig. 1)[39]. Barley is first sown in equal abundance in every plot in 12 cm wide rows and a week later, the undersown species are sown in 10 cm wide rows between the barley plants (i.e. intercropping). The undersown plant species were selected based on their functional root traits (i.e. nitrogen fixation capacity and rooting depth) and were: *Trifolium repens* L., *T. hybridum* L. (shallow rooting, nitrogen fixing), *T. pratense* L., *Medicago sativa* L. (deep rooting, nitrogen fixing), *Lolium multiflorum* Lam., *Phleum pratense* L. (shallow rooting, non-nitrogen fixing), *Festuca arundinacea* Schreb., *Cichorium intybus* L. (deep rooting, non-nitrogen fixing) (Supplementary Table 1 and Supplementary Fig. 1). The number of seeds from each undersown species at diversity level 1 (barley plus one; "D1") is calculated to reach a similar plant cover after discussion with farmers that are familiar with these cover crop species. In the higher diversity levels barley plus two ("D2"), barley plus four ("D4") and barley plus eight ("D8"), 1/2, 1/4 and 1/8 of this proportion of seeds is sown, respectively, to avoid increasing undersown abundance along the plant diversity gradient and potential competition between undersown species and barley plants. The D1 treatment is replicated in three plots for each undersown specie (totalizing 24 D1 plots); D2 treatment is replicated in 10 plots; D4 is replicated in 6 plots and D8 is replicated in 4 plots totalizing 56 planted experimental plots (Supplementary Fig. 1). Four plots are maintained as "bare fallows" without any plant cover, totalizing 60 experimental plots within the TwinWin experiment. The plots follow a randomized design distributed in four blocks (Supplementary Fig. 1). All diversity treatments are present in the four blocks and the plots are 40 m^2 (10 x 4 m).

Aboveground biomass measurements are made before the harvest of barley and before the tilling in the Spring each year and at each plot, except for 2019. At each plot, an area of 50 x 50 cm was selected to include 4 rows of barley and to best represent the general composition of the plot (e.g. isolated spots where no vegetation was present or areas which were unusually very dense with weeds are avoided). Then all the biomass (stem + leaves) was cut and separated in two categories: barley biomass and other plants biomass (undersown species + weeds). The samples were then dried for 48 h at 60 °C and weighed with a precision of 0.01 g. To understand how the distinct plant diversity treatment may influence plant biomass we compiled the biomass data measured during two consecutive years (from summer 2020 to summer 2022). and divided them between spring and summer values. Spring biomass measurements include only undersown species and weeds, while summer biomass measurements include barley, undersown species and weeds. Barley yield was assessed at the moment of harvest in early up to mid-September when all barley is harvested. Barley yield is measured from the harvest of the central 2 m

strip of the plots to reduce potential edge effects. After the barley harvest, the barley monoculture plots were tilled, while all the other plots remained untilled until shortly before the re-sowing of the barley and the undersown species the next season end of May/early June. The exact dates of sowing and harvest can vary between years.

Barley rhizosphere soil was collected during August 2020 on a subset of plots of the TwinWin experiment to capture the influence of a plant diversity gradient on the biotic interactions within the soil and soil C cycling processes. We sampled the barley monoculture, D1, D4 and D8 treatments (Fig. 1). For the D1 treatment, we sampled the rhizosphere soil from barley growing under the influence of four different undersown plant species (*L. multiflorum*, *F. arundinacea*, *T. hybridum* and *M. sativa* from all three plot level replicates (Fig. 1) representing each of the functional groups (i.e. nitrogen fixation capacity or not, and shallow or deep rooting species). We also sampled the barley rhizosphere at the D4 treatment within 4 randomly chosen plots and from all the D8 treatment plots (Fig. 1). The sampling design was performed to obtain 24 samples from each treatment (barley monoculture, D1 treatment from each of the four chosen undersown species, D4 and D8) totaling 168 rhizosphere soil samples (Fig. 1). Rhizosphere soil was defined as the soil attached to the roots of barley after excavating the barley plants and shaking them. The soil was sieved to 2 mm on site before being transported back to the lab 2 h after sampling. Immediately upon arrival at the lab, one subsample of each soil sample was dried to constant mass at 65 °C overnight to determine soil moisture content. The remaining soil was left in zip-log plastic bags at room temperature overnight. Different soil subsamples were then used for microbiological assays and soil organic matter analyses described below. The barley above ground plant biomass was dried in an oven for 48 h at 105 °C.

Respiration, growth, and CUE

We used the substrate-independent ($H_2{}^{18}O$-CUE) method[43] to evaluate soil microbial carbon use efficiency. To measure growth and respiration three replicates per soil sample of 0.3 g soil aliquots were weighed into 2 ml Eppendorf tubes, and sealed with parafilm to prevent additional moisture loss, the day after the soil was collected. Water was added to bring them to 60% water holding capacity. Two replicates received water so that 20% of the total water was present as ^{18}O-water, while the one remaining replicate received all water as ^{16}O-water to account for natural abundance of ^{18}O. The two replicate vials that received $H_2{}^{18}O$ were added to a 100 ml glass vial, sealed with a crimp cap and immediately placed in an incubator at room temperature (22 °C). Empty glass vials were also sealed in the beginning of the assay and after every 15 tubes in order to measure the starting CO_2 levels in the glass vials at the moment of starting the incubation. After 24 h, the CO_2 was measured in the glass vials using a GC gas chromatograph. Then the soil samples were placed at −80 °C until DNA extraction according to the $H_2{}^{18}O$ CUE protocol previously stablished[47].

CUE measurements using the ($H_2{}^{18}O$-CUE) method estimate the new microbial biomass produced during the incubation period based on ^{18}O-DNA enrichment. DNA was extracted from all soils using the Zymo Quick-DNA Soil Microbe 96 Kit according to manufacturer instructions. For each barley rhizosphere sample (168 samples), 2 technical replicate soil samples were incubated with $H_2{}^{18}O$ and 1 soil sample with $H_2{}^{16}O$. Each of the soil samples that received $H_2{}^{18}O$ water was split into two for DNA extraction and the control sample that received $H_2{}^{16}O$ has extracted with one single DNA extraction. Thus, 168 barley rhizosphere samples x 2 ($H_2{}^{18}O$ -water incubation for each rhizosphere sample) x 2 DNA extraction per soil sample + 1($H_2{}^{16}O$ -water incubation for each rhizosphere sample) x 1 DNA extraction per soil sample, equals 840 DNA extractions in total. The technical replicates for the DNA extraction were pooled before quantification using Qubit to improve reproducibility and avoid bias in CUE measurements[91]. DNA $\delta^{18}O$ values were measured using a Flash IRMS elemental analyzer operated in pyrolysis mode coupled to a Delta V Plus isotope ratio mass spectrometer (Thermo Fisher, Waltham, MA, USA) at the Stable Isotope Ecology Lab, University of Basel, Switzerland, and reported relative to Vienna Standard Mean Ocean Water (VSMOW) in ‰. Long term instrumental precision for the lab for non-^{18}O-enriched analyses is 0.2 ‰. Analytical precision for ^{18}O-enriched samples measured as a part of this study was 2.6 ‰ (quality control samples $n = 9$). CUE was calculated as per Spohn[49].

Soil organic matter quality and quantity

We used Ramped-thermal Rock-Eval® pyrolysis (RE) to evaluate SOM quality and quantity on another subsample of the rhizosphere soil samples. During RE, carbon oxides are quantified as they come off a soil sample subject to increasing temperatures, thereby providing a metric of SOM intrinsic thermal stability. Compounds with high thermal stability include aromatic and phenolic non-lignin compounds, while lipids and polysaccharides tend to have lower thermal stability[42]. Soil samples were first dried at 65 °C and crushed to a fine powder with a mortar and pestle. Then between 50–70 mg of soil was pyrolyzed over a temperature ramp from 200 to 650 °C, followed by combustion from 200 to 850 °C using a Rock-Eval 6 pyrolizer (Vinci technologies) at the Institute of Earth Sciences of the University of Lausanne (Switzerland). Hydrocarbons released during this process were measured by a flame ionization detector (FID). This method allows us to obtain the soil total organic carbon percent (TOC) in these soils. Additionally, the resultant thermogram also allow us to calculate the I index ("labile carbon fraction") and R index ("recalcitrant carbon fraction") as previously[41].

Bacterial and Fungal sequencing

An aliquot of the DNA extract used for the CUE estimates (described above) was used to perform 16S rRNA gene (V3-V4) and ITS region (ITS7 - ITS4) tagged amplicon sequencing using NovaSeq PE250 platform by Novogene (Cambrigde, UK). Raw sequences from amplicon sequencing were quality filtered, merged, and clustered to generate OTU's at 97% sequence similarity for bacteria and fungi using QIIME2 pipeline[92]. Diversity matrices were calculated on 47.000 reads for bacteria and 19.000 reads for fungi respectively using the vegan[93] R library. SILVA (reference database 132)[94] and UNITE (version 01.12.2017)[95] were used for the taxonomy assignment of bacteria and fungi, respectively. OTUs were filtered to exclude nonbacterial, nonfungal sequences and single-count OTUS. A total of 7.891 and 2.437 OTUs were found for bacteria and fungi, respectively. NMDS of the UNIFRAC distance matrices (weighted) were used to describe bacterial and fungal community structure. We tested the effect of treatments (plant diversity and plant diversity by plant species) with Adonis in the vegan[93] package.

Statistical analysis

Analysis was completed in R version 3.6.3 using packages reshape2[96], vegan[93], lme4[97], agricolae[98], lattice[99], MASS[100], permute[101] and r2glmm[102]. Normality of each variable was tested with the visual distribution of residuals and log or square root transformed if needed. Outliers were detected by verifying if an observation was outside the 1.5 × inter quartile range for the first and third quartiles. To check whether undersown richness explain our studied soil parameters, we fit linear-mixed effect models using the lme or lmer functions[97] and used the *r2beta* function within the r2glmm package[102] to obtain the R Squared from these linear mixed models. For these analyses the logarithm of undersown richness plus one was used, because this allowed us to include barley monoculture plots with undersown richness = 0, with location in the field (plot and/or block) as random effects. Figures were made using the ggplot2 package[103] and igraph package[104]. One-way ANOVA followed by Tukey HSD test was used to evaluate the differences between plant diversity treatments across the distinct measurements performed in this study.

Network analysis

Bacterial and fungal network analyses were performed based on co-occurrence patterns utilizing the more ubiquitous OTUs in each diversity treatment (≥ 5%). Positive and negative networks based on relative abundance data were built using stringent cut-offs (Spearman's rho > 0.6, and correcting for false discovery rate (FDR) $P < 0.05$, using OTUs which were present in at least 10% of the samples within each diversity treatment using the *igraph* package[104] as previously[53]. We also build one network for each undersown species within the D1 treatment. To compute the plant diversity impact on networks, we used network arithmetic to subtract the intersected correlations patterns between the barley monoculture network and barley monoculture, barley plus 1 with barley plus 4 and finally barley plus 4 with barley plus 8 (Supplementary Fig. 1a). With this approach we evaluate the impact of the diversity gradient in the network properties. Network topology parameters were calculated with igraph[104]. Shortly, nodes are the OTUs represented in the network, the edges between nodes represent the inferred associations between the different OTUs, the weight capturing the strength of those associations. Degree centrality is a parameter computing the connectivity among OTUs within a network. Eigen centrality is a measure comprising the relevance of the different OTUs. OTUs with high eigen centrality are those which are connected to many other OTUs which are in turn also connected to many other OTUs. After computing the network parameters for the different levels of plant diversity, we extracted these OTUs-based values and calculated a sample-specific value based on the relative abundance of the specific OTUs in the different samples. The sample-specific network-based parameters values were then used in our structure equation model (explained below) to evaluate how the microbial network properties impact microbial community physiology.

Structural equation modeling

We used structural equation modeling (SEM) to test the hypothesis that (1) plant diversity enhances positive interactions between microorganisms within the rhizosphere of barley and (2) the interactions between microorganisms have an impact on the community growth efficiency or CUE. The hypothesized path structure was based on the previous observations that diversity within the microbial community can enhance CUE under favorable conditions[25] suggesting that complementarity effects can arise from facilitation and niche differentiation that resulted from inter-species interactions increasing overall community efficiency. Here, we hypothesized that increasing plant diversity increases the niche space as different plant species present different root functional types and produce different exudates[71], microorganisms should encounter more favorable conditions to grow fostering the positive interactions in relation to the negative interactions within the microbial community with increasing plant diversity. We also completed SEM analysis including other network properties but we kept the networks that explained a higher fraction of the observed CUE (Supplementary Figs. 12 and 14). The SEM model path fit was performed using the piecewiseSEM package[105]. We kept the model that explained the most variation in CUE and had a non-significant Chi-squared test ($P > 0.05$), a low Akaike Information Criterion (AIC), and a high Comparative Fit Index (CFI > 0.9). If the test of direct separation[90] identified missing paths in our hypothesized model, we added these paths into the model. Prior of performing the SEM we run covariance analysis with the soil properties measured in these soils (i.e. pH, Ca, P, Mg, Mn, S, K, Cu, Zn, Cation exchange capacity and C/N ratio) to exclude variables that co-vary across our soil samples and therefore would provide limited new information for the SEM.

Data reproducibility

Here, we used an agricultural diversity experiment (TwinWin) to evaluate how plant diversity, plant biomass, microbial community dynamics and soil properties drive CUE. Although this experiment was performed once we used a relatively high number of biological and technical replicates for the different assays to increase reproducibility. To reduce variability during microbial community analysis, we sampled 24 samples for each plant diversity treatment and performed technical quadruplicates for DNA extraction. For this, every microcosm had 2 subsamples receiving $^{18}O–H_2O$ which were then divided in 2 technical replicates for the DNA extraction. DNA extractions were quantified and subsamples pooled prior to sending the samples to the Stable Isotope Facility and for the sequencing facility. One sample showed negative ^{18}O-atom% excess, which resulted in a negative growth value and therefore was excluded from the data analysis.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data supporting the findings presented here are available from the corresponding authors on request and from the Open Science Framework Repository (https://doi.org/10.17605/OSF.IO/QN3JC). The sequencing data are available in the NCBI repository with the identifiers PRJNA1137244 and PRJNA1136844 for bacteria and fungi, respectively. Source data are provided with this paper.

Code availability

The R code supporting the findings presented here is available from the corresponding authors on request and from the Open Science Framework Repository: https://doi.org/10.17605/OSF.IO/QN3JC.

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Author contributions
A-L.L., A.K.L. and J.H. designed the TwinWin long term farming field experiment. L.A.D-H. conceived this particular experiment to evaluate how plant-microbial interactions influence CUE. L.A.D-H, S.L.C., R.S., D.S., J.H., S.G., and D.N. conducted the experiments; L.A.D-H analyzed the data. L.A.D-H. wrote the first draft of the paper and A-L.L., S.L.C., R.S., D.S., J.H., S.G., D.N., A.K.L, A.K., P.D., and E.V. contributed to revising the manuscript.

Competing interests
The authors declare no competing interests.

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