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Interaction of Inherited Microbiota from Cover Crops with Cash Crops

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Abstract: Cover crops (CC) provide important ecosystem services that are demanded to achieve more sustainable agrosystems. However, the legacy effects of CC on the microbial community structure and its interactions with the subsequent cash crops (CaC) are still poorly understood, especially when CC mixtures are involved. In this work, five CC (3 monocultures and 2 mixtures) were selected in an experiment under semi-controlled conditions to investigate if CC monocultures and mixtures differed in their effects on soil and crop variables and if the identity of the subsequent crop modulates these effects. The two most consumed crops worldwide, wheat and maize, were sown separately after CC. The legacy effects of CC on the studied microbial variables largely depended on the interaction with the CaC. The vetch and the barley-vetch mixture stood out by providing the microbial conditions that enhanced the absorption of macro- and micronutrients, to finally seek the highest wheat biomass (>80% more than the control). In maize, the effects of CC on soil microbiota were more limited. The soil microbial responses for CC mixtures were complex and contrasting. In wheat, the barley-vetch mixture behaved like barley monoculture, whereas in maize, this mixture behaved like vetch monoculture. In both CaC, the barley-melilotus mixture differed completely from its monocultures, mainly through changes in archaea, Glomeromycota, and F:B ratio. Therefore, it is necessary to deepen the knowledge on the CC-CaC-microbial interactions to select the CC that most enhance the sustainability and yield of each agrosystem.

Keywords: wheat; cover crop mixtures; mycorrhization; fungi; bacteria

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1. Introduction

The study of cover crops (CC) is booming due to their potential to improve the sustainability of agricultural systems and to contribute to mitigation and adaptation to climate change. Thus, CC provide ecosystem services such as increasing the supply of C to the soil system [1], reducing nitrate leaching [2], decreasing albedo [3], or suppressing of weed vegetation [4,5], among others. In addition, CC enhance soil health since, on average, they increase the microbiota abundance by 27%, the activity by 22%, and the microbial diversity by 2.5% compared to bare fallow [6], besides stimulating the development of beneficial microorganisms for crops [7–9].

The influence of CC on the microbial community structure largely depends on the species or mixture of species used. Fundamentally, the CC species influences the soil microbiota by way of three mechanisms: (i) the release of characteristic root exudates [10], (ii) the symbiosis between the CC roots and organisms of specific genera, such as arbuscular mycorrhizal fungi (AMF) and/or rhizobium [11,12], and (iii) the decomposition of their fresh residues that, among other characteristics, present different C/N ratios [13].

Most CC serve as hosts for AMF and, therefore, can help to maintain or increase the inoculum potential of these important microorganisms in subsequent crops [14]. In this way, the ecosystem services provided through AMFs are enhanced, such as nutrient absorption [14,15], stress resistance, soil aggregation [16], or pathogen control [17]. Nevertheless, the benefits for AMFs also differ according to the host CC, for example, legume CC can increase the abundance of AMF [18,19], but the ability to inoculate them in the subsequent crop seems to be lower than that of grasses in a Mediterranean climate [20]. It is expected that CC mixtures of species with complementary traits can increase the multifunctionality and sustainability of the system [21,22]. For example, a mixture of grasses and legumes can increase soil organic carbon and nitrogen pools [23]. The mixture of different species influences the composition of the soil microbiota [24–26], and has positive effects such as the increase in the microbial biodiversity [22].

As well as CC identity, the identity of the CaC species, and the CC-CaC interaction can modify soil microbiology [11,22,25]. However, there is still a significant lack of knowledge about the range and the mechanisms of influence of the microbiota that are inherited after using CC on the subsequent different CaC. Therefore, it is important to carry out studies that shed light on the influence of the inherited soil microbiota after using CC on the most widespread cash crops.

With this research, we investigated how different types of CC can affect the growth and the nutrition of the two most consumed CaC, wheat and maize, mainly through altering the soil microbial community under low-input strategy. We hypothesized that: (i) CC mixtures of legumes with grasses would combine the advantages of the two families regarding beneficial microbiota enhancement; (ii) the inherited microbial population structure of CC would have a significant influence on the subsequent CaC development; (iii) each of the CaC would also interact differently with the inherited microbiota from each of the CC. Therefore, the main objective of this study was, firstly, to determine the legacy effects of different types of CC (vetch, melilotus, barley, and the binary mixtures of the legumes with the grass) on a selection of microbial, and crop variables measured in two different subsequent CaC. Secondly, assessing how effects of CC mixtures differed from its monocultures for each CaC. To do that, we established an experiment under semi-controlled conditions and low-input recourses, and measured mycorrhization variables, including the abundance of Glomeromycota, the abundances of total fungi, total bacteria and total archaea, as well as nutritional growth variables, and biomass of the subsequent wheat and maize.

2. Materials and Methods

2.1. Experimental Setup

The experiment was carried out in a greenhouse in central Spain (40°26′49.08″ N and 3°44′18.81″ W; 599 m.a.s.l), in order to reduce the common extrinsic factors of a field experiment (e.g., extreme weather conditions). The soil was extracted from the first 20 cm of the surface horizon of a Calcic Haploxeralf [27], located in Alcalá de Henares (40°30′47.89″ N and 3°18′35.47″ W. 597 m.a.s.l), with the following characteristics: pH (mean ± standard deviation) 8.35 ± 0.11 (1:2.5 soil:water suspension), loam texture (USDA) by hydrometer method [28], and organic matter content [29] of 1.13% ± 0.03. After sieving at 1 cm, the soil was mixed with autoclaved river sand (2 soil: 1 sand in volume) to promote drainage. The homogenized mixture was used to fill 18 × 18 × 25.5 cm microcosms. The first study factor was the type of CC with six levels; three species in monoculture: vetch (*Vicia sativa* L.) (VET), melilotus (*Melilotus officinalis* L.) (MEL), and barley (*Hordeum vulgare* L.) (BAR); two mixtures: barley with vetch (B+V) and barley with melilotus (B+M), in addition to a control without CC (CON). The second study factor was the type of cash crop that was sown after the CC, with two crops: maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.). The experimental design was a randomized complete block design with 5 replications. Therefore, the twelve resulting treatments (6 × 2) were randomly distributed within each of the 5 blocks, giving a total of 60 microcosms.

2.2. Crop Management

Low-input and direct seeding strategies were applied in the trial. The sowing density in the CC was 312 plants/m^2 (156 plants/m^2 per species in the CC mixtures). The CC were developed for 12 weeks and then they were terminated by mechanical mowing, leaving the residues of each microcosm on the ground. Subsequently, the CaC were sown and grown for nine weeks. An irrigation equivalent to 20 mm/month was applied per microcosm (50 mL three times a week in each microcosm). The rational fertilization [30] was calculated based on the NPK of the mean soil. The necessary fertilization to avoid the very low starting levels of NPK was applied before the CC (20-0-0). Later, a fertilization of 61-14-0 at the basal dressing of the CaC; and other of 92-21-0 at the top dressing were applied as NH_4NO_3 and $(NH_4)_2HPO_4$ in order to provide the estimated crop demands with our average soil. To control an incipient fungal infection during CC growth, Tebuconozole was applied to CC leaves at low dose (7 mL at 0.04% per microcosm). In addition, weeds were manually removed. The mean temperature during the growth period of the CC was 16.4 °C and during the CaC was 16.0 °C.

2.3. Sampling and Analysis

The principal sampling was carried out in the main phenological stage 2 for wheat and main stage 3 for maize, according to the extended BBCH scale [31]. Soil and plant samples were taken using a 3.5 cm diameter cylindrical metal sampler, which extracted the aerial part of the plant with its roots, and the adjacent soil (rhizospheric soil) from 0 to 10 cm depth. Once the shoot was separated, the roots were carefully separated from the soil, washed, and preserved for later mycorrhizal analysis. The soil was subjected to different pretreatments according to the final analysis, such as sieving at 2 mm, air drying for pH, EC and nutrient measurements, conservation at 4 °C for extraradical hyphal length, and at −20 °C for the analysis of the abundances of total bacteria, archaea and fungi, and Glomeromycota. Previously, at the time of CC termination, a similar sampling was carried out, but with a lower number of measurements, mainly CC biomass and extraradical hyphal length.

For the analysis of mycorrhizal colonization in CaC roots, we applied the method of Vierheiling et al. [32] to stain the structures containing chitin, the principal component of AMF cell walls, and the "magnified intersections method" of McGonigle et al. [33] to count the AMF structures. The length of the extraradical hyphae (or mycelium) was determined from an aqueous soil extraction and by applying the membrane filter technique described by Jakobsen et al. [34] and adapted by García-González et al. [20].

Total bacteria, total fungi, total archaea, and Glomeromycota in soil were quantified by quantitative PCR (qPCR), using specific genetic markers. DNA was extracted from the soil with the PowerSoil® DNA isolation kit (Mo-Bio laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The communities of bacteria and archaea were evaluated through the 16S rRNA genes, the abundance of the total fungal community was studied using the ITS region, and the community of AMF-phylum Glomeromycota was investigated using the subunit gene small (SSU) rRNA [35–38]. The qPCR standards for each molecular target were obtained using a serial (10-fold) dilution of plasmids, carrying a single cloned target gene or a relevant part thereof. The base DNA of the standard curve and the control without DNA were amplified in duplicate on the same plate as the environmental samples. qPCR amplifications were performed in 20 µL volumes containing 10 µL of the KAPA SYBR® FAST qPCR Master Mix Kit (2X) (Kapa Biosyistems), 4.2 µL of nuclease-free water, 0.4 µL of each primer (10 µM), and 5 µL of pre-diluted template DNA using a real-time system (LightCycler® 480-Roche). The DNA extracts were tested with a series of dilutions to identify possible inhibitions and to determine the dilution that produced the highest copy number. Standard curves were generated using dilutions of linearized cloned plasmids. The genes were cloned into P-GEM T-easy (Promega, Madison, WI, USA) and the inserts were sequenced to confirm their correct length and identity. The standard curves generated in each reaction were linear (serial dilutions of plasmids from

102 to 107 gene copies) with r^2 values greater than 0.98. The amplification efficiencies of all quantification reactions were 80–100%. The number of copies of bacteria, archaea, and total fungi were expressed as Log_{10} gen copy number $\times$ g^{-1} dry soil.

The pH and electrical conductivity of the soil was determined in a 1:2.5 suspension of soil: water. Nutrients in soil (P, K, S, Ca, Mg, B, Cu, Fe, Mn, Zn, Co, and Na) were extracted with Mehlich-3 [39] and measured by Inductively coupled plasma atomic emission spectroscopy (ICP-AES).

The percentage of germination of the CaC was obtained 15 days after emergence by visual counting. The weight of the aerial biomass per microcosm in the final sampling was obtained by drying at 60 °C for 48 h. The concentration of nutrients in the aerial part of the plants (P, K, S, Ca, Mg, B, Cu, Fe, Mn, Zn, and Na) was measured using ICP-AES in dry, homogenized, and ground samples. Total N in soil and plant was determined by using the Kjeldahl method [40]. The content of each nutrient was obtained by multiplying its concentration by the dry aerial biomass in each of the microcosms.

2.4. Statistical Analysis

A generalized linear model was used to analyze the effect of the study factors on the different variables and the interaction between these factors for a randomized block design. The normal distribution and the hypothesis of homoscedasticity of the variables were verified (Levene's test) with 95% confidence, applying the Box-Cox transformation when necessary. To compare the means of the different levels within a factor, Tukey's HSD (Honestly-significant-difference) test was applied after rejecting the null hypothesis of equality of means using the ANOVA technique. Due to the strong interaction between CC and CaC found in most of the variables, subsequent analyses were performed separately for wheat and maize. To describe the data set with a lower number of variables that explain the microbial and nutritional variability, a principal component analysis (PCA) was performed. The PCA was used as a descriptive tool and as an aid in interpreting the results obtained. To determine the variables that explain the CaC biomass production, a multiple linear regression model was carried out with the principal components of the microbial, crop, and soil variables as explanatory variables, using principal components regression. The multiple linear regression models presented predictive variables of biomass production with standardized values (mean 0 and variance 1), whose determination coefficients and standard residual error are the variables that provide reliability for the estimated models. The generalized linear models were used with the Statgraphics Centurion 18 software, and the principal component analysis and principal components regression were performed with the R program [41] using the factoextra package [42].

3. Results

Sampling, at the time of CC termination provided results on the direct effect of each of the CC in the potential AMF inoculum, measured as length of the extraradicalhyphae (hyphal length, hereinafter). BAR was the CC that promoted the longest hyphal length and VET showed the shortest values (Table S1). Meanwhile, MEL produced the least biomass compared with the rest of the CC (Table S2). We also observed that the lowest % germination observed in the cash crops (wheat and maize) was after MEL (Table S3). Subsequently, in the main sampling on cash crops, we found significant effects of both the CC and the CaC, and their interaction in most of the microbial and crop variables that were studied (Table S4). Thus, the effects of some CC differed greatly in wheat compared to maize and, consequently, the results on wheat and on maize were generally displayed separately.

3.1. Effect of Cover Crops on Microbial Variables in Cash Crops

Microbial variables were significantly affected by the type of CC in both maize and wheat (Tables 1 and 2). The variables related to mycorrhization evaluation (% colonization, hyphal length, and Glomeromycota gene abundance) did not follow a similar pattern

among CC treatments. In wheat, the percentage of colonization showed the highest values in VET and MEL, increasing by more than 80% of the control value. However, hyphal length showed its highest value in BAR (86% more than the control) and Glomeromycota abundance in B+M (33-fold the control value). All of these variables displayed their lowest value in CON, except hyphal length, whose lowest value was statistically similar in CON, MEL, and VET. Regarding the abundance of genes related to the large groups of the microbial communities (bacteria, fungi, and archaea), we found that the abundance of bacteria showed its lowest values in both CC mixtures, whereas CON and CC monocultures showed similar values. In line with colonization, fungal abundance was highest in the two legume monocultures and lowest in BAR and B+V. Archaeans showed a different behaviour, in that they presented their highest abundance in B+M, followed by its pure cultures. In general, we can say that the mycorrhization variables and the abundance of fungi, bacteria, and archaea showed a preference for the legume monocultures (MEL and VET) and for the B+M mixture (Table 1).

In maize, mycorrhization variables such as colonization percentage and hyphal length showed a very similar pattern to wheat, but Glomeromycota abundance showed its highest value in CON. The abundance of bacteria, fungi, and archaea did not follow the same pattern of similarity observed in wheat. We highlight that B+V mixture tended to exhibit the highest values of bacteria and fungi abundances, but also the lowest archaeal abundance (44% less than control). In contrast, archaeal abundance displayed its highest value at BAR, 10% more than the control and about 2-fold the B+V value (Table 2).

To explain the effects of cover crops on microbial variables in wheat and maize, the principal component analysis (PCA) technique was used to reduce the data set and explain the variability and covariance of the variables with artificial axes (principal component-PC). The PCA for wheat led to four uncorrelated PCs, with eigenvalues greater than 1, which together explained 77% of the variability of the microbial data set. The first two components explained 56% of the variability of the data (Figure 1A). The first component (horizontal axis) showed a high positive correlation with mycorrhizal colonization, abundances of total bacteria and total fungi, and a negative correlation with the hyphal length (mycelium). Thus, and according to the PCA, the treatments with legumes tended to enhance bacteria and fungi abundances, and mycorrhizal colonization. The B+M and B+V mixtures, which showed low bacteria abundance, were projected to the left side of the axis. CEB and B+M treatments, with high hyphal length, were projected to the left or negative side of the axis. The second component (vertical axis) was clearly positively correlated with four variables, i.e., Glomeromycota abundance, total fungi abundance, F:B ratio, and mycorrhizal colonization and, negatively, with the bacteria abundance. Therefore, the treatment projected onto the positive side of the axis (B+M) tended to present high values of these four variables.

The first two main components in the PCA in maize explained 75% of the data variability, and they were helpful to visualize the data set (Figure 1B). Contrary to what was observed in wheat, B+V had higher values of colonization, fungi, and bacteria than the average values in maize. The two components also showed that BAR and CON were characterized by high values of mycorrhizal colonization and fungi abundance, but low values of Glomeromycota and archaea; whereas, VET and MEL (as B+V) were characterized by having high values of colonization and fungi abundance, but low values of Glomeromycota and archaea. In contrast, B+M were characterized by having high F:B and low values of bacteria abundance and hyphal length.

Table 1. Microbial variables in wheat crop after the different cover crops.

Cover Crop	Mycorrhizal Colonization (%)	Hyphal Length (cm g^{-1})	Total Bacteria (Log Copies g^{-1})	Total Archaea (Log Copies g^{-1})	Total Fungi (Log Copies g^{-1})	Glomeromycota (Copies g^{-1})	F:B
CON	28.0 (±4.47) c	8.02 (±1.12) cd	8.20 (±0.07) a	9.23 (±0.01) d	4.70 (±0.04) ab	600 (±47) d	−3.49 (±0.05) b
VET	54.8 (±4.82) a	7.34 (±1.84) cd	8.21 (±0.17) a	9.06 (±0.31) d	4.87 (±0.03) a	2513 (±257) cd	−3.34 (±0.15) ab
MEL	51.2 (±5.40) a	6.22 (±1.52) d	8.33 (±0.09) a	11.73 (±0.19) c	4.90 (±0.29) a	8271 (±936) b	−3.61 (±0.22) b
BAR	42.8 (±3.35) b	14.91 (±2.11) a	8.14 (±0.02) a	12.56 (±0.09) b	4.53 (±0.05) b	8060 (±899) b	−3.61 (±0.06) b
B+V	40.8 (±6.72) b	9.94 (±1.69) bc	7.84 (±0.08) b	7.2 (±0.13) e	4.52 (±0.13) b	3936 (±1699) c	−3.32 (±0.16) ab
B+M	39.2 (±3.90) b	12.63 (±2.54) ab	7.91 (±0.07) b	13.78 (±0.02) a	4.72 (±0.07) ab	19,807 (±2105) a	−3.19 (±0.10) a

Analysis of variance for the mycorrhizal variables, and abundances of total bacteria (LOG), total archaea (LOG), total fungi (LOG), and total Glomeromycota in wheat plants. ± indicates the standard deviation of each mean; different lowercase letters after the means indicate significant differences with $p < 0.05$ according to HSD Tukey; CON: control without CC; VET: vetch; MEL: melilotus; BAR: barley; B+V: barley with vetch; B+M: barley with melilotus.

Table 2. Microbiological variables in maize crop after the different cover crops.

Cover Crop	Mycorrhizal Colonization (%)	Hyphal Length (cm g^{-1})	Total Bacteria (Log Copies g^{-1})	Total Archaea (Log Copies g^{-1})	Total Fungi (Log Copies g^{-1})	Glomeromycota (Copies g^{-1})	F:B
CON	34.4 (±4.77) c	12.65 (±2.79) a	7.78 (±0.09) b	12.95 (±0.09) b	4.58 (±0.36) ab	5219 (±997) a	−3.15 (±0.29) b
VET	58.0 (±7.62) a	12.00 (±1.15) a	7.83 (±0.11) b	12.05 (±0.10) c	4.85 (±0.05) a	308 (±43) d	−2.84 (±0.29) b
MEL	54.4 (±6.07) a	12.28 (±1.32) a	7.81 (±0.11) b	9.56 (±0.24) d	4.77 (±0.10) a	1343 (±255) c	−2.90 (±0.29) b
BAR	40.4 (±3.85) bc	9.80 (±2.34) ab	7.47 (±0.16) c	14.21 (±0.03) a	4.62 (±0.04) ab	3473 (±376) b	−2.93 (±0.20) b
B+V	49.2 (±5.40) ab	11.68 (±1.61) ab	8.08 (±0.07) a	7.29 (±0.06) e	4.86 (±0.15) a	28 (±3) e	−3.26 (±0.21) b
B+M	41.6 (±6.39) bc	7.96 (±1.11) b	5.44 (±0.13) d	12.69 (±0.06) b	4.35 (±0.21) b	3746 (±1357) b	−1.09 (±0.32) a

Analysis of variance for the mycorrhizal variables, and abundances of total bacteria (LOG), total archaea (LOG), total fungi (LOG), and total Glomeromycota in maize plants. ± indicates the standard deviation of each mean; different lowercase letters after the means indicate significant differences with $p < 0.05$ according to HSD Tukey; CON: control without CC; VET: vetch; MEL: melilotus; BAR: barley; B+V: barley with vetch; B+M: barley with melilotus.

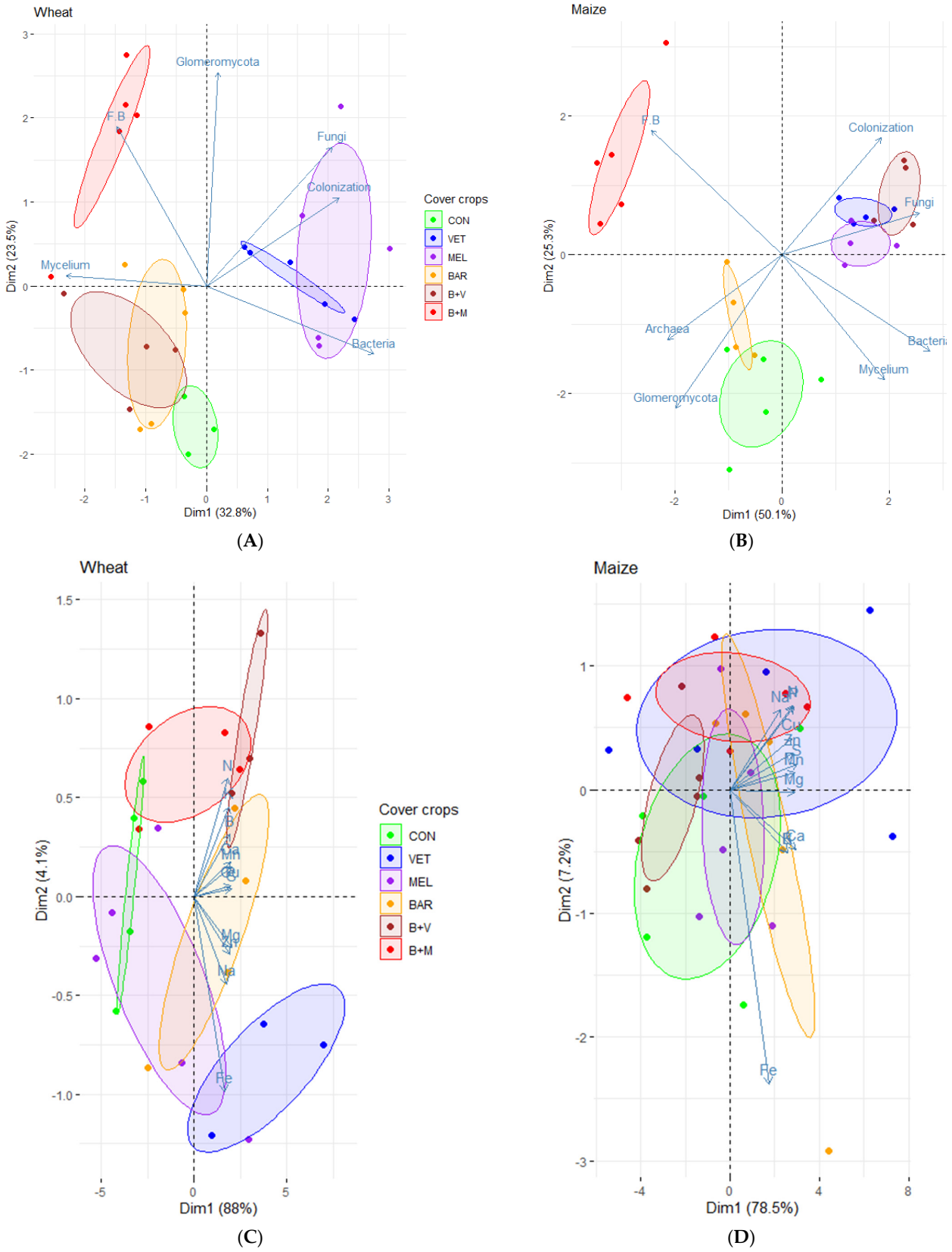

Figure 1. Analysis of principal components of the microbial variables of the soil in wheat (**A**) and in maize (**B**); and, crop variables in wheat (**C**) and in maize (**D**).

If we look at how the PCA locates the different types of CC in the quadrants according to the microbial variables (Figure 1A,B), we can identify some similar patterns in wheat and maize, while some others differ. The soil microbial response after the legume monocultures (VET and MEL) was quite similar (overlapping in the same quadrant) in both wheat and maize, and was different from that after the grass BAR. Moreover, the mixture B+M treatment differed completely from its pure cultures, BAR and MEL, each of the three being placed in a different quadrant in both CaC. In contrast, microbial responses after the B+V mixture relative to its pure cultures were different in wheat and maize. In wheat, the B+V mixture behaved like barley monoculture, and they both differed from vetch monoculture (Figure 1A). In maize, this mixture behaved like vetch monoculture, and they both were different from barley monoculture (Figure 1B).

3.2. Effect of Cover Crops on the Nutrition and Growing of Cash Crops

The type of CC affected wheat more than maize, both in terms of nutrient content and biomass. In wheat (Table 3), VET and B+V presented the highest contents of N, P, K, Ca, B, Cu, Fe, Mn, and Zn in shoots, together with the higher wheat biomass (Table 4). Thus, the treatments with vetch (VET and B+V) increased wheat biomass compared to the control by 81% and 85%, respectively. However, the control, followed by MEL, showed the lowest contents of B, Cu, Fe, Mn, and Na, as well as the lowest wheat biomass. The first two components in the PCA explained 92% of the data variability. All of the nutrients contents in wheat shoots showed positive correlations with the first component (horizontal axis in Figure 1C). The control treatment presented the lowest nutrient values because they were below the mean (negative side of the axis); whereas, VET, B+V, and BAR were significantly different from the control and tended to show the highest nutrient values.

On the contrary, in maize, the macro- and micronutrients content in shoots did not present significant differences between treatments, with the exception of Cu, which had a higher content after VET compared to the B+V mixture (111% higher) (Table S5). Consequently, the PCA in maize also did not show differences between the CC and the control (Figure 1D). Furthermore, no significant differences were observed between the CC treatments for maize biomass (Table 4).

In general, the concentrations of soil nutrients were little affected by the type of CC, which in any case were more affected in wheat than in maize, and with Na and P being more sensitive to the treatments. In wheat, VET increased soil P by 29% compared to the control; meanwhile, B+M mixture provided the highest Na, double those of BAR and B+V mixture (Table S6). In maize, BAR showed the lowest values of soil P, and B+M showed the highest, but BAR increased S by 55% relative to the control (Table S7). Finally, soil pH and electrical conductivity showed no significant differences between treatments (data not shown). According to the PCA in wheat (60% of the variability of the soil data with the first two components) no CC were different from the control, but there were differences between BAR and VET or between BAR and B+M (Figure S1A), whereas in maize (the first two components of the PCA representing which represented the 57% of the data variability) no differences were found between the treatments (Figure S1B).

We found numerous correlations between soil microbial (especially mycorrhizal variables) and nutrition variables in wheat and maize. In wheat, significant correlations were observed between mycorrhizal colonization and shoot nutrients such as Zn (r = 0.59 **), Fe (r = 0.59 **), Mg (r = 0.52 *), P (0.48 *), S (r = 0.44 *), and Cu (r = 0.43 *). Similarly, correlations were observed between colonization and soil Cu (r = −0.52 *) and between the fungi abundance and soil P (r = 0.60 **) and soil S (r = 0.46 *). In maize, mycorrhizal variables also correlated with crop nutrition variables. Thus, mycorrhizal colonization correlated with shoot P (r = 0.47 *), shoot Zn (r = 0.43 *), and shoot Cu (r = 0.41 *). Furthermore, hyphal length correlated with shoot Fe (r = 0.56 **), and Glomeromycota with shoot K (r = 0.54 **). Finally, colonization correlated with soil Mn (r = 0.47 *) in wheat, and had negative correlations with soil P in wheat and in maize (r = −0.42 *; −0.46 *, respectively).

Table 3. Macro and micronutrient contents in wheat shoots after the different cover crops.

Cover	N	P	K	S	Ca	Mg
crop			(mg microcosm^{-1})			
CON	112.6 (±26.5) c	5.4 (±2.8) c	113.7 (±18.4) bc	5.4 (±0.65) c	10.8 (±1.2) b	7.6 (±4.9) a
VET	239.5 (±64.9) a	11.9 (±4.4) a	163.9 (±41.5) ab	14.1 (±4.3) a	22.3 (±4.5) ab	16.2 (±4.5) a
MEL	136.3 (±56.2) bc	5.8 (±3.1) bc	106.4 (±47.3) c	6.7 (±03.6) bc	12.4 (±4.3) b	7.7 (±4.93) a
BAR	197.7 (±58.3) ab	7.9 (±2.8) abc	142.3 (±38.4) abc	9.3 (±2.4) ab	18.3 (±2.3) ab	10.7 (±2.7) a
B+V	242.6 (±14.2) a	10.6 (±2.2) ab	189.1 (±24.6) a	12.7 (±2.3) a	23.8 (±3.9) a	15.0 (±4.1) a
B+M	185.3 (±61.6) abc	8.8 (±4.2) abc	151.5 (±50.2) abc	10.2 (±4.1) ab	17.3 (±5.0) ab	11.6 (±6.1) a
	B	**Cu**	**Fe**	**Mn**	**Zn**	**Na**
			(µg microcosm^{-1})			
CON	24.87 (±3.44) c	18.13 (±2.14) c	364.6 (±84.44) b	151.0 (±21.44) c	46.08 (±4.77) c	0.11 (±0.08) c
VET	75.33 (±27.02) a	44.71 (±13.04) a	590.7 (±72.66) a	445.0 (±139.27) a	153.35 (±59.68) a	0.94 (±1.18) a
MEL	32.01 (±12.20) bc	22.06 (±10.03) bc	382.7 (±152.28) b	200.7 (±89.67) bc	76.45 (±45.73) bc	0.17 (±0.09) bc
BAR	46.52 (±11.12) abc	31.08 (±10.04) ab	460.5 (±52.67) ab	321.0 (±77.01) ab	81.44 (±24.39) abc	0.78 (±1.00) ab
B+V	68.05 (±19.84) a	40.72 (±7.10) a	496.0 (±109.63) ab	428.3 (±81.24) a	132.39 (±36.44) ab	0.36(±0.13) abc
B+M	53.29 (±19.37) ab	31.27 (±12.84) abc	407.8 (±170.59) ab	328.9 (±121.54) ab	73.88 (±26.41) abc	0.26 (±0.23) bc

± indicates the standard deviation of each mean. Different lowercase letters after the means indicate significant differences with $p < 0.05$ according to HSD Tukey. CON: control without CC; VET: vetch; MEL: melilotus; BAR: barley; B+V: barley with vetch; B+M: barley with melilotus.

Table 4. Shoot dry biomass in cash crops after the different cover crops treatments.

Cover Crop	Wheat (kg ha^{-1})	Maize (kg ha^{-1})
CON	1240 (±274.2) b	480 (±228.2) a
VET	2240 (±564.3) a	578 (±269.1) a
MEL	1351 (±616.3) b	505 (±131.2) a
BAR	1904 (±545.3) ab	607 (±62.2) a
B+V	2297 (±167.1) a	357 (±97.1) a
B+M	1708 (±554.3) ab	496 (±178.4) a

CON: control without CC; VET: vetch; MEL: melilotus; BAR: barley; B+V: barley with vetch; B+M: barley with melilotus; mean ± standard deviation; different lowercase letters indicate significant differences (HSD Tukey, $p < 0.05$).

3.3. Wheat and Maize Biomass Modelization

For the multiple linear regression model for wheat biomass production (dependent variable) as a function of microbial, four explanatory variables were selected: hyphal length, mycorrhizal colonization, Glomeromycota, and archaea abundance (Equation (1)) (p value < 0.05). These four response variables explained 41% of the variability in wheat biomass production.

$$\text{Wheat biomass production} = 7.44 + 0.27 * \text{hyphal length} + 0.17 * \text{Colonization} + 0.10 * \text{Glomeromycota} - 0.23 * \text{archaea} \quad (1)$$

In our experiment, which has the same fertilization for every microcosms, the soil variables (pH, EC, and available nutrients in soil) only explained 10% of the variability of wheat biomass production in the multiple linear regression model.

In maize, the multiple regression model for microbial variables explained 25% of the variability in biomass production (p-value < 0.05) and selected colonization, and the hyphal length as positive explanatory variables (Maize biomass production = 6.16 + 0.17 * Colonization + 0.18 * hyphal length). The model for biomass production in maize as a function of plant variables, which explained 92% of the variability of biomass production, only selected the shoot K content as explanatory variable (maize biomass= 6.14 + 0.13 * K) (p-value < 0.05). Regarding the soil variables, they explained 15% of the variability in maize biomass production (p-value < 0.05). In these cases, the model only selected EC as explanatory variable (Maize biomass production = 6.15 − 0.11 * EC).

4. Discussion

4.1. Interactions of Crop Identities with the Soil Microbiota

In general, the results showed important effects of the identities of the crops, CC and CaC, as well as the interactions between species within the CC mixtures on the soil microbiota. Furthermore, an interaction between the microbiota that were inherited from each of the CC and the identity of each CaC were also observed. It is noteworthy that the relationship between the roots of the crops and the microorganism of the rhizosphere is bidirectional [43]. However, while the mechanisms and effects of microorganisms on plants appear to be very varied (e.g., nitrogen fixation, P absorption, diseases, production, etc.), the influence of roots on microorganisms seems to be more centralized in only a few mechanisms, such as the secretion of root exudates [44,45], which are characteristic of each species [46] and can specifically stimulate or repress different microbial communities [47,48].

The CC made up of mixtures provided remarkable findings in this study. Firstly, the B+V mixture in wheat presented very different effects on the abundances of total bacteria, total fungi and Glomeromycota with respect to those observed in maize (Tables 1 and 2). This fact could be related with the type of photosynthesis pathway followed by each plant species, which can alter the composition of roots exudates [49] and thereby affect soil microorganisms [50]. Thus, in wheat (plant C_3 grass), B+V presented microbial variables which were similar to that of BAR (C_3), and both are overlapping (Figure 1A), whereas in maize (C_4 grass), B+V resembled that of VET (C_4 legume), which is in the opposite quadrant

to BAR (C_3 grass) (Figure 1B). In addition to the type of metabolism, stress conditions can also alter the composition of the root exudates and the rhyzospheric microbiome [51]. Thus, the different microbial response between wheat and maize could be enhanced by the temperature of the experiment (16 °C on average and with negative peaks of 5 °C), which was lower than the optimum temperature for maize. This abiotic stress could negatively affect the secretions of its root exudates, as does occur with other species [52].

The other treatment with mixture B+M, presented a microbial response that was very different from what was observed in the monoculture treatments, BAR and MEL, and completely separate from the rest of the treatments, in both wheat and maize (Figure 1A,B). Archaea and Glomeromycota in wheat and F:B in both CaC seem to explain this differential behaviour of the mixture, because, for these variables, the mixture favours them to a much greater extent than their pure cultures (Tables 1 and 2). The mixture of plants with different C:N ratio provides a variety of substrates, thus expanding the niches for a greater diversity of microorganisms [53]. However, to date, it is difficult to explain the specific interaction between the barley and the melilotus at the microbial level but, once again, root exudates seem to play an important role in plant-plant relationships, in addition to the plant-microorganism relationship [54]. The complexity of these relationships in the soil-plant system means that it is hardly possible to expect a simple response of the mixtures as a combination of the responses of their corresponding pure crops, as we had expected in our first hypothesis.

4.2. Relationship of AMF and Nutrients in Main Crops

As indicated by many authors, AMF can improve P uptake by plants under conditions of soil P deficiency [55]. Furthermore, P deficiency can induce the establishment of symbiosis with AMF in some species [56]. In our soil, P concentration was initially low (<12 ppm of P Olsen) and all of the CC increased the mycorrhizal colonization relative to the control by providing a host to these obligate symbionts. Thus, CC increased the AMF inoculum potential in the CaC period [12].

The positive effect of CC on colonization increased nutrient uptake by wheat, as evidenced by the positive correlations found between colonization and shoot nutrients such as P, Fe, Zn, Mg, S, and Cu by plants [57]. Previous studies have also shown a positive impact of AMF on the concentration of Fe, Zn, and Cu in crops, since AMF can provide the absorption of these immobilized nutrients in the alkaline pH of the studied soil, through the extra-root mycelium. The positive effect of VET on wheat root colonization seems to be the main cause for the higher content of P, K, S, Na, B, Cu, Fe, Mn, and Zn in wheat shoots after vetch, besides the increase of N content by N fixation. Thus, mycorrhizal colonization appears to be an effective mechanism for facilitating the transfer of numerous macro- and micronutrients from the soil to the crop.

Through external mycelium, AMF can also benefit the soil structure and the % of water-stable aggregates [21], as well as the absorption of water in drought conditions [58]. At the end of the CC, BAR presented the longest hyphal length, while VET presented the shortest. The greater specific root length in barley [59] is positively correlated with hyphal length [60], and can explain the better performance of barley compared to vetch regarding this AMF structure. The shortest hyphal length found after VET contrasts with its high root colonization. This behaviour has been observed in certain orders of AMF, such Glomerales that promote internal hyphae against external mycelium [61]. The shorter hyphal length of vetch against barley was detected at the end of the CC, and then largely maintained after nine weeks of wheat cultivation. Thus, the important imprint of CC on wheat was observed once again.

In maize, plant nutrition was also promoted by AMF, as shown by the positive correlations found between mycorrhizal variables and shoot nutrients, for example, colonization with Cu and P content, and the hyphal length with the Fe content. This again highlights the benefits of AMFs on plant nutrition, thanks to the extraradical hyphae, that can act as extensions of the roots in order to explore more soil volume and absorb elements that are

not very mobile [57]. The positive correlation that was found between the Glomeromycota abundance and shoot K content could be explained by the fact that Glomeromycota are K-solubilising microorganisms through the synthesis of organic acids, which dissolve the mineral K by protonation and acidification [62]. This facilitates better growth and performance of crops, such as maize [63], in low-input production systems [64]. The lesser impact of CC on nutrition in maize compared to wheat may be due, in part, to the relatively low temperatures that are negatively affecting the development of maize, which could mask the effects of treatments.

Soil pH showed positive correlations with the concentrations of Co, Fe, and Mn in wheat soil, which may indicate that these nutrients were less available to the plant, as the pH increased and they were not extracted from the soil by the crops [65]. On the contrary, the concentration of P in soil presented negative correlations with the pH in wheat and maize ($r = -0.42$; p-value < 0.05; and, $r = -0.46$; p-value < 0.05, respectively). This was partly due to the soil P absorption through mycorrhizae, which usually increases as the pH increases [66,67].

Finally, according to PCAs (Figure 1A,B), the behaviour of both legume monocultures (VET and MEL) was similar in both CaC, which was due, in part, to the predilection of AMFs to colonize legumes [56]. Nevertheless, the details of the nutrient, and biomass variables have shown that the VET provided better results than MEL under the conditions of the present study.

4.3. Bacteria, Archaea, and Fungi

As expected, the different types of CC differentially impacted the main groups of soil microorganisms, being the CC mixtures that provided more contrasting results. Thus, the lowest abundances of bacteria in wheat were observed in the CC mixtures. According to Latz et al. [68], this may be because legumes can have a negative effect on the density of active bacteria, since their presence within a community can disproportionately impact on the biocontrol traits expressed by the rhizosphere bacteria. Moreover, the combination of residues with a larger range of C:N ratio may enhance the competition between different groups of microorganisms [14], thus in our case, with the groups of bacteria and archaea being negatively affected in wheat and maize, respectively, or bacteria being promoted in maize after B+V. The mechanisms underlying these complex effects are still unclear.

The mixtures (B+V and B+M) had a markedly opposite effect on archaea. Thus, B+V gave low values and B+M gave high values, with respect to the control in both wheat and maize (Tables 2 and 3). This opposite effect could be due, in part, to the physiological and metabolic differences of vetch (C_4) and melilotus (C_3), whose exudate productions are different [69]. However, the behaviour of the mixtures does not seem to be explained solely by the study of the three involved species separately. This, therefore, reaffirms the hypothesis that there is a strong interaction between the species in the mixtures and suggests that, as with bacteria, certain plants within a community may have a disproportionate impact on archaea. Moreover, numerous archaea communities are known to increase their relative abundance in the microbial community structure under difficult or extreme edaphic conditions [70]. In this way, the treatments that showed better results (VET and B+V), also showed lower abundances of archaea.

On the other hand, the positive relationship that was found between bacteria abundance and mycorrhizal variables (i.e., colonization in wheat and hyphal length in maize) may be explained by the fact that the mycelial exudates of arbuscular mycorrhizal fungi can feed soil bacteria by stimulating their growth in the soil rhizosphere [71,72].

The abundance of total fungi presented a strong significant correlation with the concentration of P in the soil ($r = 0.60$; p-value < 0.01). This is because P can act as an important regulator of the structure of fungal communities [73]. In the same way, the total fungi showed a correlation with the S in soil ($r = 0.46$; p-value < 0.05). Regarding the F:B ratio, the highest occurred in both cash crops, after B+M, which may contribute to the increase in C sequestration in the soil [74,75] and a greater aggregation of soil particles [76].

4.4. Wheat and Maize Biomass

The type of CC, as well as the interaction between CC and CaC had an important effect on the biomass production of wheat and maize. This was in such a way that, in wheat, the biomass production after the VET and the B+V mixture was higher than the control (81% and 85%, respectively). However, in maize, no biomass increases were observed with CC (Table 4). This could be due, as indicated above, to the fact that the average ambient temperature was relatively low for maize, which can suffer adverse physiological and metabolic effects when temperatures are below 15 °C [77], causing a slowdown in growth, reduction of the leaf surface, and reduction of the absorption of P by the roots [78]. Wheat (C_3 plant), on the contrary, performs better than maize in relatively low temperatures (i.e., 5 °C) [79].

The microbial variables that were studied seem to have had an important effect on the biomass production of wheat, in such a way that they could explain 41% of the variability of the biomass, according to the multiple regression model (Equation (1)). This model confirms and quantifies the importance of the mycelium (or hyphal length) and the colonization of AMF (which belongs to the phylum Glomeromycota) for the production of wheat in the study environment, mainly due to its role in the absorption of nutrients and water [55–57]. AMFs also improve resistance to fungal pathogens that negatively affect biomass production [80].

Finally, the multiple regression model suggests a negative effect of archaea on the biomass production of wheat. This negative correlation could be due to the fact that the abundance of these microorganisms decreases with a high concentration of exudates from plants and fungi. This results in slow growth rates due to the low competitive capacity of archaea against other microorganisms such as bacteria [81].

The PCA of the crop variables (Figure 1C,D) were able to explain most of their variability of the wheat and maize biomass with only the first two components. This PCA in wheat, showed a clear difference in the VET, B+V, and BAR behaviours compared to the control. Thus, the inherited soil microbiota of these three CC helped CaC absorb different nutrients to a greater extent than the rest of the treatments.

The multiple regression model for biomass production in maize, as a function of plant variables, only selected the shoot K content as explanatory variable. Indeed, the K content could influence the biomass of maize, due to the fact that this nutrient acts in several physiological processes, such as the regulation of stomata and photosynthesis, causing a direct relationship with the production of biomass [82]; additionally, K can increase the resistance of maize to relatively low temperatures [83]. However, the concentrations of K that were analyzed in the soils were above optimum [84] although no potassium fertilizer was added.

5. Conclusions

The selection of cover crops is essential, in order to take advantage of probiotic functions and thereby improve the sustainability of agrosystems. In the conditions of this study, legacy effects of CC were more patent in wheat than in maize. In wheat, the most promising CC were those with vetch, either in monoculture or combined with barley. Thus, these CC favoured mycorrhizal colonization, the absorption of macro- and micronutrients, such as N, P, K, S, B, Mn, Cu, and Zn, and produced the highest biomass. Melilotus failed to promote nutrient absorption and cash crop biomass despite the fact that both CC legume monocultures contributed to a greater mycorrhizal colonization. In relation to their pure cultures, the CC mixtures showed a great diversity of responses. Therefore, until the underlying mechanisms behind this apparent complexity are clarified, the interaction of the species within the mixed cover crops prevents predictable results from the study of the species separately. The interaction of the heritage of some CC with wheat was opposite to the interaction with maize, especially in the microbial variables. From this perspective, the effect of the identity of each crop on the soil microbiota was evidenced. Thus, the CC selection, to a large extent, also depends on the cash crop that is going to be implanted.

Further research is needed at the controlled and field level, to better understand these interactions, especially concerning the CC mixtures, in a way that would give support to agronomic decisions that promote the sustainability of agrosystems.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11112199/s1, Figure S1: Principal component analysis for soil variables: pH, EC, macro and micronutrients in (a) wheat and (b) maize. Table S1: Microbial variables at the end of cover crops. Table S2: Shoot biomass of cover crops. Table S3: Cash crops germination after the different cover crops. Table S4: Summary of the ANOVA significance levels of the study factors and their interaction. Table S5: Macro y micronutrient content in maize shoots after the different cover crops. Table S6: Soil macro and micronutrients in wheat treatment after the different cover crops. Table S7: Soil macro and micronutrients in maize treatment after the different cover crops.

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