Long-term adoption of reduced tillage and green manure improves soil physicochemical properties and increases the abundance of beneficial bacteria in a Mediterranean rainfed almond orchard

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ABSTRACT

Soil health and function is one of the most important components for sustainable management of woody orchards. Intensive use of machinery and tillage creates heavy pressure on agroecosystems by altering the soil physicochemical and biological characteristics. In this study we aimed to assess the changes in soil physicochemical properties and the soil bacterial community structure, as well as the main drivers involved in shifts in the microbial community following 10 years implementing reduced tillage and reduced tillage plus green manure in a rainfed Mediterranean almond orchard. The treatments were: i) conventional tillage (CT); ii) reduced tillage (RT); and iii) reduced tillage and diversification with *Avena sativa* and *Vicia sativa* (RTD). After ten years, the results showed that the different managements had no significant effect on almond yields. RTD significantly increased total organic carbon (TOC), with an average content of 19.5 g kg^{-1} compared to 17.1 g kg^{-1} in CT. RTD also contributed to an increase in the fraction of soil macro-aggregates. Both RT and RTD significantly increased soil total nitrogen (NT) and particulate organic carbon (POC). However, other physical and chemical variables such as exchangeable Ca, Mg and K, bioavailable Fe, Cu, Zn and Mn, cation exchange capacity, bulk density, wilting point and field capacity were not significantly affected by the management. The bacterial community significantly changed with management: RTD showed a higher relative abundance of the family Solirubrobacteraceae and the genera *Streptomyces* and *Solirubrobacter*. The bacterial community structure was explained by changes in TOC, POC, pH, and NT values. Thus, a combination of reduced tillage and green manure could represent an appropriate sustainable management for rainfed almond orchards in very fragile environmental conditions, due to an increase in soil organic matter, total nitrogen, and the higher presence of beneficial bacteria related to soil productivity.

1. Introduction

Agricultural practices such as monoculture or crop diversification, tillage and fertilizer additions, together with crop type, are the main factors controlling soil properties, biodiversity and crop productivity in cropped areas (Kumar and Goh, 1999; Stavi et al., 2016). Soils play a major role in nutrient cycling, and are a reservoir of biodiversity (Almagro et al., 2021). Within soil components, soil microbes, and specifically soil bacteria, are essential in the provision of multiple soil functions such as biomass production, storage and cycling of nutrients, filtration and transformation of pollutants, carbon storage, water retention and infiltration, aeration, etc. (Doran, 1980; Drijber et al., 2000; Torsvik and Øvreås, 2002). Thus, it is essential to select those management practices that better contribute to increasing bacterial abundance and biodiversity and improving soil characteristics such as the organic carbon content, nutrient concentration, aggregation or water availability, in order to contribute to the long-term sustainability of agro-ecosystems (Luján Soto et al., 2021).

Tillage is a common practice which can affect soil properties and can trigger soil erosion, ultimately affecting the soil microbial community structure and activity (Lupwayi et al., 1998; Wang et al., 2020). The use of intensive tillage decreases soil compactness, increases water infiltration and aeration, and avoids the growth of weeds (Almagro et al 2017). In this line, most fruit orchards under a Mediterranean climate are submitted to intense tillage with bare soil for most of the year, in order to decrease water competition with weeds. This is done because farmers fear that competition with alley vegetation may reduce fruit production (Almagro et al., 2016, de Vente et al., 2012). This practice affects soil bacterial communities through the reduction of soil organic matter (SOM) as a source of C and nutrients, changes in soil moisture and temperature, and a decrease in the proportion of stable macro-aggregates, which provide a friendly microhabitat for bacteria (Balota et al. 2004; Dilly et al 2003). Previous studies have shown that reduced tillage significantly increases the SOM content at 0–10/30 cm depth compared with conventional tillage, regardless of the climate conditions, soil type, and cropping system (Alvarez and Alvarez, 2005; Liu et al., 2006).

The disadvantages of intensive tillage and maintaining bare soils can be minimized not only with the adoption of reduced tillage but also with the implementation of green manure (Garcia-Franco et al., 2015). Reduced tillage associated with green manure in orchards can improve soil physical, chemical, and biological properties due to the release of root exudates while the crop is growing and the incorporation of residues, leading to significant accumulations of SOM and nutrients (Almagro et al 2021; Montanaro et al 2017). Consequently, this can lead to increased land productivity with reduced fertilizer use (Serraj and Siddique, 2012). Furthermore, the incorporation of green manure promotes the improvement of the soil structure by aggregation (Bhattacharyya et al., 2009), which is an essential feature for creating microhabitats for microorganisms (Wang et al. 2015) and providing physical protection against soil degradation and erosion (Six et al., 2002).

Previous research has shown that no-tillage and reduced tillage coupled with green manure can increase microbial biomass, microbial diversity, and enzyme activity (Nivelle et al., 2016; Sofo et al., 2014; Wang et al., 2017; Zuber and Villamil, 2016), with shifts in the composition of soil microbial communities (Yin et al., 2010). However, a number of other studies have reported these practices as having no effect on those microbiological properties (Casacchia et al., 2010; Degrune et al., 2016; Navarro-Noya et al., 2013; Zheng et al., 2018b). Such contradictory results indicate that the effect of agricultural practices such as reduced tillage and green manure on soil properties and microbial communities depends to a great extent on the crop type, climatic conditions, soil type and period of implementation (van Capelle et al 2012, Mousavi and Eskandari 2011). Changes in soil properties are particularly strongly correlated with plot age, and a period of <10 years may show varying results which may not prove to be robust enough to define the most suitable strategies for sustainable management (Havlin et al., 1990; Mbuthia et al., 2015; Ramos et al., 2010). Hence, there is still insufficient information on the exact effects of reduced tillage or no tillage and crop diversification on soil physical, chemical and biological properties, including biodiversity, in the long term for specific regions and crops, to provide adequate evidence to farmers, land managers and policy-makers to foster their adoption (Soane et al., 2012). With regard to the Mediterranean region, Turrini et al. (2017) studied the effects of 10 years adoption of permanent green cover versus shallow tillage in an olive grove, reporting higher fungal abundance and microbial activity with the presence of cover crops. Luján Soto et al. (2021) assessed the long-term effects (7–13 years) of cover crops and green manure associated with no tillage or reduced tillage in several almond orchards, observing improved soil physical and chemical properties, including soil organic matter and nutrients. Almagro et al. (2021) reported that following 10 years implementing reduced tillage and green manure in almond orchards, the labile and *N*-rich plant residues from vetch and oat

used as green manure increased the microbial biomass and slightly changed the soil microbial community structure assessed by PLFA analysis, enhancing the dynamics and storage of soil organic C and N. Thus, very few studies have dealt with the long-term effects of sustainable management practices such as reduced tillage, no tillage or crop diversification in rainfed orchards, with a lack of information regarding the effects of this strategy on soil microbial communities.

The objectives of this study were to: i) investigate the long-term effects of reduced tillage and green manure on soil physicochemical properties and the soil bacterial community structure in a Mediterranean almond orchard compared to a monoculture under conventional-intensive tillage; and ii) assess the relationships between the soil bacterial community structure and physicochemical properties in response to changes in the management practices. For this purpose, three different treatments were assessed in an almond orchard: almond monocrop with intense tillage; almond monocrop with reduced tillage; and almond with reduced tillage and diversification with *Avena sativa* and *Vicia sativa*, sown as green manure. We hypothesized that long-term (10 years) reduced tillage and green manure application would result in a significant shift in the soil bacterial community, driven by a decrease in soil disturbance and an increase in vegetation cover, responding to increases in soil aggregation and SOM content.

2. Materials and methods

2.1. Study site and experimental setup

In 2009, a field experiment was established in an almond orchard (*Prunus dulcis* Mill.) of 5000 m^2 located in the Region of Murcia, SE Spain (37°51′59″N, 1°43′11″W; 700 m a.s.L.). This field experiment belongs to the Soil and Water Conservation Research Group from the CEBAS-CSIC. The almond crop is cultivated under organic rainfed conditions, without the addition of fertilizers, pesticides or other inputs. The almond trees were planted in 1999 with a tree spacing of 7 m × 7 m. The average annual temperature of the area is 16.6 °C, its mean annual precipitation is 330 mm, concentrated in spring and autumn, and the annual potential evapotranspiration is 800 mm. July and August are the driest months. The soil is a Cambic Calcisol (loamic) developed from colluviums (IUSS Working Group WRB, 2014). The field experiment consisted of a complete randomized block designed with three replicates (three blocks). Each block consisted of three treatments established in plots of 125 m^2 (5 m × 25 m), with the long side of each one following the direction of the maximum slope. Each plot contained seven almond trees. The three blocks and plots within each block were separated 14 m from each other. The average plot slope was 12 %. The treatments were: i) almond monocrop under conventional tillage (chisel ploughing 4–5 times yr^{-1} at 20 cm depth after important rainfall events) (CT); ii) almond monocrop under reduced tillage (chisel ploughing 2 times yr^{-1} at 20 cm depth (November and May-June), to control weeds) (RT); and iii) almond orchard under reduced tillage (chisel ploughing at 20 cm depth in November) diversified with *Avena sativa* L. and *Vicia sativa* L. as green manure (1:3 at 150 kg ha^{-1}) (RTD). The green manure was sown yearly in November after tillage and incorporated into the soil at 20 cm depth in May-June with a cultivator. Tillage was performed in the entire surface area of the plot, including under the tree canopies. More information about the study site and experimental designed can be found in.

2.2. Soil sampling, crop yield and above-ground biomass of alley vegetation

Soil was sampled in before tillage in November 2019 at 0–10 cm depth with an auger (10 cm diameter). Three composite random samples each derived from five subsamples were collected in each plot outside the influence of the canopy of the central five trees of each plot, in the middle of the alley. The alley was the piece of land between the tree rows. Thus, we had nine samples per treatment (3 composite samples ×

3 plots). Soil samples were separated into two aliquots, one kept at environment temperature for physicochemical analyses and the other stored in a cool box with ice for biological analyses, nitrate and ammonium. The samples were immediately taken to the lab. The soil was air-dried for one week for physicochemical analyses. One fraction of the dried soil was directly used for the determination of the aggregate size distribution, while the second fraction was sieved at <2 mm for the rest of analyses. Soil for biological analyses, nitrate and ammonium was sieved at <2 mm once in the lab and stored at −20 °C. Soil cores using steel cylinders (5 cm high × 5 cm diameter (98 cm^3)) were taken (0–5 cm depth) to determine soil bulk density (BD) (ISO, 2017).

Almond crop yield was calculated by weighing all the almonds harvested directly from the five central trees in each plot in July 2019. The dry above-ground biomass of annual vegetation growing in the alleys (spontaneous and sown green manure) was determined in May 2019 (in spring, when the biomass of the vegetation in the alleys is highest) by cutting and weighing the above-ground biomass of the vegetation (roots not included) grown in the alleys (not under the canopy) using three quadrats (1 m × 1 m) randomly placed in each plot (three quadrats per plot). Biomass was dried for 7 d at 60 °C in an oven. Spontaneous vegetation mostly consisted of *Lolium perenne* L. and *Anacyclus clavatus* (Desf.) Pers. in CT and RT, which was incorporated into the soil during the tillage operations. *L. perenne* also grew in RTD, albeit with lower coverage owing to the development of oat and vetch.

2.3. Soil physicochemical analyses

Soil water content at − 33 kPa (field capacity, SWFC) and −1500 kPa (wilting point, SWW) was measured using Soil Moisture Equipment (Corp., Santa Barbara, CA, USA) (VanGenuchten, 1980). Particle size distribution was measured using a Coulter LS200 laser diffraction device after oxidation of organic matter and dispersion of clays. Soil pH and electrical conductivity (EC) were measured in deionized water (1:2.5 and 1:5 w/v, respectively). Total carbon, total organic carbon (TOC), and total nitrogen (Nt) were determined by an elemental CHNS-O analyzer (EA-1108, Carlo Erba). TOC was measured after removal of soil carbonates by addition of HCl. Total particulate organic carbon (POC), defined as a fresh or decomposing organic material (mainly composed of fine root fragments and other organic debris) was measured as per Cambardella and Elliott (1992). Soil NH_4^+ was extracted with 2 M KCl in a 1:10 soil:extractant ratio and calorimetrically measured (Keeny and Nelson, 1982; Kandeler and Gerber, 1988). Soil NO_3^- was extracted with deionized water in a 1:10 soil:extractant ratio and measured by ion chromatography (Metrohm 861). The aggregate size distribution was determined by the wet sieving method using four aggregation classes: >2000 μm, 250–2000 μm, 53–250 μm, and < 53 μm (Elliot et al., 1986). Cation exchange capacity (CEC) and exchangeable Ca, Mg, K and Na were determined using $BaCl_2$ as exchangeable cation following the method of international standard (ISO (13536, 1995). Available Fe, Mn, Cu and Zn were extracted by chelation using DTPA (1:2 w/v) (Kabata-Pendias 2000; Reichman, 2002). Available B was extracted with deionized water (1:5 w/v) at 50 °C (Porta Casanellas et al., 1986). All nutrients were measured using ICP-MS (Agilent 7500CE).

2.4. DNA isolation and 16S sequencing

For DNA extraction, out of the nine samples collected from each treatments, the five that were the closest to the average value for each treatment in terms of soil pH, organic carbon, clay content and bulk density were selected to perform this study. We selected the samples based on these properties as they are the key drivers of soil microbial functioning and dynamics (Ji et al., 2022), so that they were representative of the treatment and discard possible outliers, without eliminating the field variability to process with statistical analyses. DNA extraction from soil was carried out using the DNeasy PowerSoil Kit (QIAGEN) according to the manufacturer's instructions using 0.5 g of soil.

Assignments of purity and concentration values were performed using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and using a Qubit® 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, USA) combined with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific), respectively.

Amplification of the bacterial 16S hypervariable regions was performed using the Ion 16S™ Metagenomics Kit (ThermoFisher Scientific). The library preparation process was carried out using the Ion Xpress™ Plus gDNA Fragment Library Preparation Kit (ThermoFisher Scientific) combined with the Ion Xpress™ Barcode Adapters kit (ThermoFisher Scientific) to enable several samples to be pooled for sequencing reactions. An Agilent 2100 Bioanalyzer™ instrument was used to evaluate the concentration, purity and size distributions of the barcoded libraries for further dilutions with the suitable Agilent High Sensitivity DNA Kit. Prepared and diluted library amplicons were processed for template preparation using Ion Sphere™ Particles (ISPs) via Ion OneTouch™ 2 System with suitable Ion PGM™ Hi-Q™ View OT2 Kit (ThermoFisher Scientific) followed by the enrichment of ISPs using Ion OneTouch™ ES. Sequencing reaction was carried out with the Ion PGM™ System, Ion PGM™ Torrent Server and the appropriate Ion PGM™ Hi-Q™ View Sequencing kit (Thermo Fisher Scientific) in competence with sequencing chips from the Ion 316™ Chip v2 kit. All purification processes between incubation and the amplification reactions during the library preparation were processed using DynaMag™ 2 magnetic racks (Thermo Fisher Scientific) and an AMPure XP Purification Kit (Beckman Coulter). Purification of ISPs after the enrichment was conducted using DynaMag™ 2 magnetic rack and Dynabeads™ MyOne™ Streptavidin C1 Beads.

2.5. Bioinformatics process of raw sequencing reads

Bacterial 16S raw reads were trimmed for barcodes and primers interfaced with the BaseCaller application. Sequences were denoised via ACACIA (Bragg et al., 2012), and low quality sequences were excluded using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). Bacterial 16S reads with a Q < 25 (quality threshold) were then removed and the remaining sequences were filtered for chimeras using VSEARCH (Rognes et al., 2016), with the ribosomal database project (RDP database). The usable reads were then consigned to Operational Taxonomic Units (OTUs) based on 97 % similarity with the SILVA 128 reference database. Low confidence OTUs were removed. The total number of reads analysed, ranging from 56 149 to 147 290, were grouped into 21 311 OTUs. Subsequently, the number of sequences was rarefied at 56 149 for each sample. Rarefaction curves showed that the sampling and sequencing was close to the plateau for the three cropping systems with a coverage value of > 0.90 (Supplementary Fig. S1).

2.6. Statistical analysis

For crop yield, above-ground vegetation biomass and soil physicochemical properties, data were checked to ensure normal distribution using the Shapiro test. Data were submitted to a one-way ANOVA followed by Tukey's HSD test at p < 0.05 to assess the differences between treatments (CT, RT, and RTD). Statistical analyses for these properties were performed with the IBM SPSS for Windows, Version 26 software package.

The rarefaction curves and the biodiversity indices Chao1 index and Shannon diversity index (H') at OTU level were calculated using R packages iNEXT v 2.0.19 (Hsieh et al., 2016) and vegan v 2.5–7 (Oksanen et al., 2012). Significant differences in Chao1 and Shannon diversity indices among treatments were assessed by means of one-way ANOVA after testing the assumptions of normality and homogeneity of variance using Shapiro and Levene (car package) tests, respectively, and significant differences were examined using Tukey's HSD test at p < 0.05. Dissimilarity of bacterial community at OTU level was visualised

with a Principal Coordinate Analysis (PCoA) with the difference among treatments being tested with the ANOSIM test using Bray-Curtis distance since variance assumption was not met (checked with the betdisper() function from vegan package). The differences between treatments in the relative abundance data at phylum, family, and genus level were calculated using one-way ANOVA after testing the assumptions of normality and homogeneity of variance using Shapiro and Levene (car package) tests, respectively, and significant differences were examined using Tukey's HSD test at p < 0.05.

A PERMANOVA with the Adonis function and the Bray-Curtis distance was used to identify the main soil physicochemical properties involved in the variations between bacterial communities. We tested the effect of the 32 measured soil physicochemical parameters on the variation of bacterial genera. Parameters with a significant contribution (p < 0.05) were then used to visualize the bacterial variability at genus level with a Redundancy Analysis (RDA) on data previously transformed by the Hellinger transformation, performed using the R software *vegan* and *ggplot* v 3.3.5 packages (Wickham, 2009). RDA was used to assess how much of the variation in bacterial data can be explained by the variation in the set of physicochemical properties. Samples with similar bacterial data profiles have similar scores and thus will group closer together when plotted. Soil physicochemical properties are represented by vectors. Vectors of greater magnitude and forming smaller angles with an axis are more strongly correlated with that axis. Statistical tests for bacterial community data were performed using R version 3.3.6.

3. Results

3.1. Crop yield, above-ground biomass and soil physicochemical properties

Crop yield and above-ground biomass of the alley vegetation were similar in all treatments, with mean values of 349 kg ha^{-1} and 120 g m^{-2}, respectively (Table 1). However, there was an upward unsignificant trend in these two parameters with the reduction of tillage.

There were no significant differences among treatments in BD, SWW, SWFC, and texture. The soil pH was significantly higher in CT and RTD, while EC was significantly higher in RT and RTD (Table 2). TOC was significantly higher in RTD compared to CT; Nt and POC were significantly higher in RT and RTD treatments compared to CT, with no significant differences concerning CEC, NH$_4^+$ and NO$_3^-$ (Table 3). There were no significant differences among treatments for exchangeable and available nutrients (Supplementary Table S1). Aggregate size distribution significantly different between treatments (Table 4). As a general pattern, RTD showed the highest proportion of macro-aggregates (>2000 μm and 2000–250 μm), while CT showed the highest proportion for micro-aggregates (53–250 μm) and RT for the silt plus clay particles (<53 μm). Furthermore, RT showed the lowest proportion of aggregates > 2000 μm.

3.2. Soil bacterial diversity

DNA concentration was significantly higher in RTD, with no

significant differences between CT and RT (Table 3). The Chao1 index (F-value = 1.04; p = 0.38) and Shannon index (F-value = 1.78; p = 0.21) showed no significant differences among treatments (Supplementary Fig. S2). Nonetheless, bacterial community at OTU level was significantly different among treatments (R-ANOSIM = 0.57; p < 0.001) (Supplementary Fig. S3).

3.3. Soil bacterial composition

The dominant bacterial phylum in all treatments was Actinobacteria, covering an average of 54.18 % of the total sequences, followed by Proteobacteria, Acidobacteria, and Gemmatimonadetes, which covered 24.64 %, 7.98 %, and 5.69 %, respectively (Fig. 1). Actinobacteria relative abundance was significantly higher in RT and RTD than in CT (p < 0.01), while Acidobacteria, Gemmatimonadetes, and Planctomycetes showed the highest significantly relative abundance in CT (p < 0.01). None of the bacterial phyla showed significant differences between RT and RTD (Supplementary Table S2).

The most dominant families were Geodermatophilaceae (20.95 %) followed by *Solirubrobacterales* bacteria 67–14 (14.26 %), Solirubrobacteraceae (10.08 %), Gemmatimonadaceae (9.04 %), Sphingomonadaceae (8.94 %), and Nocardioidaceae (7.67 %) (Fig. 2). The relative abundance of Nitrosomonadaceae was significantly higher in CT, with no significant differences between RT and RTD. Solirubrobacteraceae, on the other hand, showed a significantly higher abundance in RTD, with no significant differences between CT and RT (Supplementary Table S3).

The most dominant bacterial genera were *Blastococcus* (28.26 %), *Rubrobacter* (12.43 %), *Nocardioides* (12.06 %), and *Solirubrobacter* (11.20 %) (Fig. 3). Only three genera showed significant differences among treatments. *Blastococcus* showed a significantly higher abundance in CT and RT than in RTD (p < 0.05), while *Streptomyces* and *Solirubrobacter* showed a significantly higher abundance in RTD (p < 0.01) (Supplementary Table S4).

3.4. Interrelationship between soil bacterial community and physicochemical properties

The RDA performed showed that the first two axes can themselves explain 66 % of the total variation (Fig. 4). Axis 1, which explained 41 % of the variation, separated RTD (positive scores) from CT and RT (negative scores). Axis 2 explained 25 % of the variation, and separated CT (highest scores) from RT (lowest scores). TOC, POC, sand content, and EC explained the differences between the bacterial community between treatments, with highest scores in RTD. Nt and pH were the variables with the highest significant scores to explain the difference between the bacterial community in RT and CT, with lower pH and higher Nt content under RT. Contrary to our initial hypothesis, soil aggregate distribution was not significant at explaining the variability in the bacterial community structure. Supplementary Table S5 shows the results of the PERMANOVA test to assess the contribution of each property to explain the distribution of soil bacteria.

4. Discussion

4.1. Soil physicochemical properties

Our results have shown that reduced tillage associated to growing *A. sativa* and *V. sativa* as green manure has been successful in enhancing the TOC accumulation in soil at 0–30 cm at least, and is a sustainable practice to combat climate change by: i) reducing greenhouse gas emissions by decreasing machinery use, and ii) enhancing the soil C content, as previously reported by (Almagro et al., (2017), Martin-Gorriz et al., (2020) or, Martínez-Mena et al., (2020). It is true that we do not have evidence about the effect of reduced tillage and green manure below 10 cm, and so these data should be complemented with further

Table 1
Almond crop yield and above-ground biomass of alley vegetation. Values are mean ± standard deviation (n = 3).

Treatment[a]	Almond crop yield (kg ha^{-1})	Above-ground biomass (g m^{-2})
CT	334 ± 58	72 ± 15
RT	352 ± 358	111 ± 16
RTD	361 ± 239	152 ± 43
F-value[b]	0.015 ns	0.268 ns

[a] CT: conventional tillage; RT: reduced tillage; RTD: reduced tillage diversified with *A. sativa* and *V. sativa*.

[b] ns: not significant (p > 0.05) after one-way ANOVA.

Table 2

Bulk density, soil water content at wilting point, soil water content at field capacity, texture, pH and electrical conductivity in the soil of an almond orchard under different farming practices (0–10 cm depth). Values are mean ± standard deviation (n = 9).

Treatment[a]	BD (g cm^{-3})	SWW (cm^3 cm^{-3})	SWFC (cm^3 cm^{-3})	sand (%)	silt (%)	clay (%)	pH	EC (dS m^{-1})
CT	1.09 ± 0.15	0.20 ± 0.01	0.34 ± 0.02	36.3 ± 15.5	46.1 ± 9.3	17.7 ± 7.0	8.00 ± 0.03b	0.18 ± 0.01a
RT	1.11 ± 0.15	0.19 ± 0.02	0.35 ± 0.02	41.4 ± 13.1	43.4 ± 9.8	15.0 ± 3.2	7.90 ± 0.08a	0.20 ± 0.01b
RTD	1.07 ± 0.09	0.20 ± 0.01	0.34 ± 0.02	34.5 ± 11.9	49.1 ± 9.1	16.4 ± 2.8	8.00 ± 0.05b	0.20 ± 0.01b
F-value[b]	0.97 ns	1.42 ns	1.63 ns	0.43 ns	0.74 ns	0.07 ns	8.54**	11.67**

BD: Bulk density; SWW: Soil water content at wilting point; SWFC: Soil water content at field capacity; EC: Electrical conductivity.

[a] CT: conventional tillage; RT: reduced tillage; RTD: reduced tillage diversified with *A. sativa* and *V. sativa*.

[b] Significant at ***p < 0.001; **p < 0.01; *p < 0.05; ns: not significant (p > 0.05). Different letters indicate significant differences after Tukey's HSD test (p < 0.05).

Table 3

Total organic carbon, total nitrogen, particulate organic carbon, cation exchange capacity, ammonium, nitrate and DNA concentration in the soil of an almond orchard under different farming practices. Values are mean ± standard deviation (n = 9).

Treatment[a]	TOC (g kg^{-1})	Nt (g kg^{-1})	POC (g kg^{-1})	CEC (cmol kg^{-1})	NH$_4^+$ (mg kg^{-1})	NO$_3^-$ (mg kg^{-1})	DNA (μg g^{-1})
CT	17.09 ± 2.53 a	1.50 ± 0.17 a	6.14 ± 1.42 a	20.95 ± 1.10	2.24 ± 1.35	31.28 ± 7.90	1202 ± 375a
RT	18.21 ± 1.31 ab	1.86 ± 0.14b	10.21 ± 2.86b	21.97 ± 1.43	3.36 ± 2.14	45.57 ± 11.03	1227 ± 161a
RTD	19.49 ± 2.69b	1.75 ± 0.23b	11.65 ± 2.09b	20.53 ± 1.17	3.32 ± 1.61	42.86 ± 7.55	1677 ± 67b
F-value[b]	9.88**	18.99***	9.18**	0.41 ns	0.14 ns	3.33 ns	6.27*

TOC: Total organic carbon; Nt: total nitrogen; POC: Particulate organic carbon; CEC: Cation exchange capacity.

[a] CT: conventional tillage; RT: reduced tillage; RTD: reduced tillage diversified with *A. sativa* and *V. sativa*.

[b] Significant at ***p < 0.001; **p < 0.01; *p < 0.05; ns: not significant (p > 0.05) after one-way ANOVA followed by Tukey's HSD test.

Table 4

Aggregate size distribution in the soil of an almond orchard under different farming practices. Values are mean ± standard deviation (n = 9).

Treatment[a]	>2000 μm	250–2000 μm	53–250 μm	<53 μm
	%			
CT	2.27 ± 1.76b	40.96 ± 5.60a	31.86 ± 4.39b	24.90 ± 5.27a
RT	1.42 ± 0.73a	41.91 ± 7.74a	25.36 ± 3.50a	31.31 ± 4.38b
RTD	2.62 ± 2.18b	44.03 ± 13.44b	27.91 ± 6.34a	25.44 ± 10.39ab
F-value[b]	8.77**	5.78*	5.41*	5.69*

[a] CT: conventional tillage; RT: reduced tillage; RTD: reduced tillage diversified with *A. sativa* and *V. sativa*.

[b] Significant at ***p < 0.001; **p < 0.01; *p < 0.05; ns: not significant (p > 0.05) after one-way ANOVA followed by Tukey's HSD test.

studies along the soil profile to assess how this practice globally effects soil organic C storage in the soil. The increase in TOC was associated to increases in Nt, as previously reported in the literature (Garcia-Franco et al., 2015; Mäder and Berner, 2012). Tillage and green manure presence and addition did not influence the concentration of other soil nutrients such as Ca, Mg, K, B, Fe, Mn, Cu or Zn in this study. This may be because vegetation can only fix CO_2, while legumes can also fix N_2, which are incorporated into the soil by root exudates and plant residues. However, the level of the other nutrients is not affected, since there is no external fertilization in any of the plots (Culumber et al., 2019). This can be explained by the fact that the available nutrients from the soil are absorbed by vegetation, and returned to the soil by plant residues, with no external entrance in non-fertilized systems. Other authors have reported increases in TOC following green manure application in fruit orchards at 0–60 cm depth in the Fujian Province, China (Wang et al., 2015) and at 0–20 cm depth in the Hubei Province, China (Zhou et al., 2022), whilst a review performed on agricultural land in the USA showed that intensive tillage decreases TOC (West and Marland, 2002).

The long history of tillage in CT may have contributed to soil organic matter mineralization, with a continuous decreasing trend in TOC and Nt. Reduced tillage (RT) enabled the development of a spontaneous cover crop which, once it was incorporated into the soil, may have contributed to increase Nt compared to CT. Vegetation cover can catch mineral N forms from the soil and so prevent them from leaching after rainfall events. Once vegetation is incorporated into the soil, N stored in the plant biomass returns to the soil, thereby contributing to increase soil Nt compared to CT (Justes et al., 2012). Additionally, owing to the climate conditions of the area (high temperature and aridity) and long-term history of conventional tillage with no addition of organic amendments, the low SOM in soils, even in RT or RTD, may not have contributed to a high biomass and abundance of *N*-fixing bacteria. Therefore, the atmospheric N fixation rates by vetch in this system must be low, hindering differences with RT from being obtained. In addition, the higher abundance of bacteria belonging to the Nitrosomonadaceae family at the CT may also be involved in decreasing the soil N content, owing to their participation in the nitrification process (see section 4.2).

Treatments with reduced tillage have contributed to increase POC content in soil compared to conventional tillage. POC is formed from fresh or partly decomposed plant materials, often referred to as labile organic C, which are rapidly decomposable (Zimmermann et al., 2007). Thus, POC has a relatively shorter turnover time, and is considered as being more sensitive to management than mineral associated organic C (YEASMIN et al., 2022). As in our study, the literature has previously shown that POC usually increases under reduced tillage and when crop residues are returned to the soil (BEGUM et al., 2022; Jin et al., 2021; Samson et al., 2020). Hence, residue retention with reduced tillage can favour the accumulation of plant-derived materials near the soil surface, which can promote the formation of stable macro-aggregates (Samson et al., 2020). The POC fraction is incorporated within large soil aggregates, fostering the initial stages of aggregates formation(Gregorich et al., 2006). The progressive fragmentation of POC eventually leads to the formation of finer and more stable SOC through intimate associations with mineral particles (Dungait et al., 2012). In this line, the development of seeded vegetation in RTD contributed to increase the proportion of macro-aggregates, which can be linked to the increase in POC. In addition, reduced tillage helps stabilize POC within macro-aggregates, and protects it from decomposition (Six et al., 2004). Macro-aggregation is an indicator of improved soil health, because of higher macro-porosity, and so facilitates soil micro- and meso-fauna movement, water and air fluxes, and root growth (Knoepp et al., 2000; Rabot et al., 2018). In line with our results, Chen et al. (2014) observed that SOM loss in cultivated areas was associated with a decrease in the fraction of macro-aggregates, linked to the lowest abundance of plant residues in soil. In addition, the largest aggregate
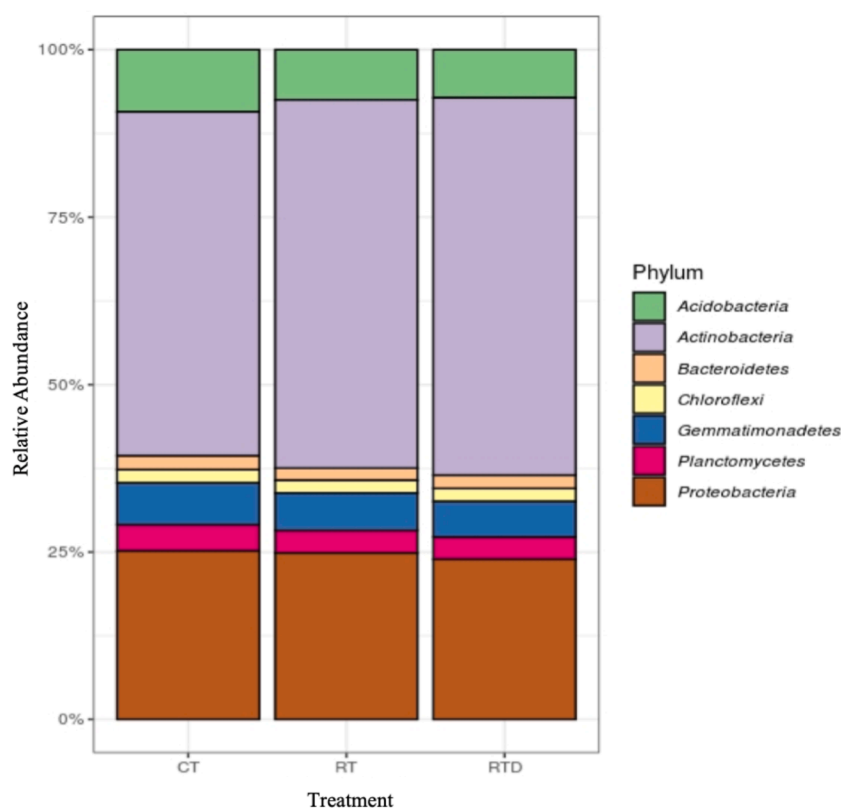
Fig. 1. Relative abundance (>1%) of bacterial phyla in the three treatments. CT: conventional tillage; RT: reduced tillage; RTD: reduced tillage diversified with *A. sativa* and *V. sativa.*
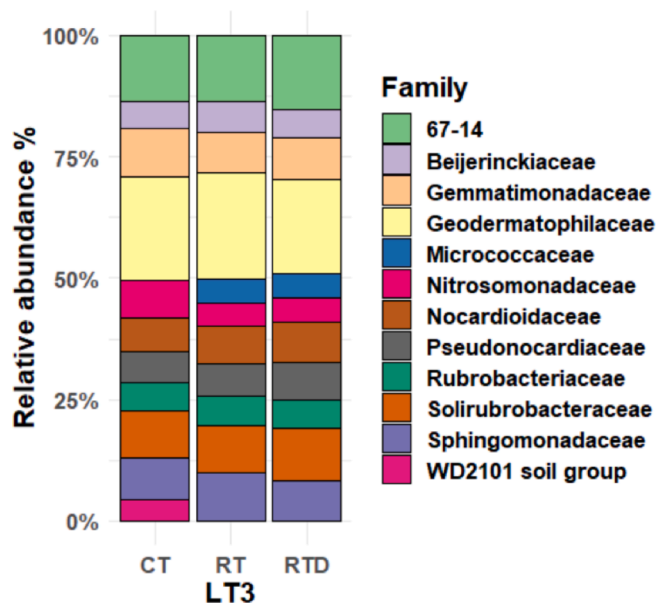

Fig. 2. Relative abundance (>1%) of bacterial families in the three treatments. CT: conventional tillage; RT: reduced tillage; RTD: reduced tillage diversified with *A. sativa* and *V. sativa.* "67–14" and "WD2101 soil group" are undescribed families.

sizes have been previously associated with higher TOC content (Rabot et al., 2018). Thus, the RTD strategy may be promoting soil C sequestration and storage by also promoting the formation of stable macroaggregates, mostly because tillage affected not only the total quantity of C, but also the quality by increases in POC.

Reduced tillage and green manure has traditionally been considered by farmers from Mediterranean rainfed orchards as an unsustainable practice because of the high risk of soil compactness and competition for water and nutrients between the cover crops and the trees, which farmers fear will lead to decreases in fruit yields (Morugán-Coronado et al., 2020). However, the present study confirms that reduced tillage and green manure applied for several years and incorporated as green manure, not only improved soil physicochemical properties, but can also keep the almond yield at the same level as in the traditional system with intensive tillage, so production costs can decrease whilst maintaining yields, and therefore increased farm profitability (Martínez-Mena et al., 2021; Martin-Gorriz et al., 2020). Other studies have reported that reduced tillage and green manure application neither increased nor had any effect on fruit yields in the long term (de Leijster et al., 2019). However, Martínez-Mena et al. (2021) observed a negative effect on the almond crop yield in a Mediterranean orchard during the first years of implementation of reduced tillage plus green manure, but stated that the negative effect tended to revert with time.

4.2. Soil bacterial community

Biodiversity indexes for soil bacterial populations showed no significant differences between treatments. In this line, great controversy currently surrounds the Shannon and Chao1 indexes with regard to assessing differences in bacterial diversity evaluated by next-generation sequencing. According to the review performed by Gihring et al. (2012), the standard errors of these indexes range from 12 % to 96 % depending on the difference in read numbers of individual samples. Thus, the diversity indexes obtained for this type of analyses may be misleading (Gihring et al., 2012; Kunin et al., 2010). Nonetheless, in our study the standard errors for these indexes ranged between 1 and 3 %, indicating that the data presented low variability. Thus, rather than alfa biodiversity indices, the study of the relative abundance of the different taxa
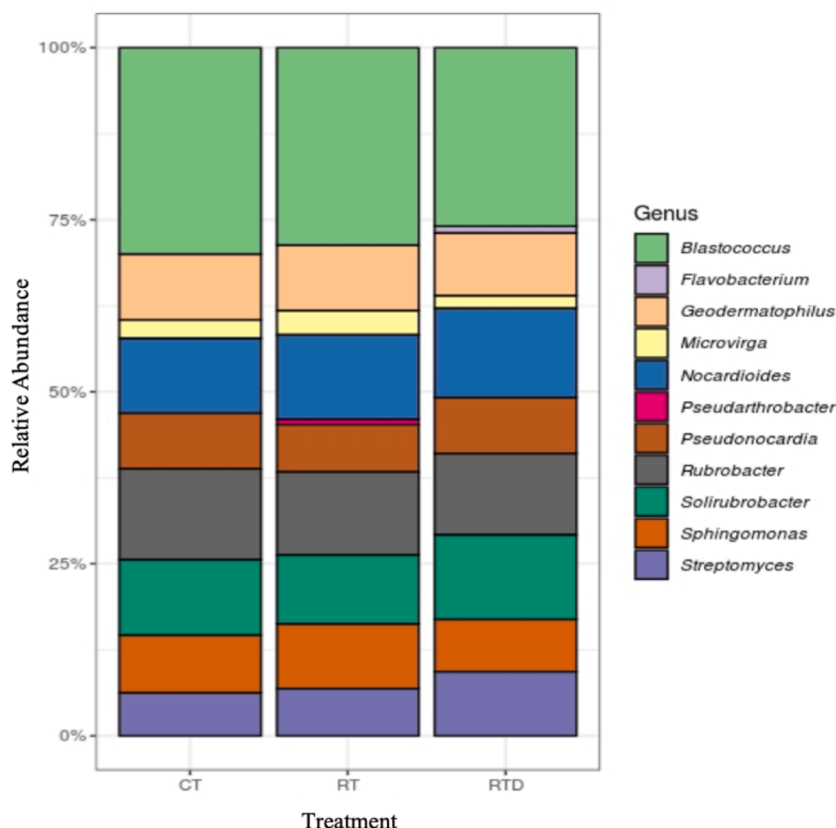
Fig. 3. Relative abundance (>1%) of bacterial genera in the three treatments. CT: conventional tillage; RT: reduced tillage; RTD: reduced tillage diversified with *A. sativa* and *V. sativa*.
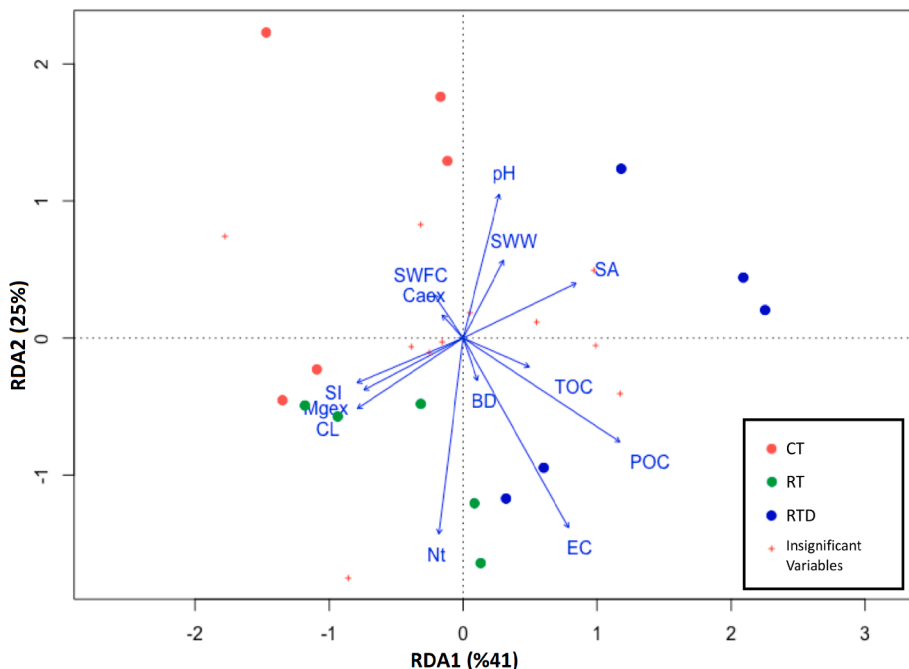

Fig. 4. Redundancy analysis (RDA) of the soil bacterial community, showing the significant contribution of soil physicochemical properties in explaining variations in the bacterial community: Nt: total nitrogen; BD: bulk density; EC: electrical conductivity; POC: particulate organic C; TOC: total organic C; SA: sand; SWW: soil water content at wilting point; SWFC: soil water content at field capacity; Caex: exchangeable Ca; SI: silt; Mgex: exchangeable Mg; CL: clay. Treatments: CT: Conventional Tillage; RT: Reduced Tillage; RTD: Reduced tillage diversified with *A. sativa* and *V. sativa*.

(Figs. 2 and 3) would seem a reliable strategy for assessing the effects of agricultural management on the bacterial community structure (Hermans et al., 2020). Zheng et al. (2018a) also observed that green manure application for eight years in an apple orchard had no significant effect on biodiversity indices, but did change the bacterial community structure and the relative abundance of different taxonomic groups.

The ANOSIM test showed that the soil bacterial community structure significantly differed among treatments. Thus, the soil bacterial community has changed owing to both a reduction in tillage as well as the growth of green manure, incorporated into the soil once a year.

Specifically, the bacterial community in RTD is associated to increases in TOC, POC and Nt because of the long-term management. Reduced tillage and especially green manure application have previously been identified as strategies to increase TOC, POC, and Nt paralleled by an according change in soil bacterial communities in orchards from the Mediterranean basin at 0–20 cm depth (Almagro et al., 2021; García-Orenes et al., 2013; Sofo et al., 2014).

With regard to the different taxonomic groups, Actinobacteria, which was the most abundant phylum in the three treatments, showed the highest relative abundance in the treatments involving reduced tillage. Actinobacteria participate in the decomposition of organic matter and in major C cycling pathways, and are essential for soil function (Anandan 2016). Bacteria belonging to this phylum are easily activated by root exudates, thereby supplying plants with easily assimilable nutrients, and play a key role in controlling root pathogens (Govaerts et al., 2008). Hence, the highest vegetation cover for most of the year in our long-term trial, whether it was spontaneous as in RT or cultivated as in RTD, compared to CT, may be behind the increases in this bacterial group. The Nitrosomonadaceae family (the highest in CT) that belongs within the proteobacteria phylum, are mostly ammonia oxidizers. These are responsible for oxidizing ammonia to nitrite, followed by further oxidation to nitrate by other bacterial families (Prosser et al., 2014a). Nitrosomonadaceae lead to a loss of ammonium, nitrous oxide production, and nitrate pollution of waters by leaching (Prosser et al., 2014b). Thus, the higher abundance of the Nitrosomonadaceae family in CT may be contributing to greater nitrification processes which may result in soluble nitrate that may be lost by leaching in a soil devoid of vegetation that can absorb this nutrient. The Solirubrobacteraceae family, with the highest abundance in RTD, is a member of the Actinobacteria phylum and many members have yet to be characterized (Anandan, 2016b; Whitman and Suzuki, 2015). These bacteria are associated to SOM mineralization and the release of available nutrients to plants, increasing soil fertility and productivity (Anandan, 2016b; Singleton et al., 2003; Whitman and Suzuki, 2015), such as *Solirubrobacter*, with a higher abundance in RTD, which has positive impacts on the development of vegetation (Anandan, 2016b).

With regard to genera, *Blastococcus*, with the highest proportion in CT and RT, is one of the most abundant bacteria in bare soils or soils with low vegetation cover, due to its resistance to detrimental environmental factors (Chouaia et al., 2012; Normand et al., 2014a). Thus, its higher presence in the treatments with no seeded green manure may be related to the greater environmental stress supported under this management. The increased relative abundance of *Streptomyces* in RTD can be considered as a positive indicator of soil productivity and biocontrol. *Streptomyces* is highly associated with soil fertility, plant growth and high crop yields (Kämpfer, 2006; Olanrewaju and Babalola, 2019). This genus is considered to be a natural biofertilizer and biocontrol element and contributes to the control of soil-borne diseases through invasive species suppression due to their highly competitive nature with antibiotic secretion (de Lima Procópio et al., 2012; Olanrewaju and Babalola, 2019; Zhou et al., 2017). *Streptomyces* has a high capacity to decompose plant residues and thus contributes to increase SOM (Normand et al., 2014b; Olanrewaju and Babalola, 2019; González-Ubierna et al., 2012; Zornoza et al., 2007; Almagro et al., 2021; García-Orenes et al., 2013; Sofo et al., 2014; Souza et al., 2016).

5. Conclusions

Reduced tillage associated with green manure for 10 years promoted an increase in soil organic carbon and total nitrogen contents without negatively affecting almond yields, contributing to the overall sustainability of the agro-ecosystem. Bacterial biodiversity indexes showed no differences among monoculture, reduced tillage, and reduced tillage with the adoption of green manure. However, the adoption of reduced tillage and green manure has changed the soil bacterial community structure. Soil bacteria were significantly associated with a variation in soil organic carbon, with an increased presence of beneficial bacteria related to soil productivity and fertility with higher soil organic matter content. Thus, reduced tillage and green manure can be considered a sustainable management practice in Mediterranean rainfed orchards to improve soil health, increase soil C sequestration and storage, and promote beneficial bacteria. The long-term application of green manure and reduced tillage practices in similar areas may significantly contribute to increased farm profitability and sustainability by reducing CO_2 emissions through agricultural machinery.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Raul Zornoza reports financial support was provided by European Commission.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.geoderma.2022.116218.

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