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Biochar + AD exerts a biostimulant effect in the yield of horticultural crops and improves bacterial biodiversity and species richness in the rhizosphere

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ABSTRACT

Organic fertilisers are gaining prominence in advanced agri-systems due to the need for alternatives to the most pollutant agricultural inputs, contributing to sustainable agriculture. The objective of this study was to analyse the agronomic effect of a biochar non-additivated and additivated with anaerobic digestate (AD) on the soil microbiome in melon and pepper crops at the field scale, hypothesising that the synergy between biochar and the additive confers additional benefits to the crop. Two doses of biochar (250 and 500 kg ha^{-1}) and two doses of additive with respect to biochar (5 and 10% v:w) were tested. The highest yield was observed for a reduced dose of mineral fertilisation (NPK −20%) with biochar + AD at the highest dose of additive: a biochar dose of 250 kg/ha with 10% AD for the melon crop and a biochar dose of 500 kg ha^{-1} with 10% AD for the pepper crop. Specifically, the yield increase compared with the control, which only received NPK, was a 33% increase in melon and 18% in pepper. The microbiome of the bulk soil was not modified by biochar + AD, but the composition of the rhizosphere microbiome changed, emerging plant growth-promoting rhizobacteria (PGPR) or increasing its relative abundance (e.g. *Arthrobacter, Mitsuaria* or *Bacillus* genus). We have demonstrated a positive correlation between yield and fruit quality parameters, and the presence of cluster of bacteria with predominance of known PGPR genera, that have been boosted by the treatments with biochar + AD. Thus, we hypothesize that the improved yield and fruit quality is in part due to the rhizosphere bacteria community enhancement.

1. Introduction

The demand for agricultural products is increasing because of world population growth (Fukase and Martin, 2020). According to some predictions, agricultural productivity must increase globally and at least double by 2050 to meet the projected food demand (Beltran-Peña et al., 2020). Mineral fertilisers significantly increase crop yields, but as a result have disrupted natural environments, where nutrient over-enrichment produces a loss of species richness and functionality, which in turn changes the services that ecosystems supply (Backer et al., 2018; Wezel et al., 2018). Therefore, to maintain crop yield improvements while adhering to sustainability criteria, it is necessary to reduce the levels of chemical inputs that could potentially harm natural ecosystems (Besset-Manzoni et al., 2018). Organic fertilisers combined with mineral fertilisers have been presented in recent years as a promising solution to solve the problem of excess chemical inputs contributing to sustainable agriculture.

Bio-residues from the agri-forestry sector and from the food chain have been applied to soils as crop amendments; however, they need to be treated to avoid problems in the soil when they are not properly managed. The circular economy not only aims to increase resource efficiency by implementing cascading uses of raw materials and bio-residues to deliver high-value products and services (Escobar and Laibach, 2021) but also involves the conditioning of bio-residues intended for use as agricultural inputs (De Corato, 2020). Two outstanding environmentally friendly technologies to treat bio-residues are anaerobic digestion and pyrolysis; the latter is used to obtain biochar. Anaerobic digestate (AD) contains ample amounts of plant nutrients, mainly nitrogen, phosphorous and potassium and is one of the most stable treated organic residues (Tsapekos et al., 2021); therefore, it can be used as a fertiliser (Hammerschmiedt et al., 2022). Biochar is a carbon-rich solid product (Lehmann et al., 2011) widely used in agriculture because, as a soil amendment, it improves crop yield (Dorner et al., 2022), plant growth and soil properties (Liao et al., 2021), water retention capacity (Kumar et al., 2022), and bioavailability of nitrogen and phosphorous (Chen et al., 2018), immobilises contaminants (Cao

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Table 1
Mineral N and organic fertiliser received by controls and treatments carried out in the field trial for melon and pepper crops.

Treatment Code	Mineral N fertiliser		Organic fertiliser		
	Dose (kg N ha^{-1})	Type	Dose (kg ha^{-1})	Type	Additive Percentage of AD in the biochar (v:w) (%)
C 0	0	–	0	–	–
C 80	176 (80% of full dose)	NH_4NO_3 (27% N)	0	–	–
C 100	220 (full dose)	NH_4NO_3 (27% N)	0	–	–
B 250	176	NH_4NO_3 (27% N)	250	Biochar	0
B 500	176	NH_4NO_3 (27% N)	500	Biochar	0
B 250 + AD 5	176	NH_4NO_3 (27% N)	250	Biochar + additive	5
B 250 + AD 10	176	NH_4NO_3 (27% N)	250	Biochar + additive	10
B 500 + AD 5	176	NH_4NO_3 (27% N)	500	Biochar + additive	5
B 500 + AD 10	176	NH_4NO_3 (27% N)	500	Biochar + additive	10

et al., 2011; Liu et al., 2012; Kumar et al., 2022), acts as a carbon sink (Yuan et al., 2017; Yang et al., 2020), and reduces greenhouse gas emissions (CO_2, CH_4, and N_2O) (El-Naggar et al., 2018; Yang et al., 2020). Conversely, little information is available about the effects of biochar on soil physicochemical and biological properties, especially in the soil microbiome, which plays a central role in mediating nutrient cycling in soils (Liao et al., 2021). In this sense, metagenomic analysis has been used to detect the microbiome composition and its response to different treatments (Bulgarelli et al., 2015; Xu et al., 2018). The combination of AD and biochar (Martin et al., 2014; Doyeni et al., 2022; Gulyás et al., 2022; Mickan et al., 2022) and the triple mix of AD, biochar and mineral fertiliser (Vanden-Nest et al., 2021) have been studied on the laboratory and greenhouse scales to assess their effects on plants and soil parameters. However, there is a lack of studies in real field conditions about the environmental and agronomic effects of the combination of biochar additivated with AD and mineral fertilisation.

Melon is considered one of the ten most popular cultivated fruits in the world (Weng et al., 2021), and Spain stands out as the highest producer of melons in Europe, as well as the top exporter and eighth largest producer globally (FAOSTAT, 2022). Additionally, Spain is a leading European pepper grower and the fifth largest in the world (FAOSTAT, 2022).

This work aims to fill the mentioned knowledge gap about the environmental and agronomic effect of technologically improved organic fertilisers consisting of biochar additivated with AD, combined with a 20% reduction of the standard mineral fertilisation dose in horticultural crops, such as melon and pepper. We hypothesised that the synergy between biochar and AD would confer additional benefits to the crop, such as improved nutrient use efficiency and physiological conditions, resulting in increased yield and fruit quality. However, environmental improvement would involve increased microbiome biodiversity. To our knowledge, this is the first overall investigation testing this objective in horticulture crops at the field scale in Spain.

2. Materials and methods

2.1. Description of the products tested and their production

The additivated biochar consisted of two components, i.e., biochar and AD. Biochar was obtained from the wood of vine shoots (Table S1) by slow pyrolysis in a pilot pyrolizer with an electrically heated reactor and a semi-continuous feeding system. The system for biochar production and the characteristics of the pyrolizer are described in Rosas et al. (2015). The AD was obtained from a 25 L anaerobic continuously stirred tank reactor (CSTR) treating organic residues from local hotels, restaurants and cafes (HORECA channel). The average composition of the feed consisted of fruit peels: pineapple and apple (31.8%); vegetables: pumpkin peels, sweet pepper and cauliflower (46.7%); and meat (9.3%), fish (4.2%) and bread (8%). The material was crushed and homogenised to attain a particle size of less than 1 cm. The reactor worked under semi-continuous operation at 35 °C and with a hydraulic retention time (HRT) of 30 days. The reactor was supplemented with NH_4Cl and KH_2PO_4 with the weekly addition of these compounds dissolved in a solution containing micronutrients with the composition proposed by Gonzalez-Gil et al. (1999). The final composition of the AD is shown in Table S2. The AD, as obtained from the reactor, was homogenised and ground to further reduce the particle size and obtain a liquid stream with solid particles less than 3 mm in size. The mix of both components was performed in a rotary drum and stored at 8 °C until incorporation in the field assay.

2.2. Field trial design

The melon cultivar 'Piel de sapo' and the pepper cultivar 'Medrano' were used for the trial conducted in Rambla Salada (37°20.11″N, 2°1627.095″W; Mazarrón) for melon and El Moaire (37°20.11″N, 2°1627.095″W; Blanca) for pepper in 2018. Trials in 2019 were conducted in Los Lorentes (37°20.11″N, 2°1627.095″W; Mazarrón) for melon and Rambla Salada for pepper. For the melon trial, the elementary plot was 35 m^2 with one row 17.5 m in length, 2 m row spacing and a space between plants of 0.8 m for a total plantation density of 6250 plants/ha. For the pepper trial, the elementary plot was 20 m^2 with one row 20 m in length, 1 m row spacing and a space between plants of 0.4 m for a total plantation density of 25,000 plants/ha. The experimental design was a randomised complete block with three blocks at each location.

A description of the treatments and their corresponding controls is shown in Table 1.

The organic fertilisers (Table 1) were applied by hand before transplantation, and the corresponding dose was spread on each row at 25 cm on each side of the drip line and incorporated into the soil with a motorised hoe. Subsequently, the plants were transplanted on 25th April for melon and 18th May for pepper in 2018 and 21st May for melon and 19th June for pepper in 2019. The crop was drip-irrigated, maintaining the field's soil moisture capacity between 80% and 100% during the field assay. The dose per treatment described in Table 1 was used to apply the mineral N fertiliser in the form of ammonium nitrate (27% N). The application schedule was designed to emulate fertirrigation and consisted of 10 applications throughout the crop cycle, each corresponding to one-tenth of the full dose. Mineral P and K fertilisers were applied by fertirrigation at the same dose for all treatments in the following doses: P_2O_5 at 38 kg ha^{-1} in melon in 2018, 126 kg ha^{-1} in pepper in 2018, 100 kg ha^{-1} in melon in 2019 and 42 kg ha^{-1} in pepper in 2019; K_2O at 216 kg ha^{-1} in melon in 2019 and 0 kg ha^{-1} in the rest treatments. The approach indicated by Urbano-Terrón (2008) was used to calculate the P and K requirements. This methodology considers the soil characteristics (Table S3) and the expected yields of 31,000 kg ha^{-1} for the melon crop and 42,000 kg ha^{-1} for the pepper crop when calculating the doses. The climatic conditions at each location and plot are provided in Table S4.

2.3. Plant and fruit sampling, variables measured, and data analysis

The dependant variables of the trial for both crops were yield and its components, fresh and dry aerial biomass, chlorophyll content (measured in 25 leaves per treatment and plot with a portable chlorophyll metre: CCM-200, ADC BioScientific Ltd., Hoddesdon, U.K.), conductivity (measured with a conductivity metre: PCE-CM 41, PCE instruments, Albacete, Spain), fruit contour (expressed in centimetres for the melon fruit and in millimetres for the pepper fruit), and solute concentration (measured with a portable digital metre) in the fruit juice. Twenty-five fruits were analysed per treatment and plotted. Furthermore, fruit penetrometry (with a penetrometer: PCE-PTR 200 N, PCE Holding GmbH & Co, Hamburg, Germany) and flowering time were measured in melons. The number of plants in phenological stage 61 was counted to determine the flowering time, and the results were expressed as the percentage of plants that reached that stage. Harvesting started at crop phenological stage 74 on the *Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie* (BBCH) scale and ended at stage 78 for melons, while for peppers it began at stage 71 and was completed at stage 75 (Feller et al., 1995).

For the dependant variables, the mean values for the combined treatments (biochar dose and additive dose) were compared with the Dunnett test, using the C 80 treatment as a reference for comparison (Figs. 1–4). Furthermore, analysis of variance (ANOVA) was performed using the treatments (the biochar dose on the one hand and the additive dose on the other) as fixed factors and the location, year and plot as random factors. The effects of the biochar dose, the additive dose and the interaction between both were analysed, and Tukey's test was used for mean comparisons (Tables 2–5 and S5–S8), using IBM-SPSS v.26.0 (IBM Corporation, Armonk, NY, USA).

2.4. Soil sampling, DNA extraction, sequencing and data analysis

For the melon crop during 2019, rhizosphere and bulk soil were collected to isolate total soil DNA in the following treatments: C 0, C 80, B 500 and B 500 + AD 10 for metagenomic analysis. Two plants per treatment from the central block were extracted from the soil five days after the flowering measurement to sample the rhizosphere soil. To prevent cross-contamination, the rhizosphere soil in contact with the roots was collected using sterilised brushes, sieved (2 mm), homogenised and stored as three different samples in Falcon tubes at −80 °C until DNA extraction. Additionally, bulk soil was also sampled from each treatment, sieved, homogenised and stored as three separated samples under the same conditions. Three samples of each type of soil were collected per treatment containing 300 mg of soil each and used for total microbial DNA extraction with the DNeasy Power Soil kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

Paired-end amplicons of the 16S rRNA were sequenced using the Illumina MiSeq high-throughput sequencing platform at Molecular Research DNA (MR DNA) (www.mrdnalab.com Shallowater, TX, USA) (accessed on 5 April 2022) to analyse the composition of soil bacterial communities. The primer set used was 515F (50-

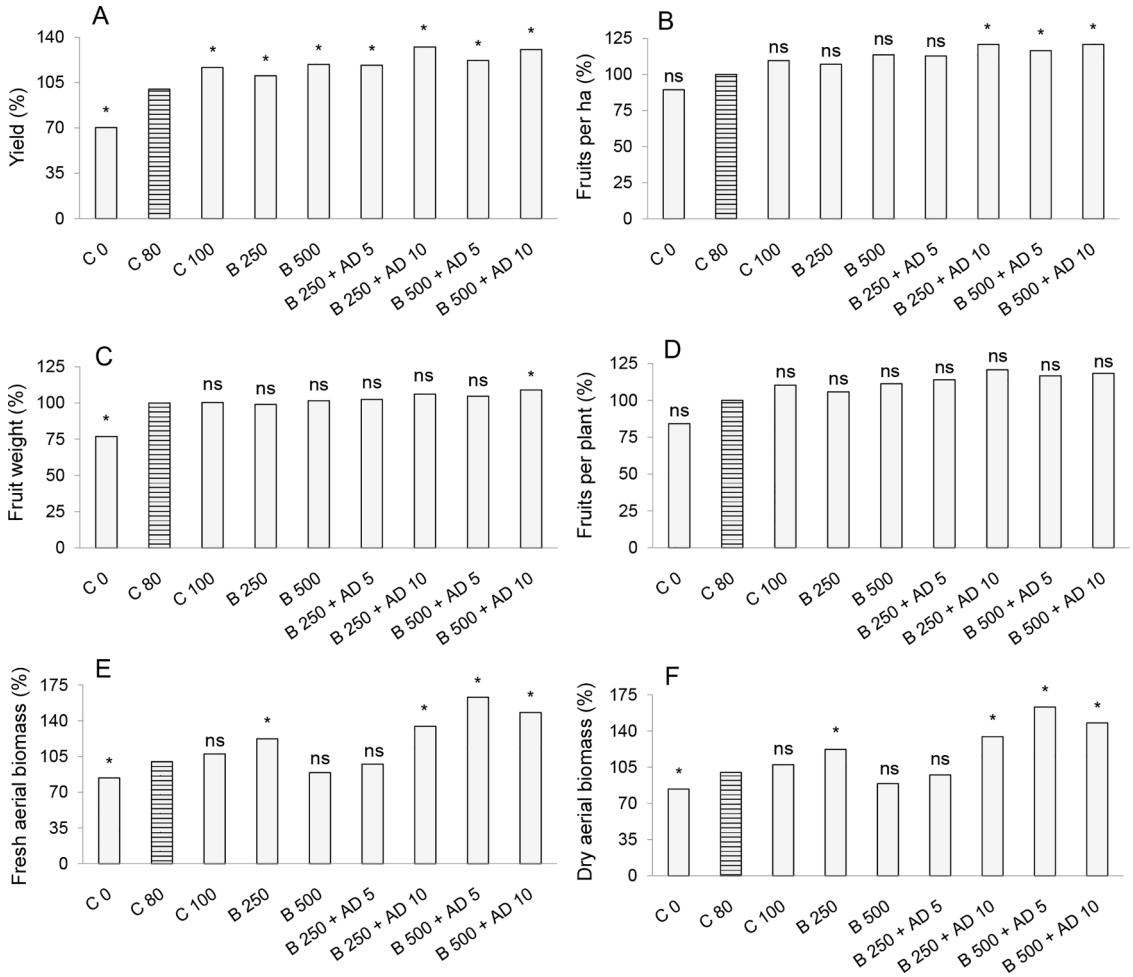

Fig. 1. Response of several agronomic variables to the treatments applied in the field trial for the melon crop: yield (A), its components (B–D), fresh aerial biomass (E) and dry aerial biomass (F). The values are expressed as relative values in percentages, using C 80 as a reference (100%). Comparison against C 80 was performed with the Dunnet test (* means a significant difference from C 80 at $p \leq 0.05$, ns means no significant difference from C 80 at $p \leq 0.05$).

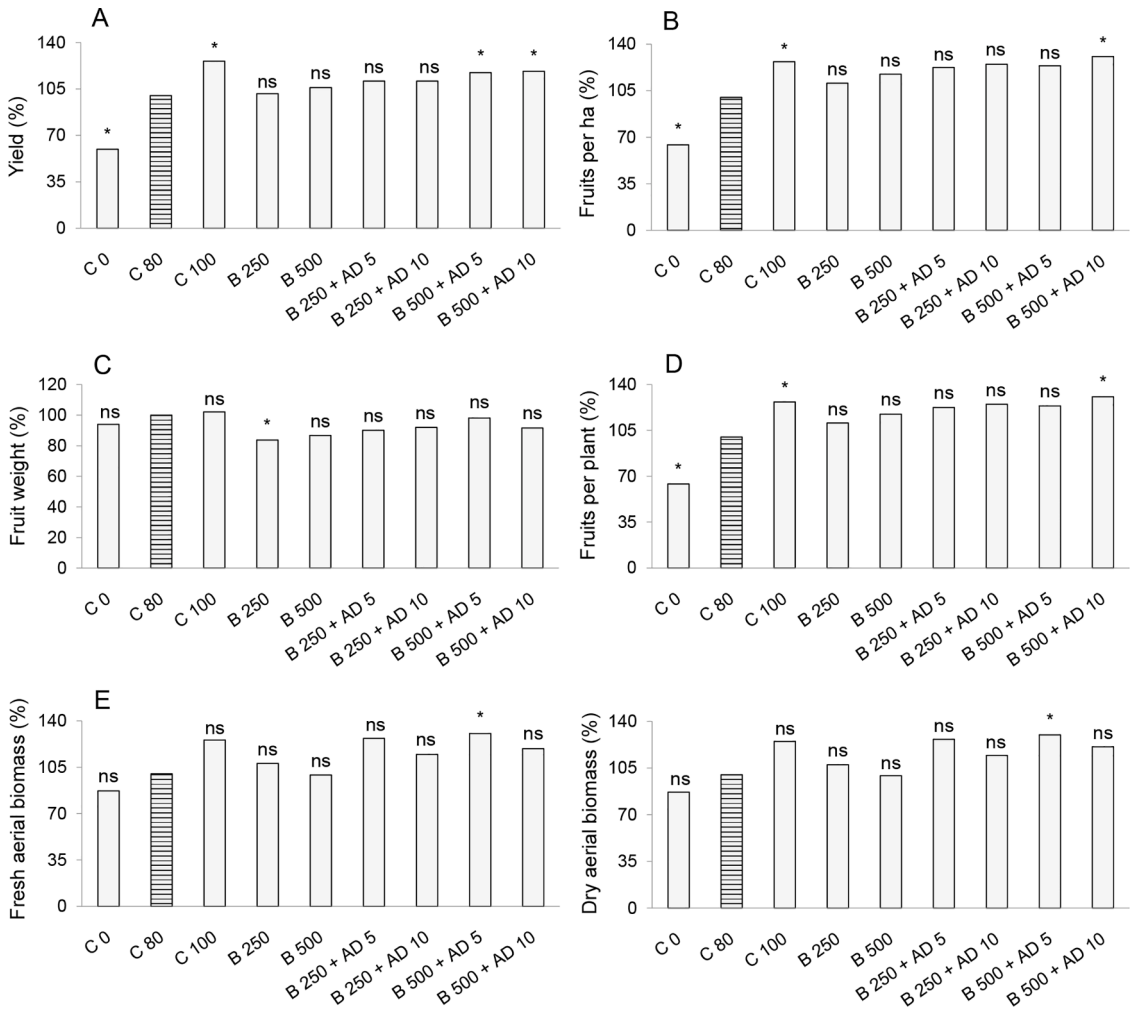

Fig. 2. Response of several agronomic variables to the treatments applied in the field trial for the pepper crop: yield (A), its components (B–D), fresh aerial biomass (E) and dry aerial biomass (F). The values are expressed as relative values in percentages, using C 80 as a reference (100%). Comparison against C 80 was performed with the Dunnet test (* means a significant difference from C 80 at $p \leq 0.05$, ns means no significant difference from C 80 at $p \leq 0.05$).

GTGCCAGCMGCCGCGGTAA-30) and 806R (50-GGACTACHVGGGTWTCTAAT-30), which are specific to the V4 region of 16S rRNA. The MR DNA pipeline (MR DNA, Shallowater, TX, USA) was used to process the sequence data, removing the primers, short sequences <150 bp and sequences with doubtful base calls. Sequences were quality filtered at Q25 using a maximum error threshold of 1.0 and subsequently dereplicated and denoised. Sequences with PCR point errors, singletons and chimaeric sequences were removed to obtain denoised sequences or amplicon sequence variants (ASV). Taxonomy was then assigned using the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov, accessed on 18 May 2022) and BLASTn against a database derived from Ribosomal Database Project II (RDPII, http://rdp.cme.msu.edu, accessed on 18 May 2022). Raw sequences obtained are available at the Sequence Read Archive (SRA) of NCBI under accession number PRJNA942119.

Primer v7 and PERMANOVA+ software were used to analyse the bacterial community and create the associated plots (Clarke and Gorley, 2015). Diversity metrics identified as the species richness, the Shannon diversity index and the number of reads of the soil microbial communities were determined for four treatments (C 0, C 80, B 500 and B 500 + AD 10), and boxplots were used to visualise the distribution of diversity indices. ANOVA was run to determine the impact of soil position (rhizosphere or bulk) and treatment (C 0, C 80, B 500 and B 500 + AD 10) on the diversity metrics of the obtained ASV. The post-hoc Tukey's test was then used to determine specific changes amongst experimental conditions, and the significance level was fixed for p-values below 0.05. ANOVA and post-hoc tests were performed using IBM-SPSS v.26.0. (IBM Corporation, Armonk, NY, USA).

Stacked bar charts were used to represent the relative abundances of microbial taxa at the phylum level, while heat plots at the ASV level were used to show the detailed organisation of the bacterial communities. Heatplots were used for clustering the treatments according to Bray–Curtis dissimilarity and for stacking the 50 most frequent genera according to hierarchical clustering based on the index of association. The variation in the composition of the bacterial communities after the application of the different treatments for the rhizosphere and bulk soil was evaluated using the non-metric multidimensional scaling (nMDS) of the Bray–Curtis dissimilarity matrix. The significance of pairwise comparison between the responses to the treatment within each type of soil for the bacterial community was verified with Permutational Multivariate Analysis of Variance (PERMANOVA) for 999 permutations; dissimilarity was performed using Bray–Curtis with treatment and soil considered fixed factors.

To assess the influence of rhizospheric microbial consortia on yield and fruit parameters correlation analysis were carried out. Identification of main bacterial consortia in rhizospheric soil were obtained by contingency matrix of metagenomic data based on correlation and ordered by Hierarchical clustering. Analysis were restricted to the 70 more frequent taxa. Correlation was made using spearman rank methods to avoid bias of non-normal distribution of bacterial frequencies and

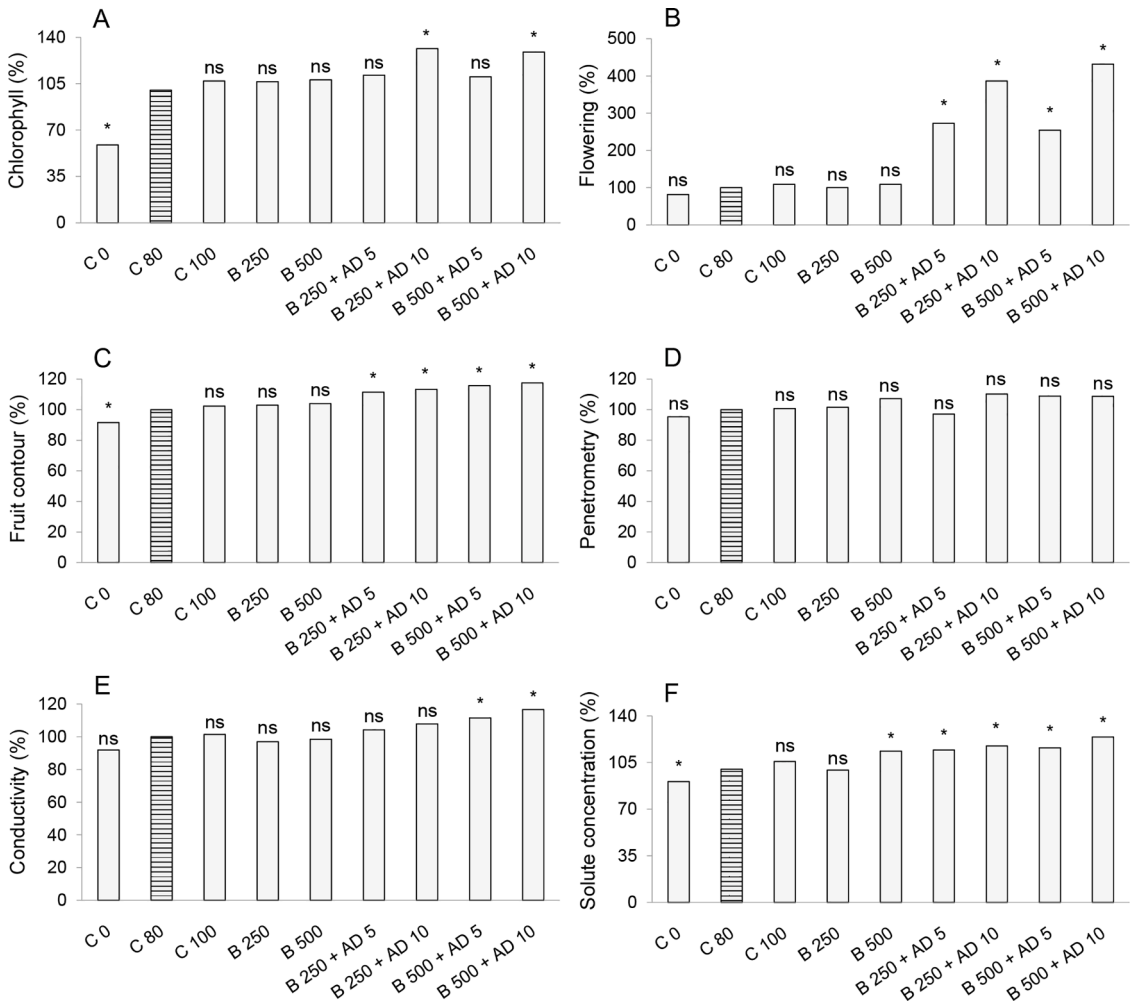

Fig. 3. Response of other variables to the treatments applied in the field trial for the melon crop: chlorophyll (A), flowering (B), penetrometry (C), fruit contour (D), conductivity (E) and solute concentration (F). The values are expressed as relative values in percentages, using C 80 as a reference (100%). Comparison against C 80 was performed with the Dunnet test (* means a significant difference from C 80 at $p \leq 0.05$, ns means no significant difference from C 80 at $p \leq 0.05$).

hierarchical clustering on correlation values were produced by complete linkage methodology. Only data with positive correlation threshold above 0,5 were considered, data were visualized by mean of heatmap. The resulting cluster were than used for a second correlation analysis between identified microbial consortia and 12 crop variables using Spearman rank methodologies between centroids of each cluster and agronomic data that were log transformed before the analysis. Results were showed in heatmap and agronomic data organized by hierarchical clustering based on contingency matrix calculated by Euclidean distances.

3. Results

3.1. Yield, yield components and biomass production

In the melon crop (Fig. 1), all treatments showed significantly higher yields than C 80, and in the case of aerial biomass, all treatments, except for B 500 and B 250 + AD 5 produced significantly higher biomass than C 80. The combination of biochar and AD improved the yield between 19 and 33% compared with C 80, depending on the combination between the biochar dose and additive dose (Fig. 1A). The highest yield corresponded to the B 250 + AD 10 and B 500 + AD10 treatments, with an increase of 33% and 31%, respectively, compared to the control, while the highest aerial biomass was for B 500 + AD 5 (Fig. 1E,F). The combination of biochar and AD produced a significantly higher number of fruits per ha than the C 80, with the exception of B 250 + AD 5 (Fig. 1B). Conversely, for fruit weight, only treatment B 500 + AD 10 produced significantly higher values than the C 80, with a 9% increase (Fig. 1C). For fruits per plant, no biochar treatment produced significantly higher values compared to C 80 (Fig. 1D). In summary, the best-performing treatments were B 250 + AD 10 and B 500 + AD10 for yield and its components and B 500 + AD 5 for fresh and dry aerial vegetative biomass.

ANOVA for the factors of biochar dose and additive dose in the melon crop are presented in Table 2. The two factors and the interaction between the biochar and the additive doses were significant for the aerial biomass variables, and only the factor additive dose produced significant differences in yield and individual fruit weight. For the biochar dose of 250 kg ha^{-1}, the additive dose of 10% produced significantly higher values than other additive doses (0 and 5%) for all dependant variables except the fresh and dry aerial biomass, in which the additive dose of 5% produced significantly lower values than 0 and 10% and fruits per plant, with no differences between AD doses (Table S5). For the biochar dose of 500 kg ha^{-1}, the two additive doses (5 and 10%) produced significantly higher values than 0% for the fresh and dry aerial biomass, while only the additive dose of 10% produced a significantly higher yield and fruit weight (Table S5).

In the pepper crop (Fig. 2), compared to C 80, the yield was significantly improved by the treatments with biochar at their highest dose combined with AD (17% increase for B 500 + AD5 and 18% for B 500 +

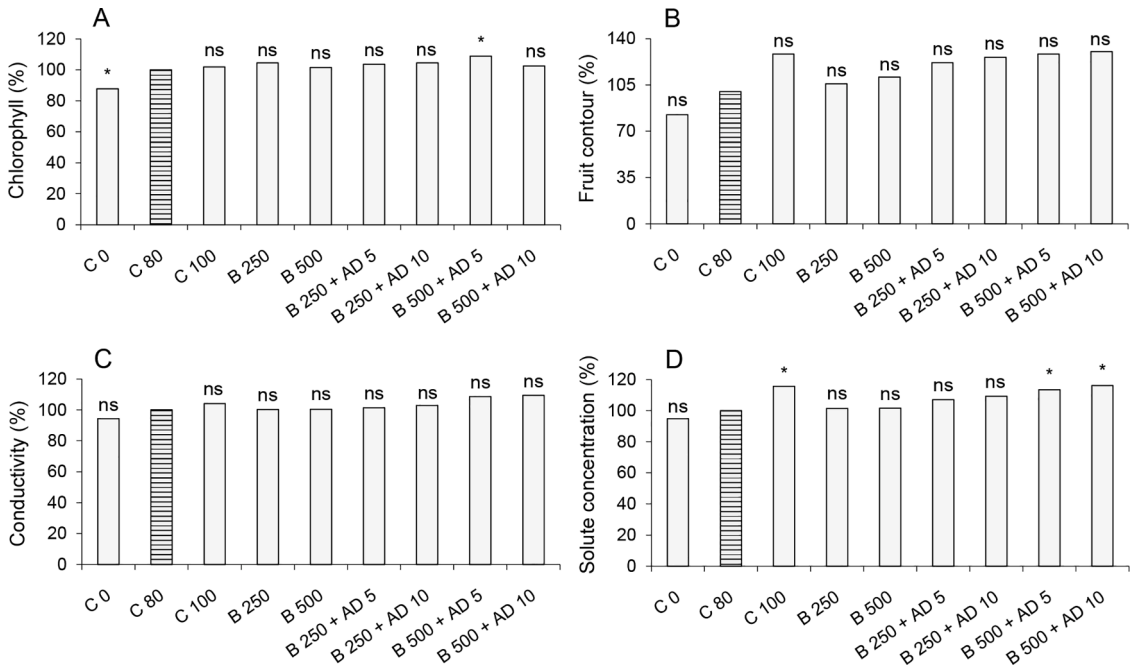

Fig. 4. Response of other variables to the treatments applied in the field trial for the pepper crop: chlorophyll (A), fruit contour (B), conductivity (C) and solute concentration (D). The values are expressed as relative values in percentages, using C 80 as a reference (100%). Comparison against C 80 was performed with the Dunnet test (* means a significant difference from C 80 at $p \leq 0.05$, ns means no significant difference from C 80 at $p \leq 0.05$).

Table 2
ANOVA for biomass production, yield and yield components in the melon crop. The location, year and plot were considered random factors, while the biochar dose and additive (anaerobic digestate) dose were fixed factors (significance level: *** $p \leq 0.001$; ns, not significant).

Doses	Fresh Aerial Biomass (g Per Plant)		Dry Aerial Biomass (g Per Plant)		Yield (kg ha^{-1})		Number Fruits Per ha		Fruit Weight (g)		Number Fruits Per plant	
	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic
Biochar dose (kg/ha)	314,782	21.4 ***	198,294	21.1 ***	11,165,675	3.47 ns	0.026	0.140 ns	26,678	3.42 ns	0.026	0.140 ns
Additive dose (%)	593,194	40.4 ***	373,992	39.7 ***	90,959,362	28.3 ***	0.292	1.59 ns	74,040	9.48 ***	0.292	1.59 ns
Biochar dose × Additive dose	1088,155	74.1 ***	686,267	72.9 ***	8824,489	2.75 ns	0.039	0.212 ns	139,852	0.018 ns	0.039	0.212 ns

AD10; Fig. 2A); however, the yield of the control fertilised with mineral N at the standard dose (C 100) increased by 26% compared to C 80. Conversely, the treatments did not increase the fruit weight compared to C 80. For the number of fruits per ha and fruits per plant, only treatment B 500 + AD10 and the C 100 showed significantly higher values than C 80, reaching the maximum increase (31%) for B 500 + AD10. In the case of aerial biomass (fresh and dry), only treatment B 500 + AD 5 produced significantly higher values (30% increase) than C 80.

The ANOVA results for biochar dose and additive dose in the pepper crop are presented in Table 3. Both factors produced a significant effect on crop yield, but interestingly, only the additive dose produced a significant effect on one of the yield components (fruit weight). For aerial biomass (fresh and dry), only the additive dose produced a significant effect. The interaction of both doses was not significant for the dependant variables measured. For the biochar dose of 250 kg ha^{-1}, no additive doses produced significant differences for the different dependant variables (Table S6). However, for the biochar dose of 500 kg ha^{-1}, both additive doses (5% and 10%) produced significantly higher values than no additive dose for the yield and the fresh and dry aerial biomass, but no significant increases for the yield components (Table S6).

3.2. Chlorophyll content, flowering and fruit parameters

In general terms, for the melon crop, treatments with biochar + AD produced significantly higher values than C 80, except for the parameter penetrometry with no significant differences (Fig. 3A–F). In brief, compared to C 80, the highest increase in chlorophyll content was observed for the treatment B250 + AD10 (31% increase) and in the rest of the parameters for treatment B 500 + AD10 (332% increase in flowering time, 18% in fruit contour, 17% in conductivity and 24% in solute concentration).

The ANOVA results for the melon fruit parameters are presented in Table 4. The biochar dose produced significant differences for the penetrometry, conductivity and solute concentration, while the additive dose produced significant differences for all parameters except penetrometry. The interaction between the two factors was not significant for any parameter. For the biochar dose of 250 kg ha^{-1}, the AD dose of 5% produced significantly higher values than the biochar alone for three parameters (flowering, fruit contour and solute concentration), while the dose of 10% produced significantly higher values for all parameters (Table S7). For the biochar dose of 500 kg ha^{-1}, the 5% AD dose produced significantly higher values for three parameters (flowering, fruit contour and conductivity) and the 10% AD dose produced significantly

Table 3
ANOVA for biomass production, yield and yield components in the pepper crop. The location, year and plot were considered random factors, while the biochar dose and additive (anaerobic digestate) dose were fixed factors (significance level: ** $0.001 < p \leq 0.01$; * $0.01 < p \leq 0.05$; ns, not significant).

Doses	Fresh Aerial Biomass (g Per Plant)		Dry Aerial Biomass (g Per Plant)		Yield (kg ha^{-1})		Number Fruits Per ha		Fruit Weight (g)		Number Fruits Per plant	
	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic
Biochar dose (kg/ha)	3.26	0.002 ns	9.43	0.010 ns	54,195,149	5.39 *	4282,237,469	0.715 ns	82.5	1.68 ns	6.85	0.715 ns
Additive dose (%)	10,602	6.96 **	6606	6.83 **	72,359,713	7.19 **	$1.351 \bullet 10^{10}$	2.26 ns	180.7	3.68 *	21.6	2.26 ns
Biochar dose × Additive dose	880.5	0.578 ns	646.9	0.669 ns	896,235	0.089 ns	590,668,385	0.099 ns	37.3	0.758 ns	0.945	0.099 ns

Table 4
ANOVA for chlorophyll content, flowering and several fruit parameters in the melon crop. The location, year and plot were considered random factors, while the biochar dose and additive (anaerobic digestate) dose were fixed factors (significance level: *** $p \leq 0.001$; ** $0.001 < p \leq 0.01$; * $0.01 < p \leq 0.05$; ns, not significant).

Doses	Chlorophyll (CCI)		Flowering (%)		Penetrometry (kg)		Fruit Contour (cm)		Conductivity (μS cm^{-1})		Solute Concentration (mg l^{-1})	
	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic
Biochar dose (kg/ha)	0.357	0.073 ns	11.1	0.192 ns	0.119	4.19 *	15.4	3.49 ns	0.722	9.10 **	115,657	12.5 ***
Additive dose (%)	121.0	24.6 ***	2340	40.4 ***	0.066	2.34 ns	85.1	19.3 ***	1.55	19.5 ***	145,262	15.7 ***
Biochar dose × Additive dose	0.840	0.171 ns	25.7	0.444 ns	0.062	2.20 ns	1.77	0.402 ns	0.102	1.28 ns	28,430	3.07 ns

Table 5
ANOVA for chlorophyll content and several fruit parameters in pepper crop. The location, year and plot were considered random factors, while the biochar dose and additive (anaerobic digestate) dose were fixed factors (significance level: ** $0.001 < p \leq 0.01$; * $0.01 < p \leq 0.05$; ns, not significant).

Doses	Chlorophyll (CCI)		Fruit Contour (mm)		Conductivity (μS cm^{-1})		Solute Concentration (mg l^{-1})	
	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic	Mean Square	F Statistic
Biochar dose (kg/ha)	0.652	0.074 ns	2.85	0.131 ns	0.309	4.18 *	61,504	3.30 ns
Additive dose (%)	16.1	1.84 ns	15.3	0.703 ns	0.183	2.47 ns	136,935	7.33 **
Biochar dose x Additive dose	42.4	4.83 *	0.041	0.002 ns	0.073	0.990 ns	13,889	0.744 ns

higher values for all parameters except penetrometry (Table S7).

For the pepper crop, the treatments with biochar + AD produced significantly higher values than C 80 for all parameters except for conductivity, for which the differences were not significant (Fig. 4A–D). In the case of chlorophyll, the best treatment was B 500 + AD 5, with a 9% increase, and for the fruit contour, conductivity and solute concentration, the best treatment was B 500 + AD10, with increases of 30, 9 and 16%, respectively.

Table 5 shows the ANOVA results for the pepper fruit parameters. The biochar dose produced significant differences in conductivity, as did the AD dose for the solute concentration. An interaction was detected only for the chlorophyll parameter. For the 250 kg ha^{-1} biochar dose, the addition of AD to the biochar had no effect on any dependant variable measured. For the biochar dose of 500 kg ha^{-1}, the addition of AD at 5% produced a significant increase in chlorophyll, and conversely, the 10% additive dose produced a significant increase in solute concentration (Table S8).

3.3. Bacterial diversity in soil

Bacterial diversity varied across the experimental samples (Fig. 5). For the indexes species richness (S) and Shannon index (H') in the rhizosphere soil, the treatment with biochar alone (B 500) presented the significantly highest values, followed by control C 0 and treatment biochar + AD, both with similar values; conversely, the significantly lowest value was for C 80. In the case of the bulk soil, index S showed the highest value for biochar + AD and the lowest value for C 80. The index H' showed similar values for all treatments and controls. Conversely, regarding the number of reads in the rhizosphere soil, the highest value was for control C 80, which did not differ from the biochar alone or that additivated with AD; in the bulk soil, the highest value was for biochar + AD, which did not differ from the other treatments and the controls.

3.4. Composition of bacterial soil communities

At the phylum level, for a given treatment, the composition of the bacterial communities from the rhizosphere and from the bulk soil showed consistent differences (Figs. S1 and S2). Furthermore, regardless of the treatment, the abundance of the phyla Proteobacteria, Actinobacteria and Bacteroidetes was higher in the rhizosphere soil, while in the bulk soil Gemmatimonadetes, Acidobacteria, Verrucomicrobia, Plantomycetes and Chloroflexi dominated the bacterial community. Comparing the effect of the treatments in the bacterial communities, depending on the soil position, it was negligible in the bulk soil. However, in the rhizosphere soil, the composition of the bacterial community in C 80 differed from the other treatments (Figs. S1 and S2).

At the ASV level, the bacterial community differed between rhizosphere soil and bulk soil (Fig. 6), although *Arthrobacter, Acidobacterium, Sphingomonas* and the group Others were present in both soil types. Regardless of the treatment, the bacterial community was characterised

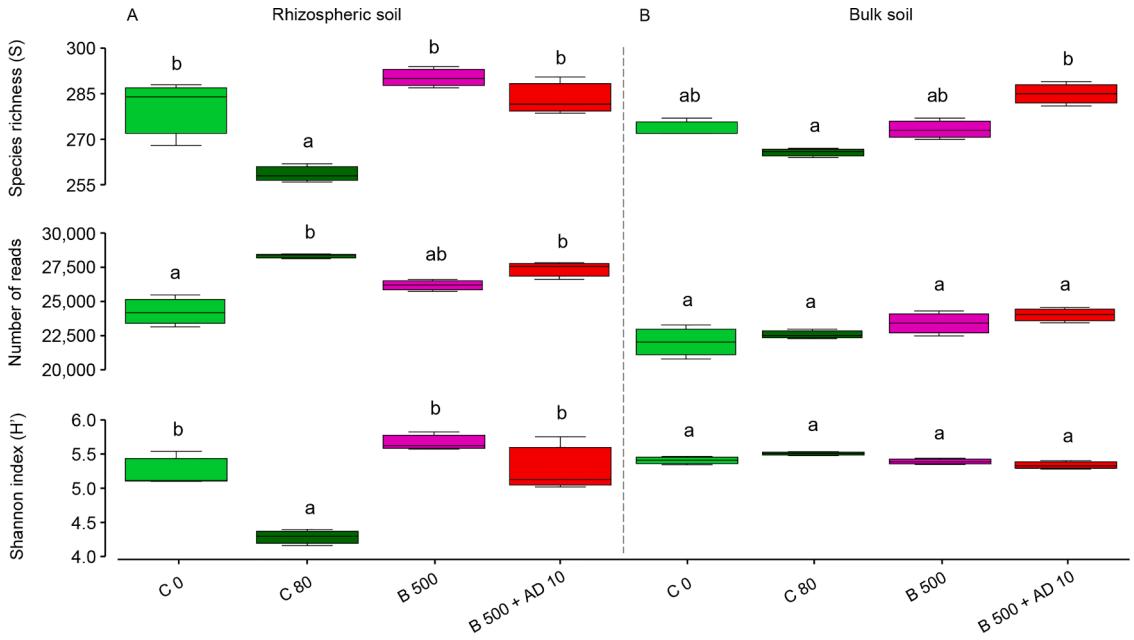

Fig. 5. Box plots showing the distribution of diversity indices for the bacterial community in each treatment in (A) rhizosphere and (B) bulk soil. S: Nº of ASV/number of amplicon sequence variants; number of reads for the bacterial community; H': Shannon index. The lower and upper bounds of the boxplots show the first and third quartiles (the 25th and 75th percentiles); the middle line shows the median; and the whiskers above and below the boxplot indicate interquartile ranges. Different letters indicate significant differences for p-values below 0.05 according to Tukey's HSD test.

by the high presence of *Acinetobacter, Sphyngopyxis, Arthrobacter, Novosphingobium, Sphingobium, Pseudomonas* and *Bacillus* in the rhizosphere soil and by *Acidobacterium, Pelobacter* and *Gemmatimonas* in the bulk soil (Fig. 6).

In the rhizosphere soil, the genus *Mitsuaria* was present only in the treatment with biochar + AD, and the genus *Arthrobacter* showed a much higher relative abundance (around 50%) in biochar + AD than in the treatment with biochar alone and in the controls. The relative abundance of the genus *Bacillus* was similar for all treatments with biochar + AD and biochar alone and for control C 0. Conversely, the genera *Pseudomonas* and *Pseudoxanthomonas* appeared in the treatment with biochar alone, with a relative abundance of around 20% each, but it was not found in the other treatments or controls. Concomitantly, the genus *Acinetobacter* was observed only in C 80. The correlation between rhizospheric microbial consortia, i.e. the obtained clusters and agronomic and fruit data revealed that the clusters A and B were linked to low yield and scarce fruit quality indicators. Oppositely, clusters D and E were correlated with high yield and good values for fruit parameters. The

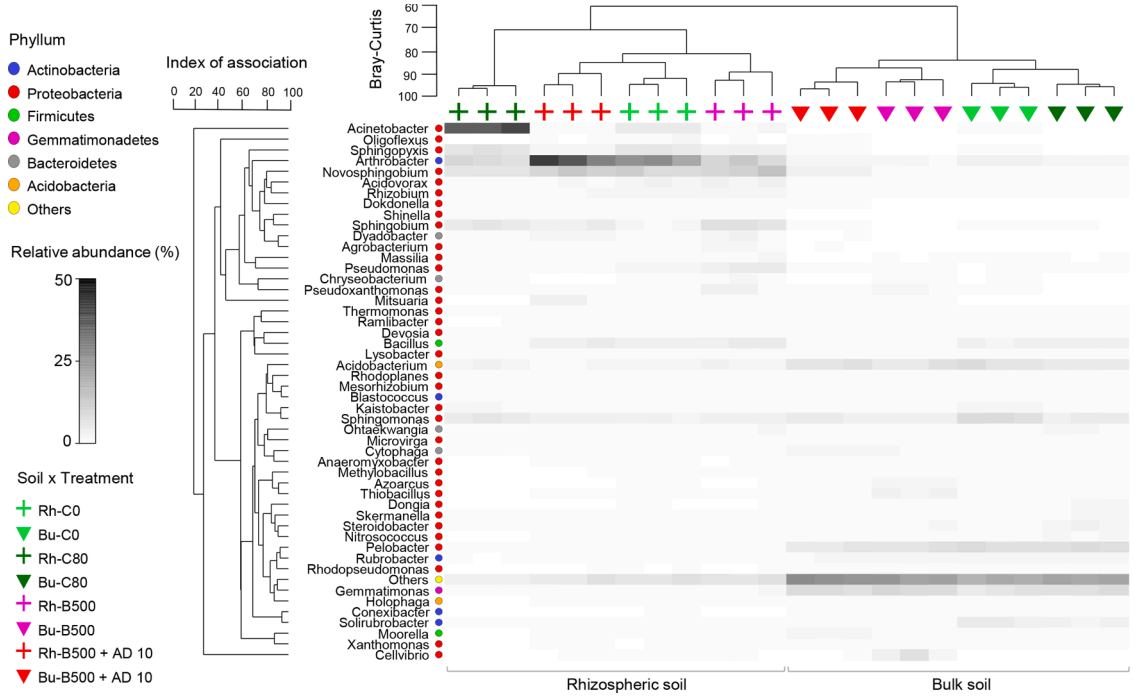

Fig. 6. Heat plot showing the relative abundance of the 50 most frequent taxa in the bacterial community. In the column legend, the same colour and shape indicate replicates of the same treatment and the same type of soil (Rh, Rhizosphere; Bu, Bulk).

clusters C and F showed scarce or no effect in those parameters (Figs. S3 and S4)

In the bulk soil, only a few changes in the relative abundance of several genera were observed, namely the presence of *Novosphigobium* in the biochar + AD but not in the other treatments or controls and the absence of *Bacillus* and *Solirubrobacter* in the biochar + AD and in the biochar alone, while they were present in the controls at a low level of relative abundance. In brief, the treatment affected the relative abundance of the different genera in the rhizosphere soil, while negligible changes were observed in the bulk soil.

NMDS ordination confirmed that the bacterial community of the rhizosphere soil showed a response to the treatments, while there was no response in the bulk soil, as deduced by the degree of similarity (Fig. 7). Notwithstanding, for the rhizosphere soil, control C 0 did not show significant differences in the pairwise comparison either with the treatment biochar + AD or with the treatment with biochar alone (Table 6 and Fig. 7). However, significant differences were found in pairwise comparisons between C 80 and control C 0, biochar + AD and biochar alone (Table 6 and Fig. 7).

Table 6
PERMANOVA significance test across treatments within each type of soil in the bacterial community. Treatment and soil were used as fixed factors (number of permutations: 999). The test of significance was based on Bray–Curtis similarity values. The significance level was fixed for a p-value equal to 0.001 (significance p-values are in bold).

	Bacteria			
	Rhizospheric Soil		Bulk Soil	
	Pseudo-F/t	p-Value	Pseudo-F/t	p-Value
C0 vs. Control 80	8.24	0.001	3.84	0.004
C0 vs. B500	3.38	0.006	4.74	0.002
C0 vs. B500+AD10	2.99	0.012	5.54	0.004
Control 80 vs. B500	8.32	0.001	3.60	0.008
Control 80 vs. B500+AD10	8.07	0.001	4.85	0.002
B500 vs. B500+AD10	3.50	0.008	3.16	0.004

4. Discussion

4.1. Effect of the application of biobased materials in agronomic and fruit parameters

For the two crops, melon and pepper, the combination of biochar + AD with a reduced dose of mineral fertiliser (C 80) produced a significant increase in yield and biomass production compared to the control, which only received the same reduced dose of mineral fertiliser (C 80). Moreover, for the melon crop, the yield obtained in the treatment with biochar + AD and a reduced dose of the mineral fertiliser was even higher than the yield obtained in the control that received the full mineral dose (C 100) (between 2 and 16% higher). Furthermore, we demonstrated that the incorporation of AD improved the yield compared with the use of biochar alone for both crops, with the yield increase ranging between 7.5 and 16%.To the best of our knowledge, this is the first work that tests, at field scale, the triple combination of a reduced dose of mineral fertiliser, biochar and AD. The double combination of mineral NPK fertilisers and biochar has been tested by Qian et al. (2014), resulting in an increased crop yield and reduced greenhouse gas emissions compared to mineral fertilisers alone. Additionally, Joseph et al. (2013) and Yao et al. (2015) observed improved nutrient availability to the crop as a consequence of biochar addition. However, our results have demonstrated that the effect of biochar can be improved by adding AD in a proportion as low as 5 or 10%. Although Gulyás et al. (2022) had previously combined biochar and AD and observed a significant increase in plant yield compared to treatments with only biochar, their experiment was on a laboratory scale. Moreover, Mickan et al. (2022) performed a test in pots combining biochar and AD with substratum to produce tomato plants. In our work, we used biochar as a carrier for the AD (5 and 10% AD volume relative to the biochar weight), resulting in a solid product, whereas they used biochar and AD as independent supplements for the pots' substratum with a much higher proportion of AD relative to the biochar quantity. In such conditions, Mickan et al. (2022) observed that digestate with biochar lagged, but the plant growth was not reduced at low digestate rates, which in their case, accounted for 20% AD volume relative to biochar weight. They explained it as a consequence of the biochar action in nitrogen metabolism; however, their conclusions cannot be extrapolated to a field experiment and thus cannot be compared with our results. Doyeni et al. (2022) and Vanden-Nest et al. (2021) also combined pig manure digestate (PMD) with biochar in wheat plants at the lab scale, but they only found a significant yield increase for the treatment PMD + biochar

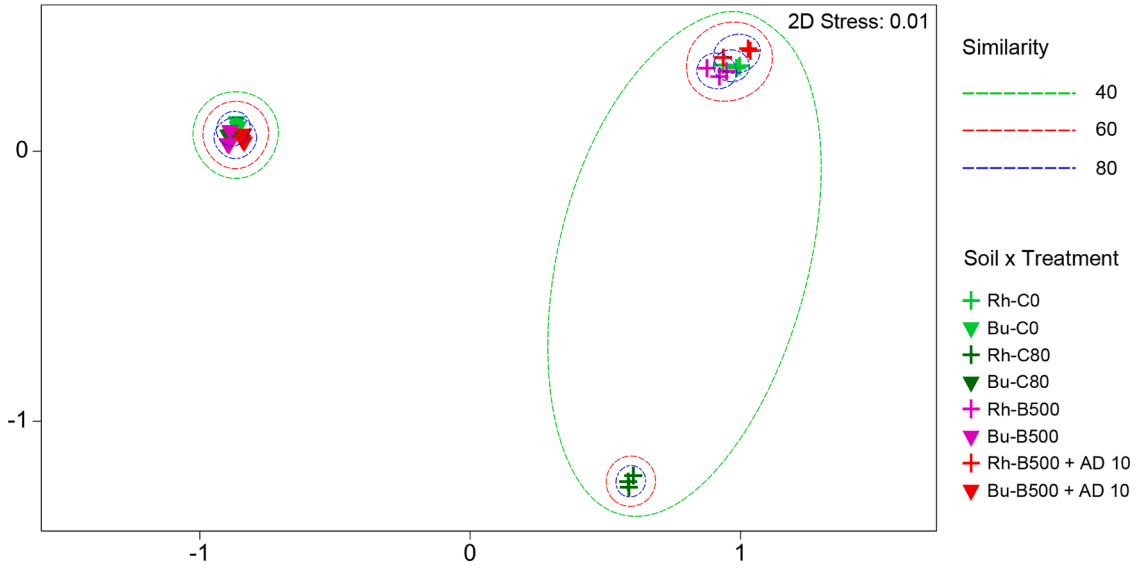

Fig. 7. Non-metric multidimensional scaling (nMDS) plots of bacterial community according to treatment applied. Each point represents the microbiome of one replicate of soil according to soil type. MDS axis 1 and MDS axis 2 represent the two axes of the two-dimensional ordination space. The stress level shown indicates how well the individual distances are represented (the closer to 0, the better are the original data points represented in the ordination space). All ordinations were performed using ASV-level data ($p = 0.001$ based on 999 permutations).

compared with the unfertilised control but not with the treatment fertilised with mineral fertilisers

The chlorophyll content depends on the concentration of nitrogen available to plants (Tekaya et al., 2016) because N stimulates the chlorophyll *b*iosynthesis process (Akram and Ashraf, 2009). As expected, the treatment with the full mineral dose (C 100) showed higher chlorophyll content than the treatment with the reduced dose 80% (C 80) for both crops. Interestingly, in melon, the treatment with 80% mineral fertiliser plus biochar + AD at the higher dose of AD (10%) showed a higher chlorophyll content than the control that received the full nitrogen dose (C 100); however, in pepper, the effect of biochar + AD was not so clear. Considering that the content of nitrogen applied with AD was much lower than 45 kg ha^{-1} N, which is the difference between C 80 and C100, the increase in the chlorophyll content triggered by the biochar + AD must be due to an increased N use efficiency or to other effects possibly related to the hormonal activity in the plant.

For the melon crop, and to a lesser extent for the pepper crop, all fruit parameters showed higher values for the treatments with biochar + AD than for the rest of the treatments and controls (C 80, C 100, B 250 and B 500), with significant differences for the melon crop for all the parameters except for penetrometry. The higher values of conductivity and solute concentration could be due to higher concentrations of sugars and minerals, which results in a higher fruit quality; this could be induced by higher gibberellin production (Radhakrishnan and Lee, 2016), which could have been produced by the plants as a response to the treatment with biochar + AD or by the new rhizosphere microbiota resulting from the treatment (see Section 3.4). However, the confirmation of any of these hypotheses requires further research. These hypotheses could be reinforced by the fact that the increase in nutrient availability in C 100 did not increase the values reached by the fruit parameters (Radhakrishnan and Lee, 2016)

4.2. *Agronomic effects resulting from the addition of biobased materials explained from soil microbiome outlook*

As expected, we observed sharp changes in the bacterial community between the rhizosphere and bulk soil. This is a common situation that has also been described in other works (Schmidt et al., 2019; Glick and Gamalero, 2021; Ortiz-Liébana et al., 2022). Moreover, all treatments modified the rhizosphere soil bacterial community but not the bulk soil bacterial community, as previously observed by Ortiz-Liébana et al. (2022), who concluded that where the plant cannot intervene directly, the treatments do not modify the soil bacterial community; moreover, those authors assigned to the plant an active selection of the beneficial bacterial taxa that arise in the soil as a consequence of the treatment.

Biochar provides a small amount of nutrients required by the crop as reported by several authors (e.g. Al-Wabel et al., 2018; Dorner et al., 2022; Jílková and Angst, 2022); in our case the Nitrogen provided by biochar itself ranged between 1.20% of the crop need (understanding as crop need, the full N dose according to Table 1) for the biochar dose of 250 kg ha^{-1}, and 2.3% for the biochar dose of 500 kg ha^{-1}. Concomitantly, the Nitrogen provided by the combination of biochar + AD ranges between 3% (for B 250 + AD 5) and 7% (for B 500 + AD10) of the crops needs. For assimilable Phosphorus and Potassium the percentages were similar or even lower. Thus, we hypothesize that the significant increase of yield and the improved values of fruit parameters observed in the treatments with biochar + AD could be partially due to the changes in the rhizosphere bacterial community exerted by the treatment, as discussed below.

In the first-place, the mineral fertiliser reduced the values of bacterial diversity and richness compared with the non-fertilised control (C 0); the treatments B 500 and B 500 + AD 10 (both fertilised with mineral fertiliser at the same dose) restored the lost values of bacterial diversity and richness. High biodiversity and species richness in the crop rhizosphere are important from the agronomic side because they improve crops performance for several reasons (Mickan et al., 2022): i) greater species richness will increase the overall use of resources, as different species use different resources, ii) species-rich communities are highly productive, as they likely contain species with a considerable influence on ecosystem functioning (Bell et al., 2005), and iii) genetically diverse soils are usually more resilient with greater functional redundancy (Stockdale et al., 2013). Ortiz-Liébana et al. (2022) obtained similar results regarding rhizosphere bacteria diversity and richness in a non-fertilised soil versus a soil fertilised with mineral fertiliser and another with the addition of the mineral fertiliser that received compost + biochar + one *Bacillus* strain. Conversely, Mickan et al. (2022) found less bacterial diversity and richness when adding biochar or AD. Nevertheless, most studies claim that the addition of either biochar or AD enhances soil microbial diversity (Kim et al., 2007; García-Sánchez et al., 2015), which supports our data (Mickan et al., 2022; Bell et al., 2005; Stockdale et al., 2013).

In the second place, the composition of the rhizosphere soil bacterial community has also been modified by the treatments with biochar + AD, with an increase of certain taxa that are considered PGPR. At the phylum level, we observed a large increase in the relative abundance of Actinobacteria in the treatment with B 500 + AD 10. This phylum is well characterised as being able to degrade complex molecules, aiding in the mineralisation of organic substrates into plant-available nutrients (Sapp et al., 2015). Regarding to genera, the biochar + AD treatments produced a large increase in the relative abundance of the *Arthrobacter* genus and also but less of the *Mitsuaria* genus in the rhizosphere. Both genera are well-known plant growth-promoting rhizobacteria (PGPR). *Arthrobacter* exerts a positive effect on shoot and root length, fresh and dry plant weight, synthesis of plant hormones, plant nutrient uptake, and yield (Chhetri et al., 2022); moreover, *Arthrobacter* protects plants from abiotic stress (Sziderics et al., 2007; Tiwari et al., 2011; Qin et al., 2014), and it is antagonistic against pathogenic bacteria (Munaganti et al., 2016). Besides, several species of the genus *Mitsuaria* help plants cope with some abiotic stress conditions (Glick, 2005; Huang et al., 2017), reduce pathogen growth (Benítez and McSpadden Gardener, 2009) and produce ACC deaminase, which enhances plant tolerance to both biotic and abiotic stressors (Glick, 2005; Huang et al., 2014; Huang et al., 2017). Another PGP genera as *Bacillus* (Barquero, 2014; Pastor-Bueis et al., 2017) is present in C 0, B 500 + AD 10 and in the treatment with biochar alone (B 500), and this can be interpreted as another sign that the use of biochar prevents the alteration of the rhizosphere as a consequence of mineral fertilisation, especially regarding the beneficial associated bacteria and in particular those from the *Bacillus* genus.

In order to assess if the presence of *Arthrobacter, Mitsuaria* and *Bacillus* in the rhizosphere are related with high yield and good fruit parameters, the correlation between rhizospheric microbial consortia and agronomic and fruit data was assessed. We observed a positive correlation between the yield and fruit quality indicators for genera in clusters D and E. Those include, amongst others, the genera *Arthrobacter, Mitsuaria* and *Bacillus*. Other PGPR genera like *Dyadobacter* (Zhang et al., 2019) and *Shinella,* a Nitrogen fixer and IAA producer (Taulé et al., 2012) also belonged to cluster E. Intriguinly *Agrobacterium* is also in cluster E; in spite of the presence of plant pathogenic species, this genera has been recognized as a PGPR and even proposed as inoculant to improve Phosphorus assimilation (Ejaz et al., 2020). However, although *Dyadobacter, Shinella* and *Agrobacteriun* are linked to cluster E, only *Arthrobacter* and *Mitsuaria* were clearly increased by the treatments with biochar + AD.

Interestingly, in the treatment with biochar alone (B 500), two other genera, *Pseudomonas* and *Pseudoxanthomonas*, emerged compared with the treatment biochar + AD and controls C 0 and C 80. Both genera include PGPR species but also pathogens. However, as we observed better yield, yield components and fruit quality parameters in B 500 compared with C 80, it is expected that *Pseudomonas* and *Pseudoxanthomonas* arose as a result of the B 500 treatment, as PGPR species predominate within these genera (Hayat et al., 2010; Nayaka et al.,

2019; Thierry et al., 2004).

Finally, for the control that only received a reduced mineral dose (C 80), the relative abundance of Actinobacteria *Arthrobacter, Mitsuaria, Pseudomonas* and *Pseudoxanthomonas* decreased or even disappeared, and conversely, the relative abundance of the *Acinetobacter* genus increased. The *Acinetobacter* genus is represented by more than 50 species, and even if some are non-pathogenic organisms naturally present in the environment (Kizheva et al., 2022), many strains produce dangerous risks to human health, such as *Acinetobacter baumannii, Acinetobacter calcoaceticus* and *Acinetobacter woffii* (Dijkshoorn, 1992). *Acinetobacter baumannii* is a pathogen that has been detected in soil (Dekic et al., 2020; Manchanda et al., 2010), produces infections in people in contact with infected soil (Scott et al., 2007) and is resistant to all antimicrobial agents (Towner, 2009); *A. calcoaceticus* has been associated with a wide range of diseases, including pneumonia, bacteremia, urinary tract infections (Glew, 1977) and nosocomial infection outbreaks (Buxton et al., 1978). *Acinetobacter* is in cluster A in the correlation between rhizospheric microbial consortia and agronomic and fruit data; the cluster A is strongly negatively correlated with the yield and fruit quality indicators.

In brief, our results indicate that biochar application to the soil (alone or additivated with AD) produced effects in the holobiont, namely an increase in bacterial species diversity and richness and an improvement in the relative abundance of bacteria with PGP properties (e.g. *Arthrobacter, Bacillus*). This contrasts with the impoverishment of the bacterial community and the reduction of the relative abundance of Actinobacteria when the crop is exclusively fertilised with mineral fertiliser. Our results indicate that the improved agronomic performance of the crops that received biochar + AD can be related with the changes in the rhizospheric community, and further research is needed to elucidate the mechanisms involved in the improvement of the rhizosphere bacterial composition and to elucidate the mechanisms by which the species associated with the rhizosphere improve the crops performance.

4.3. Environmental implications explained from soil microbiome outlook

In the nMDS analysis, C 0, B 500 and B 500 + AD 10 were plotted closely with no significant differences in the pairwise analysis via PERMANOVA. Whilst C0 was not fertilized at all, B 500 and B 500 + AD 10 were fertilized with a reduced dose of mineral fertilisers (80% of the total dose). Thus, this result could be interpreted in terms of the very low environmental impact of fertilisation with a reduced dose of mineral fertilisers if combined with the treatment with biochar or with biochar supplemented with AD. The environmental benefits of the use of the biostimulant based on biochar + AD encompass the reduction of the dose of mineral fertilisers, the improvement of the bacterial soil biodiversity and the richness and valorisation of bio-residues contributing to the circular economy and sustainable agriculture. For horticulturists, the proposed technology can reduce production costs by reducing mineral fertilisers in a scenario of rising prices.

5. Conclusions

The agricultural use of biochar + AD produced a significant yield increase compared with the control, which received the same dose of mineral fertiliser (C 80). Such an increase was 33% in melon and 18% in pepper. As a result, the best combination to optimise yield in melon was a reduced dose of mineral fertiliser (80%) combined with biochar + 10% AD (volume of AD : weight of biochar), and the two doses of biochar tested produced a similar yield, which was even higher than the yield obtained with a full mineral dose (100%) and without biochar. In the case of pepper, the highest yield was with small differences for the control with the full mineral dose (C 100) and for the reduced dose of mineral fertiliser (80%) combined with the highest dose of biochar (500 kg ha^{-1}) + AD (10%, volume of AD: weight of biochar). The fruit parameters showed the best values in terms of quality for the highest doses of biochar and additive in both crops.

A new insight has been proposed to explain the improved agronomic results, based on the effect of the treatments in the bacteria rhizopsheric community. The treatments with biochar increased the bacterial diversity and richness of the crops' rhizosphere. Interestingly, the treatments with biochar increased the relative abundance of PGPR genera in the rhizosphere, remarkably in the case of biochar + AD, in which an increase in the relative abundance of *Arthobacter* and *Mitsuaria* was observed.

This work has demonstrated, at the field scale, that the use of the plant biostimulant based on the proposed combination of biochar + AD improves crop yield and fruit quality at a reduced dose of mineral fertiliser. The two environmental benefits achieved are the reduction of the mineral fertiliser dose by 20% and the improvement of soil bacteria biodiversity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Data availability

Data will be made available on request.

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Author Contributions

N O-L conceptualization, investigation, formal analysis, writing original draft; M Z formal analysis and writing review and editing; M B writing review and editing; FG-A conceptualization, formal analysis, writing review and editing, funding acquisition. All authors have read, revised and agreed to the published version of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2023.112277.

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