Towards Nutrition-Sensitive Agriculture: an evaluation of biocontrol effects, nutritional value, and ecological impact of bacterial inoculants

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Abstract: Nutrition-Sensitive Agriculture (NSA) is a novel concept in agriculture that ~~does not~~ considers not only yield, but also nutritional value of produce~~the food~~, sustainability of ~~the~~ production, and ~~the~~ ecological impact of agricultur~~eal practices~~. In accordance with its goals, NSA would benefit from applying microbial-based products as they are deemed more sustainable than their synthetic counterparts.

This study ~~aims to~~ characterized ~~the effect of~~ 3 plant-beneficial bacterial strains (*Paenibacillus pasadenensis* strain R16, *Pseudomonas syringae* strain 260-02, *Bacillus amyloliquefaciens* strain CC2) on their biocontrol activity and effect on ~~the~~ nutritional and texture quality of romaine lettuce plants (*Lactuca sativa*) ~~and in containing pathogens~~ in ~~controlled conditions~~ ~~(~~greenhouse~~)~~. The pathogens used in the trials are *Rhizoctonia solani* and *Pythium ultimum*.

The obtained results indicate that strain R16 had a significant ability to cause a statistically significant reduction in the ~~e~~ symptoms caused by both *P. ultimum* (reduction of 32%) and *R. solani* (reduction of 42%)~~analyzed pathogens~~, while the other two strains showed a less efficient biocontrol ability.

Indices of the nutritional quality (~~photochemical activity of photosystem II,~~ content in phenols, carotenoids and chlorophyll) were ~~largely~~ unaffected by the treatments, indicating that the product was equivalent to that obtained without using the bacteria, while the texture of the leaves benefits from the biocontrol treatments. In particular, the mechanical resistance of the leaves was significantly higher in non-treated plants affected by *R. solani* but was restored to the values of healthy plants when the bacterial inoculants were present as well.

The ecological impact was evaluated by characterizing the bacterial microbiota in bulk soil, rhizosphere, and root ~~microbiota~~ in the presence or absence of the inoculants.

The composition of the microbiota, analyzed with a Unifrac model to describe beta-diversity, was radically different in the rhizosphere and the root endosphere among treatments, ~~but~~ while

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the bulk soil formed a single cluster regardless of treatment~~remained practically unchanged~~, indicating that the use of these treatments did not have an ~~large-scale~~ ecological impact outside of the plant~~effect~~.

Keywords:
Nutrition-sensitive agriculture; Biocontrol; Microbiota; Lettuce; Texture; Nutritional value;

Abbreviations:
CFU: Colony-forming units
G%: Germination percentage
GLV: Green leafy vegetables
I%I: Infection percentage index
NSA: Nutrition-sensitive agriculture
NSR: Roots grown in non-sterilized soil
NT: Non-treated
OTU: operational taxonomic unit
PDA: Potato-dextrose agar
PI: Performance index
PNA: Peptide-nucleic acids
PU: *Pythium ultimum*
RH: Rhizosphere
RS: *Rhizoctonia solani*
S: Bulk soil
SR: Roots grown in sterilized soil

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1. Introduction

In recent years, the subject of Nutrition Sensitive Agriculture (NSA) is becoming gradually more widespread and relevant. While the name itself of NSA makes it clear that it gives great relevance to the nutritional quality of food obtained from agriculture, the concepts of NSA take into account also health-related properties, food security, sustainable agricultural production, and maintaining biodiversity in the agroecosystem (Jaenicke and Virchow, 2013).

Plant diseases are a major threat to worldwide food security, causing severe yield loss in all known crop species, and their management is one of the main concerns regarding the sustainability of agriculture: in order to control pathogens and pests, several pesticides are employed, and these can have a high environmental impact (Berg, 2009). The use of more sustainable methods to manage plant diseases is thus a very important step towards making the goals of NSA a reality. One of the most promising alternative strategies to the use of synthetic pesticides for a more environmental-friendly control of diseases is the use of biocontrol microorganisms (Albouvette et al., 2009). Biocontrol can be defined as the exploitation of organisms, or molecules they produce, capable of reducing or eliminating the damage caused by pathogens, either by direct antagonism or by enhancing the plant's defenses against the pathogens (Junaid et al., 2013). In most cases, biocontrol does not achieve the same level of protection of the crops as synthetic pesticides do and faces the problem of having inconsistent results when used in field scale (Barret et al., 2011), but it is still a promising, more sustainable technique that can be employed in agriculture (Berg, 2009). Despite this, there are several questions regarding the use of biocontrol and its respect of the concept of NSA. For example,

the effect of biocontrol treatments on the health-oriented quality and perceived quality of the produce is a little-investigated topic, as is the effect of these treatments on the microbiota of the plant and soil in which they are inoculated. The safety and ecological impact of these inoculants is still a matter of debate in the scientific community (Deising et al., 2017; Koch et al., 2018; Lugtenberg, 2018) and the full extend of the effect of inoculants on non-target organisms is an important point to investigate and define. This is especially true regarding the endophytic communities of the treated plants, in contrast with the effect on the rhizosphere community which has been more extensively investigated (Grosch et al., 2012, Erlacher et al., 2014, Cipriano et al., 2016)

Regarding the nutritional and sustainable aspects of NSA, green leafy vegetables (GLV) are important crops to consider thanks to their high nutritonal value and for being consumed mostly raw, keeping intact most of their properties. Among GLV, lettuce (*Lactuca sativa* L.) is one of the most important and common raw edible plants and constitutes a good source of healthy compounds such as polyphenols, carotenoids and vitamins (Becker et al., 2014, Peirez-Loipez et al., 2014). Despite its popularity as a vegetable, lettuce still faces great risks from hard-to-manage diseases caused by soilborne fungal pathogens which can cause devastating losses in field, in particular *Pythium ultimum* and *Rhizoctonia solani* (Van Beneden et al., 2009). The difficulty in managing these pathogens is a limitation both in organic and in conventional farming. For example, *R. solani* was once kept under control by applying methyl bromide, which use was then forbidden because of its ozone-depleting effect and high toxicity (UNEP, 1999), leaving the farmers with few tools that could be used against this pathogen (Martin, 2003), which can survive in the soil for many years. Since these fumigants are no longer allowed, ~~and~~ the pathogens either form sclerotia that can survive in the soil for several years (for *R. solani)* or are often resistant to fungicides (in the case of *P. ultimum*), and R. solani is one of the most problematic pathogen to contain in both organic (Termorshiuzen et al., 2006) and integrated farming (Bonanomi et al., 2018), novel tools in the management of these diseases are necessary (Fatouros et al., 2018).

The present study investigated the biocontrol ability of three different bacterial strains on two different fungal, soilborne pathogens of lettuce, *Pythium ultimum* and *Rhizoctonia solani*. The study did not only evaluate the effect of the inoculated bacteria on their ability to reduce the symptoms induced by the pathogens, but also evaluated some physiological traits of the plants, and consequent macroscopic texture attributes, which are related both to the quality and to defense responses against pathogens, to compare the quality of the produce between treatments. Furthermore, the bacterial communities of endophytes in the roots (grown in sterilized or non-sterilized soil), rhizosphere, and bulk soil either non-treated or inoculated with bacteria were described and compared, to define the effect of these treatments on the microbial diversity inside the root tissues.

2. Materials and methods

2.1. Microbial strains

In this study, three bacterial strains were used as candidate biocontrol and plant-growth promoting agents: *Paenibacillus pasadenesis* strain R16, which has been already described as a potential antifungal agent in Passera et al., 2017; *Pseudomonas syringae* strain 260-02, which has been already described as a potential biocontrol and plant-growth promoting agent on Solanaceae plants in Passera et al., 2019; and *Bacillus amyloliquefaciens* strain CC2. Both strains were cultivated on LB High Salt Agar plates (tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 10 g/L, agar 15 g/L) at 25 °C and were stored in a 20% glycerol solution at -80 °C for long conservation periods.

Two soilborne fungal isolates were used in antagonism assays with bacterial strains: *Rhizoctonia solani* (Cooke) Wint, strain RS1 (which will be identified as RS for the rest of the study), isolated from millet (*Pennisetum glaucum* L.) kernels in 2012; and *Pythium ultimum* Trow, strain DSM 62987 (which will be identified as PU for the rest of the study). The fungal strains were conserved in the fungal culture collection of the Mycology Laboratory at the Department of Agricultural and Environmental Sciences (DiSAA), University of Milan, Italy. The isolates were ~~as~~ cultivated on potato dextrose agar (PDA, Difco™) at 20 °C and stored at 4°C.

2.2. Inoculum with bacterial strains and plant cultivation

Two weeks old seedlings of romaine lettuce (*Lactuca sativa* L. var. longifolia) were inoculated with the bacterial strains by soil drenching: the plants were transplanted in 13 cm in diameter pots containing potting soil and in each pot was added either a suspension of bacterial cells of strain R16, 260-02, or CC2 (10^5 CFU/ml in Ringer's solution) or sterile Ringer's solution for the non-treated control. The potting soil used in these trials was previously sterilized by autoclaving 3 times at intervals of approximately 16 hours between each sterilization. The plants were grown in greenhouse at a temperature between 25 °C and 28 °C with 14 hours of light per day and were harvested after three weeks from transplant for further biochemical and molecular analyses. Each treatment was carried out on 7 plants to obtain biological replicates.

In parallel, a different trial was set up utilizing the same methods, number of plants, and bacterial treatments, but were carried out in soil which was not sterilized. These plants and soil were used for DNA extraction only.

2.3. Biocontrol effect against soilborne pathogens

Biocontrol assays against the fungal pathogens PU and RS were carried out, based on the methods described by Fatouros *et al.*, 2018, with some modifications, in parallel to the cultivation of healthy lettuce plants, and used either plants inoculated with strain R16, 260-02, or CC2, or non-treated controls. For the biocontrol assay, a further positive control was used, treating the potting soil with a *Trichoderma* ssp-based product (indicated as TH from now on) commercially available and indicated for the biocontrol of these soilborne pathogens on horticultural crops; these treatments were performed following the manufacturer's instructions for the utilization of the product. These assays were carried out on 7 plants per treatment.

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The fungal inoculums used in these assays was obtained by air drying active cultures of either PU or RS, incubated on pearl millet at 26 °C for 3 weeks. These inoculums were mixed with the sterile potting soil at a concentration of 20g/kg of soil on the day of the transplant.

The plants were visually examined for the presence of symptoms induced by either PU or RS once a week for a period of 3 weeks after transplant and, at the end of the experiment, the roots were examined as well.

For both pathogens the symptoms included mild to severe leaf and architecture deformation, stunted growth, root damage and, only for RS, crown rot. Root damage could be evaluated only at the end of the trials when the plants were uprooted. The symptoms were evaluated through symptom classes (0: healthy plant, 1: mild leaf deformation, 2: mild leaf and architecture deformation and stunted growth, 3: leaf and architecture deformation and stunted growth, 4: severe leaf and architecture deformation, stunted growth and root damage, 5: dead plant, caused by crown rot) and these classes were then converted to an infection percentage index (I%I) using the formula proposed by Townsend and Heuberger (1943).

An additional assay was carried out to test the biocontrol effect of strains R16, 260-02, CC2, and TH, used as a positive control, against RS on lettuce seedlings. This assay was carried out using the methods described by Liu *et al.*, 2018, with some modifications. In detail, pathogen inoculum was carried by mixing potting soil with dried RS inoculum one week before sowing, at a concentration of 20g/kg of soil. Inoculation with strains R16, 260-02, and CC2 was carried out at the same time as sowing by soil drenching using a suspension with a concentration of 10^5 CFU/mL, pouring 1 L every 1.5 kg of soil. For TH the treatment was performed following the manufacturer's instructions for the utilization of the product. Non-treated control (NT) was obtained inoculating the soil with sterile solution, without the bacterial inoculum. Each treatment was carried out in either soil without RS (Control), or with RS inoculum, in 4 replicates of 50 seeds each. Germination percentage (G%) was evaluated 5 days after sowing. For this evaluation, seedlings that emerged but died due to damping-off were not considered as successfully germinated.

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2.4. Evaluation of physiological responses in lettuce leaves

2.4.1. *In vivo* chlorophyll *a* fluorescence measurement

Immediately before harvesting, the efficiency of the photosynthetic apparatus was evaluated non-destructively, by measuring the chlorophyll *a* fluorescence *in vivo*.

This analysis was performed on dark-adapted leaves using a portable fluorimeter (Handy PEA; Hansatech, Kings Lynn, UK). After 30 minutes of dark adaptation, leaf surface was exposed to a saturating light intensity of 3000 µmol m^{-2} s^{-1} emitted by three diodes. The fluorescence emission was then measured by a fast-response PIN photodiode with an RG9 long pass filter (Technical manual, Hansatech, Kings Lynn, UK). The parameters measured were the maximum quantum efficiency of photosystem II (F_v/F_m) and the performance index (PI) derived from the JIP test.

Analysis was performed on six independent replicates.

2.4.2. Quantification of functional health-oriented parameters of lettuce leaves

At harvest, total chlorophylls (a+b) and total carotenoids were extracted from lettuce leaf tissue (around 40 mg) using 5 mL of methanol 99.9% as solvent. Samples were then kept overnight in a dark room at 4 °C. Absorbance readings were taken at 665.2 and 652.4 nm for chlorophyll pigments and 470 nm for carotenoids. Chlorophylls and carotenoids concentrations were calculated by Lichtenthaler's formula (Lichtenthaler, 1987).

For the extraction of phenolic compounds and anthocyanins, lettuce leaf tissue (around 40 mg) were placed in 4 mL of acidified methanol (1 % HCl V/V) and maintained overnight in the dark. The phenolic index was calculated as the absorbance at 320 nm of the diluted extracts, normalized to fresh weight (Ke and Saltveit, 1989). Total anthocyanins were determined spectrophotometrically at 535 nm using an extinction coefficient (ε) of 29,600 mM^{-1} cm^{-1} and expressed as cyanidin-3-glucoside equivalents and (Klein and Hagen, 1961).

Each analysis was performed on three independent replicates.

2.4.3. Evaluation of leaf texture

In order to evaluate the objective textural properties of romaine lettuce leaves grown in all the assayed experimental conditions (with or without biocontrol inoculants and/or pathogens), a

mechanical bending test was performed with the TA.TX2 Stable Micro Systems texture analyzer (Stable Micro Systems, Godalming, UK) as reported in Roversi et al. (2016). A single leaf was fixed on an annulus-bounding fixture plate with a central testing area of 7 mm diameter. A round-ended stainless steel plunger of 4 mm diameter was moved to the leaf surface at 10 mm/s constant speed until the probe passed through the specimen. During the test the imposed mechanical loading develops a state of flexural stress which causes the leaf to undergo an uniaxial deformation up to failure. A uniform one-dimensional stress distribution within the film thickness was assumed. Results of the mechanical test were expressed in force/distance coordinates. From the recorded curves, mechanical discrete parameters were extracted by means of Texture Exponent Exceed TEE32 (Stable Micro Systems, Godalming, UK) software. The force needed to bend the leaf sample up to failure F (N) was measured as a function of the displacement of the probe (mm). The mechanical properties of lettuce leaves were evaluated at room temperature at harvest time. For each treatment combination, seven specimens were analyzed.

2.5. Sampling, DNA extraction and 16S sequencing

The following samples were collected for DNA extraction: roots from plants grown in sterile and non-sterile soil, rhizosphere from plants growing in non-sterile soil, and non-sterile soil. For each kind of sample, 7 samples were collected from each treatment (NT, CC2, 260-02, and R16).

For roots, both grown in sterile and non-sterile soil, samples were collected after three weeks from transplant, cleaned from soil and surface sterilized (3' 70% ethanol, 2' 5% bleach, 3' 70% ethanol, 3 washings with sterile water). Starting from 1 gram of each of these samples, total nucleic acids were extracted, following the protocol described by Bulgari and colleagues (2012).

For rhizosphere, the roots were carefully extracted from soil and cleaned from loosely attached bulk soil. The remaining, thin layer of soil clinging to the roots, identified as rhizosphere, was collected in falcon tubes containing sterile water. The suspension was then centrifuged and the supernatant was discarded. Rhizosphere pellet was then stored at -30 °C until DNA extraction.

For soil samples, a 2 grams aliquot of soil was taken from each pot and stored at -30 °C until DNA extraction.

DNA from soil and rhizosphere samples was extracted using the DNeasy PowerSoil kit (QIAGEN), following the manufacturer's instructions.

DNA from the sampled roots was sent to an external service (Personal Genomics, Verona (VR), Italy) for sequencing of the hypervariable V3–V4 region of the 16S rRNA gene using a MiSeq1000 sequencer, utilizing a PNA blocker for organellar 16S rDNA amplification (Lundberg et al., 2013). The obtained reads (deposited in EMBL-ENA under accession number PRJEB35767) were analyzed using the QIIME pipeline in order to assign them to OTUs and determine the richness of species in the different samples. Reads that mapped on plant-derived sequences (mitochondria, chloroplasts), and reads with low quality, were filtered out. ~~Alpha diversity indexes (Chao-1, Shannon and PD) were calculated for each sample.~~

2.6. Microbiota analysis

The OTU table obtained from the sequencing analysis was analyzed in R (version 3.6.0) using the R Phyloseq package (McMurdie and Holmes, 2013). ~~The sequencing data were~~

~~analyzed in different ways.~~ A first stage of analysis included the identification of OTUs that were unique to certain treatments or compartments, opposed to shared or "core" OTUs, considering only OTUs with 10 or more counts per sample type to determine the shared or unique OTUs. These data were visually represented as Venn's diagrams using the online software Venny (Oliveros, 2007-2015).

The calculations for alpha- and beta-diversity were carried out as described by Pietrangelo et al., 2018, except as follows: the beta-diversity was calculated exclusively using the weighted Unifrac index, and that 10000 permutations were used with the adonis function.

The composition of the bacterial community, expressed as relative abundance, was defined at the Phylum level and at Family level, with 1% cutoff threshold.

~~The beta-diversity among the samples was determined with a Unifrac model using the Phyloseq package for R. The significance of the effect of the treatments and/or compartments on the observed diversity was tested with the ADONIS algorithm, employing 10000 permutations.~~

2.7. Statistical analyses

The data obtained from the biocontrol assays (described in section 2.3.) were analyzed as follows: (i) the values obtained for I%I among the different treatments throughout the 3 weeks of observation were compared by performing a general linearized model test, optimized for repeated measures, followed by Tukey's exact post-hoc test ($p < 0.05$); (ii) the values obtained for G% were compared between treatments and pathogen by One-Way ANOVA followed by Bonferroni~~Tukey's exact~~ post-hoc test ($p < 0.05$).

The data obtained from the functional health-oriented quality parameters quantification (described in section 2.4.) were analyzed as follows: results obtained in different conditions were compared by a two-way ANOVA followed by Bonferroni~~Tukey's~~ multiple comparisons test. Statistics were performed using GraphPad Prism version 6 for Windows, GraphPad Software, La Jolla, California, USA (www.graphpad.com).

3. Results

In the present study the effect of bacterial inoculants on lettuce plants was examined taking into consideration different aspects: biocontrol against relevant soilborne pathogens of lettuce (*P. ultimum* and *R. solani*), effects on the physiology and nutritional quality of the leaves, and the effect on the bacterial microbiota associated to the soil and roots of the plants.

The plants were grown in greenhouse conditions, either in healthy soil or in soil experimentally inoculated with the pathogens, to assess the biocontrol efficacy of the selected bacterial inoculants in providing biocontrol. Biocontrol efficacy against *R. solani* was also assessed in a different experiment which involved planting seeds of lettuce in healthy soil or soil inoculated with the pathogen, determining how the bacterial inoculants affected germination of the seedlings, a development stage of lettuce that is particularly susceptible to the attack by *R. solani*. All these biocontrol assays included controls that were not treated with any biocontrol inoculant, and control that were treated with a commercial biocontrol product which uses *Trichoderma* spp. as the biocontrol agent. These results are reported in section 3.1.

Effects on the physiology of the plant and nutritive content was carried out in two stages: during the biocontrol assay, parameters related to photosynthetic efficiency were measured; at the end of the experiment (3 weeks after transplant and inoculation) the leaves were sampled from these plants to quantify chlorophyll content, phenols content, and total carotenoid. The values obtained from these

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experiments were compared between plants grown with different combinations of pathogens and bacterial inoculants to determine if statistically significant differences could be identified, highlighting differences in the physiology of the treated plants. These results are reported in section 3.2.
Lastly, the effect on the bacterial microbiota was evaluated in four different compartments (bulk soil, rhizosphere, roots grown in steriled soil, roots grown in non-sterilized soil) related to the healthy plants grown either without inoculation or with one of the bacterial inoculants. The abundance and identity of OTUs was compared among all compartments and treatments; beta-diversity and OTU abundance analyses were carried out to determine which differences could be caused by the treatments. These results are reported in section 3.3.

3.1. Biocontrol effect against soilborne pathogens

The plants grown in soil inoculated with either PU or RS developed symptoms starting from one week after transplant. While the most common symptoms that these pathogens inflict on seedlings (e.g. damping off) were not observed on these plants, symptoms regarding the plant architecture were observed on most plants and, in the case of RS, four plants out of 35 died due to crown rot. Comparison between the I%I in different treatments shows that, for both pathogens, the non-treated plants showed a more severe symptomatology (Fig 1 A and B) compared to the treated plants. In particular, the treatment with strain R16 managed to significantly reduce the I%I for both pathogens, while CC2 and 260-02 reduced the symptom severity but without any significant difference with the non-treated control. Likewise, the treatment with TH managed to reduce the symptom severity compared to the non-treated plants, but the difference is not statistically significant.

The results of the germination trial in presence of RS follow the same general trend as the experiment carried out on two-weeks-old plants, except for the treatment with TH which showed an effective biocontrol effect in this trial. The seeds grown without RS in the soil show a high G%, ranging from 62% to 83% and, while there is an increase of G% with the treatments (in particular CC2 and TH), this difference is not statistically significant (Fig 1C). Seeds grown in the presence of RS instead show differences between the treatments: in the NT seeds, average germination drops from 71% to 26%, the seeds treated with CC2 and 260-02 show an average germination above 30%, while those treated with R16 maintain a higher germination rate of 53%, which is statistically different from those of other treatments with RS, and comparable to that of seeds sowed in soil without RS (Fig 1C). The best result in this assay is obtained by TH, which shows a G% over 70% also in the presence of RS.

3.2. Evaluation of physiological responses

The chlorophyll *a* fluorescence-related parameters indicated that the maximum efficiency of the photosystem II was generally maintained in all the experimental conditions (Fig. 2A). The only exception was represented by lettuce leaves inoculated with RS and treated with the strains R16 and 260-02, which showed a slight but statistically significant decrement in the Fv/Fm ratio, compared to untreated leaves. (Fig 2B).

Considering the health-related compounds, at harvest there were no marked differences among treated and non-treated plants, although some specific combinations of bacterial inoculant and pathogen showed some results of relevance. For example, the phenolic index showed a significant increment in leaves of non-treated plants challenged with RS (Fig. 3A), while the leaves of plants treated with different strains and challenged with RS did not show the same trend. Interestingly, to note that these physiological values are mirrored in the results obtained from the analysis of leaf texture, with the NT-RS plants having leaves with a significantly tougher firmness (force at break = 1.46 $\pm$ 0.09 N) compared to all other treatments

(force at break = 1.12 ± 0.12 N) (data not shown). Significant changes were observed in the total carotenoid contents. In fact, plants treated with the strains R16 and 260-02 and challenged with RS had a higher content compared to the plants treated with the same bacterial strains but without the pathogen (Fig 3C). The same trend was observed in the case of total chlorophyll content, which reached the maximum level in leaves treated with the strain R16 and inoculated with RS as well (Fig 3D).

3.3. Description of bacterial community

Sequencing of partial 16S gene on the surface-sterilized roots (grown in sterilized or non-sterilized soil), rhizosphere, and bulk soil produced, after filtering out organellar sequences, a total of 2.47 million sequences belonging to 7205 different OTUs. Number of sequences and OTUs obtained from each compartments and treatment are reported in Table 1. Of these OTUs, 42 were shared among all compartments and treatments. Comparison between the different compartments in the non-treated controls or treated samples showeds a high variability among the different compartments: on average, only 5.7%8.4% of the OTUs weare shared among all 4 examined compartments in each treatment, ranging from 8,4% of shared OTUs between the compartments for NT (Fig 4A) to 3.9% for CC2 (Fig 4C). The highest amount of unique OTUs was registered in the rhizosphere (RH) compartment in all treatments, but it is of note that in the treated plants the amount of OTUs specific to the rhizosphere was around two times higher than in the NT control. , while values above 10% are found for the OTUs unique to the rhizosphere, soil, and roots compartment when the latter are grown in non-sterilized soil. On the contrary, roots grown in sterilized soil showed very few unique OTUs (0.5<1%). The only two compartments which showeding a highr level of similarity are rhizosphere and bulk soil, sharing approximately 25.2% of all the OTUs identified in 3 conditions out of 4 (Fig 4A, B, D), this percentage was lower in plants treated with CC2, only 16%, but this seems to be due to the higher number of OTUs shared not just among rhizosphere and soil, but also in the root endosphere (Fig 4C). Comparisons between the same compartment among different treatments showed that, regardless of the compartment, the shared OTUs between treatments weare slightly above 30%, while the remainder is affected by treatment. In particular, in roots of plants grown in non-sterilized soil (NSR), the highest number of non-core OTUs are those unique to plants treated with strain CC2 (Fig 54BA); in roots of plants grown in sterilized soil (SR) the highest number of non-core OTUs waiis found in non-treated plants (Fig 5B4C); in rhizosphere samples (RH) the highest number ofn non-core OTUs weare those shared by the three bacterial treatments, but missing in the non-treated control (Fig 5C4D); in the soil, the highest number of non-core OTUs are those unique to the 260-02 treatment (Fig 5D).

Analysis of Beta-diversity calculated with the Unifrac model highlight a vast difference between compartments: root endosphere, regardless of soil sterilization, is different from the rhizosphere and soil, which are very similar between them, although clustering separately (Fig 65A). It is interesting to note that, for the non-treated samples, there is no clear separation between bulk soil and rhizosphere, while these two compartments form are clearly separated for the treated samples. Also, for the non-treated samples, there is no distinction between bulk soil and rhizosphere, while the two compartments form separate clusters for all the treated samples.

Performing beta-diversity analysis only between the root endosphere samples highlights that there are differences between the microbiota of roots grown in sterilized soil and in non-sterilized soil (Fig 65B). Also, it is possible to see that while the non-treated samples are generally found on the left side of the graph and the treated samples are found on the middle and right side, there is no clear clustering between the different treatments.

Abundance analysis at phylum level shows that all compartments and all treatments are dominated by Proteobacteria, with other relevant phyla being Verrucomicrobia, Bacteroidetes, Actinobacteria, and Acidobacteria (Fig 6A). In particular, in root endosphere samples there is a higher abundance of Proteobacteria and lower abundance of the other four aforementioned phyla compared to soil and rhizosphere.

Moving to family level allows to better discriminate between different compartments and treatments. Regarding the Proteobacteria, which are highly abundant in all compartments, it can be seen that in the rhizosphere and soil compartments they are mostly composed by Hyphomicrobiaceae, Caulobacteraceae, and Xanthomonadaceae, while the root endosphere is dominated by Burkholderiaceae, but showing also presence of Xanthomonadaceae.

The main difference between treated and non-treated samples in the root endosphere and rhizosphere compartments is the family Oxalobacteraceae: bacteria of this family are relevantly present in root endosphere of non-treated plants and are absent in the rhizosphere of non-treated plants; on the contrary, they are present only in the rhizosphere of treated plants, and absent from the root endosphere of those plants.

Lastly, in none of the treated samples can be observed an increase in OTUs belonging to the taxonomy of the bacteria used for the inoculation (Pseudomonadaceae for 260-02, Bacillaceae for CC2, or Paenibacillaceae for R16).

Table 1. Sequencing of 16S sequencing, reporting the number of reads and OTUs (expressed as reads - OTUs) produced for each kind of sample analyzed in this study. Each row indicates a compartment (roots grown in non-sterilized soil, NSR; roots grown in sterilized soil, SR; rhizosphere, RH; bulk soil, S) while each column indicates a different treatment (non-treated, NT; strain 260-02; strain CC2; strain R16).

	NT	260-02	CC2	R16
NSR	181’906 – 2’131	107’365 – 1’572	106’560 – 1’997	88’911 – 1’525
SR	22’434 – 1’191	14’064 – 842	10’898 – 680	13’786 – 977
RH	301’272 – 2’413	254’580 – 3’357	280’308 – 3’444	247’405 – 3’682
S	217’595 – 2’007	217’721 – 1’997	177’479 – 2’226	226’864 – 2’678

4. Discussion

While the overall lower environmental impact of biological control compared to synthetic pesticides is well-established (Berg, 2009), in order to conform to the guidelines of NSA these treatments should also guarantee the quantity and quality of production, while having a minimal impact on the biodiversity found in the agroecosystem.

Regarding the yield, no direct evaluation was carried out in this specific study, but the results obtained in the biocontrol assays can indicate how effective the tested inoculants are in comparison to a commercially-available product. The experiments carried out in this study highlighted a positive biocontrol effect of one of the three assayed bacterial strains, R16, against the fungal pathogens *P. ultimum* and *R. solani*. The other treatments carried out, including a commercial *Trichoderma*-based product, managed to reduce the symptoms induced by both pathogens, although not in a statistically significantly way. Results obtained in the seed-germination assay with *R. solani* showed similar results to those obtained on grown seedlings, with strain R16 managing to cause a statistically significantly reductione in the symptoms. Interestingly, in this assay also the *Trichoderma*-based product managed to cause a statistically significantly reductione in the damage caused by the pathogen, restoring conditions similar to those of healthy plants, suggesting that the development stage of the plant can influence the effect of this product. These results are of particular relevance because, while there are several

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biocontrol agents (BCA) reported in literature as being able to antagonize either *P. ultimum* or *R. solani*~~known to control one of these soilborne pathogens are very abundant~~, BCA effective on both are very rare. Only two similar cases are currently reported in literature: *Gliocladium virens* strain G20 (Lumsden and Locke, 1989) and *Paenibacillus alvei* strain K165 (Fatouros et al., 2018). The fact that both strains R16 and K165 belong to the *Paenibacillus* genus might be an indication that further research in broad-range biocontrol strains against soilborne pathogens could become more successful by focusing on bacteria of this genus.

Despite slight variations in the quantum efficiency of the photosystem II in plants treated with different bacterial strains and pathogens, the leaves functionality was maintained in all the experimental conditions tested. In fact, in all the plants the Fv/Fm values were always above the value 0.83, which, in the case of leafy vegetables, is generally considered as a threshold between non-stressed and stressed conditions (Björkman and Demmig, 1987).

Regarding the quality of the product, the biochemical and technological analyses carried out give strong indication that there is no loss of quality related to the treatments. Chlorophyll content in treated plants was generally increased, suggesting a possible positive physiological effect on carbon fixation and visual appearance of the produce.

The only relevant change in the observed parameters is the quantity of phenols in the leaves of non-treated plants challenged with RS, which is higher than that of all other plants. This difference can be explained by an activation of defense pathways by the plants in response to the infection by RS, causing the accumulation of phenolic compounds (Cruickshank and Perrin, 1964, Toffolatti et al., 2012). In contrast, the plants inoculated with the beneficial bacteria showed a lower level of phenolic compounds, comparable to the healthy plants, as the stress caused by the pathogen is not perceived as strongly. The increase of phenolic compounds is also an important nutritional aspect, since increase in the antioxidant capacity has beneficial effects on human health. Results indicated that growing management can contribute to the enrichment of bioactive compounds in produce (Toscano et al., 2019) while protecting the crops.

This difference is also reflected in the texture of the leaves, an important feature for salads tissues which are sensitive to stressful events that can determine a loss of instrumental firmness, or crispness in sensorial terms. Considering that textural properties are one of the main perceived quality attributes of salads (Dinnella et al., 2014), it is interesting to note that the treatment with the bacterial strains aided the leaves in maintaining the textural properties of fresh salads even in the presence of pathogens.

These results suggest that the obtained biocontrol effect therefore comes at a very little, if any, cost on the plant's metabolism, supported by the lack of an increase in phenolic compounds, which are higher in plants that activate resistance pathways (Cruickshank and Perrin, 1964, Toffolatti et al., 2012). The results obtained would suggest that this response is in fact activated by exposure to RS in non-treated plants, which show a higher abundance of phenolic compounds and a tougher leaf surface, both values that could explain the development of mild *Rhizoctonia*-induced symptoms that did not lead to plant death. On the other hand, plants that were treated with bacteria and exposed to RS developed even milder symptoms without undergoing drastic changes in their metabolism.

The effect on bacterial biodiversity, both in the soil and associated to the plant, was analyzed through the 16s amplicon sequencing, producing several interesting results.

The least expected one was that, even though there was no enrichment in OTUs belonging to the bacteria used in the inoculation, the bacterial community of the treated plants was markedly different from that of the non-treated plants. This result indicates that the

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employed bacteria were either unable to colonize the plants or did so in a transient way, as is often the case with single strains inoculated in a complex microbial community. In spite of this, the effects on both the plant-associated microbial community and the symptoms caused by the pathogens are relevant. These results suggest that a high rhizosphere competence and ability to colonize the host plant's tissues may not be essential to the development of a beneficial effect, in contrast with previous research that report direct colonization of the host as a necessary step to obtain effective biocontrol (Barret et al., 2011, Ghirardi et al., 2012, Schreiter et al., 2018). The results obtained with three different inoculums show that, while the microbiota associated to the plants faces a shift when exposed to these external bacteria inoculations, the soil microbiota remains largely unaffected, suggesting that the impact on the soil biodiversity is minimal.

One explanation for the reduced symptoms that were recorded could be a direct biocontrol effect against the pathogen, expressed in the early period after the inoculation. Both strains R16 and 260-02 have been reported to have antifungal effect both in *in vitro* and *in vivo* assays (Passera et al., 2017; Passera et al., 2019) and strain CC2 belongs to the *Bacillus amyloliquefaciens* species, for which many strains are known as antifungal agents (Yu et al., 2002, Chowdhury et al., 2013).

Another explanation is that the effect was caused by the shift of microbial community in the rhizosphere and roots. It is possible that the presence of the bacteria either induced directly this shift or caused it through interactions with the plant host. The composition of the rhizosphere microbiota in the treated plants suggests that the biocontrol could be mostly mediated by an activation of the native microbiota since there is a relevant increase of Oxalobacteraceae, bacteria previously reported to have an antifungal activity and which abundance has been reported to be positively correlated with soil suppressiveness towards soilborne fungal pathogens (Cretoiu et al., 2013; Li et al., 2015).

The facts that (i) bacteria belonging to this family are not found in the bulk soil and in the non-treated rhizosphere, (ii) they are present in the non-treated endosphere, (iii) they are found in the rhizosphere of treated plants, (iv) they are not found the endosphere of treated plants, would suggest the possibility that the treatment could cause these Oxalobacteraceae to translocate from the inside of the roots to the rhizosphere, rather than being recruited from the soil.

A third hypothesis on this effect could be made regarding the increase in bacteria belonging to the Burkholderiaceae family in the endosphere of treated roots, a phenomenon which is particularly evident for the roots grown in non-sterile soil and treated with strains R16 or 260-02. This family includes the genera *Burkholderia* and *Paraburkholderia* which are well-known for their plant-beneficial effects (Depoorter et al., 2016), and have recently been described as contributing to soil suppressiveness towards *R. solani* (Carrion et al., 2018)

~~The results obtained with three different inoculums show that, while the microbiota associated to the plants faces a shift when exposed to these external bacteria inoculations, the soil microbiota remains largely unaffected, suggesting that the impact on the soil biodiversity is minimal.~~

Lastly, it is interesting to note that the composition of the salad-associated microbiota described in this study is quite different from that reported in some previous publications. For example, Cipriano and colleagues (2016) describe a lettuce-associated rhizosphere microbiota in which the most abundant genus is *Bacillus*, while in our data the whole Firmicutes phylum comes in a negligible amount. This diversity can be explained by having worked on different soils and different lettuce genotypes, stressing once again the role of the environment and of the host's genetic background when describing microbial communities (Bulgarelli *et al.*, 2015).

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For this reason, studies aimed at describing the effect of host-pathogen-microbiota, especially in the scope of sustainable production, must keep in mind that also the crop genotype, deeper than species level, is an essential variable in determining the success or failure of a biocontrol agent inoculation.

5. Conclusion

In conclusion, our study provides useful elements for the evaluation of the use of bacterial inoculants as biocontrol agents for the production of salads in a NSA-driven perspective, indicating an effect against symptoms induced by soil-borne pathogens, without showing adverse effects for the plant physiology or the biodiversity of the soil.

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Figure Captions

Figure 1. Results of the biocontrol assays. A) symptoms observed in plants challenged with *Pythium ultimum* PU. B) symptoms observed in plants challenged with *Rhizoctonia solani* RS. In both graphs, the Y-axis reports the infection percentage index (I%) while the X-axis reports the different weeks of observation. The black dotted line represents the NT plants, the green line with square-shaped dots represent plants treated with strain CC2, the blue line with rhomboid dots represent plants treated with Trichoderma, the yellow line with triangle-shaped dots represent plants treated with strain 260-02, and the red line with circular dots represent plants treated with strain R16. Different letters (a,b) on the right side of the lines indicate statistically significant differences in the results throughout the three weeks of observation, determined by a general linear model, optimized for repeated measures, followed by the Bonferroni~~Tukey's exact~~ post-hoc test ($P < 0.05$). C) graph representing the results obtained in the germination assays. The Y-axis reports the germination percentage of the seeds, while the X-axis represents the different treatments. Dark grey bars represent plants grown in healthy soil without RS, while the white bars represent plants grown in soil containing RS. Different letters (a, b, c) on top of the bars indicate statistically significant differences among the results, according to a One-Way ANOVA followed by the Bonferroni~~Tukey's exact~~ post-hoc test ($P < 0.05$).

Figure 2. Photosynthetic efficiency. The chlorophyll *a* fluorescence indexes measured *in vivo* on dark-adapted romaine lettuce (*Lactuca sativa* L. var. longifolia) leaves grown under different growing conditions. A: performance index (PI), B: maximum quantum efficiency of photosystem II (Fv/Fm). Values are means ± SE (n = 6). Different letters (a,b) indicate statistically significant differences among the results, according to a Two-Way ANOVA followed by the Bonferroni post-hoc test ($P < 0.05$).~~($P < 0.05$).~~

Figure 3. Physiological parameters. Phenolic index (A), total carotenoids (B) and total chlorophyll (C), measured in Romaine lettuce (*Lactuca sativa* L. var. longifolia) leaves grown under different growing conditions. The values are means ± SE (n = 3). Different letters (a,b,c,d)

indicate statistically significant differences among the results, according to a Two-Way ANOVA followed by the Bonferroni post-hoc test ~~among different cycles~~ ($P < 0.05$).

Figure 4. Microbiota: OTU distribution within the same treatments. Venn diagrams showing the comparative distribution of OTUs in the different ~~treatments and/or~~ compartments analyzed for the same treatment. Each circle is labeled with the compartment (NSR – endosphere of root grown in non-sterilized soil, in blue; SR – endosphere of root grown in sterilized soil, in yellow; RH – rhizosphere, in green; S – soil, in red) and treatment (NT – non-treated; CC2 – inoculated with strain CC2; 260-02 – inoculated with strain 260-02; R16 – inoculated with strain R16). A) Comparison between all four investigated compartments in non-treated plants; B) Comparison between all four investigated compartments in plants treated with strain 260-02;~~comparison between the NSR compartment in all four treatments;~~ C) Comparison between all four investigated compartments in plants treated with strain CC2;~~comparison between the SR compartment in all four treatments;~~ D) Comparison between all four investigated compartments in plants treated with strain R16;~~comparison between the RH compartment in all four treatments; E) comparison between the S compartment in all four treatments~~.

Figure 5. Microbiota: OTU distribution within the same compartment. Venn diagrams showing the comparative distribution of OTUs in the different treatment for each compartment analyzed. Each circle is labeled with the compartment (NSR – endosphere of root grown in non-sterilized soil; SR – endosphere of root grown in sterilized soil; RH – rhizosphere; S – soil) and treatment (NT – non-treated, in green; CC2 – inoculated with strain CC2, in yellow; 260-02 – inoculated with strain 260-02, in blue; R16 – inoculated with strain R16, in red). A) Comparison between the NSR compartment in all four treatments; B) comparison between the SR compartment in all four treatments; C) comparison between the RH compartment in all four treatments; D) comparison between the S compartment in all four treatments.

Figure ~~6~~5. Microbiota: Beta-diversity. Graphs reporting the distribution of the samples according to beta-diversity calculated with a weighted Unifrac index~~UNIFRAC model~~. A) graph with all samples; different shape of the markers indicates different compartments and different colors indicate different treatments, as reported in the legend. B) graph reporting only root endosphere samples; different shape of the markers indicates roots grown in sterilized or non-sterilized soil, different colors indicate different treatments, as reported in the legend. Circles were added to highlight the different clusters of samples.

Figure ~~7~~6. Microbiota: relative abundance. Graphical representations of the abundance at different taxonomic levels of the microbiota. Stacked bar plots representing the relative abundance of each taxonomical unit (graph A, clustered at phylum level; graph B, clustered at family level) among the samples. Y-axis reports the relative abundance of the considered taxonomic order, while the X-axis reports the different treatments, divided by compartments in the grid. For ease of interpretation, each graph reports only the most abundant taxonomical groups (cutoff 1%); when a stacked bar does not reach 100%, the missing values belong all to taxonomical groups with abundance lower than the cutoff threshold.

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