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Use of bacterial strains antagonistic to *Escherichia coli* for biocontrol of spinach: A field trial

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ABSTRACT

To counteract global food safety hazards related to raw consumption of ready-to-eat leafy vegetables, a method to improve bacterial status using antagonistic bacteria was studied under field conditions. This is the first study to identify potential *Escherichia coli* antagonists from the native microbiota on leafy green vegetables and evaluate their effect in an industrial field production setting. Bacterial strains were isolated from different types of leafy green vegetables and selected upon their effect against *E. coli in vitro*, and out of 295 tested bacterial strains, 37 showed an antagonistic effect. Four of those antagonistic strains were coated in separate treatments onto spinach seeds and planted in the field. Both seeds and plants were analyzed by Illumina MiSeq next generation sequencing (NGS), and it was seen that the microbiota of the plants contained lower relative abundance of plant and human pathogenic genera. Higher β-diversity was observed for the samples treated with *Bacillus coagulans* LMG P-32205 and *B. coagulans* LMG P-32206 compared to control, indicating that those strains have induced substantial changes in the native microbiota of the leaves. A reduction of *Escherichia-Shigella* was seen for two of the isolates (*Pseudomonas cedrina* LMG P-32207 and *Pseudomonas punonenis* LMG P-32204) as the seeds developed into plants. Seeds inoculated with two of the strains (*B. coagulans* LMG P-32205 and *B. coagulans* LMG P-32206) had increased levels of *Lactobacillaceae*, and treatment with *B. coagulans* LMG P-32206 resulted in lower levels of *Pantoea* (from 31.4 to 12.2%).

These results encourage the usage of bacterial antagonists as part of a global solution to reduce the risk of human pathogens on leafy green vegetables.

1. Introduction

Leafy green vegetables are included in a large number of diets around the world and considered a nutritious and healthy food component. However, as the demand of fresh and convenient vegetables increases, so does the number of food-borne illness outbreaks worldwide (Harris et al., 2006; Long, Adak, O'Brien, & Gillespie, 2002; Parish et al., 2003). Contamination with human pathogens can occur in the whole production chain, and as the product is eaten raw, microorganisms are difficult to remove with tap water (Castro-Rosas et al., 2012; Doyle & Erickson, 2008; Uhlig et al., 2017). The efficacy of chlorine treatments aimed to reduce the bacterial load is questioned and believed to pose health and environmental risks. The use of chlorine is also forbidden in many countries (Beuchat, 1998; Gil, Selma, López-Gálvez, & Allende, 2009; McKellar, 2007; Parish et al., 2003; Stiles, 1996). The principle of biopreservation, or biological control, is to instead use antagonistic bacteria or their metabolites in order to control the growth and survival of pathogens. Antagonistic bacteria can be used as a preventative approach to change the native microbiota in a healthier direction for human consumption, reducing the need for preservatives in fresh produce. The posed mechanisms behind the antagonistic effect are for example competition of nutrients or physical space, or production of antagonistic compounds (Ananou, Maqueda, Martínez-Bueno, & Valdivia, 2019; Johnston, Harrison, Morrow, & R., 2009; Lopez-Velasco, Tydings, Boyer, Falkinham, & Ponder, 2012). In previous studies, biological control has been used with bacteria isolated from fresh produce to prevent *Salmonella* Chester and *Listeria monocytogenes* on green pepper discs (Liao & Fett, 2001). Mixed isolates of the native microbiota of the endive were found to protect against L. *monocytogenes* (Carlin, Nguyen-The, & Morris, 1996) and strains of lactic acid bacteria inhibited

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Aeromonas hydrophilia, *L. monocytogenes*, *Salmonella enterica* and *Staphylococcus aureus* (Vescovo, Torriani, Orsi, Macchiarolo, & Scolari, 1996). In a study performed by Oliveira et al. (2015), five genera from whole and fresh-cut vegetables were also found to reduce *S. enterica* more than 1 log unit on lettuce discs. Although *E. coli* is one of the most common and severe pathogens that can be found on leafy greens, only a few investigations have been performed to prevent its growth through biopreservation. In two previously performed studies, *Enterobacter cloacae* reduced *E. coli* O157:H7 by 1 log CFU/g on lettuce (Jablasone, Warriner, & Griffiths, 2005), and G. Lopez-Velasco et al. (2012) found a range of bacteria that inhibited the growth of *E. coli* O157:H7 on sterile spinach leaves. In a third study, bacteria isolated from iceberg lettuce and spinach that were shown to inhibit *E. coli* O157:H7, mostly belonged to the genera *Pantoea*, *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Aeromonas* and *Burkholderia* (Johnston et al., 2009). However, in biopreservation is it important, to focus on antagonistic bacteria that are non-pathogenic, concerning both humans and plants. Up to the present time, the effect of antagonistic isolates on human pathogens have not been tested on live plants, and especially not in real production settings, which is crucial in order to evaluate the potential for commercial use.

The aim of this project was to isolate *E. coli* antagonistic bacteria from leafy green vegetables and study their potential as biocontrol agents for modification of the microbiota of plants in the field.

2. Materials and methods

2.1. Isolation, in vitro testing and identification of antagonists

To isolate potential antagonistic bacteria, five bags (65 g fresh weight) of iceberg lettuce (*Lactuca sativa* var. *capitata nidus tenerimma*), Swiss chard (*Beta vulgaris* subsp. *cicli*), spinach (*Spinacia oleracea*), mangold, spinach and rocket (*Eruca sativa* ssp. *sativa*) were purchased at a local supermarket in Lund, Sweden. Additionally, 30 bags of rocket were collected from a local leafy-greens producer. Microbial culturing was performed according to Uhlig et al. (2017) using the following growth media: Tryptic Soy Agar (TSA, Sigma-Aldrich, St. Louis, MO, USA), Casamino Acids Yeast Extract Dextrose agar (YCED: yeast extract, 0.3 g; casamino acids, 0.3 g; D-glucose, 0.3 g; K_2HPO_4, 2.0 g; agar, 18 g; distilled water 1000 ml), Water Yeast Extract agar (WYE: yeast extract, 0.25 g; K_2HPO_4, 0.5 g; agar, 18 g; distilled water 1000 ml), Raffinose-histidine agar (Raffinose, 10 g; L-histidine, 1 g; $MgSO_4*7H_2O$, 0.5 g; $FeSO_4*7H_2O$, 0.01 g; NaCl, 20 g; agar, 18 g; distilled water, 1000 ml), Casein-salt agar (Casein, 0.3 g; KNO_3, 1.25 g; NaCl, 47.25 g; K_2HPO_4, 1.25 g; $MgSO_4*7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4*7H_2O$, 0.01 g; agar, 18 g; distilled water, 1000 ml), ISP2 (yeast extract, 4 g; malt extract, 10 g; D-glucose, 4 g; agar, 18 g; distilled water, 1000 ml), and malt agar (Sigma-Aldrich, St. Louis, MO, USA).

Incubation proceeded for 3 days at 30 °C for TSA, 7 days at 30 °C for YCED, WYE, Raffinose, Casein, ISP2 and ISP5 agar, and 7 days at 20 °C for malt agar. Colonies were isolated, pure cultured and stored in freezing medium (K_2HPO_4, 0.85 g; KH_2PO_4, 0.2; Tri-sodium-citrate-dehydrate, 1.5 g; $MgSO_4x7H_2O$, 0.25 g; glycerol (99.5%), 121.5 ml; distilled water, 875 ml (Ahrné, Molin, & Ståhl, 1989)) at −80 °C. Isolates were tested for antagonism against *E.coli* CCUG 29300^T with the perpendicular streak method (Egorov, 1985) on TSA plates. The culture stored in freezing medium was streaked directly on the plate in a line and incubated for 3 days at 30 °C. After incubation, *E. coli* from freezing culture was streaked perpendicularly to the isolate and the plate was further incubated for 24 h at 30 °C. The antagonistic effect was read as the presence of an inhibition zone larger than 3 mm, or a gradient on the line of *E. coli*. This test was performed for 295 isolates. Examples of the two cases can be seen in Fig. 1.

The same test was repeated six years later to evaluate whether the effect changes during storage at −80 °C. Two isolates recently isolated from edible plants, *Bacillus coagulans* LMG P-32205 and *B. coagulans* LMG P-32206, were tested against *E. coli* by the agar plug diffusion method described by Balouiri, Sadiki, and Ibnsouda (2016), due to the inability of *B. coagulans* to grow on the same nutrient agar as *E. coli*. Shortly, the *B. coagulans* isolates were grown in dense mats on MRS agar (Merck KGaA, Darmstadt, Germany) for 7 days at 37 °C, and *E. coli* on Tryptic Soy Agar (Sigma-Aldrich) for 24 h at 37 °C. Plugs (1 cm^2) of *B. coagulans* were cut out and transplanted into the *E. coli* plates. The antagonistic effect was read as the presence of circle around the *B. coagulans* plug where *E. coli* could not grow.

2.1.1. DNA purification and Sanger sequencing of antagonistic isolates

Isolates that showed antagonism *in vitro* were DNA purified and sequenced by Sanger sequencing according to Uhlig et al. (2017). In short, purified isolates were suspended in physiological saline following a bead beating step according to Ahrné et al. (1989), and the supernatant was used for PCR, amplifying an approximately 1500 bp long fragment of the rRNA gene (16S) using the forward primer ENV1 (5'-AGAGTTT-GATIITGGCTCAG-3′) and the reverse primer ENV2 (5'-CGG ITA CCT TGT TAC GAC TT-3′) (Eurofins Genomics, Ebersberg, Germany, (Pettersson et al., 2003). The results were confirmed by gel electrophoresis. The PCR products were sent for Sanger sequencing at Eurofins Genomics (Ebersberg, Germany) on an ABI 3130xl Genetic analyzer (Applied biosystems, Foster City, CA, USA) using ENV1 as sequencing primer. The sequenced genes were trimmed to between 590 and 788 bp depending on sequence quality and compared to type strain sequences at the Ribosomal Database project (RDP) by the Seqmatch software (Cole et al., 2013).

2.2. Field trial

Four strains displaying *in vitro* inhibition of *E. coli* were deposited under the Budapest treaty in Belgian coordinate collections of microorganisms/Labroatorium voor Microbiologie-Bacterenverzamleling (BCCM/LMG) and were assigned the following accession numbers: *Pseudomonas cedrina* LMG P-32207, *P. punonenis* LMG P-32204,

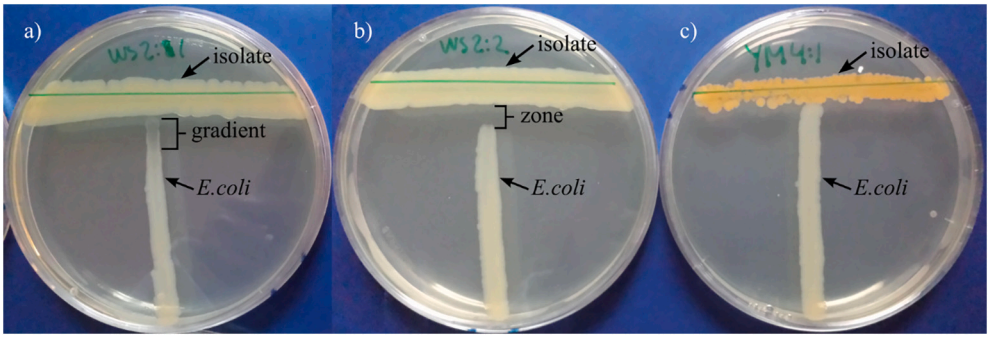

Fig. 1. Perpendicular streak method according to Egorov (1985). a) Antagonistic effect against *E. coli* shown as a gradient where *E. coli* is less prone to grow. b) Antagonistic effect against *E. coli* is shown as a measurable inhibition zone where *E. coli* is not able to grow. c) A negative result, no inhibition zone can be visualized.

B. coagulans LMG P-32205 and *B. coagulans* LMG P-32206. These strains were evaluated with regard to their impact on the microbial community of seeds and leaves in a commercial field setting with spinach as model crop. Seed inoculum was prepared by incubating 1 µl pure culture with 50 ml Tryptic Soy Broth (Sigma-Aldrich) for 24 h at 30 °C for *Pseudomonas* isolates and MRS broth (Merck KGaA) for 6 days at 38 °C for *Bacillus coagulans* isolates. After incubation, the isolates were washed in physiological saline and diluted to a total volume of 40 ml in freezing media, and stored at −80 °C until seed coating treatment.

Spinach seeds (Spinach 'Yuma' F1/PV1245, Batch # 366948 2018/2019, Pop Vriend Seeds, Andijk, The Netherlands) were divided into six treatment groups of 1100 g each as follows; normal control (NC) contained untreated seeds, coated control (CC) with seeds coated only with freezing media; group A with seeds coated with *P. cedrina* LMG P-32207 ($3.6*10^9$ cfu/g seed), group P2 with seeds coated with *P. punonensis* LMG P-32204 ($8.2*10^6$ cfu/g seed), group B1 with seeds coated with *B. coagulans* LMG P-32205 ($9.1*10^5$ cfu/g seed) and group B2 with seeds coated with *B. coagulans* LMG P-32206 ($9.1*10^5$ cfu/g seed). Seeds were coated with a laboratory coating machine SATEC Concept ML 2000 (SATEC Equipment GmbH, Elmshorn, Germany) for 2 min and dried with a laboratory drier SUET 819214/002 (SUET Saat- und Erntetechnik GmbH, Eschwege, Germany) for 4 min at room temperature. One-hundred g of seeds from each group were taken out after treatment for NGS sequencing.

The field trial was carried out in cooperation with an industrial partner in the south of Sweden between 3 and 23 of August 2019 (Air temperature: 23–26 °C daytime, 11–16 °C nighttime, average day length 15 h, 15 min). The crops were sown in one raised bed (length: 120 m, breadth: 1.3 m), with each treatment group dedicated to 20 m, in which six plots of 1 m^2 were evenly staked out (Fig. 2). Each 1 m^2 plot was considered one replicate. Harvesting was performed 20 days later according to Darlison et al. (2019) using aseptic technique by cutting the spinach leaves within the plots 1.5 cm above the soil with disinfected scissors and gloves. The leaves were transferred to plastic bags, sealed and immediately put in a cool box until analysis in the laboratory. All equipment was disinfected between replicates.

2.2.1. Next generation sequencing of bacterial biota on seeds and plants

DNA extraction of leaves and seeds was performed according to Tatsika, Karamanoli, Karayanni, and Genitsaris (2019), with some modifications. Samples of 2 g of plant material were placed in test tubes with 20 ml PBS (Oxoid Ltd., Blastingstoke, UK) and thereafter sonicated for 10 min. The plant material was removed and the remaining liquid centrifuged 20 min at 11600 xg. The supernatant was discarded and the remaining pellet stored at −18 °C until further processing by DNA purification. DNA purification was performed using the Nucleiospin® Soil Kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. DNA concentration was measured using Qubit ™ 1× ds DNA HS Assay Kit (Life Technologies Corporation, Eugene, OR, USA). The PCR primers B969F (ACGCGHNRAACCTTACC) and BA1406R (ACGGGCRGTGWGTRCAA) (Eurofins Genomics, Ebersberg, Germany) were used with PCR reagents Kapa HiFi Hotstart Ready Mix (Kapa Biosystems Pty (Ltd), Salt River, Cape Town, South Africa) to amplify 470 bp of the V6-V8 hyper variable region of the 16S rRNA gene with the following PCR program: 25 cycles at 95 °C for 30 s, 25 °C for 30 s and 72 °C for 30 s, and a hold period of 5 min at 72 °C. The PCR products were purified using AMPure XP beads (Beckman Coulter Genomics, Brea, CA, USA). An index PCR was run at 95 °C for 3 min followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and the products were again measured with Qubit (Life Technologies). The length of the amplified fragments was measured on random samples (Tatsika et al., 2019) with Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbrunn, Germany) and Agilent DNA kit (Agilent Technologies, Vilnius, Lithuania), according to manufacturer's instructions after both PCR runs. The indexed samples were diluted to 4 nM with resuspension buffer (Illumina, San Diego, CA, USA) and sequenced on Illumina MiSeq with MiSeq reagent kit v3 (600-cycle) according to manufacturer's instructions. PhiX (Illumina) was used as internal control. The final loading volume was 600 µl.

The produced reads were demultiplexed by Illumina CASAVA 1.8 (Illumina) and filtered using DADA2 (Callahan et al., 2016) in Qiime 22,020.6 (Bolyen et al., 2019) and then further processed in R (R Team, 2006). The total number of reads after filtering was 3,128,913 and the mean number of reads per sample was 65,186. The sequences were trimmed at the ends at 25 bp left and 275 bp right. Samples containing <5000 reads were removed. Taxonomic classification of the remaining reads was made using the SILVA 132 database (Quast et al., 2013). Reads identified as eukaryotic, mitochondria or chloroplasts were removed.

2.3. Calculations and statistical analysis

From the NGS data, relative abundance levels were calculated by the sum of operational taxonomic units (OTUs) for all replicates divided by total number of OTUs. Due to low replicate numbers among a few groups, analyses were performed with non-parametric methods. Relative abundance levels and α-diversity, defined as the variation of species within a sample was calculated byChao1 and Shannon indices and compared groupwise by Kruskal-Wallis one-way analysis of variance on ranks. Two groups were compared with Mann-Whitney rank sum test. β-diversity, defined as the variation of species between samples, was calculated with weighted UniFrac and compared by permutational multivariate analysis of variance (PERMANOVA). The differential abundance between groups were analyzed on genus level by DESeq2 (Anders & Huber, 2010) with a *p*-value cut-off at 0.001. All calculations and statistical analysis, except β-diversity, were performed using R version 3.6.3 (R Team, 2006), Vegan package version 2.6 (Oksanen et al., 2019). β-diversity was calculated using Qiime 22,020.6 (Bolyen et al., 2019).

3. Results

3.1. Isolation, in vitro testing and identification

Out of 295 isolates tested with the perpendicular streak method, at the first test in year 2014, 18 showed a measurable, clear zone (> 3 mm) where the *E. coli* could not grow, and in 21 isolates, a gradient on the *E. coli* line could be observed. The isolates that showed antagonistic

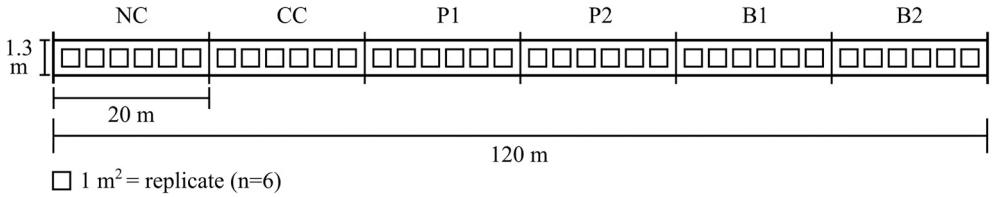

Fig. 2. Experimental design of field trial. Group NC: untreated seeds, CC: coated control, P1: *Pseudomonas cedrina* LMG P-32207, P2: *P. punonensis* LMG P-32204, B1: *B. coagulans* LMG P-32205 and B2: *B. coagulans* LMG P-32206. Each 1 m^2 plot was considered one replicate. Each replicate was distanced 15 cm to the edge of the bed on both sides, and 20 cm to the next replicate within the same group. The distance to a replicate belonging to a different group was 40 cm.

effect were sequenced by their 16S rRNA gene and their putative identities are presented Table 1, together with their antagonistic effect in 2014 and in 2020. The antagonistic isolates belonged primarily to the families *Micrococcaceae*, *Bacillaceae* and *Pseudomonadaceae*. The *Bacillus* isolates mostly exhibited antagonism in the form of a clear zone that did not disappear during storage. The isolated *Pseudomonas* sp. showed both zone and gradient, and about half of the isolates still displayed inhibition of *E. coli* after 6 years of storage, whereas no inhibition of *E. coli* was found by any of the isolated *Arthrobacter* isolates after storage. For the two *B. coagulans* isolates, tested by the agar plug diffusion test, a gradient on the *E. coli* plate was observed before and after storage.

3.2. Field trial

To evaluate the microbiota changes of seeds and plants *in vivo* in the field upon administration of selected antagonists, spinach seeds were coated with *Pseudomonas cedrina* LMG P-32207, *P. punonensis* LMG P-32204, *B. coagulans* LMG P-32205 and *B. coagulans* LMG P-32206 separately. A visual inspection of the plants at the time of harvest did not show any quality imperfections. Bacterial DNA extracted from both seeds and plants were sequenced by Illumina MiSeq NGS sequencing. Due to low sequencing depth, 7 of 18 seed replicates, and 2 of 36 replicates had to be removed. On both seeds and leaves, the most abundant phylum was Proteobacteria, 67.6% and 67.4% respectively. On seeds, the second most abundant phylum was Bacteriodetes (19.3%), followed by Actinobacteria (9.2%), but on leaves the second most common phylum was Firmicutes (31.1%) followed by Bacteriodota (1.0%).

The data on family level is visualized in the taxa bar plot in Fig. 3. The most abundant taxa on seeds were *Pseudomonadaceae* (22.4%) followed by *Erwiniaceae* (17.1%), *Springomonadaceae* (10.0%), *Weeksellaceae* (8.9%) and *Sphingobacteriaceae* (8.7%). On leaves, the most abundant taxa were *Erwiniaceae* (34.5%), *Exiguobacteriaceae* (28.6%) and *Pseudomonadaceae* (13.2%). No statistical differences were found on family level for leaf samples NC_L, $P1_L$, $P2_L$, $B1_L$ and $B2_L$ compared to coated control (CC_L). The most abundant genera on seeds were *Pseudomonas* (22.0%), followed by *Pantoea* (11.9%), and on leaves, the most abundant were *Exiguobacterium* (28.6%) *Pantoea* (20.1%) and *Pseudomonas* (13.2%).

The differential abundance of NC, P1, P2, B1 and B2 *vs.* CC on genus level can be seen in the log 2 fold change plots in Fig. 4. The abundance of amplicon sequence variants (ASVs) belonging to *Pseudomonas* on the seeds were higher in uncoated seeds compared to coated control (NC_S *vs* CC_S). Seeds coated with *P. cedrina* and *P. punonensis* ($P1_S$ and $P2_S$ *vs* CC_S) had a higher ASV abundance of *Escherichia-Shigella*, while coating with *B. coagulans* strains ($B1_S$ and $B2_S$ *vs* CC_S) raised the ASV abundance of *Pseudomonas* and *Lactobacillus*.

The seed coating process also rendered many changes in the microbiota of the leaves, among others *Pantoea* and *Pseudomonas* species. Higher ASV abundance of *Erwinia* was observed in coated control (CC_L) compared to leaves from untreated seeds (NC_L). All antagonistic stains on the leaves lower the ASV abundance of *Erwinia* or *Pectobacterium* compared to coated control, and group $B2_L$ (*B. coagulans* LMG P-32206) displayed a lower ASV abundance of *Pantoea*.

When comparing seeds and leaves from the same group (Fig. 5), a large range of genera were in higher ASV abundance on the seeds compared to leaves. In groups P1 and P2, *Escherichia-Shigella* was in higher ASV abundance on seeds than on leaves.

Due to technical limitations (lack of sequencing depth), it was not possible to identify the antagonistic strains on seeds and leaves.

The Chao1 index, indicating species richness, was between 217.0 and 303.3 for seeds and between 82.0 and 98.0 for leaves. The Shannon index was between 4.3 and 4.7 for seeds and between 2.4 and 2.8 for leaves. Chao1 and Shannon indices were neither significantly different within seed groups (NC_S, CC_S, $P1_S$, $P2_S$, $B1_S$ and $B2_S$) and leaf groups (NC_L, CC_L, $P1_L$, $P2_L$, $B1_L$ and $B2_L$), nor when comparing leaf and seed samples for the same treatment.

Table 1
Putative identification by 16S rRNA gene Sanger sequencing of isolates exhibiting inhibition on the growth of *E.coli* CCUG 29300^T *in vitro* year 2014 and after six years of storage at −80 °C in 2020. *B. coagulans* isolates were tested against *E. coli* by the agar plug diffusion method, and all other isolates were tested with the perpendicular streak method.

ID no.	Closest type strain name and original designation	Similarity (%)	*In vitro E. coli* antagonism year 2014[a]	*In vitro E. coli* antagonism year 2020[a]
Family: *Micrococcaceae*				
MR2z	*Arthrobacter arilaitensis* (T) CIP 108037	99	++	–
RR4a	*A. arilaitensis* (T) CIP 108037	100	+	–
RR8y	*A. arilaitensis* (T) CIP 108037	99	+	–
WM4.1	*Arthrobacter chlorophenolicus* (T) A-6	99	+	–
RR8x	*Arthrobacter oxydans* (T) DSM 20119	100	+	–
Family: *Bacillaceae*				
RR10b	*Bacillus aerophilus* (T) 28 K	99	++	++
MR2y	*Bacillus amyloliquefaciens* (T) FZB42	100	++	–
YC19.1	*B. amyloliquefaciens* (T) FZB42	100	+	++
LMG P-32205#	*Bacillus coagulans* (T) NBRC 12583	100	+	+
LMG P-32206#	*B. coagulans* (T) NBRC 12583		+	+
MR6x2	*Bacillus invictae* (T) Bi.FFUP1	98	++	++
RR10a	*Bacillus safensis* (T) FO-036b	100	++	++
RR9b	*B. safensis* (T) FO-036b	100	++	++
Family: *Comamonadaceae*				
TS39.1	*Delftia tsuruhatensis* (T) T7	100	+	–
Family: *Paenibacillaceae*				
TSI4.2a	*Paenibacillus amylolyticus* (T) NRRL NRS-290 T	100	++	–
Family: *Erwiniaceae*				
YM2.2	*Pantoea agglomerans* (T) DSM 3493	100	+	–
RR9y	*Pantoea eucalypti* (T) LMG24197	99	+	+
Family: *Pseudomonadaceae*				
YC39.2	*Pseudomonas azotoformans* (T) IAM1603	100	+	–
LMG P-32207#	*Pseudomonas cedrina* (T) CFML 96–198	100	++	++
RR1.2b	*P. cedrina* (T) DSM 14938	100	++	–
WS2.2	*Pseudomonas constantinii* (T) CFBP 5705	100	++	–
WS5.1	*Pseudomonas extremaustralis* (T) CT14-3	100	+	–
TM3.2	*Pseudomonas fulva* (T) AJ2129	100	+	++
TS20.1	*P. fulva* (T) AJ2129	100	+	–
TS5.2	*P. fulva* (T) AJ2129	100	++	++
YC27.1	*P. fulva* (T) AJ2129	99	+	–
WM5.1	*Pseudomonas grimontii* (T) CFML 97-514 T	98	+	++

(*continued on next page*)

Table 1 (*continued*)

ID no.	Closest type strain name and original designation	Similarity (%)	*In vitro E. coli* antagonism year 2014[a]	*In vitro E. coli* antagonism year 2020[a]
	Pseudomonas helmanticensis (T)			
TS13.1	OHA11	100	+	–
	Pseudomonas libanensis (T) CIP			
WS2.1	105460	99	++	+
	Pseudomonas orientalis (T) CFML			
TS36.2	96–170	100	++	–
	P. orientalis (T) CFML			
TSI4.2	96–170	100	+	–
	Pseudomonas			
WY39.2	*protegens* (T) CHA0	100	++	++
LMG P-32204#	*Pseudomonas punonensis* (T) LMT03	100	++	+
Family: *Rhizobiaceae*				
	Rhizobium nepotum			
I2R6x	(T) 39/7	99	++	–
	Rhizobium nepotum			
WS1.1	(T) 39/7	98	+	–
	Rhizobium nepotum			
WS3.1	(T) 39/7	100	+	+
	Rhizobium nepotum			
WS5.2	(T) 39/7	100	+	++
Family: *Corynebacterineae*				
	Rhodococcus cerastii			
MR5x	(T) C5	100	++	++
Family: *Xantomonadaceae*				
	Xanthomonas arboricola (T) LMG			
MR2x	747 T	100	++	–

[a] + Antagonism shown as gradient, ++ Antagonism shown as a clear zone >3 mm

Isolates selected for field trial.

The β-diversity of the leaf samples which can be seen in Fig. 6 is higher in groups B1 and B2 compared to coated control, CC.

4. Discussion

The present study aimed to investigate changes in the bacterial community composition of spinach seeds and field grown spinach leaves after seed inoculation with isolated and selected strains displaying inhibition of *E. coli* CCUG 29300 T under *in vitro* conditions. The study does not provide information on the interactions with STEC-pathovars *in vitro* or *in vivo*.

Isolation of antagonistic isolates were performed from bagged products, since it is important that they are able to survive the production processes to be used as biocontrol agents. Therefore, isolating viable strains from products in the form that it reaches the consumer is preferable to ensure their viability in the products. Identification of the 16S rRNA gene of the antagonistic isolates revealed that most belonged to the *Micrococcaceae*, *Bacillaceae* and *Pseudomonadaceae* families. *Pseudomonas* spp. are well known to exhibit antagonistic properties to human pathogens, particularly towards *E.coli* O157:H7 (Duffy, Whiting, & Sheridan, 1999; Janisiewicz, Conway, & Leverentz, 1999; McKellar, 2007; Schuenzel & Harrison, 2002), *Listeria monocytogenes* (Buchanan & Bagi, 1999; Carlin et al., 1996; Liao & Fett, 2001) and *Salmonella* (Liao & Fett, 2001; Matos & Garland, 2005). McKellar (2007) reported that the growth of *E. coli* O157:H7 was repressed in depleted medium from *P. fluorescens*, and replenishment of the media or its components reversed the effect, concluding that nutrient limitation was the mechanism of action. *Pseudomonas* spp. are also known for their ability to produce siderophores, iron chelating metabolites, which could be of advantage in iron-poor environments (Faraldo-Gómez & Sansom, 2003) and have an antagonistic effect, but it could also be due to production of unidentified antimicrobial substances (Fett, 2006; Liao & Fett, 2001). *Bacillus* spp. have also proven to be efficient biocontrol agents against human pathogens such as L. *monocytogenes* and *Candida albicans* (Shafi, Tian, & Ji, 2017) as well as *Yersinia enterocolitica*, *Salmonella enterica* and *E. coli* O157:H7 (Liao, 2009). It is also worth noting that the antagonistic capability was different between isolates that belong to the same species, meaning that antagonism seem to be strain specific.

The antagonistic isolates were tested again after being stored at −80 °C for 6 years without interruption. Some of them, mostly belonging to the *Arthrobacter* and *Rhizobium* genera, had lost their effect, possibly indicating a plasmid carried trait. Some isolates kept the same ability to counteract *E. coli* over time, most of them belonging to the *Bacillaceae* family. These results strongly suggest that it is important to verify that the antagonistic effect is stable over time when considering a strain for commercial application.

Inoculating seeds with specific bacterial strains to enhance plant growth and suppress plant pathogens has previously been performed (Hultberg & Waechter-Kristensen, 1998; Jaber, 2018; Merriman, Price, & Baker, 1974; Zalila-Kolsi et al., 2016). Inoculating seeds with bacteria to reduce human pathogens is much less studied. In a study by Rossi and Lathrop (2019), alfalfa seeds inoculated with a mix of *Lactiplantibacillus plantarum*, *Pediococcus acidilactici*, and *Pediococcus pentosaceus* decreased the concentration of *Salmonella* and L. *monocytogenes* significantly with 1.0 log CFU/g and 4.5 log CFU/g respectively, and the inoculation did not affect sprout quality. Shen, Mustapha, Lin, and Zheng (2017) found that seed inoculation with three strains of *Bacillus subtilis* from lettuce and mung beans could significantly reduce the internalization of *S. enterica* and enterohemorrhagic *E. coli* in mung bean sprouts.

In this study, the impact of *E. coli* antagonistic isolates on the bacterial community composition of spinach seeds and leaves was tested in a commercial field production system. Spinach seeds were coated in a commercial system with four different isolates that showed antagonistic effect *in vitro* (Table 1), *P. cedrina* LMG P-32207 (group P1), *P. punonensis* LMG P-32204 (group P2), *B. coagulans* LMG P-32205 (group B1 and B2) and *B. coagulans* LMG P-32206 (group B2).

The 16S metagenomics sequencing with Illumina MiSeq rendered low sequencing depth from some samples, and only samples with high quality data was considered. The analysis revealed that the most prevalent phyla on spinach leaves and seeds were Proteobacteria, coherent with other NGS studies on leafy green vegetables (Jackson, Randolph, Osborn, & Tyler, 2013; G. Lopez-Velasco, Carder, Welbaum, & Ponder, 2013; Gabriela Lopez-Velasco, Welbaum, Boyer, Mane, & Ponder, 2011; Tatsika et al., 2019).

To investigate the microbiota changes upon administration of the *E. coli* antagonistic isolates in commercial field production conditions, we used a natural setting without artificial spreading of *E. coli* which might otherwise have required special permission from the authorities. A study in a natural field production setting mirrors the exposure to normally occurring potential pathogens and evaluate the capacities of the antagonistic isolates to influence the microbiota of the leaves in a complex ecosystem. The trial was based on an allocated area of 156 m^2, and the six replicates per treatment was based on a study performed by Söderqvist et al. (2019) where the prevalence of *E. coli* and *Enterobacteriaceae* was mapped from the same area where the present trial was conducted. A trial in a real production setting is valuable prior to a standardized trial with inoculation of selected pathogenic strains, to prove that the antagonists will be able to affect the microbiota under normal cultivation conditions.

During analysis, attention was drawn to the relative abundance of native *Escherichia* species on seeds and leaves. On seeds, the relative abundance was higher ($p < 0.001$) in group P1 (3.3%) and P2 (5.6%) than the coated control (CC) (0.01%). When comparing the uncoated seeds (NC_S) to coated control (CC_S), the only differences in relative ASV

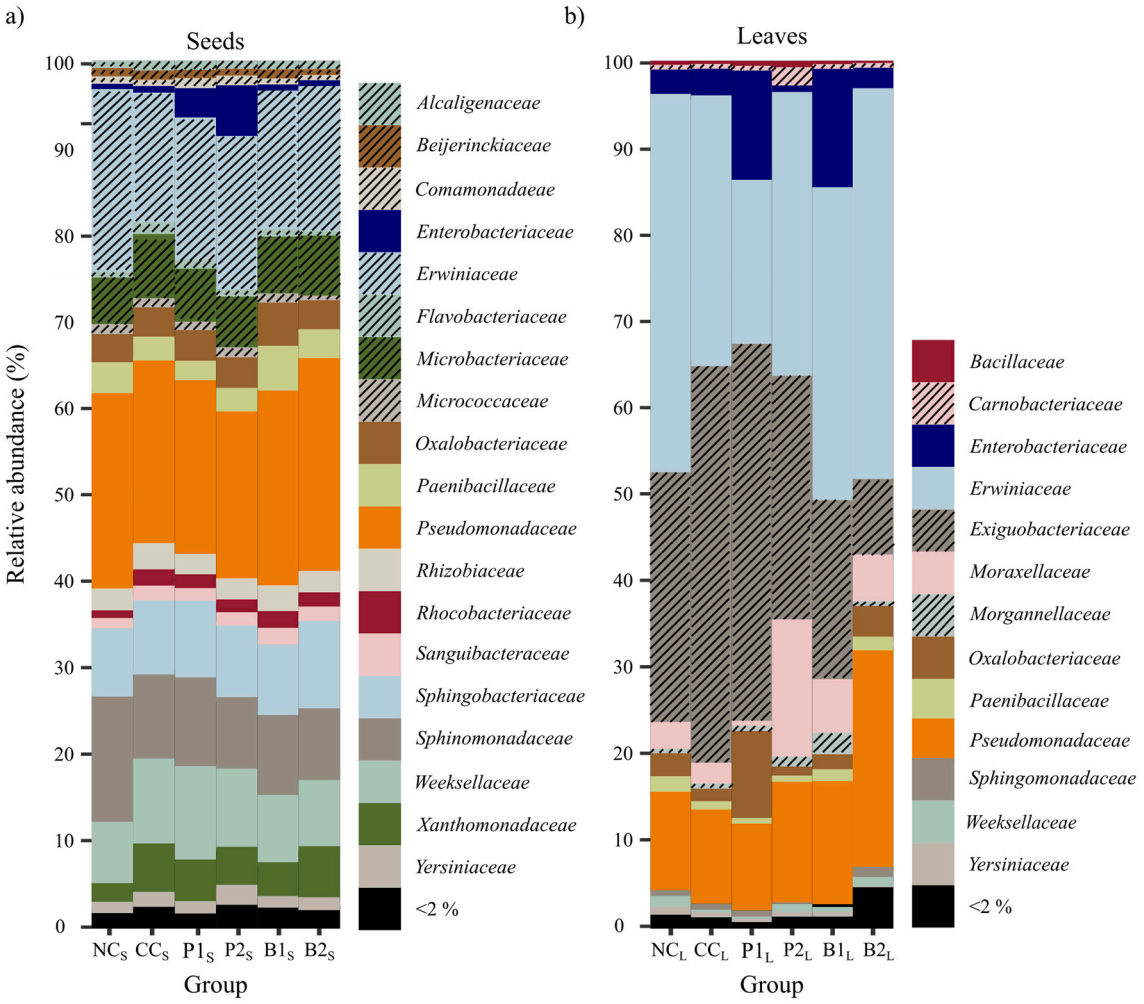

Fig. 3. Relative bacterial abundance at family level. a) seeds coated with *E. coli* antagonistic bacteria. NC_S = untreated seeds (n = 2), CC_S = coated control (n = 2), $P1_S$=*Pseudomonas cedrina* LMG P-32207 (n = 2), $P2_S$=*P. punonensis* LMG P-32204 (n = 2), $B1_S$=*B. coagulans* LMG P-32205 (n = 1) and $B2_S$ = *B. coagulans* LMG P-32206 (n = 2). b) plants from the seeds in a). NC_L (n = 5), CC_L (n = 6), $P1_L$ (n = 5), $P2_L$ (NC = 6), $B1_L$ (n = 6) and $B2_L$ (n = 6).

abundance was found in *Pseudomonas* species, indicating that it is unlikely that a contamination of *Escherichia-Shigella* happened during seed coating. Moreover, the Relative ASV abundances of *Escherichia-Shigella* differ within groups, and it was below detection limit on group B1 and B2. It is more likely that there was a difference in the microbiota on the seeds before coating, which is a natural artifact. When comparing seeds to leaves from the same group, a higher relative abundance of *Escherichia-Shigella* was found on the seeds for group P1 and P2, and when looking at the relative abundance, *Escherichia-Shigella* was below the detection limit on the leaves (<0.001%). Seed coating of these antagonistic isolates (*P. cedrina* and *P. punonensis*) may therefore have potential to prevent the presence of *Escherichia-Shigella* on seeds to propagate to the leaves.

There is a difference in the relative abundance of taxa between seeds and leaves. Within the group, the relative abundance of different genera present on seeds are higher than on leaves. This phenomenon from seed to plant was observed in all groups, including the uncoated control (NC). *Acinetobacter*, *Bacillus*, *Erwinia*, *Exiguobacterium* and *Pseudomonas* were on the other hand found to thrive on the leaves in almost all groups. As the survival from seed to leaf is paramount, it is important to consider this selection process when choosing a bacterial species aimed for biological control.

In our case, to estimate whether the antagonists have survived the coating process and are able to establish in the native microbiota on the leaves, we analyzed the relative abundance of *Pseudomonas* in samples P1 and P2 and *Bacillus* in samples B1 and B2. Changes in *Pseudomonas* species were plentiful and higher relative abundances could be observed for different species in all samples, both on seeds and leaves. Since for technical limitations it was not possible to identify the antagonists on species level, it was also not possible to determine whether they have survived and established on the leaves. However, the antagonistic effect that was seen in group P1 and P2 through lower relative abundances of *Escherichia-Shigella* might indicate their presence. Similarly, *Bacillus* species were in higher relative abundance on both seeds and leaves in group B2. In conclusion, direct identification of the added strains was not possible, but at the same time it can be seen that the antagonistic strains have induced different changes in the microbiota, also indicating that microbiota modification of spinach is strain specific.

Moreover, all antagonistic stains imposed lower relative abundance of *Erwinia* and/or *Pectobacterium*, both genera containing plant pathogenic species. This is a positive result, since it is important that the antagonistic strains do not promote the growth of plant pathogens. Group B2 displayed a lower relative abundance of *Pantoea*, a genus including opportunistic human pathogens. Seeds of both group B1 and B2 inoculated with *B. coagulans* had higher relative abundance of *Lactobacillaceae* compared to control. It has previously been shown that lactic acid bacteria and their metabolites may increase the safety and shelf-life of Lamb's lettuce (Siroli et al., 2015), and therefore, the increase of the relative abundance of *Lactobacillaceae* in the present study can be interpreted as a positive trait. As *B. coagulans* can form

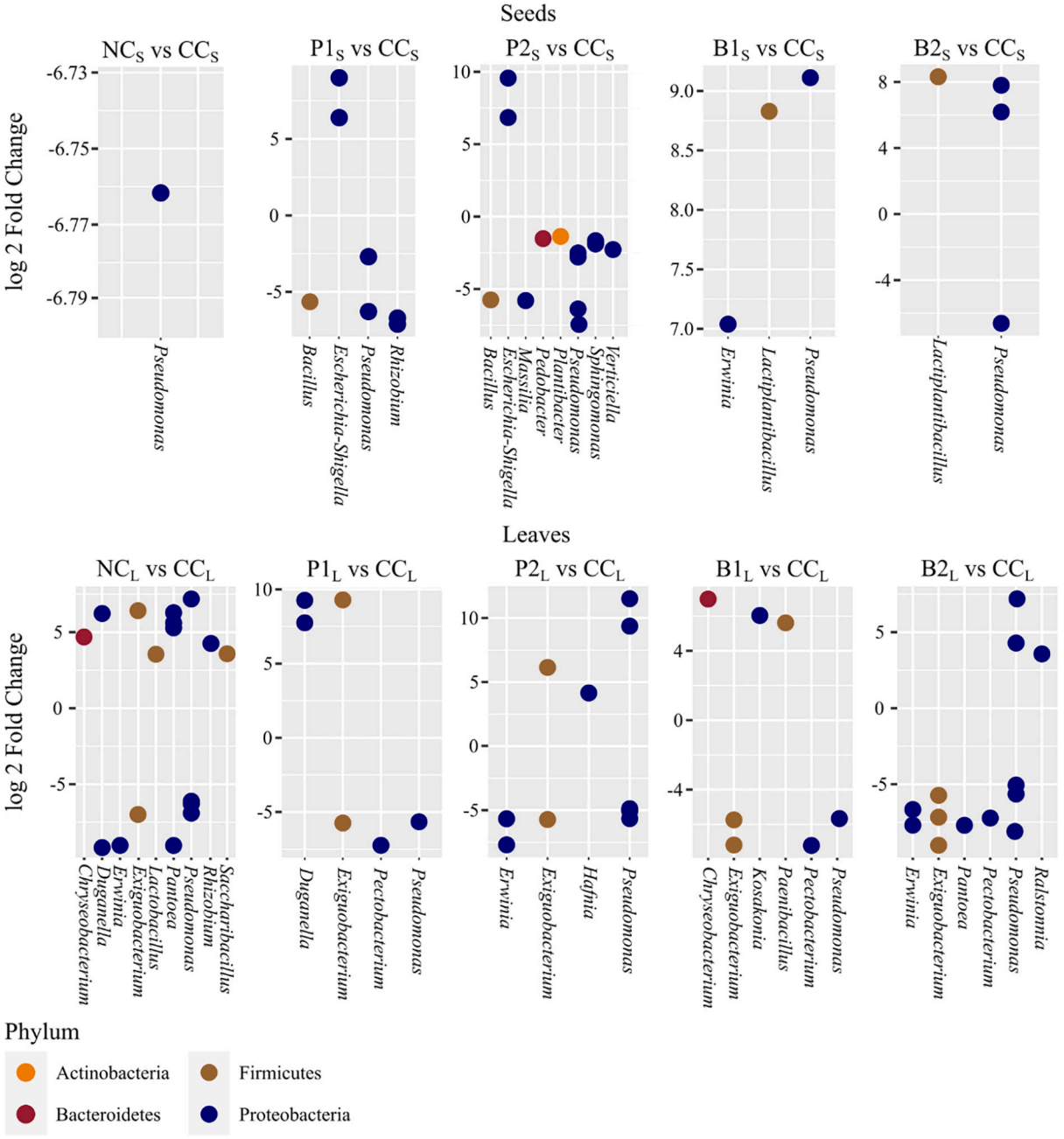

Fig. 4. Log 2 fold change plots of leaves and seeds at genus level analyzed group-wise by DESeq2 with *p*-value cutoff at 0.001. All groups are compared to coated control (CC). NC_S = untreated seeds (n = 2), CC_S = coated control (n = 2), $P1_S$ = seeds coated with *Pseudomonas cedrina* LMG P-32207 (n = 2), $P2_S$ = seeds coated with *P. punonensis* LMG P-32204 (n = 2), $B1_S$ = seeds coated with *B. coagulans* LMG P-32205 (n = 1) and $B2_S$ = seeds coated with *B. coagulans* LMG P-32206 (n = 2). NC_L = leaf sample from untreated seeds (n = 5), CC_L = leaf sample from coated control seeds (*n* = 6), $P1_L$ = leaf samples from seeds inoculated with *P. cedrina* LMG P-32207 (n = 5), $P2_L$ leaf samples from seeds inoculated with *P. punonensis* LMG P-32204 (n = 6), $B1_L$ = leaf samples from seeds inoculated with *B. coagulans* LMG P-32205 (n = 6) and $B2_L$ = leaf samples from seeds inoculated with *B. coagulans* LMG P-32206 (n = 6). Note that the y-axes display different intervals. Positive log2 fold change values indicate higher relative ASV abundance in the treatment group (or NC), and negative values indicate higher relative abundance in the coated control group (CC). Multiple symbols within the same genus represent different ASVs assigned to the same genus.

endospores, and as such have extraordinary resistance against environmental factors and thus also high storage stability, a great advantage in products such as seed coatings.

The α-diversity did not change by the coating treatments, but β-diversity calculated by weighted unifrac method was higher in plants from seeds inoculated with the two strains of *B. coagulans*, indicating that the relative microbial abundance in those groups are phylogenetically different compared to control. An increased β-diversity has previously been linked to a positive outcome of the tea leaf microbiome using biocontrol (Cernava et al., 2019).

From a safety perspective, the strains selected for the field trial were chosen not only on the basis of their performance in the antagonism tests and for how easy they were to cultivate, but also because they are not documented in scientific literature as pathogenic to neither humans, animals, nor plants. *B. coagulans* has been reported as safe by the US Food and Drug Administration (FDA) and the European Union Food Safety Authority (EFSA) and is on the Generally Recognized As Safe (GRAS) and Qualified Presumption of Safety (QPS) list (EFSA Panel on

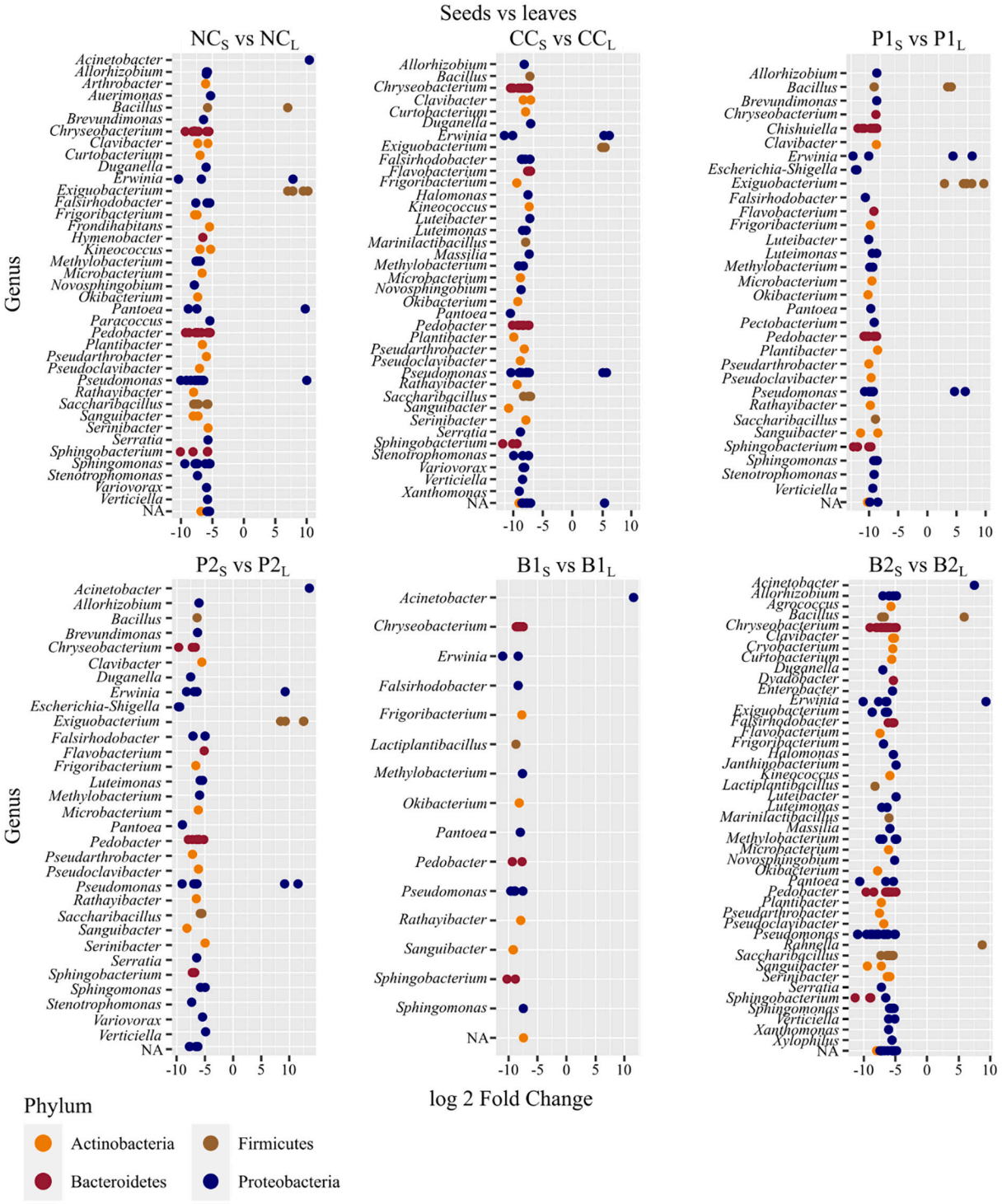

Fig. 5. Log 2 fold change plots of leaves and seeds compared within the same group by DESeq2 with p-value cutoff at 0.001. NC_S = untreated seeds (n = 2), CC_S = coated control (n = 2), $P1_S$ = seeds coated with *Pseudomonas cedrina* LMG P-32207 (n = 2), $P2_S$ = seeds coated with *P. punonensis* LMG P-32204 (n = 2), $B1_S$ = seeds coated with *B. coagulans* LMG P-32205 (n = 1) and $B2_S$ = seeds coated with *B. coagulans* LMG P-32206 (n = 2). NC_L = leaf sample from untreated seeds (n = 5), CC_L = leaf sample from coated control seeds (n = 6), $P1_L$ = leaf samples from seeds inoculated with *P. cedrina* LMG P-32207 (n = 5), $P2_L$ leaf samples from seeds inoculated with *P. punonensis* LMG P-32204 (*N* = 6), $B1_L$ = leaf samples from seeds inoculated with *B. coagulans* LMG P-32205 (n = 6) and $B2_L$ = leaf samples from seeds inoculated with *B. coagulans* LMG P-32206 (n = 6). Positive log2 fold change values indicate higher Relative ASV abundance in seeds, and negative values indicate higher abundance in leaves. Multiple symbols within the same genus represent different ASVs assigned to the same genus.

Biological Hazards, 2013). It is also used in several commercial products world-wide (Konuray & Erginkaya, 2018). *Pseudomonas* species are dominant residents on leafy greens (Jackson et al., 2013; Gabriela Lopez-Velasco et al., 2011; Rastogi et al., 2012) which means that large quantities of these bacteria are normally ingested by consumption of leafy greens. Furthermore, *Pseudomonas* have been studied and used commercially for decades as biocontrol agents (Stockwell & Stack, 2007; Weller, 2007).

This is the first study to evaluate the changes in bacterial community composition after seed inoculation of *E. coli* antagonistic isolates on live

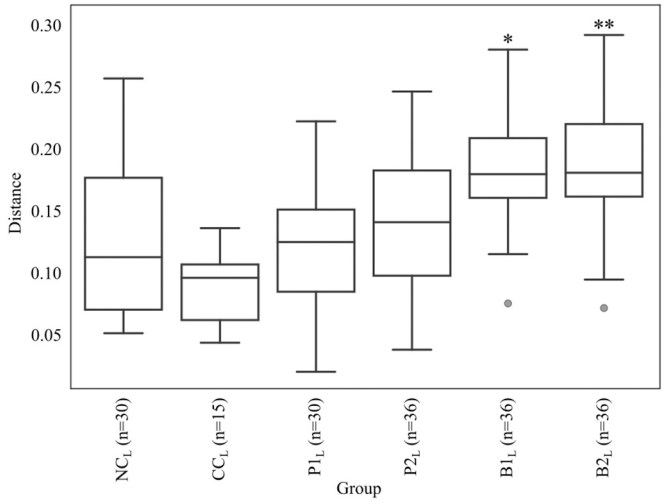

Fig. 6. β-diversity of leaf samples calculated by weighted unifrac and all groups are compared pairwise with coated control (CC) using Permutational multivariate analysis of variance (PERMANOVA). NC = leaves from untreated seeds, CC = coated control, P1 = leaves from seeds coated with *Pseudomonas cedrina* LMG P-32207, P2 with *P. punonensis* LMG P-32204, and B1 with *B. coagulans* LMG P-32205 and B2 with *B. coagulans* LMG P-32206. * $p < 0.05$, ** $p < 0.01$ compared to coated control, CC. n = number of comparisons.

plants in a commercial field setting. Previous studies have only been performed on sterilized leaf systems, where the native microbiota has been removed (Jablasone et al., 2005; Johnston et al., 2009; G. Lopez-Velasco et al., 2012). Competition for space and coexistence in an already established ecosystem is one of the most important challenges for survival and growth of a potential antagonist, which can only be measured *in planta*. Through seed coating with antagonistic bacteria, the present study has shown that the leaf microbiota contained less relative abundance of potential human pathogens. In that way the risk of pathogenic multiplication and spread on leafy green vegetables can be reduced, and the consequences of their related outbreaks in the future can be mitigated.

CRediT authorship contribution statement

E. Uhlig: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **A. Kjellström:** Data curation, Formal analysis, Software. **N. Nurminen:** Investigation. **C. Olsson:** Conceptualization, Writing – review & editing. **E. Oscarsson:** Investigation. **P. Canaviri-Paz:** Investigation. **L. Mogren:** Conceptualization, Project administration. **B. Alsanius:** Conceptualization, Funding acquisition. **G. Molin:** Conceptualization, Writing – review & editing. **Å. Håkansson:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing

Declaration of Competing Interest

No conflict of interest to declare.

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