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Plant variety and soil type influence *Escherichia coli* O104:H4 strain C227/11ϕcu adherence to and internalization into the roots of lettuce plants

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ABSTRACT

Human disease outbreaks caused by pathogenic *Escherichia coli* are increasingly associated with the consumption of contaminated fresh produce. Internalization of enteroaggregative/enterohemorrhagic *E. coli* (EAEC/EHEC) strains into plant tissues may present a serious threat to public health. In the current study, the ability of the fluorescing Shiga toxin-negative *E. coli* O104:H4 strain C227/11ϕcu/pKEC2 to adhere to and to internalize into the roots of *Lactuca sativa* and *Valerianella locusta* grown in diluvial sand (DS) and alluvial loam (AL) was investigated. In parallel, the soil microbiota was analyzed by partial 16S rRNA gene sequencing. The experiments were performed in a safety level 3 greenhouse to simulate agricultural practice. The adherence of C227/11ϕcu/pKEC2 to the roots of both plant varieties was increased by at least a factor three after incubation in DS compared to AL. Compared to *V. locusta*, internalization into the roots of *L. sativa* was increased 12-fold in DS and 108-fold in AL. This demonstrates that the plant variety had an impact on the internalization ability, whereas for a given plant variety the soil type also affected bacterial internalization. In addition, microbiota analysis detected the inoculated strain and showed large differences in the bacterial composition between the soil types.

1. Introduction

Contamination of fresh produce including lettuce, spinach and sprouts with pathogenic *Escherichia coli* represents an emerging risk to public health as produce is mainly consumed raw. Especially bacteria that invade the plant tissues are difficult to remove by the addition of disinfectants into the wash water (Seo and Frank, 1999; Solomon and Sharma, 2009). Washing will only eliminate pathogens that adhere to the surface as these are exposed to the disinfectants, whereas internalized bacteria are protected by the plant tissue itself. The possible routes of contamination present an important issue as plant-based food may also be directly contaminated on field *via* feces, irrigation water, manure, and surface water.

Within the last years, outbreaks of human disease caused by enterohemorrhagic (EHEC) and enteroaggregative/enterohemorrhagic (EHEC/EAEC) *E. coli* strains were frequently traced back to contaminated fresh produce such as spinach, and bagged salad (Grant et al., 2008; Greig and Ravel, 2009; Marder et al., 2014). One of the latest reported outbreaks was recorded in the U.S.A. by the Center for Disease Control and Prevention from April to June 2018 (CDC; https://www.cdc.gov/). In this case, romaine lettuce contaminated with *E. coli* O157:H7 was identified as the source of the multistate outbreak. The romaine lettuce was traced back to several farms within the same geographical region.

The occurrence and persistence of fecal pathogens such as enteric *E. coli*, Shiga-toxin producing *E. coli* (STEC), and *Salmonella*, in water and soil as well as the persistence of STEC in leafy greens have been investigated in several studies (Abberton et al., 2016; Bolton et al., 2011; Ceuppens et al., 2015; Fremaux et al., 2008; Sharma et al., 2009; Thurston-Enriquez et al., 2005; Wright et al., 2017). Thurston-Enriquez et al. (2005) detected high levels of *E. coli* in cattle manure which is often used as organic fertilizer and soil amendment. This direct deposition of manure on soil represents one important route of contamination. Moreover, upon heavy rainfalls, *E. coli* may be carried over to nearby fields or pastures. Indeed, STEC were detected in soil sampled from pastures located near bovine farms (Bolton et al., 2011). In the

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tested soil, STEC were able to persist several months (Bolton et al., 2011). Since STEC are able to colonize leafy greens when grown in contaminated soil (Chitarra et al., 2014; Solomon et al., 2002), these findings emphasize the significance of manure and soil as a route of contamination. Especially as another study demonstrated that *E. coli* O157:H7 B6-914 is able to internalize into the roots of certain tomato cultivars and to migrate further into the ripe fruits posing a serious threat to potential consumers (Deering et al., 2015). Additionally, during harvest, root internalized bacteria may contaminate the harvest equipment which can then result in cross-contamination of the produce and other fields if the harvest equipment is not sufficiently sanitized before being moved to the next field (Matthews, 2013). Furthermore, an international study investigated the occurrence of *Salmonella* and STEC in primary production of leafy greens and strawberries in Belgium, Brazil, Egypt, Norway, and Spain (Ceuppens et al., 2015). Produce on field was more prone to contamination with pathogens than soil. Interestingly, irrigation water showed the highest potential for containing pathogens (Ceuppens et al., 2015). In 0.7% of all tested samples STEC were detected. Collected rainfall water used for irrigation was the major source of isolation followed by soil and leafy greens (Ceuppens et al., 2015).

In contrast, EAEC are the major cause of protracted diarrhea in children in developing countries (Nataro, 1998), but are also emerging pathogens in industrial countries that already caused several outbreaks in Europe (Kaur et al., 2010). In 2011, a large outbreak of diarrhea and hemolytic uremic syndrome was recorded in Germany and Western Europe caused by fenugreek sprouts that were contaminated with a novel type of *E. coli* O104:H4 (King et al., 2012; Robert-Koch-Institute, 2012). The identified *E. coli* O104:H4 isolates were able to produce Shiga-toxin 2a but shared more genetic similarities with EAEC strains than with classical EHEC strains (Brzuszkiewicz et al., 2011). Therefore, the term enteroaggregative hemorrhagic *E. coli* (EAHEC) was introduced (Bielaszewska et al., 2011; Brzuszkiewicz et al., 2011), which is equivalent to enteroaggregative/enterohemorrhagic *E. coli* (EAEC/EHEC).

The main reservoir of EAEC is controversially discussed as this group of *E. coli* can be found in various animal species, but these isolates differ from EAEC found in humans, suggesting that the animal reservoir is highly unlikely to be associated with human infection (Uber et al., 2006; Vijay et al., 2015). Nevertheless, Beutin et al. (2013) provided evidence that EAHEC O104:H4 strains acquired stx_{2a}-encoding phages from the bovine reservoir. The interaction of EAEC/EHEC strains with plants is poorly understood. The EAEC/EHEC O104:H4 strain TW16133, which was isolated during the 2011 outbreak, was found to be able to adhere to the leaves of spinach and romaine lettuce, and to form aggregates on them in aggregative adherence fimbriae (AAF) mediated manner (Nagy et al., 2016). Berger et al. (2009) investigated the ability of several clinical EAEC isolates of different serotypes to adhere to leaves of *Eruca vesicaria*. All of the tested isolates were able to attach to the leaves. Furthermore, it was demonstrated for EAEC O44:H18 strain 042 that flagellin as well as AAF are involved in leaf colonization (Berger et al., 2009). Attachment to the stomatal guard cells was found to be flagellin-mediated whereas AAF were involved in adherence to the epidermis of the leaves (Berger et al., 2009). However, the ability of EAEC and EAEC/EHEC to internalize into plant tissue, *e.g.* leaves or roots, was not assessed.

In the present study, we analyzed the capability of *E. coli* O104:H4 strain C227/11ϕcu/pKEC2 to colonize the roots of different lettuce types grown in diluvial sand (DS) and alluvial loam (AL). Strain C227/11ϕcu is a *stx2a*-phage cured derivative of *E. coli* O104:H4 C227/11, which was isolated during the large outbreak in 2011 (Zangari et al., 2013). *Valerianella locusta* and *Lactuca sativa*, also known as lamb's lettuce and lettuce, respectively, present lettuces with the highest revenue in Germany according to the German Federal Ministry of Food and Agriculture (https://www.bmel.de/EN/). DS and AL differ in texture, clay content, nutrient content, water holding capacity, and autochthonous microbiota composition (Ruehlmann and Ruppel, 2005; Schreiter et al., 2014). The use of these soil types addressed the question of the impact of distinct soil compositions on the adherence and internalization behavior of the tested *E. coli* strain. To mimic natural contamination *via* irrigation water as closely as possible, unsterile lettuce seeds were grown in potting soil, and transferred to DS and Al. The *in vivo* experiments were performed in a biosafety greenhouse meeting the safety requirements for biosafety level 3 according to appendix 4 of the Swiss Containment Ordinance (ESV)(https://www.admin.ch/opc/en/classified-compilation/20100803/index.html). Adherence and internalization of *E. coli* O104:H4 C227/11ϕcu/pKEC2 to and into the roots were analyzed by qualitative and quantitative analysis to elucidate the influence of the different tested conditions on distinct aspects of root colonization. Furthermore, the autochthonous soil microbiota was analyzed by next generation sequencing in the presence and absence of *E. coli* O104:H4 C227/11cu/pKEC2.

2. Material and methods

2.1. Bacterial strain and growth conditions

The *tagrfp-t* containing plasmid pKEC2 (Eißenberger et al., 2018) was transformed into Stx2a-negative *E. coli* O104:H4 strain C227/11ϕcu (Zangari et al., 2013) as described previously (Eißenberger et al., 2018), yielding *E. coli* O104:H4 strain C227/11ϕcu/pKEC2 which is chloramphenicol-resistant and expresses the red fluorescent protein (RFP). This strain was routinely grown in LB medium (10% (w/v) tryptone, 10% (w/v) NaCl, 5% (w/v) yeast extract, pH 7.0) at 37 °C on a rotary shaker with 180 rpm. Chloramphenicol (Roth, Germany) was added to a final concentration of 20 μg/ml.

2.2. Plasmid stability in E. coli O104:H4 C227/11ϕcu/pKEC2 grown in soil

The stability of pKEC2 in strain C227/11ϕcu in the different soil types was investigated according to previously published persistence experiments (Eißenberger et al., 2018) with minor modifications. Briefly, *E. coli* strain C227/11ϕcu/pKEC2 was grown overnight in LB medium supplemented with 20 μg/ml chloramphenicol at 37 °C. Cells were harvested at 6,000 × *g* at 4 °C for 8 min and resuspended in 10 mM $MgCl_2$. Diluvial sand (DS) or alluvial loam (AL) were thoroughly blended with bacterial suspension to a final inoculum level of 10^8 colony forming units (cfu) per gram soil. Soil blended with 10 mM $MgCl_2$ only served as negative control. Both soils were kindly provided by Dr. Rita Grosch (Leibniz Institute of Vegetable and Ornamental Crops, Groβbeeren, Germany). DS was described as an Arenic-Luvisol with less silty sand and 5.5% clay (Ruehlmann and Ruppel, 2005; Schreiter et al., 2014). AL was described as Gleyic-Fluvisol with heavy sandy loam and 27.5% clay (Ruehlmann and Ruppel, 2005; Schreiter et al., 2014). Samples were incubated at 22 °C for up to 14 days and analyzed after 0, 2, 4, 7, and 14 days of incubation. The 14 days sample was investigated to ensure long term plasmid stability in the soil environment.

To recover *E. coli* cells from soil for quantification, 9 ml 0.5 × Murashige-Skoog (MS) medium (2.165 g/l Murashige & Skoog Medium, Duchefa Biochemie, Netherlands, pH 5.8) were added to the samples followed by extensive blending. Appropriate serial decimal dilutions were plated on TBX chromogenic agar (Roth, Germany) and on TBX agar supplemented with 20 μg/ml chloramphenicol. Because of its glucuronidase activity, strain C227/11ϕcu/pKEC2 appeared as blue/green colonies when grown on TBX agar, and these were therefore easily distinguished from other soil bacteria as those appeared as whitish colonies. The viable counts per gram soil were calculated after incubation at 37 °C overnight. Three independent experiments were performed.

2.3. Seeding and propagation of lettuce plants

Seeds of *Valerianella locusta* (L.) „Verte á coeur plein“ (Select, Wyss Seed and Plants AG, Switzerland) and seeds of *Lactuca sativa* “Tizian” (Syngenta, Switzerland) were grown in Floradur® A Press potting soil (Floragard, Germany) for approximately two weeks in seed trays (50 × 30 × 5 cm) with 150 slots. After reaching the second leaf stage (first leaf rosette or growing of the third leaf), the plants were carefully excavated and freed of soil before being repotted in plant pots (9 cm in diameter) containing DS or AL. Each pot then contained three plants. Plants were used for contamination experiments after a two-day adaption phase.

2.4. Analysis of adherence to and internalization into lettuce roots

Strain C227/11ϕcu/pKEC2 was grown overnight (ca. 18 h) as described above. Inoculation of plants was performed as described previously by dispensing 20 ml of bacterial suspension into the soil (Eißenberger et al., 2018). As negative control, a 10 mM $MgCl_2$ solution without bacteria was used. After four days of incubation at 21 °C with 12 h day-/night-cycle and 20% relative humidity in a biosafety level 3 greenhouse, the plants were analyzed quantitatively and by fluorescence microscopy regarding adherence and internalization of strain C227/11ϕcu/pKEC2 as described previously (Eißenberger et al., 2018). Briefly, the plants were lifted and freed from remaining soil particles by washing for 10 min on a rotary shaker with 50 rpm in 0.5 × MS medium. Plants assigned for internalization experiments were additionally surface sterilized by washing in 0.5 × MS medium supplemented with 50 μg/ml gentamicin for 20 min. Surfaced sterilization was confirmed by placing the disinfected roots onto a TBX agar plate containing 20 μg/ml chloramphenicol for 10 s. These so-called “imprint plates” were then incubated overnight at 37 °C. Following washing, and surface sterilization, the root systems were carefully removed from the plants using sterile tweezers. For qualitative analysis, the removed root material was mounted on an object slide with 30 μl of 0.5 × MS medium. Cover slides were then sealed with nail polish. At least 20 microscopic fields were investiagted per root. For quantitative analysis, the roots were transferred in a reaction tube containing ~15 glass beads (1–3 mm in diameter) and 500 μl PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2.0 mM KH_2PO_4, pH 7.4). The weight of the roots was determined. The roots were then homogenized at 25 Hz for 5 min using a mixer mill (MM200, Retsch, Germany) and subsequently, appropriate dilutions of the homogenate were spread plated on TBX agar supplemented with 20 μg/ml chloramphenicol. After overnight incubation at 37 °C, the viable counts per gram root were calculated. Per soil type/lettuce variety combination, a total of 36 roots each were investigated microscopically and analyzed quantitatively. For subsequent analysis of the soil, approximately 5 g of contaminated and uncontaminated (negative control) soil was sampled for each experiment and replicate. Soil samples were immediately stored at −20 °C. For each lettuce variety and soil combination, three independent experiments were performed in triplicate each, resulting in 288 samples in total.

2.5. Total microbial community DNA extraction

Total microbial community DNA was extracted from 4 g soil of 72 collected soil samples using a DNeasy® PowerMax® Soil Kit (Qiagen, Netherlands) according to the manufacturer's instructions with minor modifications. Total DNA was eluted in 2 ml of C6 solution (Qiagen, Netherlands). To obtain amplifiable DNA samples, the extracted DNA was concentrated with Amicon Ultra 0.5 Centrifugal Filter Units with a molecular weight cut-off of 3 kDa (Merck KGaA, Germany) to a final volume of 30 μl following the manufacturer's instructions. With the same device, the concentrated DNA samples were subsequently washed five times with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Concentrated and washed DNA samples were then diluted 1:50 to 1:200 in PCR-grade water and stored at −20 °C until further use.

2.6. Partial sequencing of 16S rRNA genes

For all 72 samples, fragments of the 16S rRNA gene were amplified from total microbial community DNA using primers 515F and 806R targeting the V4 region (Caporaso et al., 2011; D'Amore et al., 2016). The V4 region was chosen, since it is included in the MiSeq SOP, as this region is known to be the most reliable 16S rRNA gene region for representing the full-length 16S rRNA sequences in the phylogenetic analysis of most bacterial phyla (Yang et al., 2016). The PCR reactions as well as the sequencing of the hypervariable V4 region of the 16S rRNA gene were conducted by the Center for Genomic Research at the University of Liverpool (Great Britain). Sequencing was performed using the Illumina MiSeq Sequencing platform according to standard protocols (Illumina, USA).

2.7. Analysis of sequencing data

Sequences were clustered to operational taxonomical units (OTUs) using the Mothur software v1.40.5 as described in the MiSeq standard operating procedure (SOP) (Kozich et al., 2013). The SILVA 102 bacterial database (Quast et al., 2013) was used as reference for sequence alignment. Cut-offs and considered sequence lengths are described in detail in the MiSeq SOP (Kozich et al., 2013). Unclassified bacteria were removed and redundant OTUs were summarized. Only OTUs with an overall relative abundance higher than 0.0001% were included in further analyses. Sequence reads were normalized by total sum normalization and transformed by square root transformation. All OTU-based statistical analysis was performed using Calypso v8.84 (Zakrzewski et al., 2016). Single samples were grouped according to the soil type and lettuce variety combination. Rarefaction analysis was performed to estimate the coverage of microbial diversity by the obtained sequence data. Alpha diversity was assessed by Shannon diversity index. To evaluate differences in global community composition among the groups, the intragroup and intergroup Jaccard distances between community profiles were compared by Wilcoxon rank test. The impact of the soil type, the lettuce variety, and the combination of both on the microbial soil composition was evaluated by redundancy analysis. $p < 0.05$ was considered significant. By linear discriminant analysis (LDA) effective size (LEfSe) analysis, OTUs were identified which were significantly associated with particular lettuce variety/soil type combinations (Segata et al., 2011) based on $p < 0.05$ and LDA score ($\log_{10}$) > 2.0.

2.8. Statistical analysis

Data from the infection and plasmid stability experiments were analyzed with the Brown–Forsythe test for variance homogeneity, followed by either one-way ANOVA and two-tailed student's *t*-test with Benjamini-Hochberg correction, or by Welch's ANOVA and two-tailed Welch *t*-test with Benjamini-Hochberg correction if homogeneity of variance was violated. $p_{corrected} < 0.05$ was considered significant.

2.9. Data availability

Sequencing data were deposited at the European Nucleotide Archive (ENA) under the study accession number PRJEB30456.

3. Results

3.1. Stability of plasmid pKEC2 in E. coli O104:H4 strain C227/11ϕcu/pKEC2 incubated in soil

In order to analyze the adherence of red-fluorescing *E. coli* strain C227/11ϕcu/pKEC2 to root tissues, microscopic analysis was carried

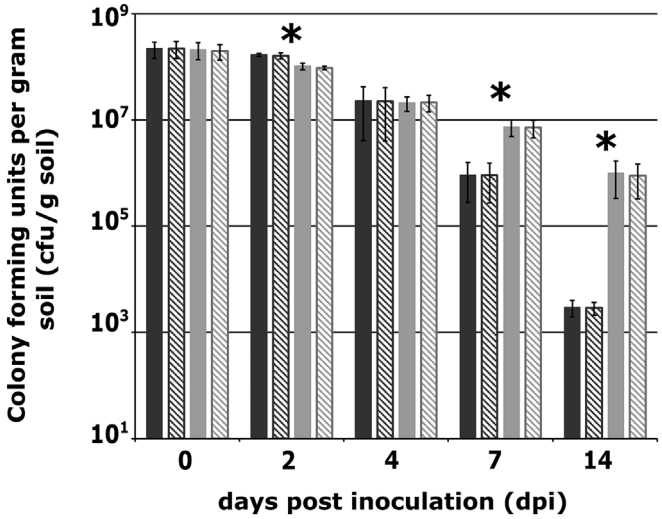

Fig. 1. Stability of plasmid pKEC2 in *E. coli* O104:H4 C227/11ϕcu/pKEC2. DS (dark grey) and AL (light grey) were inoculated with 1.0×10^8 cfu *E. coli* O104:H4 C227/11ϕcu/pKEC2 per gram soil and incubated for up to 14 days. Soil was sampled 0, 2, 4, 7, and 14 days post infection (dpi), and investigated concerning total counts on TBX agar (filled bars) and TBX agar with chloramphenicol (striped bars). Data are means ± standard deviations of three independent experiments. * indicates $p < 0.05$ between the tested soil types.

out. Since the red fluorescing protein (RFP) is encoded on plasmid pKEC2 and since recombinant plasmids can get lost during growth without selection pressure, the stability of pKEC2 in strain C227/11ϕcu without antibiotic selection was investigated. Therefore, C227/11ϕcu/pKEC2 was inoculated in both, DS and AL, at room temperature. Samples were taken at days 0, 2, 4, 7, and 14. To determine the proportion of *E. coli* cells that contained pKEC2, the inoculated cells were recovered from the soil and the samples were plated on TBX agar with and without 20 µg/ml chloramphenicol.

When incubated in DS, the total viable counts of *E. coli* O104:H4 C227/11ϕcu/pKEC2 growing on TBX agar with and without chloramphenicol declined over time, starting at app. 2.2×10^8 cfu/g soil (Fig. 1). After four days, app. 2.3×10^7 cfu/g soil were still detected in both soils. Fourteen days after inoculation, only 3.0×10^3 cfu/g soil and 2.9×10^3 cfu/g soil (Fig. 2) could be detected on TBX agar and TBX agar containing chloramphenicol, respectively. The viable cell counts did not differ significantly for both agars, meaning that plasmid pKEC2 has not been lost during the 14-day incubation period in DS.

For experiments performed in AL, the total viable counts on TBX agar and TBX agar containing chloramphenicol were 2.0×10^8 cfu/g soil and 1.8×10^8 cfu/g soil at day 0, respectively (Fig. 1). The decline over time in AL was different compared to the experiments conducted in DS (Fig. 1). After seven days, significantly higher viable cell counts on both agars were detected in AL (Fig. 1). This difference was also seen after 14 days with 1.0×10^6 cfu/g soil on TBX and 9.0×10^5 cfu/g soil on TBX with chloramphenicol. Compared to DS, this represents a statistically significant difference of about 2.7 log cfu/g soil. These results clearly indicate that plasmid pKEC2 was stable during the tested period of time without antibiotic selection in both soils. Moreover, the results of the experiments have shown that AL obviously supports survival of C227/11ϕcu/pKEC2.

3.2. Microscopic analysis of C227/11ϕcu/pKEC2 adherence at and internalization into the roots of L. sativa and V. locusta

First, we investigated the ability of strain C227/11ϕcu/pKEC2 to adhere to the roots of *L. sativa* and *V. locusta* grown in AL and DS. After four days of incubation, contaminated and uncontaminated roots of both varieties were analyzed by microscopic analysis. Red fluorescent bacteria were not detected at or in uncontaminated roots (Fig. 2 A–D, I–L). In contrast, red fluorescent cells of strain C227/11ϕcu/pKEC2 were detected at the contaminated roots of both, *L. sativa* and *V. locusta*, under all conditions tested (Fig. 2E–H). Per microscopic field, 8 to 17 fluorescing bacterial cells were found.

Next, the internalization was investigated accordingly. As no bacterial growth was detected on the imprint plates, surface sterilization was considered successful. The surface disinfection procedure has been evaluated previously and should not damage bacterial cells within the plant root tissues (Eißenberger et al., 2018). Cells of strain C227/11ϕcu/pKEC2 were still detected after surface disinfection in the roots of both lettuce types grown in both soil types (Fig. 2M–P). However, the number of bacterial cells per microscopic field decreased to a range of one to two cells (Fig. 2M–P). By this technique, bacterial cells were found preferentially at the edge of rhizodermal cells or between them (Fig. 2 E–H, O–P). Still, several *E. coli* cells seemed to localize rather at the center of individual rhizodermal cells (Fig. 2 G–H, M–N).

Even though differences between adherence and internalization experiments were observed with fluorescence microscopy, this provides mainly a qualitative information. For a quantitative analysis, cultural techniques were applied.

3.3. Quantitative analysis of adherence of C227/11ϕcu/pKEC2 to lettuce roots of plants grown in DS and AL

For quantitative analysis of the influence of soil and lettuce type on the adherence of strain C227/11ϕcu/pKEC2 to lettuce roots, contaminated and uncontaminated roots from the same experimental setup that has been used for the microscopic analysis (see 3.2) were weighed and homogenized. The homogenates were then diluted and spread-plated on TBX agar containing 20 µg/ml chloramphenicol. After incubation, the number of colony forming units per gram root was calculated.

At the roots of *L. sativa* 2.6×10^6 cfu/g root and 8.7×10^5 cfu/g root were detected when the plants were grown in DS and AL, respectively (Fig. 3). The approximately 0.5 log lower amount of adherent cells in AL is statistically significant ($p = 0.006$). A similar observation was made for the roots of *V. locusta*. The numbers of adherent bacterial cells ranged from 2.4×10^6 cfu/g root after growth in DS to 6.0×10^5 cfu/g root when plants were grown in AL (Fig. 3). This reduction of 0.6 log cfu/g root is also statistically significant ($p = 0.007$).

Adherence of C227/11ϕcu/pKEC2 to both lettuce varieties grown in the same soil did not show statistically significant differences. However, irrespective of the lettuce variety, the viable counts found at roots of plants grown in DS were significantly higher compared to those detected at AL grown lettuce roots. These results indicate that plant growth in DS enhances the ability of C227/11ϕcu/pKEC2 to adhere to the roots of both lettuce varieties.

3.4. Quantitative analysis of internalization of strain C227/11ϕcu/pKEC2 into the roots of L. sativa and V. locusta

After surface disinfection, 5.0×10^3 cfu/g root and 2.6×10^4 cfu/g root of strain C227/11ϕcu/pKEC2 were detected in the roots of *L. sativa* after growth in DS and AL, respectively (Fig. 4). In contrast, the numbers of internalized bacteria were lower in the roots of *V. locusta*, and ranged from 4.1×10^2 cfu/g root to 2.4×10^2 cfu/g root in DS and AL, respectively (Fig. 4). This equals only 0.02% and 0.04% of the adherent cells, respectively. Comparing the numbers for *L. sativa* and *V. locusta*, the detected viable counts represent a decrease in internalization between the lettuce varieties of a factor 12 in DS and 108 in AL, respectively.

Statistical analysis revealed that the observed differences between the lettuce types, *i.e.* less internalization into the roots of *V. locusta*, are significant. Therefore, we conclude that internalization of strain C227/11ϕcu/pKEC2 into the roots of lettuce plants occurs in a plant type-

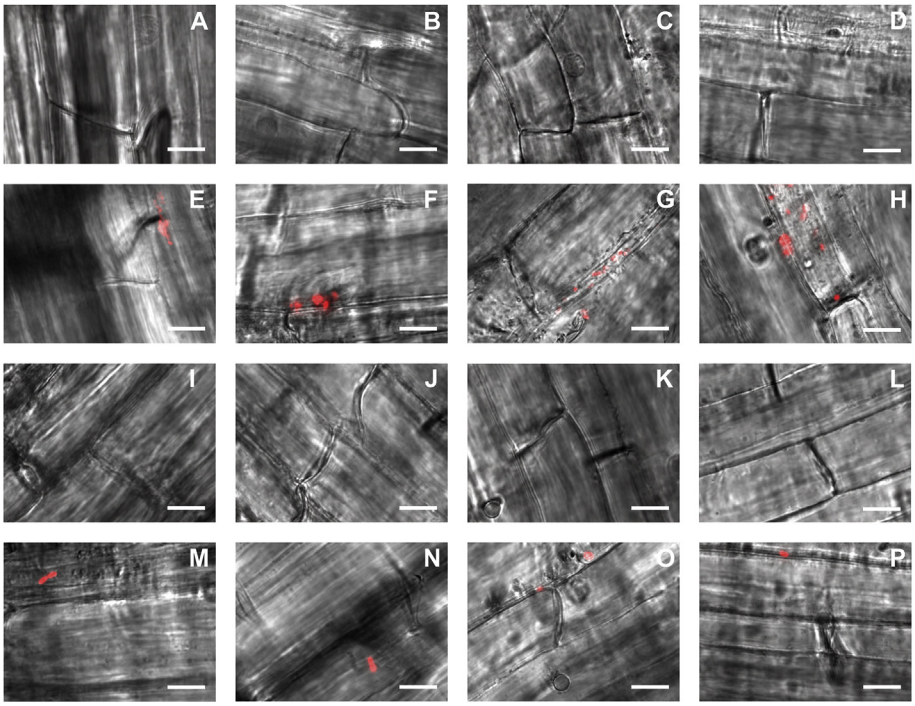

Fig. 2. Microscopic analysis of red fluorescent bacteria at and in the roots of *L. sativa* and *V. locusta* grown in different soils. Microscopy was performed either after 10 min of washing in 0.5 × MS medium to investigate adherence (A–H) or after 10 min of washing in 0.5 × MS medium and 20 min of surface disinfection in 0.5 × MS medium supplemented with 50 μg/ml gentamicin in order to analyze internalization (I-P). Depicted are overlays of brightfield and RFP signal (607/70 emission wavelengths). The panels A–D and I-L depict uncontaminated control roots. In panels E–H and M-P, the roots were contaminated with *E. coli* O104:H4 C227/11ϕcu/pKEC2. Starting from the left, the first column shows the results for DS grown *L. sativa* roots, the second for AL grown *L. sativa* roots, the third for DS grown *V. locusta* roots, and the fourth for AL grown *V. locusta* roots. Per soil type/lettuce combination 36 roots were analyzed. Bars are 10 μm. Magnification is 100-fold. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

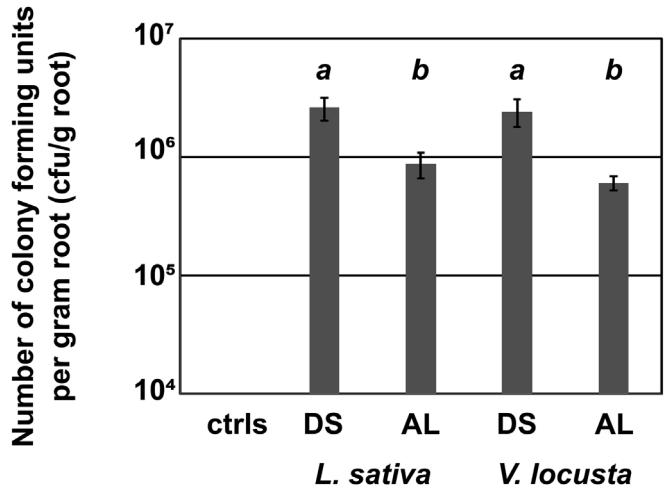

Fig. 3. Adherence of *E. coli* O104:H4 C227/11ϕcu/pKEC2 to the roots of *L. sativa* and *V. locusta* grown in different soil types. Viable counts of adherent bacteria at the roots in cfu/g root. Plants were grown in DS or AL, inoculated with 1.0×10^8 cfu/g soil and incubated for 4 days. Under all conditions tested, no bacteria could be detected at non-contaminated roots (summarized as ctrls). Data are means ± standard errors of three independent biological experiments performed in technical triplicates. Columns with different letters are statistically significantly different ($p < 0.05$).

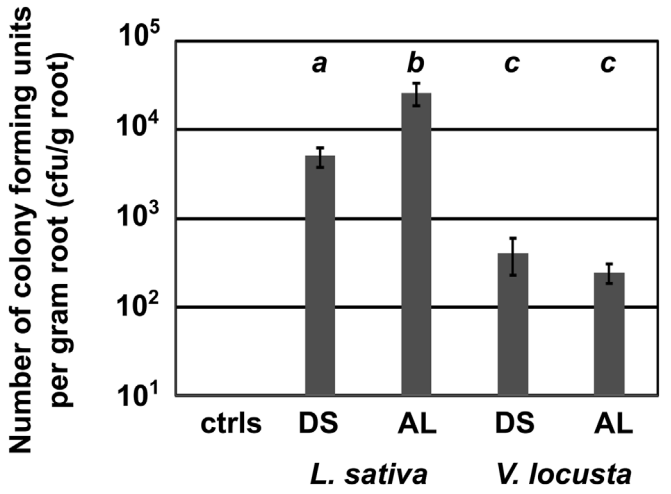

Fig. 4. Internalization of *E. coli* O104:H4 C227/11ϕcu/pKEC2 into the roots of *L. sativa* and *V. locusta* grown in different soil types. Viable counts of internalized bacteria at the roots in cfu/g root. Plants were grown in DS or AL, inoculated with 1.0×10^8 cfu/g soil and incubated for 4 days. No bacteria could be detected in non-contaminated roots under all tested conditions (summarized as ctrls). Data are means ± standard errors of three independent experiments performed in triplicates. Different letters indicate statistical significance ($p < 0.05$).

dependent manner.

For *V. locusta*, the numbers of internalized C227/11ϕcu/pKEC2 cells were 0.2 log cfu/g root lower after growth in AL compared to DS (Fig. 4). However, this difference is not statistically significant ($p = 0.64$). Therefore, the soil type obviously did not affect the internalization of *E. coli* O104:H4 C227/11ϕcu/pKEC2.

When internalization experiments were performed with *L. sativa*, growth in the two soil types led to different results. Upon growth in DS, 5.0×10^3 cfu/g root were found after surface disinfection (Fig. 4), which is equivalent to 0.2% of the originally adherent cells. In contrast, 2.6×10^4 cfu/g were detected in the roots after disinfection of the root surface of *L. sativa* grown in AL, showing that approximately 3.0% of the adherent cells internalized into the roots. This means that 0.7 log cfu/g root more internalized bacteria were found when *L. sativa* plants were grown in AL compared to DS. The observed difference is statistically significant ($p = 0.001$) and led us to conclude that the soil type plays an important role for internalization of *E. coli* O104:H4.

Taken together, the results of the internalization experiments demonstrated that the extent of internalization is affected by the lettuce type. Moreover, in a given lettuce type, internalization is also influenced by the soil type.

3.5. Microbial community analysis of the soils from adherence and internalization experiments

In order to investigate the linkage between soil type, lettuce type, and the autochthonous microbiota, partial sequencing of 16S rRNA genes of the total microbial community of 72 samples was performed.

Analysis of sequence data identified a total of 678 different operational taxonomic units (OTUs). After filtering of the sequences, 518 different OTUs were included in further analysis (Supplementary Data). As indicated by rarefaction curves, the majority of the soil microbial communities was represented in the remaining sequencing data (Fig. S1). Altogether, 187 OTUs were identified as *Proteobacteria*, 103 as *Actinobacteria*, 103 as *Firmicutes*, and 47 as *Bacteroidetes*. *Acidobacteria* (22 OTUs), *Chloroflexi* and *Verrucomicrobia* (13 OTUs each), and *Planctomycetes* (10 OTUs) were determined to a lesser extent, while all other phyla showed even lower numbers of OTUs (Supplementary Data).

The 40 most abundant OTUs were investigated in more detail. Among them, ten OTUs were *Actinobacteria*, five *Acidobacteria*, two each *Bacteriodetes* and *Verrucomicrobia*, one each *Chloroflexi, Gemmatimonadetes*, *Planctomycetes* and TM7, while almost half of the OTUs (17) were assigned to the phylum *Proteobacteria*. Among those was OTU013 (genus *Escherichia* or *Shigella*), which was mainly detected in soil samples taken from EHEC/EAEC contaminated soil (Fig. 5, Supplementary Data). Interestingly, OTU013 showed the highest abundance of approximately 7.5% of the reads for DS grown *L. sativa*, followed by DS grown *V. locusta* (3.6%) and AL grown *L. sativa* (3.0%). Of the EHEC/EAEC contaminated soil samples, the lowest abundance of OTU013 was detected for AL grown *V. locusta* (0.3%). Quantitative visualization of the 40 most abundant OTUs emphasized the expected differences in the microbial composition between the soil types (Fig. 5). For instance, OTU010 (genus *Arthrobacter*) was more prominent in DS compared to AL, whereas OTU007 and OTU028 (both phylum *Verrucomicrobia*) were more abundant in AL than in DS.

The observation that the microbial composition differed particularly between the soil types was corroborated by redundancy analysis (RDA). Furthermore, RDA revealed clustering of the samples according to soil type/lettuce variety combination (Fig. 6) and showed that soil type, lettuce variety, the lettuce/soil type combination, and the presence of strain C227/11ϕcu/pKEC2 had a statistically significant impact on the microbial soil composition with p values of 0.001, 0.005, 0.016 and 0.026, respectively.

The alpha diversity of the soil type/lettuce variety groups was indicated by the Shannon diversity index. This index varied from 3.7 to 4.2 (Fig. 7) with the highest value observed for AL in combination with *L. sativa* followed by AL in combination with *V. locusta*, with a Shannon index of 4.1. Diluvial sand in combination with *L. sativa* and *V. locusta* resulted in Shannon indices of 3.8 and 3.7, respectively (Fig. 7). Generally speaking, the Shannon indices imply a higher alpha diversity in AL compared to DS. Thus, the richer microbial diversity of AL might be hypothesized to pose a more stable community structure. This might also account for the observed lower changes in microbiota composition

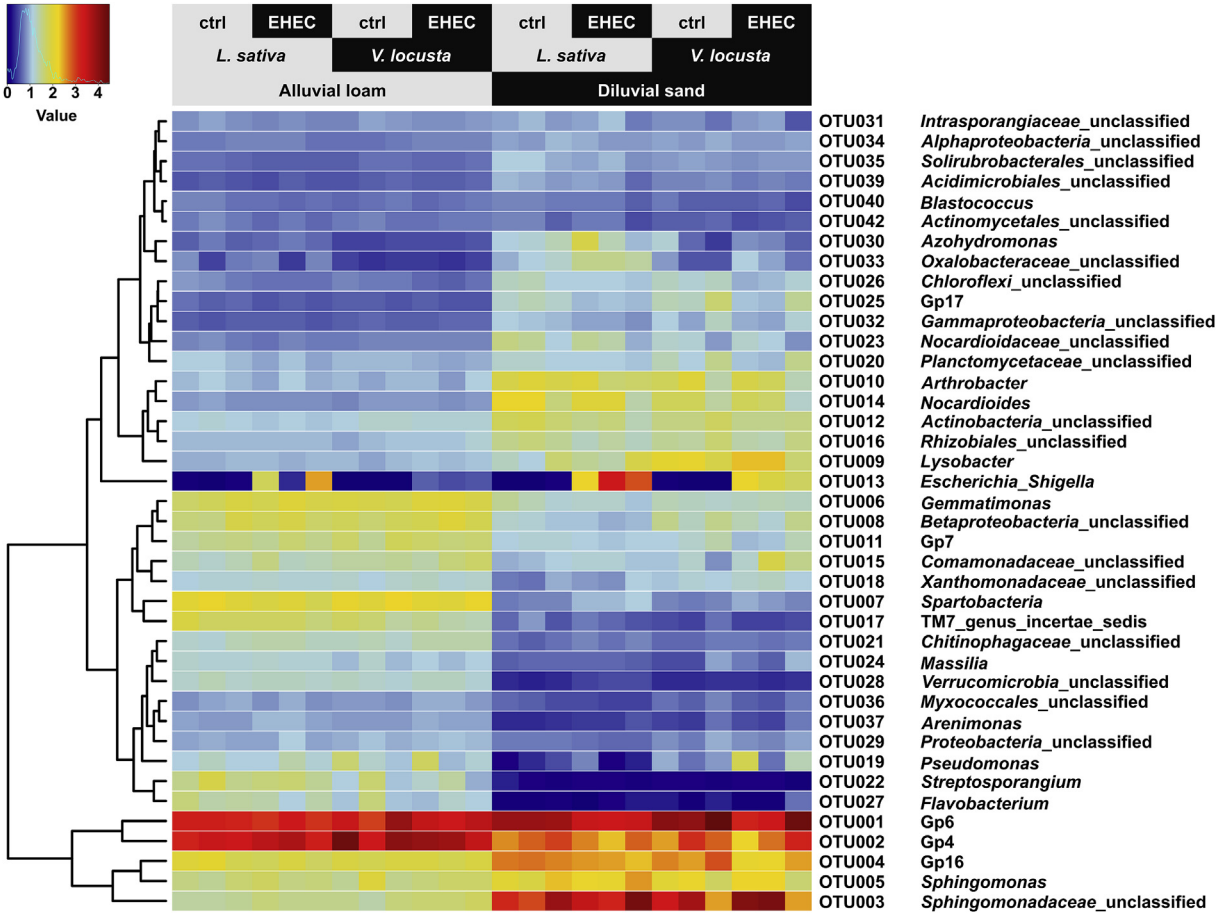

Fig. 5. Relative abundance of the 40 most dominant operational taxonomic units (OTUs). The heatmap indicates differences in the relative abundance of OTUs in the distinct soil type/lettuce variety combination. The individual samples are means of technical triplicates for each biological replicate. These are depicted as vertical columns and ordered by soil type/lettuce variety combination, uncontaminated (ctrl) and EHEC contaminated (EHEC) samples. Horizontal rows represent OTUs and assigned genera ordered by hierarchical clustering. The color code grades from dark blue (rare or not detected) over light blue, yellow, red to dark red (highly abundant). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

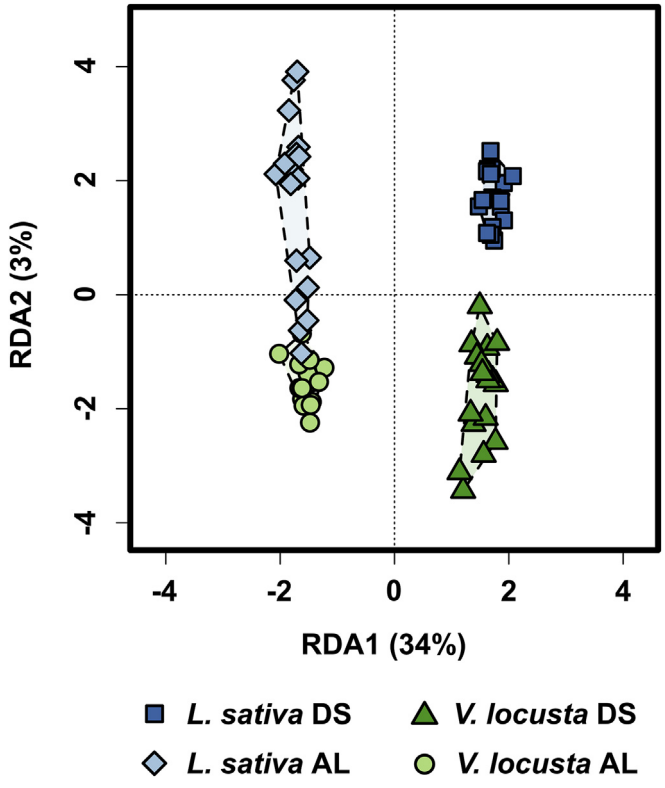

Fig. 6. Redundancy analysis (RDA) of the bacterial soil community composition. Multivariate RDA displays influence of soil type and lettuce variety on the microbial soil composition.

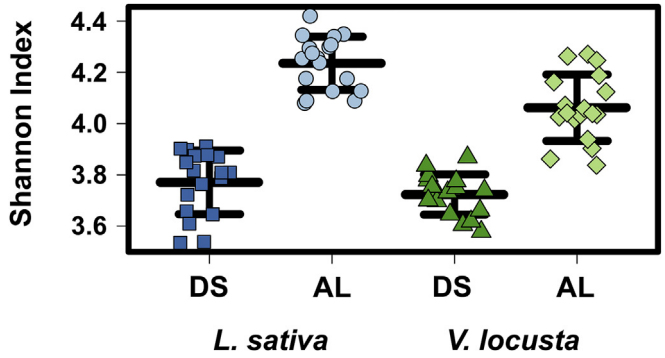

Fig. 7. Diversity within and between the soil type/lettuce variety combination. Alpha diversity given as Shannon index depicts differences in soil microbial community structure between the distinct combinations of soil type and lettuce variety.

due to lettuce variety and the presence/absence of EHEC/EAEC.

The microbiomes of the investigated lettuce/soil type combinations varied significantly regarding global community composition as measured by the Jaccard distance ($p < 0.001$; Supplementary Data). Similar differences in global community composition were detected for the following intergroup comparisons: *L. sativa* in DS compared to same lettuce variety in AL (0.48), *L. sativa* in DS compared to *V. locusta* in AL (0.48), *L. sativa* in AL compared to *V. locusta* in DS (0.47), and *V. locusta* in DS compared to the same lettuce type in AL (0.45). When comparing L. *sativa* in DS with *V. locusta* in the same soil type, and when comparing AL grown *L. sativa* with *V. locusta* in AL, the differences in global microbial composition were smaller with intergroup median Jaccard distances of 0.28 and 0.21, respectively. These findings further corroborate that the lettuce variety/soil type combination impacts the global community composition.

Moreover, LEfSe analysis revealed that 240 OTUs were significantly associated with particular lettuce variety/soil type combinations. Of these OTUs, 74 were connected to DS grown *L. sativa*, 61 to *L. sativa* grown in AL, 56 and 49 to *V. locusta* grown in AL and DS, respectively (Supplementary Data). For instance, OTU003 (family *Sphingomonadaceae*), OTU012 (order *Actinobacteria*), and OTU033 (family *Oxalobacteraceae*) were associated with DS grown *L. sativa*, whereas OTU017 (order TM7), OTU022 (genus *Streptosporangium*), and OTU027 (genus *Flavobacterium*) were significantly connected to *L. sativa* grown in AL. Significant association with DS grown *V. locusta* was found for OTU001 (*Acidobacteria* subdivision Gp6), OTU009 (genus *Lysobacter*), and OTU025 (*Acidobacteria* subdivision Gp17). OTU002 (*Acidobacteria* subdivision Gp4), OTU007 (class *Spartobacteria*), and OTU019 (genus *Pseudomonas*) were connected to *V. locusta* in AL.

The presence of EHEC/EAEC induced comparatively minor changes in the microbial community compositions. On average, the percentages of sequence reads declined for *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, and TM7 OTUs in the presence of EHEC/EAEC, while the percentages for other than *E. coli Proteobacteria* OTUs increased. This indicates that EHEC/EAEC does indeed influence the microbiota of the rhizoshere, but that the effect depends also on initial composition of the autochthonous microbiota, as more pronounced effects were found in DS than in AL, independent of the lettuce variety. Taken together, the analysis of the microbial composition confirmed that both, soil type and lettuce variety, as well as their combination affect the microbial soil composition, and showed that microbial diversity of the distinct lettuce/soil type combinations varied significantly. These differences in turn could impact on the colonization of the lettuce plants by *E. coli* O104:H4 C227/11ϕcu/pKEC2.

4. Discussion

In the current study, adherence and internalization properties of the *E. coli* O104:H4 strain C227/11ϕcu/pKEC2 at and into plant roots with regard to different plant types was analyzed. Furthermore, NGS sequencing was applied to investigate the influence of the inoculated *E. coli* strain on in the rhizosphere microbial community structure.

E. coli O104:H4 strain C227/11ϕcu/pKEC2 showed enhanced survival in AL compared to DS (Fig. 1), but interestingly, adherence was higher at the roots of plants grown in DS (Fig. 3). The applied soil types vary in texture, nutrient composition, and water holding capacity (Ruehlmann and Ruppel, 2005; Schreiter et al., 2014). The content of nutrients such as carbon, nitrogen and phosphorus, is higher in AL, and this soil has a higher clay content and higher water holding capacity than DS. As demonstrated by several studies, organic carbon content, total nitrogen content, and clay content have a positive effect on the survival and the fitness of *E. coli* in soil (Gagliardi and Karns, 2002; Wang et al., 2014b, 2014a; Zhang et al., 2017), whereas a high sand content shows negative effects (Wang et al., 2014b). In addition, the current study confirmed that the autochthonous microbiota composition (Figs. 5–7) differed significantly in both soils.

We could also demonstrate that the plant type significantly alters the internalization efficacy of C227/11ϕcu/pKEC2 (Fig. 3) with more internalized bacteria detected in *L. sativa* than in *V. locusta*. Interestingly, the viable counts of C227/11ϕcu/pKEC2 per gram root found at or in the roots of DS grown lamb's lettuce were similar to those of recent experiments performed for enterohemorrhagic *E. coli* O157:H7 strain Sakai under the same conditions (Eißenberger et al., 2018). At least for *V. locusta* grown in DS, the detected numbers of adherent and internalized bacteria seemed to be consistent for different pathogenic *E. coli* strains.

Several studies demonstrated that internalization of human pathogens varies between plant hosts (Deering et al., 2015; Erickson et al., 2010; Golberg et al., 2011; Wright et al., 2017). Plant variety, surface properties of the leaf, including morphology, chemical constituents, and metabolic activities are factors known to affect bacterial

colonization of the phyllosphere (Beuchat, 2002; Heaton and Jones, 2008; Leveau, 2009; Lindow and Brandl, 2003; Quilliam et al., 2012; Yadav et al., 2005). It might be speculated that *L. sativa* and *V. locusta* vary to some degree regarding the surface properties of their roots resulting in differences in internalization. Moreover, Quilliam et al. (2012) reported that distinct lettuce cultivars differentially influence the metabolic activity of *E. coli* O157:H7 in the phyllosphere and the rhizosphere.

In addition to the observed host effect, internalization of C227/11ϕcu/pKEC2 into the roots of *L. sativa* was affected by the soil type. Significantly more bacteria internalized when *L. sativa* was grown in AL than in DS (Fig. 2). Strikingly, this is the opposite effect than observed for adherence. Apparently, adherence to and internalization into plant roots follow distinct mechanisms. Neumann et al. (2014) reported that growth of *L. sativa* in DS or in AL differentially affects a) the root morphology in terms of length and root size in diameter, and b) the composition of the root exudates. In AL, the proportion of fine roots is higher than in DS (Neumann et al., 2014). Potentially, finer roots are more vulnerable and therefore easier to access for microorganisms. This would explain why significantly more *E. coli* cells internalized into the roots of AL grown *L. sativa* even though significantly less *E. coli* cells were adherent to the roots under these conditions.

Another reason may be the different composition of root exudates secreted from *L. sativa* in different soil types. Root exudates of *L. sativa* contain less nutrients in AL than in DS (Neumann et al., 2014). For instance, the amounts of sugars and amino acids in the exudates were lower in AL than in DS, whereas the amount of organic acids is higher in AL. Moreover, the tested soil types also differ in their sugar and amino acid composition, and under both conditions, *L. sativa* secretes antimicrobial and antifungal agents like benzoic and lauric acid (Lee et al., 2006; Neumann et al., 2014; Tangwatcharin and Khopaibool, 2012; Walters et al., 2003). Combining soil and root exudate composition, the roots of *L. sativa* grown in AL may be less attractive to *E. coli* compared to the bulk soil, and internalization may be more beneficial than adherence under these conditions. Nevertheless, it remains unclear why this soil effect is not observed during internalization experiments performed with *V. locusta*. Possibly, *V. locusta* responds differently to the changes in soil composition than *L. sativa*.

The adherence experiments did not demonstrate differences between plant hosts but between soil types. It should be taken into account that besides the chemical composition, soil types also differ regarding their microbiota. AL and DS showed variations in the microbial composition of bulk soil as well as of the rhizosphere of *L. sativa* (Schreiter et al., 2014). In the present study, the soil microbiota was significantly affected by the soil type, the lettuce variety, and the lettuce/soil type combination resulting in differences concerning the microbial diversity between the distinct combinations. As indicated by the Shannon index, DS/lettuce combinations showed lower alpha diversity than AL/lettuce combinations. These differences in soil microbiota probably have effects on *E. coli*. As OTU013 (genus *Escherichia* or *Shigella*) was more abundant in contaminated DS samples compared to contaminated AL samples (Fig. 5), it might be speculated that lower alpha diversity favors the establishment of *E. coli* within the soil community, thus facilitating adherence to the roots. Colonization of lettuce and spinach leaves by *E. coli* O157:H7 was shown to be differentially affected by distinct epiphytic bacteria (Cooley et al., 2006; Lopez-Velasco et al., 2012). Lopez-Velasco et al. (2012) identified 18 genera of spinach phylloepiphytic bacteria that influenced the growth of *E. coli* O157:H7 *in vitro*. Of these genera, 16 showed growth inhibiting effects, and two exhibited growth promoting effects. In our study, we detected 15 of the 18 described genera in the rhizosphere of which eight, *Bacillus* (OTU122), *Brevibacillus* (OTU241), *Brevundimonas* (OTU082), *Microbacterium* (OTU073), *Paenibacillus* (OTU135), *Pseudomonas* (OTU019), *Stenotrophomonas* (OTU041) and *Flavobacterium* (OTU027), were significantly associated with distinct lettuce/soil type combinations. OTU027, OTU041, OTU073, and OTU082 were associated with AL grown *L. sativa*, whereas OTU122 and OTU019 were connected to *V. locusta* in AL. OTU241 and OTU135 were associated with DS grown *L. sativa* and *V. locusta*, respectively. However, these genera were found in all lettuce variety/soil type groups. Thus, it was not possible to draw conclusions from the presence and number of described antagonists about the viable counts of EHEC/EAEC at or in the roots. However, the interactions between these *E. coli* antagonists and the remaining soil microbiota, and the resulting effects of these interdependencies on *E. coli* remain unknown. Soil is a highly complex and dynamic system, and the interactions within the soil microbiota are equally complex. Therefore, the soil microbiota may influence plant/root colonization in an eclectic way.

Taken together, the present study showed that *E. coli* O104:H4 C227/11ϕcu/pKEC2 is able to a) maintain its plasmid during incubation in different soil types for at least 14 days, b) adhere to the roots of different lettuce varieties grown in different types of soil, and c) internalize into these roots under the tested conditions. The plant host predominantly affected the success of *E. coli* internalization whereas the soil type only showed an effect for *L. sativa*. The observed differences in adherence and internalization are most probably not due to the physicochemical soil properties *per se* but result from a complex interplay of these properties, the different microbial compositions, and both root structural and physiological properties.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103316.

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