Uptake of enterohemorrhagic *Escherichia coli* into the roots of lettuce plants

Dissertation for Obtaining the Doctoral Degree of Natural Sciences (Dr. rer. nat.)

Faculty of Natural Sciences

University of Hohenheim

Institute of Food Science and Biotechnology

submitted by

Kristina Eißenberger

from Treuchtlingen, Germany

2020

Dean:	Prof. Dr. Uwe Beifuß
1st Reviewer:	Prof. Dr. Herbert Schmidt
2nd Reviewer:	Prof. Dr. David Drissner
Date of submission:	October 14, 2019
Date of oral examination:	January 23, 2020

This thesis was accepted by the Faculty of Natural Sciences at the University of Hohenheim on December 12, 2019 as "Dissertation for Obtaining the Doctoral Degree of Natural Sciences".

Eidesstattliche Versicherung über die eigenständig erbrachte Leistung gemäß § 18 Absatz 3 Satz 5 der Promotionsordnung der Universität Hohenheim für die Fakultäten Agrar-, Natur- sowie Wirtschafts- und Sozialwissenschaften

1. Bei der eingereichten Dissertation zum Thema "Uptake of enterohemorrhagic *Escherichia coli* into the roots of lettuce plants" handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

3. Ich habe nicht die Hilfe einer kommerziellen Promotionsvermittlung oder -beratung in Anspruch genommen.

4. Die Bedeutung der eidesstattlichen Versicherung und der strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt.

Die Richtigkeit der vorstehenden Erklärung bestätige ich. Ich versichere an Eides Statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

_____ _____

Ort, Datum Unterschrift

Acknowledgements

I am grateful to Prof. Dr. Herbert Schmidt for giving me the opportunity to work on this interesting project, his advice and support throughout the last years.

I would also like to thank Prof. Dr. David Drissner from the Albstadt-Sigmaringen University for his support and advice throughout my doctoral project, especially throughout the greenhouse experiments, and for acting as second reviewer.

I am thankful to the German Federal Ministry of Food and Agriculture (BMEL), more particularly, the Federal Office for Agriculture and Food (BLE) for funding (2813HS028).

I would like to express my gratitude to Dr. Daniel Rigling, Dr. Andreas Zurlinden and Hélène Blauenstein from the Swiss Federal Institute for Forest, Snow and Landscape (WSL) in Birmensdorf, Switzerland, for the opportunity to conduct these experiments in the new BSL3 greenhouse and laboratory containment, and for assistance during the experimental periods.

Next, I would like to thank Dr. Agnes Weiss for sharing her expertise, her encouragement and for her support.

I am grateful to the former AG Drissner at Agroscope for the support and the amicable working atmosphere during the experimental periods. Special thanks go to Dipl. Ing. Jürgen Krauss and Carmela Total for the assistance during plant propagation.

I would also like to thank the staff from Albstadt-Sigmaringen University for the support and understanding during the last year.

Furthermore, I would like to express my gratitude to all members of the Department of Food Microbiology and Hygiene for the help and support. Working with you guys was never boring and always instructive. I am especially grateful to Dr. Maike Krause and Laura Heinisch for their moral and scientific support, all the critical scientific discussions we had, and their friendship. It was a pleasure working with you.

Finally, I owe gratitude to my nearest and dearest for moral support, encouragement, and faith throughout the last years. I couldn’t have done it without you!

Contents

Abstract

Within the last 10 years, the annual numbers of human infections with enterohemorrhagic *Escherichia coli* (EHEC) in Germany increased by a factor of 2.4. The peak was reached during the large German outbreak in 2011. Intriguingly, the source of the outbreak was supposedly traced back to organic fenugreek sprouts. Moreover, the number of EHEC outbreaks traced back to plant-based foods, e.g. fresh produce, increased also in the United States. This trend poses a serious threat to public health as fresh produce is mostly consumed raw. Also, these observations gave rise to investigate the interactions of plants and human pathogens in more detail especially as fresh produce may be contaminated directly on the field.

In the present thesis, the capability of different EHEC strains and an enteroaggregative/enterohemorrhagic *E. coli* (EAEC/EHEC) strain, to adhere to and to internalize into the roots of different lettuce plants was investigated. These studies conducted within the scope of this dissertation focused on different aspects of the mentioned processes, such as different bacterial strains, the bacterial genetic equipment, and different environmental conditions, such as plant variety, soil type used for plant growth, and the soil microbiota. To mimic the natural conditions as close as possible, plant seeds were not disinfected prior to sowing and plants were grown in unsterile soil under greenhouse conditions.

In the first publication, the overall ability of *E. coli* O157:H7 strain Sakai to adhere to and internalize into the roots of *Valerianella locusta*, also known as lamb's lettuce, grown in diluvial sand soil was described. It was demonstrated that *E. coli* O157:H7 strain Sakai is indeed able to attach to and internalized into the lettuce roots under the conditions tested. Moreover, this paper shed light on potentially important intrinsic bacterial factors, i.e. genes/proteins, which are putatively involved in adherence and/or internalization. Therefore, deletion mutants lacking *hcpA* and/or *iha*, were also investigated regarding adherence to and internalization into the lamb's lettuce roots. Both genes, coding for the major subunit of the hemorrhagic coli pilus HcpA and the adhesin Iha, respectively, are supposed to be associated with adherence and therefore called "adherence factors". However, deletion mutants lacking one or both of these genes did not show significant differences in root attachment compared to the wild-type strain. Regarding internalization, deletion of either of these genes resulted in significantly lower numbers of internalized bacteria clearly indicating that both of these genes – or the proteins encoded by these genes – play an important role during invasion of *E. coli* O157:H7 strain Sakai into the roots of lamb's lettuce. Interestingly, deletion of both genes did not result in further reduction of internalization compared to single deletion mutants. Hence, *hcpA* and *iha* encode rather internalization factors than adherence factors which do not show synergistic effects. Moreover, internalization does not solely depend on these two factors.

Abstract

The second paper focused on the influence of lettuce varieties and soil type on the adherence and internalization behavior of *E. coli* O104:H4 strain C227/11ϕcu. In this study, the lettuce varieties *Valerianella locusta* and *Lactuca sativa*, also known as lamb's lettuce and lettuce, respectively, were both grown in two different soil types, diluvial sand (DS) and alluvial loam (AL), to address the impact of plant host and environment on bacterial attachment and invasion into lettuce roots. To approach the latter aspect in more detail, the composition of the soil microbial community was analyzed simultaneously by partial 16S rRNA gene sequencing. Adherence to the roots was positively influenced by the soil type as the number of adherent *E. coli* O104:H4 strain C227/11ϕcu bacteria significantly rose by a factor of three to four when the plants were grown in DS compared to AL. However, when grown in the same type of soil, no statistically significant differences in attachment were detected between the distinct lettuce varieties. On the other hand, internalization significantly differed predominantly between the two types of lettuce. Internalization into the roots of *L. sativa* compared *V. locusta* was found to be increased by a factor of 12 upon growth in DS, and by a factor of 108 when the plants were grown in AL. Moreover, internalization into the roots of *L. sativa* was five-times higher in AL than in DS. Consequently, the lettuce variety significantly influences to ability of *E. coli* O104:H4 strain C227/11ϕcu to internalize into the lettuce roots, while the soil type affected bacterial invasion only at the roots of *L. sativa* under the conditions tested. Moreover, by microbiota analysis, the inoculated strain was found within the soil microbiota, and this analysis demonstrated that soil type, lettuce variety, and the combination of both result in large differences in the composition of the soil microbiota.

Zusammenfassung

In den letzten 10 Jahren hat sich die jährliche Fallzahl der Infektionen mit enterohämorrhagischen *Escherichia coli* (EHEC) in Deutschland mehr als verdoppelt. Den Höhepunkt der EHEC-Erkrankungen in Deutschland stellte der große Ausbruch von 2011 dar. Interessanterweise waren wahrscheinlich Bio-Bockshornkleesprossen die Infektionsquelle. Zusätzlich steigt auch in den Vereinigten Staaten die Anzahl der EHEC-Ausbrüche, die auf pflanzliche Lebensmittel, wie beispielweise Salat, zurückzuführen sind. Diese Entwicklung stellt eine ernstzunehmende Gefahr für die öffentliche Gesundheit dar, weil Frischwaren wie Salat meist roh verzehrt werden. Diese Beobachtungen gaben außerdem Anlass die Interaktion von Pflanzen und Humanpathogenen genauer zu untersuchen, vor allem da Frischwaren direkt auf dem Feld kontaminiert werden können.

In der vorliegenden Arbeit wurden verschiedene EHEC-Stämme und ein enteroaggregativer/enterohämorrhagischer *E.coli* (EAEC/EHEC) Stamm, untersucht, hinsichtlich ihrer Fähigkeit an die Wurzeln unterschiedlicher Salatsorten zu adhärieren und in diese zu internalisieren. Die Studien, die im Rahmen dieser Dissertation durchgeführt wurden, fokussierten sich dabei auf unterschiedliche Aspekte dieser Prozesse, wie etwa die unterschiedlichen Bakterienstämme, die genetische Ausstattung der Bakterien, und verschiedene Umweltbedingungen, wie Salatsorte, für die Anzucht verwendeter Bodentyp und die Bodenmikrobiota. Um die natürlichen Bedingungen möglichst genau nachzuahmen, wurde das Saatgut vor der Aussaat nicht desinfiziert und die Pflanzen wurden in nicht sterilem Boden in einem Gewächshaus angepflanzt.

Die erste Veröffentlichung befasste sich mit der grundsätzlichen Fähigkeit von *E. coli* O157:H7 Stamm Sakai an die Wurzeln von *Valerianella locusta*, also Feldsalat, zu adhärieren und in diese zu internalisieren, wobei der Salat in Diluvialsand gewachsen ist. Es konnte gezeigt werden, dass *E. coli* O157:H7 Stamm Sakai unter den getesteten Bedingungen tatsächlich adhärieren und internalisieren kann. Dar-über hinaus wurde die Rolle von potentiell wichtigen intrinsischen Bakterienfaktoren untersucht, d.h. Gene bzw. Proteine, die möglicherweise in Adhärenz und/oder Internalisierung involviert sind. Dazu wurden Deletionsmutanten mit *hcpA*- und/oder *iha*-negativen Genotyp generiert. Diese wurden ebenfalls hinsichtlich Adhärenz und Internalisierung an bzw. in die Wurzeln von Feldsalat untersucht. Die beiden Gene kodieren die Hauptuntereinheit des hämorrhagischen Coli Pilus bzw. das Adhesin Iha und werden „Adhärenzfaktoren“ genannt, da sie im Zusammenhang mit Adhärenz stehen sollen. Al-lerdings zeigten weder die beiden Einzel- noch die Doppelmutante signifikante Veränderungen im Ad-härenzverhalten im Vergleich zum Wildtyp. Hinsichtlich der Internalisierung führte die Deletion eines der beiden Gene zu einer signifikant geringeren Zahl von internalisierten Bakterien, was darauf schlie-ßen lässt, dass diese Gene – oder die Proteine, die sie codieren – eine wichtige Rolle spielen bei dem

Eindringen von *E. coli* O157:H7 Stamm Sakai in die Wurzeln von Feldsalat. Interessanterweise resultierte die gleichzeitige Deletion beider Gene nicht in einer weiteren Reduktion der Internalisierung verglichen mit den Einzelmutanten. Folglich kodieren *hcpA* und *iha* eher Internalisierungfaktoren als Adhärenzfaktoren, die keine syngergistischen Effekte zeigen. Außerdem hängt Internalisierung nicht ausschließlich von diesen beiden Faktoren ab.

Die zweite Veröffentlichung fokussierte sich auf den Einfluss von Salatsorte und Bodentyp auf das Adhärenz- und Internalisierungsverhalten von *E. coli* O104:H4 Stamm C227/11ϕcu. In dieser Studie wurden die beiden Salatsorten *Valerianella locusta*, d.h. Feldsalat, und *Lactuca sativa*, d.h. Kopfsalat, in verschiedenen Bodentypen, Diluvialsand (DS) und Alluviallehm (AL) angepflanzt, um Auswirkungen von Pflanzenwirt und Umwelt auf die Anheftung und das Eindringen von Bakterien zu untersuchen. Um den Umweltaspekt genauer zu beleuchten wurde parallel die Zusammensetzung der Bodenmikrobiota mittels partieller 16S rRNA Gensequenzierung untersucht. Die Adhärenz an die Wurzeln wurde positiv vom Bodentyp beeinflusst. Die Zahl der adhärenten *E. coli* O104:H4 Stamm C227/11ϕcu Bakterien stieg um den Faktor drei bis vier bei Pflanzenwachstum in DS verglichen mit Wachstum in AL. Es zeigten sich aber keine statistisch signifikanten Unterschiede zwischen den Salatsorten, wenn diese im gleichen Bodentyp angepflanzt wurden. Die Internalisierung hingegen unterschied sich hauptsächlich zwischen den Salatsorten. Bei Pflanzenwachstum in DS wurde in den Kopfsalatwurzeln das 12-fache an internalisierten Bakterien gefunden verglichen mit den Wurzeln von Feldsalat. Bei Wachstum in AL war es gar das 108-fache. Zudem war die Internalisierung in die Wurzeln von *L. sativa* in AL fünfmal höher als in DS. Folglich hat die Salatsorte einen signifikanten Einfluss auf die Internalisierungsfähigkeit von *E. coli* O104:H4 Stamm C227/11ϕcu, wohingegen der Bodentyp unter den getesteten Bedingungen nur das Eindringen in Kopfsalatwurzeln beeinflusste. Darüber hinaus konnte der inokulierte Bakterienstamm mittels Mikrobiotaanalyse innerhalb der Bodenmikrobiota detektiert werden und diese Analyse zeigte, dass sowohl der Bodentyp als auch die Salatsorte als auch die Kombination von beiden zu großen Unterschieden in der Zusammensetzung der Bodenmikrobiota führen.

List of publications

Parts of this thesis were published in specialist journals or presented at conferences. The first author and presenting person are highlighted by underlining.

Original articles published in peer-reviewed journals

Eißenberger, K., Moench, D., Drissner, D., Weiss, A., Schmidt, H., 2018. Adherence factors of enterohemorrhagic *Escherichia coli* O157:H7 strain Sakai influence its uptake into the roots of *Valerianella locusta* grown in soil. Food Microbiol. 76, 245–256.

Eissenberger, K., Drissner, D., Walsh, F., Weiss, A., Schmidt, H., 2019. Plant variety and soil type influence Escherichia coli O104:H4 strain C227/11ϕcu adherence to and internalization into the roots of lettuce plants. Accepted for publication by Food Microbiology (available online; Food Microbiol. 86)

Magazine article (non-peer review)

Eißenberger, K., Drissner, D., Schmidt, H., 2017. In vivo-Kontamination von Feld- und Kopfsalat mit EHEC O157:H7 im Gewächshaus. Journal für Kulturpflanzen 69, 183-186

Oral presentations at conferences

Eissenberger, K., Drissner, D., Weiss, A., Schmidt, H.,2018. Internalization of enterohemorrhagic *Escherichia coli* into the roots of growing lettuce plants. 3rd International Workshop on Interactions between Crop Plant and Human Pathogens, Berlin, Germany.

Eissenberger, K., Drissner, D., Weiss, A., Schmidt, H., 2018. Internalisation of *Escherichia coli* O104:H4 into the roots of lettuce plants grown in different soil types. FoodMicro 2018, Berlin, Germany

Eissenberger, K., Drissner, D., Walsh F., Weiss, A., Schmidt, H., 2019. Einfluss von Bodenart und Salatsorte auf die Adhärenz von *Escherichia coli* O104:H4 C227/11ϕcu/pKEC2 an Salatwurzeln. 18. Fachsymposium Lebensmittelmikrobiologie, Kiel, Germany

Poster presentations at conferences

Eißenberger, K., Drissner, D., Saile, N., Weiss, A., Schmidt, H., 2017. Enterohemorrhagic *Escherichia coli* O157:H7 strain Sakai can be internalized into the root tissues of corn salad and lettuce following contamination of the soil under greenhouse conditions. Microbiology and Infection 2017, Würzburg, Germany.

Eißenberger, K., Drissner, D., Weiss, A., Schmidt, H., 2018. The adherence factors HcpA and Iha of enterohemorrhagic *Escherichia coli* O157:H7 strain Sakai influence its internalization into the roots of *Valerianella locusta* grown in soil under greenhouse conditions. 70. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie e. V., Bochum, Germany.

Eißenberger, K., Drissner, D., Weiss, A., Schmidt, H., 2018. HcpA and Iha influence the internalization of enterohemorrhagic *Escherichia coli* O157:H7 strain Sakai into the roots of *Valerianella locusta*. 10th International Symposium on Shiga Toxin (Verocytotoxin) producing Escherichia coli infections, Florence, Italy.

Further scientific publications

In addition to the above-mentioned publications, further scientific contributions were published which are not part of the present thesis.

Original articles published in peer-review journals

Saile, N., Schwarz, L., Eißenberger, K., Klumpp, J., Fricke, F.W., Schmidt, H., 2018. Growth advantage of *Escherichia coli* O104:H4 strains on 5-*N*-acetyl-9-*O*-acetyl neuraminic acid as a carbon source is dependent on heterogeneous phage-borne NanS-p esterases. Int. J. Med. Microbiol. 308:459-468.

Oral presentations at conferences

Saile, N., Schwarz, L., Eißenberger, K., Klumpp, J., Fricke, F., Schmidt, H., 2018. Competitive growth of *E. coli* O104:H4 strain with 5-N-acetyl-9-O-acetyl neuraminic acid as a carbon source. 70. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie e. V., Bochum, Germany.

Information on own contribution to publications

Within the scope of this thesis two manuscripts were prepared which fulfilled the requirements for a cumulative dissertation. Several co-authors were involved in the preparation. **Herbert Schmidt** was the applicant for the BLE project (2813HS028). He was significantly involved in the conception of the experiments described in the manuscripts (chapter 2 and 3), assisted the writing and correction of the manuscripts. **David Drissner** was involved in the funding proposal (2813HS028), the conception of the experiments described in the manuscripts (chapter 2 and 3), assisted the writing and correction of the manuscripts. **Agnes Weiss** was involved in the conception of the experiments described in the manuscripts (chapter 2 and 3), assisted the writing and correction of the manuscripts.

Manuscript I (chapter 2)

Kristina Eissenberger. I was involved in the conception of the experiments described in the manuscript. I conducted all experiments described in this publication except for the preliminary experiments performed with *E. coli* DH5α/pWRG435. In particular, I constructed the described plasmids, prepared isogenic deletion mutants, performed the plasmid stability experiments, conducted adherence and internalization experiments including all statistical analysis. I wrote and edited the manuscript.

Doris Moench conducted all steps of the preliminary experiments performed with *E. coli* DH5α/pWRG435.

Manuscript II (chapter 3)

Kristina Eissenberger. I was involved in the conception of the experiments described in the manuscript. I performed all experiments described in this publication except for the partial 16S rRNA gene sequencing. In particular, I performed the plasmid stability experiments, conducted the adherence and internalization experiments including all statistical analysis, performed total microbial community DNA extraction and analyzed the provided sequences in accordance to Fiona Walsh's recommendations. I wrote and edited the manuscript.

Fiona Walsh was involved in the conception of the partial 16S rRNA gene sequencing experiments including sequencing and bioinformatic analysis, and assisted the writing and correction of the manuscript.

_____ _____

Place, Date Signature of supervisor

1. Introduction

1.1 Shiga toxin producing *Escherichia coli* and enterohemorrhagic *Escherichia coli*

First described in 1885, *Escherichia coli* show a long history of research. This bacterial species shows high diversity comprising apathogenic and pathogenic strains (1). Apathogenic *E. coli* strains are commonly found in the gastrointestinal tract of humans and animals where they are part of the natural microbiota. Pathogenic *E. coli* strains can be grouped according to their genetic similarities, especially their virulence factors, and symptoms associated with infections (1, 2). Adherent-invasive *E. coli* (AIEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC) are only a subset of the described human pathogenic *E. coli* groups. Enteroaggregative *E. coli* (EAEC) and enterohemorrhagic *E. coli* (EHEC) are two more human pathogenic groups. The former is primarily associated with protracted diarrhea in children in developing countries (3), but yet has caused several food-associated outbreaks in Europe (4). The latter, again, is a subgroup of Shiga toxin producing *E. coli* (STEC). STEC comprise *E. coli* strains that carry at least one gene coding for Shiga toxin which shows similarities to *Shigella dysenteriae* type 1 toxin (1). Shiga toxin producing *E. coli* strains that are known to cause hemorrhagic colitis and the hemolytic uremic syndrome are classified as EHEC (5). Additionally, *E. coli* can be categorized to serotypes based on the major surface antigens “O” and “H”. In this case, “O” is part of the lipopolysaccharide layer and “H” is part of the flagellar apparatus, namely the flagellin encoded by *fliC* (1).

1.1.1 Virulence factors

Shiga toxin producing *E. coli* strains harbor an extensive repertoire of virulence factors which are mostly encoded by mobile genetic elements such as prophages, pathogenicity island and plasmids (6). The main virulence factors are introduced in the following chapter.

1.1.1.1 Shiga toxin

The major virulence factor of STEC is Shiga toxin (Stx). It can be classified into two major groups, Stx1 and Stx2. Both of them contain multiple subtypes. The Stx1 group comprises currently three subtypes, Stx1a, Stx1c and Stx1d, whereas seven subtypes were described for Stx2, namely Stx2a to Stx2g (7). STEC strains may carry a single *stx* variant, both variants, or a combination of *stx2* subtypes. The genes of both groups are encoded on chromosomally integrated lambdoid prophages (8). Shiga toxin is an AB_5 toxin. It is composed of one A-subunit, which exhibits N-glycosidase activity, and a pentamer of B-subunits which mediates binding to globotriaosylceramide, a glycolipid receptor present on the sur-

face of endothelial cells (9–11). Upon binding, the Stx holotoxin is endocytosed and trafficked retrogradely to the endoplasmic reticulum via the Golgi apparatus. When the A subunit is released into the cytoplasm, it removes an adenine residue from the 28S rRNA. This irreversible removal leads to the inhibition of the protein synthesis and ultimately, results in the death of the affected cell (12–15).

1.1.1.2 Locus of enterocyte effacement

The locus of enterocyte effacement (LEE) is a pathogenicity island which is frequently found in STEC strains, but also in EPEC strains (16). It contains genes coding for a type three secretion system (T3SS) and several effector proteins (17). Strains that contain the LEE are consequently referred to as LEE-positive strains. Due to its syringe-like form, T3SS acts as injectisome which translocates effector molecules directly into eukaryotic host cell. The translocated effector proteins can interact with the host actin structure and change the host cytoskeleton which leads to the formation of so-called attaching and effacing (A/E) lesions (6, 18, 19). For the formation of A/E lesions intimate contact between bacteria and host cell is necessary. Effector proteins which contribute to the required proximity are intimin, encoded by *eae*, (20) and the translocated intimin receptor Tir, which is the first translocated effector molecule (21). Upon translocation, Tir inserts into the host cytoplasmic membrane. As intimin is expressed on the bacterial surface, it can bind to Tir and ensure close contact between bacteria and host cell (21). Besides its important function in human infection, the T3SS was also shown to be involved in the colonization of plant leaves (22, 23). These findings indicate that the mechanism of animal and plant infection by *E.* coli may be conserved which was already described for *Salmonella enterica* (24).

1.1.1.3 Enterohemolysin

The heat instable toxin enterohemolysin (E-Hly) is a toxin which can cause lysis of various eukaryotic cells and erythrocytes (25–27). This toxin does not exclusively occur as free form but can also be associated with outer membrane vesicles (OMVs). Interestingly, these different forms show distinct modes of action (28, 29). Free E-HylA can directly lyse cells by binding and subsequent pore formation, whereas the OMV-associated form acts intracellularly. The target cells can take up OMVs by endocytosis. Separation of E-Hly from the vesicles is mediated by lysozymes and E-Hly then translocates into the mitochondria where it triggers capase-9-mediated apoptosis (28, 29). Therefore, E-Hly is able to cause cell damage on multiple levels.

1.1.1.4 Iron-regulated gene A (IrgA) homologue adhesin

Among virulence factors, the IrgA homologue adhesin (Iha) occupies a special position. It is widely distributed among STEC strains and further *E. coli* pathotypes, such as uropathogenic *E. coli* (UPEC), EAEC and ETEC, to name but a few (30–33). As the name "adhesin" implies, Iha mediates adherence to a number of different human and animal cells lines (34–36), although it functions as enterobactin siderophore receptor (31). In different animal models of distinct *E. coli* pathotypes, namely EHEC and UPEC, Iha was shown to be important for successful colonization of ligated pig ileal loops and the murine urinary tract (34, 36). Based on its wide distribution and the demonstrated pathogenic potential in animal models, it cannot be excluded that Iha may contribute to *E. coli* caused human disease, although the exact mode of action remains unknown.

1.1.1.5 Hemorrhagic coli pilus

The hemorrhagic coli pilus (HCP) is a type 4 pilus that was shown to harbor multiple features. Several studies showed that HCP is involved in adherence to various animal and human cell lines as well as plant leaves (22, 37–40). Xicohtencatl-Cortes et al. (39) observed that HCP is able to bind the extracellular matrix proteins laminin and fibronectin. Additionally, the same study indicates that HCP of one EHEC cell may interact with HCP of another cell thereby mediating bacterial binding. Moreover, the deletion of the gene coding for the major HCP subunit, *hcpA*, resulted in reduced biofilm formation and invasion into epithelial cells *in vitro* (39). As HCP is capable to induce proinflammatory cytokine secretion in different human intestinal epithelial cell lines (37), a potential role in pathogenicity upon human infection cannot be excluded.

1.1.2 Hosts and reservoirs

Ruminants are the best known reservoir for STEC and EHEC (41). However, further organisms and ecological niches need also to be taken into account. This chapter will discuss animals, plants, environmental niches and humans as reservoirs and host of STEC.

1.1.2.1 Animals as reservoir

As reviewed by Persad and Lejeune (41), STEC have been detected in a variety of animals, such as birds, fish, insects and mammals. Interestingly, most animals carry STEC asymptomatically which means that STEC do not cause any disease in these animals. However, STEC may replicate within the gastrointestinal tract of these animals or may be carried passively/transiently without noteworthy replication in the host (41).

From an epidemiological point of view, different animals display distinct routes of contamination and infection. Ruminants, especially cattle, are regarded as the primary reservoir of STEC (41). These animals also carry STEC asymptomatically. They represent versatile vehicles as they are able to shed high quantities of STEC and transmit STEC to the environment, other animals, such as house flies, and to humans (41). Transmission to human may also occur due to the consumption of undercooked beef. Multiple studies found high number of STEC in cattle feces (42–44). As cattle manure is often used as organic fertilizer, STEC are directly deposited on soil and therefore, transferred to the environment.

At farms, house flies live in close contact with cattle and therefore, may easily incorporate STEC. As house flies are not limited to livestock only, it is possible that they can spread STEC directly to the environment and indirectly to humans by contamination of food. Several studies demonstrated that flies in general and house flies in particular are able to take up STEC and to spread STEC to leafy greens (45–47). Moreover, a study published by Kobayashi et al. (48) found that in 1996 during an EHEC outbreak in a nursery school, the causative EHEC strain was isolated from both patients and house flies. These findings may indicate that house flies play a role in STEC and EHEC transmission.

Besides livestock, wild animals and insects, pets such as dogs and cats are also under debate as transmissive vehicle. Several studies showed that the mentioned pets can shed a variety of STEC serotypes (41, 49). As these animals usually live in close contact to their owners, STEC may be easily transferred to humans. However, to the present day, only one canine-associated outbreak of human disease was reported (50), and another study suggests that transmission from pets to owner is rather unlikely (51).

1.1.2.2 Plants as hosts for STEC

Although animals are recognized as their primary reservoir, STEC are also capable to colonize plants and to persist within plants (52–58). Apparently, plants have several entry points for bacteria such as leaf and root lesions, the leaf stomata and sites of lateral roots emergence (59). Possibly, STEC can invade plants through any of these entry sites.

Although, STEC are not pathogenic to plants, they are still recognized by the plant immune system (60–62). Nevertheless, several studies showed that STEC and additional human pathogens can attach to and internalize into seedlings, roots, and leaves of a wide range of plant species (59, 63, 64). These studies mainly focused on edible plants such as leafy greens, microgreens, and lettuce, which are predominantly consumed raw. Therefore, contamination of fresh produce with STEC presents a serious threat to the public.

Erickson and colleagues showed that *E. coli* O157:H7 are able to internalize into the leaves and the roots of spinach, lettuce, and parsley (53, 54). Chitarra et al. (52) demonstrated that STEC were able

to internalize into the roots of leafy greens and to migrate from the roots to the leaves where STEC persisted for several weeks. Interestingly, Wright and colleagues (57, 58) showed that a variety of plants such as *Nicotiana benthamiana*, lettuce, spinach, tomato, and microgreens, can be colonized by *E. coli* O157:H7 strain Sakai when the plants were grown in growth substrate or soil contaminated or irrigated with this *E. coli* strain. Moreover, the tested strain depicted active growth within the plant hosts (57, 58). These findings emphasize that plant can serve as actual hosts for STEC and not only as transmissive vehicle.

Moreover, several studies demonstrated that particular bacterial factors are involved in the internalization process (22, 23, 65, 66). For instance, *E. coli* mutants which were no longer able to secrete effector molecules showed decreased internalization into spinach leaves (22, 23). At lettuce leaves, *S. enterica* mutants with defects regarding chemotaxis depicted reduced stomata penetration (66). For both pathogens, motility was shown to play an important role during internalization into the roots of *Arabidopsis thaliana* as well as into the leaves of spinach, and lettuce (23, 65, 66). In summary, the aforementioned studies showed that internalization requires active movement of the bacterial towards natural entry points. Other than that, knowledge regarding the mechanism of bacterial root and leaf invasion is limited, and requires further investigations.

1.1.2.3 Environmental reservoirs

Besides animals and plants, STEC were already detected in soil and water as well (67). An international study showed that water and soil were even more prone to contain STEC than fresh produce on the field (68). Detection of STEC in soil and water may be linked to the proximity to agricultural holdings. As demonstrated by Bolton et al. (69), STEC can be found in pasture soil located near bovine farms. On the other hand, Tanaro et al. (70) found that the virulence profiles of *E. coli* O157 isolated from bovine livestock, and from surface water in cattle breeding areas exhibited similar genotypes. These findings indicate that STEC can easily spread from cattle to the environment.

Dissemination of STEC from cattle to soil can be achieved by applying bovine manure as organic fertilizer as already described in section 1.1.2.1. Upon heavy rainfall, surface runoff containing STEC can then flood nearby fields, enter close-by natural waters and wells by what STEC may end up in the ground waters and the drinking water supply. Multiple outbreaks of human disease were already linked to the exposure to contaminated water (67, 71) and to the exposure to contaminated soil (72, 73). Water exposure included swimming in and drinking of contaminated water. In the latter case, heavy rainfalls most likely carried over STEC from a field of a nearby farm, that was recently fertilized with manure, to a drinking water well (74). Several studies showed that *E. coli* can survive in water

and soil for several weeks up to several months (69, 75). This is of special interest as STEC were able to colonize plants when grown in contaminated soil (52, 53, 58).

1.1.2.4 Human host

Transmission of STEC or EHEC to humans can occur by contaminated food, contaminated water or by person-to-person contact (19). In humans, STEC colonize the lower gastro-intestinal tract where they are competing with the natural gut microbiota for nutrients (76). Although, ingestion of STEC and EHEC is known to cause disease in humans (1.1.3), people may also serve as reservoirs for STEC and therefore, play an important role in the epidemiology of STEC infections (41, 77, 78).

Person-to-person transmission via asymptomatic human carriers represent a serious threat to public health and has already been shown to be involved in several outbreaks (77, 78). Asymptomatic shedding of STEC was observed for children in day-care facilities (79) and also for dairy farm and abattoir workers (80, 81). For farm and abattoir workers, it sounds reasonable that they acquired STEC during work when taking into account that (fattened) cattle is the primary reservoir, as discussed in section 1.1.2.1, and that STEC can be found in plants and soil as well.

1.1.3 Infection and disease process

Human infection with STEC and EHEC may occur by ingestion of these human pathogens via contaminated food and water or by person-to-person contact. For human infection STEC and EHEC may use the same routes as for transmission to humans. In Germany, human infection with EHEC is recognized as notifiable disease that is monitored by the Robert-Koch-Institute (RKI) (82). The typical incubation period for EHEC infections is three to four days, even though longer incubation periods of up to eight or even twelve days are described as well (19, 78, 83).

Watery diarrhea accompanied with nausea and vomiting are the first clinical symptoms. In about 90% of the cases, bloody diarrhea occurs and the patients experience severe abdominal cramps and possibly fever (83). Approximately 5 to 10% of patients infected with EHEC develop the hemolytic-uremic syndrome (HUS) which manifests as triad of anemia, thrombocytopenia, and renal failure (19, 76), but can be accompanied also by central nervous manifestations (78), and may even lead to death (76).

Treatment of EHEC infections is carried out in a symptom-oriented manner as the application of antibiotics shows off as contraindicated (78). Instead, treatment may include fluid and electrolyte replacement to counteract dehydration, dialysis, erythrocyte and whole-blood transfusions (78, 83). In 2017, in total 2020 cases of human EHEC infections and 72 confirmed cases of HUS were reported in Germany of which eight people died (82). The number of cases account for more than 40 outbreaks that

mainly occurred in private households. Approximately 43% of the reported HUS cases were caused by *E. coli* O157, although this serotype accounted only for 11% of the EHEC infections (82).

1.2 Foodborne disease outbreaks associated with EHEC

As the human infectious dose is suggested to be very low (84), it is not surprising that EHEC are associated with numerous outbreaks. Especially as raw or undercooked meat and dairy products are two of the main vehicles responsible for transmission to humans. Nevertheless, the number of outbreaks associated with infection sources of non-animal origin are rising (85). This chapter gives a brief overview about EHEC associated outbreaks with known infection sources.

In 1982, the first disease outbreak associated with food contaminated by EHEC was reported in the United States (86). During this outbreak, 47 people in Oregon and Michigan showed the classical symptoms linked to EHEC caused disease. At this time, the symptoms of initially watery diarrhea followed by bloody diarrhea, abdominal cramps, and little to no fever represented an unusual gastrointestinal illness. Due to intensive epidemiologic and laboratory investigations, the origin of the outbreak was traced back to restaurants belonging to the same fast-food chain (86). Moreover, undercooked meat was found be contaminated with *E. coli* O157:H7 strain EDL933 and the same strain was also isolated from patients' stool samples (86). This outbreak was the first described EHEC associated outbreak of human disease.

In 1996, the largest outbreak worldwide in terms of number of cases was reported in Japan. In contrast to the first described outbreak, the infection source was not traced back to animal-based food, but plant-based food (87). The consumption of contaminated radish sprouts resulted in more than 9000 confirmed cases and 12 deaths. The causative agent was identified as *E. coli* O157:H7 strain Sakai (87). Intriguingly, most of the patients were elementary schoolchildren as the relevant sprouts were part of their school lunch. Preparing the same menu for all schools in a certain area is an essential part of the unique school lunch system. This allowed the contaminating agent to infect many children simultaneously (87). Still, this outbreak presents the largest outbreak reported worldwide.

The most serious outbreak was reported in Germany in 2011. Between May and June 2011, a total of 3842 cases were recorded of which 855 patients developed HUS, and 53 patients died (77). The source of the outbreak was traced back to organic fenugreek sprouts (88). These sprouts were supposed to be contaminated with Shiga toxin producing *E. coli* O104:H4. *Escherichia coli* O104:H4 strain LB226692 and *E. coli* O104:H4 strain C227/11 were isolated from patients during this outbreak and characterized in detail (89, 90). Interestingly, molecular biological studies revealed that these strains carried *stx2a* but were not representatives of "classical" EHEC strains such as *E. coli* O157:H7 strain EDL933 or strain

Sakai (91). The 2011 outbreak isolates rather shared genetic similarities with EAEC strains and, therefore, are referred to as either enteroaggregative/enterohemorrhagic E. coli (EAEC/EHEC) strains or enteroaggregative hemorrhagic *E. coli* (EAHEC) (91, 92). Interestingly, besides primary transmission, ingestion of contaminated sprouts, secondary transmission played an important role. In this case, secondary transmission means that EAEC/EHEC O104:H4 were spread by humans, either directly by person-to-person contact or by transmission of the bacteria to food by asymptomatic carrier, recovered patients or infected individuals such as catering personnel (77). The 2011 outbreak represents the deadliest outbreak on global level.

From October 2018 until December 2018 and January 2019, the most recent outbreak was reported in Canada by the Public Health Agency of Canada (PHAC; https://www.canada.ca/en/public-health) and in the United States by the Center for Disease Control and Prevention (CDC; https://www.cdc.gov/), respectively. In Canada, a total 29 cases of *E. coli* infections were recorded in 4 provinces. Of these 29 patients, two developed HUS (93). In the United States, the outbreak was reported from 15 states and the District of Columbia. A total of 62 cases were confirmed of which 25 were hospitalized and two developed HUS (94). Both, the infections reported in Canada and the infections reported in the United States, were linked to romaine lettuce contaminated with an *E. coli* O157:H7 strain. Investigations of the outbreak indicated that the romaine lettuce was harvested in the Central Coastal growing regions of northern and central California. Moreover, during trace back investigations, 8 farms were identified as potential origins of the causative *E. coli* strain. A strain of *E. coli* O157:H7 was found in the sediment within an agricultural water tank located on one of the 8 identified farms. Intriguingly, the *E. coli* strain found within the water reservoir was genetically closely related to the *E. coli* O157:H7 strain isolated from ill patients. Therefore, the water tank marks a probable source of contamination. Currently, it is not clear how *E. coli* O157:H7 entered the agricultural water tank. As a precautionary measure, the identified farm recalled various fresh produce harvested between November 27 and 30, 2018, as announced by the CDC (94).

1.3 Representatives of the group of STEC

STEC and EHEC can be regarded as genetically heterogenous as they comprise numerous *E. coli* serotypes, and a wide range of genomic profiles. One of the most established distinctions is based on the presence and absence of the LEE. The following paragraph will introduce representatives of both, LEE-positive and LEE-negative strains, which were investigated in this dissertation.

1.3.1 *Escherichia coli* O157:H7 strain Sakai

Escherichia coli O157:H7 strain Sakai was isolated during a large outbreak in Japan in 1996 (87). It is a "classical" LEE-positive EHEC strain which carries two prophages coding for Stx1 and Stx2a, respectively (95). Virulence genes that are typically associated with and encoded by EHEC O157 strains, such as strain Sakai, are the intimin encoding gene *eae*, genes coding for the T3SS, E-*hlyA* encoding enterohemolysin A, and *fliC* (95). Moreover, strain Sakai carries *hcpA* and *iha*. Both genes code for proteins that may be considered as virulent traits (section 1.1.1). Besides the outbreaks mentioned in section 1.2, EHEC O157 strains caused a large number of outbreaks of human disease (1, 96–98) and according to the World Health Organization (WHO), EHEC O157:H7 is the most important *E. coli* serotype when it comes to public health (99). Therefore, *E. coli* O157:H7 strain Sakai serves as representative of this serotype which already demonstrated it pathogenic potential in the largest outbreak worldwide.

1.3.2 *Escherichia coli* O104:H4 strain C227/11

Escherichia coli O104:H4 strain C227/11 was isolated during the German 2011 outbreak (90). It is a STEC strain as it harbors a prophage coding for Stx2a and also is able to express this toxin. As it also caused hemorrhagic colitis and the hemolytic-uremic syndrome, it can be regarded as EHEC strain following the classical specification (5). However, this strain also showed aggregative, so-called "stacked-brick", adherence to human intestinal epithelial cells which is a phenotype that is typical for EAEC (92). Moreover, the 2011 outbreak strain encoded multiple genetic elements that are associated with virulence of EAEC strains (92). Intensive molecular biological investigations revealed that this strain harbors two virulence plasmids. One is similar to the pAA plasmids of EAEC strains, which contain aggregative adhesion fimbrial operons, whereas the other one carries genes associated with extended-spectrum β-lactamases (91). Consequently, *E. coli* O104:H4 strain C227/11 shares genetic similarities of both EHEC and EAEC strains. The combination of both, EHEC and EAEC, features is supposed to be reason for the high virulence of this strain as EAEC-like adherence was shown to enhance inflammation and Stx2a translocation (100, 101). This strain will be referred to as enteroaggregative/enterohemorrhagic E. coli (EAEC/EHEC) throughout this dissertation.

In this thesis, *E. coli* O104:H4 strain C227/11 was chosen as a representative of the emerging portion of non-O157 STEC strains. As the ingestion of this strain resulted in the deadliest outbreak reported so far, the *stx2a*-negative derivative *E. coli* O104:H4 strain C227/11ϕcu, which lacks the *stx2a* harboring prophage (102), was used for safety reasons.

1.4 Research objectives

The present thesis was conducted within the scope of the "Colonization of plants by *Escherichia coli* and *Salmonella enterica* - plantinfect" project. This project was funded by the Federal Office for Agriculture and Food (BLE; https://www.ble.de/EN/; 2813HS028). Between April 2015 and September 2018, a consortium of four research groups accompanied by a project advisory group investigated the colonization of crop plants by *E. coli* and *S. enterica*. The research project focused on four aspects: the validation and optimization of specific detection methods for *S. enterica* and *E. coli* in plant and soil samples, the impact of agricultural growth conditions, such as soil type and fertilizer, plant variety, and the genetic equipment of the relevant pathogens on the colonization of crop plants, the analysis of the distribution of *S. enterica* and *E. coli* within the distinct plant tissues, such as root, shoot, and leaves, by application of microscopic, DNA-based and culture-dependent methods and techniques and a final risk assessment.

The present thesis surveyed a subset of the aspects addressed in the "plantinfect" project. The aim of this thesis was to investigate the adherence and internalization behavior of different enterohemorrhagic *E. coli* strains at the roots of lettuce plants.

First, the impact of the genetic equipment of *E. coli* O157:H7 strain Sakai on its adherence to and internalization into the roots of *Valerianella locusta*, also known as lamb's lettuce, was addressed. In particular, the influence of two genes, *hcpA* and *iha*, was investigated in order to analyze intrinsic bacterial factors potentially involved in plant colonization and to shed light on the underlying mechanism of this process. The results of this study are depicted in Chapter 2.

Second, the impact of agricultural growth conditions was investigated, especially the influence of soil the type and lettuce variety, on the adherence and internalization behavior of *E. coli* O104:H4 strain C227/11ϕcu. Moreover, soil microbial composition was analyzed by next generation sequencing to determine the influence of the lettuce variety, soil type, and lettuce variety/soil type combination on the soil microbiota, and to determine the impact of the soil microbiota on adherence and internalization behavior of the investigated *E. coli* strain. The results of these investigations are described in Chapter 3.

Chapter 2

Adherence factors of enterohemorrhagic *Escherichia coli* O157:H7 strain Sakai influence its uptake into the roots of *Valerianella locusta* grown in soil

Kristina Eißenberger[a], Doris Moench[a], David Drissner[b,c], Agnes Weiss[a], and Herbert Schmidt[a]#

[a]Institute of Food Science and Biotechnology, Department of Food Microbiology and Hygiene, University of Hohenheim, Germany

[b]Microbiology of Plant Foods, Agroscope, Waedenswil, Switzerland

[c]Swiss Federal Institute for Forest, Snow, and Landscape Research WSL, Birmensdorf, Switzerland

Published in Food Microbioloy Volume 76, pages 245–256.

DOI: 10.1016/j.fm.2018.05.016 . Courtesy of Elsevier

This publication contains supplementary figures which can be found at http://dx.doi.org/10.1016/j.fm.2018.05.016

Food Microbiology 76 (2018) 245–256

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Adherence factors of enterohemorrhagic *Escherichia coli* O157:H7 strain Sakai influence its uptake into the roots of *Valerianella locusta* grown in soil

Kristina Eißenberger[a], Doris Moench[a], David Drissner[b,c], Agnes Weiss[a], Herbert Schmidt[a,*]

[a] Institute of Food Science and Biotechnology, Department of Food Microbiology and Hygiene, University of Hohenheim, Germany
[b] Microbiology of Plant Foods, Agroscope, Waedenswil, Switzerland
[c] Swiss Federal Institute for Forest, Snow, and Landscape Research WSL, Birmensdorf, Switzerland

ARTICLE INFO

Keywords:
Enterohemorrhagic *E. coli* O157:H7
Lamb's lettuce
Internalization
hcpA
iha
Greenhouse

ABSTRACT

Increasing numbers of outbreaks caused by enterohemorrhagic *Escherichia coli* (EHEC) are associated with the consumption of contaminated fresh produce. The contamination of the plants may occur directly on the field *via* irrigation water, surface water, manure or fecal contamination. Suggesting a low infectious dose of 10 to 10^2 cells, internalization of EHEC into plant tissue presents a serious public health threat. Therefore, the ability of EHEC O157:H7 strain Sakai to adhere to and internalize into root tissues of the lamb's lettuce *Valerianella locusta* was investigated under the environmental conditions of a greenhouse. Moreover, the influence of the two adherence and colonization associated genes *hcpA* and *iha* was surveyed regarding their role for attachment and invasion. Upon soil contamination, the number of root-internalized cells of EHEC O157:H7 strain Sakai exceeded 10^2 cfu/g roots. Deletion of one or both of the adherence factor genes did not alter the overall attachment of EHEC O157:H7 strain Sakai to the roots, but significantly reduced the numbers of internalized bacteria by a factor of between 10 and 30, indicating their importance for invasion of EHEC O157:H7 strain Sakai into plant roots. This study identified intrinsic bacterial factors that play a crucial role during the internalization of EHEC O157:H7 strain Sakai into the roots of *Valerianella locusta* grown under the growth conditions in a greenhouse.

1. Introduction

Enterohemorrhagic *E. coli* (EHEC) O157:H7 strains can cause serious human diseases such as diarrhea, hemorrhagic colitis and the hemolytic-uremic syndrome (HUS) (Kaper, 1998). *Escherichia coli* O157:H7 strains produce an arsenal of pathogenicity factors that enable them to be competitive and cause serious human diseases and outbreaks. The most important ones are the production of one or more Shiga toxins and the expression of the locus of enterocyte effacement (LEE) (Kaper, 1998), enabling the bacteria to translocate type III effectors into the cytosol of target cells. *Escherichia coli* O157:H7 is mainly transmitted to humans by raw or undercooked meat and dairy products but during the last years infection sources of non-animal origin were increasingly reported representing ~20% of EHEC-caused infections (Greig and Ravel, 2009). The human infectious dose was estimated in a range of 10 to 10^2 cfu for ground beef (Tuttle et al., 1999).

So far, the biggest EHEC O157 outbreak occurred in Sakai, Japan, in 1996, with more than 9000 confirmed cases and 12 deaths. The identified agent were radish sprouts contaminated with EHEC O157:H7 strain Sakai (Michino et al., 1999). Several studies have already demonstrated that *E. coli* O157:H7 strains are able to colonize the leaves and roots of lettuce and other leafy greens (Erickson et al., 2010; Seo and Frank, 1999; Solomon et al., 2002), and to persist for several days to weeks (Chitarra et al., 2014; Wright et al., 2017). Surface structures such as pili, flagella, the type III secretion system (T3SS), as well as proteins involved in quorum sensing were found to be involved in successful adherence to spinach leaves and leaves of red oak lettuce (Macarisin et al., 2012; Nuebling et al., 2017; Saldana et al., 2011) under laboratory conditions. Various factors such as surface appendages, outer membrane proteins, extracellular polysaccharides (Frank, 2001), cell surface hydrophobicity and charge (Fletcher and Loeb, 1979) are supposed to be generally involved in attachment.

The capability of EHEC to colonize the roots of leafy greens such as lettuce, parsley and spinach was shown by recent studies (Erickson et al., 2014; Solomon et al., 2002; Wright et al., 2017). These studies were performed mostly in environmental growth chambers and focused on the influence of external factors. Solomon et al. (2002) investigated the impact of different inoculation strategies comparing contamination *via* manure or irrigation water on the internalization of *E. coli* O157:H7 into lettuce seedling grown in an environmental growth chamber. The

* Corresponding author.
E-mail address: herbert.schmidt@uni-hohenheim.de (H. Schmidt).

https://doi.org/10.1016/j.fm.2018.05.016
Received 19 December 2017; Received in revised form 27 April 2018; Accepted 30 May 2018
Available online 31 May 2018
0740-0020/ © 2018 Elsevier Ltd. All rights reserved.

different treatments had only little effects. The impact of the plant growth substrate on bacterial internalization was investigated multiple times leading to contradicting results (Franz et al., 2007; Hora et al., 2005; Macarisin et al., 2014; Sharma et al., 2009). Some studies showed that invasion of *E. coli* O157:H7 into spinach roots was enhanced when plants were grown in hydroponic medium compared to soil (Sharma et al., 2009). These authors hypothesized that a hydroponic solution provides better motility leading to increased internalization compared to soil (Sharma et al., 2009). By contrast, other studies demonstrated that the occurrence of internalization events into the roots of spinach was higher in soil-grown plants than in hydroponically grown plants (Franz et al., 2007; Macarisin et al., 2014). Presumably, this is due to augmented damage of the roots as this is more likely to occur when plants are grown in soil. For plants grown in hydroponic medium, damage of the plant roots was shown to act as promoting factor for internalization (Macarisin et al., 2014). In contrast, studies investigating the connection between root damage and frequency of internalization events that were performed under growth chamber conditions did not observe increased bacterial invasion on mechanically or biologically disrupted spinach plants (Hora et al., 2005). Bacterial internalization seems to be a complex process that needs further research. To our knowledge, it remains unclear which intrinsic factors of EHEC strains are important for colonization of plant roots.

Hence, the present study focused on the role of two adherence factors, Iha and HcpA, during root colonization. The IrgA homolog adhesion (Iha) is encoded by *iha* and functions as enterobactin siderophore receptor (Rashid et al., 2006). It was first described in *E. coli* O157:H7 (Tarr et al., 2000). Its expression is repressed by the ferric uptake regulation protein Fur (Rashid et al., 2006) and triggered by short-chain fatty acids (Herold et al., 2009). In contrast to other siderophore receptors, it harbors the unique feature of contributing to adherence to different human and animal cell lines (Johnson et al., 2005; Tarr et al., 2000; Yin et al., 2009). Introduction of *iha* into non-adhering *E. coli* strains is sufficient to confer attachment capability to these strains (Tarr et al., 2000). It is widely distributed among different *E. coli* pathotypes, such as enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and uropathogenic *E. coli* (UPEC) (Schmidt et al., 2001). For the latter and extraintestinal pathogenic *E. coli* (ExPEC), Iha was found to be a virulence factor during urinary tract infections (Johnson et al., 2000, 2005; Leveille et al., 2006). Interestingly, in Shiga-toxin producing *E. coli* (STEC) *iha* was found in 57,3% of the STEC strains isolated from food of animal origin (Slanec et al., 2009).

The *hcpA* gene encodes the pilin subunit of an adhesive type IV pilus called hemorrhagic coli pilus (HCP) (Xicohtencatl-Cortes et al., 2007) and was formerly called prepilin peptidase-dependent gene (*ppdD*) (Ledesma et al., 2010). The hemorrhagic coli pilus was shown to be involved in adherence to a variety of mammalian cell lines (Xicohtencatl-Cortes et al., 2007), and to leaf surfaces (Nuebling et al., 2017; Saldana et al., 2011). However, contradicting results were gained when investigating attachment to leaf surfaces. Upon deletion of *hcpA* decreased adherence was observed on spinach leaves (Saldana et al., 2011). Interestingly, its deletion resulted in enhanced attachment to red oak leaf lettuce leaves (Nuebling et al., 2017). As these two studies used different incubation periods, the obtained results may indicate that adherence is a time-dependent process and hinges on the target surface. Moreover, deletion of *hcpA* was shown to lead to decreased internalization into HT-29 cells (Xicohtencatl-Cortes et al., 2009). For the same cell line, it was demonstrated that HCP (HcpA) induces the activation of proinflammatory cytokines in polarized HT-29 cells (Ledesma et al., 2010). Hence, HCP can be considered as a virulence factor.

As both Iha and HcpA were shown to be involved in pathogenicity in mammalian model systems, we hypothesize that they may also play a role in successful colonization of plant roots. This is supported by observations of Schikora et al. (2011), who reported that for *Salmonella* there is a high degree of conservation of the infection mechanisms in plants and animals. Thus, the role of the adherence factors Iha and HcpA during root colonization was investigated using *Valerianella locusta*, also known as lamb's lettuce, as a host. *Valerianella locusta*, mostly cultivated in greenhouses during winter, is a fall and winter lettuce which stands out due to its short leaves that are predestinated for infections starting from the roots. According to the German Federal Ministry of Food and Agriculture, lamb's lettuce is one of the lettuces with the highest revenue in Germany (https://www.bmel.de/EN/). For analysis of the *in vivo* capacity of EHEC O157:H7 strain Sakai to adhere to and internalize into the roots of cultivated plants after irrigation with contaminated water, and whether selected typical adherence factors were involved, an experimental setup was chosen under environmental conditions in a biosafety greenhouse meeting the safety requirements for biosafety level 3 according to appendix 4 of the Swiss Containment Ordinance (ESV). In order to shed light on different aspects of colonization, plant roots were analyzed concerning adherence and internalization of EHEC O157:H7 strain Sakai.

2. Material and methods

2.1. Bacterial strains

All bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown in LB medium (10% (w/v) tryptone, 10% (w/v) NaCl, 5% (w/v) yeast extract, pH 7.0) at 37 °C with shaking at 180 rpm unless indicated differently. When needed, antibiotics were added to the following final concentrations: 100 µg/ml ampicillin, 50 µg/ml kanamycin and 20 µg/ml chloramphenicol.

2.2. Preparation of electrocompetent bacterial cells and electroporation

Electrocompetent bacterial cells were prepared, and electroporation was performed as described previously (Saile et al., 2016).

2.3. Plasmid construction

Plasmid pKEC2 was constructed by amplifying the *cat* gene plus 375 bp upstream using pCP20 as template, and cloning the PCR product into pWRG435 after digesting the PCR product and the backbone plasmid with PvuI. For PCR, restriction digestion, ligation, transformation and plasmid isolation standard protocols were applied as described by Maniatis et al. (1985). Plasmid DNA was isolated from *E. coli* DH5α using a QIAprep Spin Miniprep kit (Qiagen, Netherlands) following the manufacturer's instructions, and screened for the insert's identity and orientation by sequencing using the following primer: P-cat PvuI for, cat PuvI rev, cat 124 for and cat 225 rev (Table 2).

2.4. Construction of isogenic gene deletion mutants

Gene deletions were performed according to the method of Datsenko and Wanner as described previously (Datsenko and Wanner, 2000; Saile et al., 2016). The primers applied for mutagenesis are listed in Table 2. Verification of deletions was performed by PCR and sequencing.

2.5. Cloning of adherence factor genes

For plasmid-based complementation of the knock-out strains, genomic DNA (gDNA) of *E. coli* O157:H7 strain Sakai was isolated using DNeasy Blood & Tissue Kit (Qiagen, Netherlands) following the manufacturer's instructions. To amplify the genes *hcpA* and *iha* plus 400 bp upstream of the start codon, appropriate primers – hcpA-MscI-f and hcpA-BamHI-r for *hcpA*, and iha-HindIII-f and iha-XhoI-r for *iha* – as listed in Table 2 were used. The isolated gDNA served as template. After treatment with the restriction enzyme DpnI to cleave the parental

Table 1
E. coli strains & plasmids used in this study.

Strain or plasmid	Characteristics	Origin
Strains		
E. coli DH5α	Laboratory strain	Hanahan, 1983
E. coli DH5α/pWRG435	Labelled with RFP, amp^R	this study
E. coli O157:H7 Sakai	Wildtype O157:H7 isolate from 1996 outbreak associated with white radish sprouts, *stx*+	Outbreak strain from Japan 1996, (Hayashi et al., 2001)
E. coli O157:H7 Sakai/pKEC2	Labelled with RFP, referred to as wildtype strain, cam^R	this study
E. coli O157:H7 Sakai/pKEC2/pOKD4	Labelled with RFP, carries pOKD4, cam^R, kan^R	this study
E. coli O157:H7 Sakai Δ*hcpA*/pKEC2	Labelled with RFP, deletion of *hcpA*, cam^R	this study
E. coli O157:H7 Sakai Δ*hcpA*/pKEC2/pOKD4	Labelled with RFP, deletion of *hcpA*, carries pOKD4, cam^R, kan^R	this study
E. coli O157:H7 Sakai Δ*hcpA*/pKEC2/pKEC5	Labelled with RFP, deletion of *hcpA*, complemented, cam^R, kan^R	this study
E. coli O157:H7 Sakai Δ*iha*/pKEC2	Labelled with RFP, deletion of *iha*, cam^R	this study
E. coli O157:H7 Sakai Δ*iha*/pKEC2/pOKD4	Labelled with RFP, deletion of *iha*, carries pOKD4, cam^R, kan^R	this study
E. coli O157:H7 Sakai Δ*iha*/pKEC2/pKEC4	Labelled with RFP, deletion of *iha*, complemented, cam^R, kan^R	this study
E. coli O157:H7 Sakai Δ*hcpA*Δ*iha*/pKEC2	Labelled with RFP, deletion of *hcpA* and *iha*, cam^R, kan^R	this study
Plasmids		
pKD46	Encodes for red recombinase, temperature-sensitive, amp^R	Datsenko and Wanner, 2000
pKD4	Carries kan^R flanked by FRT sites	Datsenko and Wanner, 2000
pCP20	Encoding for FLP recombinase, temperature-sensitive, cam^R/amp^R	Cherepanov and Wackernagel, 1995
pKEC2	pWRG345 backbone with cam^R instead of amp^R, encoding for RFP	this study
pOKD4	Expression plasmid, kan^R, p15A origin of replication	Dzivenu et al., 2004
pKEC4	pOKD4 backbone with iha plus 400bp upstream	this study
pKEC5	pOKD4 backbone with *hcpA* plus 400bp upstream	this study
pWRG435	P_{rpsM}:*tagRFP-T*, amp^R, pBR322 origin of replication	Bender et al., 2013

methylated gDNA, the PCR products and the vector pOKD4, a gift from Prof. Hao Wu (Harvard Medical School; Addgene plasmid #17214; https://www.addgene.org/), were digested with the corresponding restriction enzymes, ligated and transformed into chemically competent *E. coli* DH5α. The resulting plasmids pKEC4 and pKEC5 were prepared from *E. coli* DH5α using a QIAprep Spin Miniprep kit (Qiagen, Netherlands) following the manufacturer's instructions and verified by sequencing using the following primer combinations: hcpAdel-rev/hcpA-BamHI-r/hcpA-50-rev and iha-I/iha-II/iha-HindIII-f/iha-XhoI-r/iha-134up-for/iha-1754-rev/iha-1598-for (Table 2).

2.6. Plasmid stability in EHEC strain Sakai grown in soil

The stability of pKEC2 in EHEC strain Sakai was examined in the same manner as persistence experiments published by Fornefeld et al. (2017) with minor modifications. Briefly, EHEC strain Sakai/pKEC2 was grown overnight in LB medium supplemented with 20 µg/ml chloramphenicol at 37 °C with aeration. Cells were pelleted at 6000 × *g* at 4 °C for 8 min and resuspended in 10 mM $MgCl_2$. Inoculation was conducted by thoroughly mixing of soil and bacterial suspension to a final inoculum level of 10^8 cfu per g soil. As a control, soil was mixed only with 10 mM $MgCl_2$. Inoculated samples were incubated at 22 °C for 14 days and analyzed 0, 2, 4, 7, and 14 days post infection (dpi). EHEC cells were recovered from soil for quantification by the addition of 9 ml 0.5 × Murashige-Skoog (MS) medium (2.165 g/L Murashige & Skoog Medium, Duchefa Biochemie, Netherlands, pH 5.8) and subsequent extensive mixing. Serial decimal dilutions were plated on TBX chromogenic agar (Roth, Germany) and on TBX agar supplemented with 20 µg/ml chloramphenicol. After incubation overnight at 37 °C, the cfu per gram soil were calculated. Three independent experiments were performed.

2.7. Propagation of Valerianella locusta from seeds

For the propagation of seeds, seed trays (50 × 30 × 5 cm) with 150 slots were used. Seeds of *Valerianella locusta* (L.) „Verte á coeur plein“

Table 2
Oligonucleotides used in this study.

Name	Sequence (5' – 3')	Function	Reference
G70 *hcpA*-P1	**TGGACAAGCAACGCGGTTTTACACTTATCGAACTGATGGTGGT**GTAGGCTGGAGCTGCTTCG	Mutagenesis	Nuebling et al., 2017
G71 *hcpA*-P2	**GTCATCAAAGCGGAAGACATCTTCGCAGGCTTGCTGCAATGC**CATATGAATATCCTCCTTAG	Mutagenesis	Nuebling et al., 2017
del*iha*-rev	**ATGCGAATAACCACTCTGGCTTCCGTAGTCATTCCCTGTCT**GTAGGCTGGAGCTGCTTCG	Mutagenesis	this study
del*iha*-for	**CATATCCTGTTGTTGATGATCCCGTCTGGAAGTAATCACC**CATATGAATATCCTCCTTAG	Mutagenesis	this study
*hcpA*del-for	ATCTCAATACGTTTGGTGG	Confirmation of mutagenesis	Nuebling et al., 2017
*hcpA*del-rev	CGAAATAAAAAACCTCGG	Confirmation of mutagenesis	Nuebling et al., 2017
iha-I	CAGTTCAGTTTCGCATTCACC	Confirmation of mutagenesis	this study
iha-II	GTATGGCTCTGATGCGATG	Confirmation of mutagenesis	this study
hcpA-MscI-f	CTC*TGGCCA*ATCATGCTGGATAACTT	Complementation	this study
hcpA-BamHI-r	TAG*GGATCC*TTAGTTGGCGTCATCAA	Complementation	this study
iha-HindIII-f	GGG*AAGCTT*GGTCGACTGAATAAGGT	Complementation	this study
iha-XhoI-r	CGG*CTCGAG*GATAGCGTTTTGTTATTA	Complementation	this study
iha-134up-for	GCCGAGGCAGTCGTTATTTATA	Confirmation of complementation	this study
iha-1754-rev	AAATACCGACCAGCTTTTCTGC	Confirmation of complementation	this study
iha-1598-for	GGAATCGAACCTTATCCTGAATTC	Confirmation of complementation	this study
hcpA-50-rev	CCAATAACCACCATCAGTTCGA	Confirmation of complementation	this study
P-*cat* PvuI for	ATA*CGATCG*AGCGCTGATGTCCGGC	Exchange of resistance	this study
cat PuvI rev	ATA*CGATCG*TTACGCCCCGCCCTGCCA	Exchange of resistance	this study
cat 124 for	GGCCTTTTTAAAGACCG	Confirmation of exchange	this study
cat 225 rev	CATACGGAATTCCGGATG	Confirmation of exchange	this study

The homologous regions for recombineering are highlighted in bold, letters in italics indicate restriction sites.

(Select, Wyss Seed and Plants AG, Switzerland) were first grown in Floradur® A potting soil (Floragard, Germany) for approximately two weeks until reaching the second leaf stage (first leaf rosette). The plants were then carefully excavated and freed of soil before being repotted in plant pots (9 cm in diameter) containing diluvial sand soil, kindly provided by Dr. Rita Grosch (Leibniz Institute of Vegetable and Ornamental Crops, Groβbeeren, Germany). The DS soil (diluvial sand) was described as an Arenic-Luvisol with less silty sand and 5.5% clay (silty sand) (Ruehlmann and Ruppel, 2005; Schreiter et al., 2014). Each pot then contained three plants. After a two-day adaption phase, plants were ready-to-use for infection experiments.

2.8. Experimental setup

Preliminary experiments with the non-pathogenic gfp-expressing *E. coli* strain DH5α/pWRG435 were conducted to test the overall experimental set-up before performing the actual plant infection experiments with EHEC O157:H7 strain Sakai in the greenhouse. Lettuce plants were inoculated with 10 ml bacterial suspension ranging from 1.0×10^5 cfu/ml to 1.0×10^9 cfu/ml. Plants were then incubated at 21 °C with 12 h day-/night-cycle and 20% relative humidity for 4 days and subsequently analyzed qualitatively by fluorescent microscopy, where at least 20 microscopic fields were surveyed per root. The number of observed bacteria varied from 0 to 4 bacteria per microscopic field.

2.9. Determination of inoculum level and incubation time

To determine the inoculum level and incubation time suitable for the experimental set-up, experiments were conducted with *E. coli* O157:H7 strain Sakai/pKEC2 in duplicate. Plant pots were inoculated by dispensing 20 ml of bacterial suspensions into the soil, followed by incubation in a biosafety level 3 greenhouse at 21 °C with a 12 h day-/night-cycle for 2, 3, and 4 days. Bacterial suspensions contained either 5.0×10^7 cfu/ml or 5.0×10^8 cfu/ml. Plant pots were irrigated with 20 ml of a 10 mM $MgCl_2$ solution prior to inoculation in order to prevent the soil from drying out throughout the experiment.

2.10. Inoculation of Valerianella locusta plants

For inoculation of the *Valerianella locusta* plants, the following strains were used: *E. coli* O157:H7 Sakai/pKEC2, *E. coli* O157:H7 Sakai/pKEC2/pOKD4, *E. coli* O157:H7 Sakai *Δiha*/pKEC2, *E. coli* O157:H7 Sakai *Δiha*/pKEC2/pOKD4, *E. coli* O157:H7 Sakai *Δiha*/pKEC2/pKEC4, *E. coli* O157:H7 Sakai *ΔhcpA*/pKEC2, *E. coli* O157:H7 Sakai *ΔhcpA*/pKEC2/pOKD4, *E. coli* O157:H7 Sakai *ΔhcpA*/pKEC2/pKEC5, *E. coli* O157:H7 Sakai *ΔhcpAΔiha*/pKEC2. The bacterial strains were grown overnight (~18 h) in LB medium supplemented with 20 µg/ml chloramphenicol or 20 µg/ml chloramphenicol and 50 µg/ml kanamycin at 37 °C with aeration at 180 rpm. Prior to inoculation, cells were harvested at 6000 × *g* at 4 °C for 8 min and resuspended in 10 mM $MgCl_2$. OD_{600} was measured and the samples were adjusted to an OD_{600} of 1.0 (corresponding to ~ 5.0×10^8 cfu/ml). Plants were inoculated with 20 ml of bacterial suspension by careful pipetting in order to avoid contamination of the leaves. To guarantee sufficient humidity of the soil throughout the experiment, plant pots were irrigated with 20 ml of a 10 mM $MgCl_2$ solution prior to inoculation. Subsequently the plants were incubated in the biosafety level 3 greenhouse at 21 °C with 12 h day-/night-cycle for 4 days. As negative control, plants irrigated solely with 10 mM $MgCl_2$ were used.

2.11. Analysis of roots

After incubation, the plants were carefully excavated using sterile tweezers. The plants were then washed for 10 min on a rotary shaker with 50 rpm in 0.5 × MS medium to remove soil particles. In case of internalization experiments, the plants were surface-sterilized by washing in 0.5 × MS medium supplemented with 50 µg/ml gentamicin for 20 min prior to analysis. To validate efficient disinfection, the surface-sterilized roots of the first experiment were placed onto an agar dish containing TBX agar with the corresponding antibiotics for approximately 10 s before further processing. These plates, called "imprint plates", were then incubated at 37 °C overnight. In case of adherence assays, the root systems were aseptically removed from the plant using sterile scissors directly after washing. The roots were investigated by fluorescence microscopy for qualitative analysis as well as by homogenization and spread plating in order to determine the number of adherent or internalized bacteria, respectively. For microscopic analysis, the root systems were aseptically removed from the plants and mounted on an object slide with 30 µl 0.5 × MS medium. After applying the cover slip, the microscopic preparation was sealed with nail polish and analyzed using an inverted fluorescence microscope Axio Vert.A1 (Zeiss, Germany) equipped with a Axiocam 105 color camera. Pictures were taken with 100 × /1.25 oil N-Achroplan objective and the filter set 43 (AF 546; Zeiss, Germany) for detection of RFP signals. Pictures were processed with ZEN 2 lite software (Zeiss, Germany) and analyzed using ImageJ (Schindelin et al., 2012, 2015). Per field of microscopy, a maximum of two bacterial cells could be detected and formation of microcolonies was not observed. For quantitative analysis, the root systems were aseptically removed from the plants after washing or disinfection and 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 reaction tubes were weighed before and after adding the roots to calculate the roots' weight. Subsequently the roots were homogenized at 25 Hz for 5 min using a mixer mill (MM200, Retsch, Germany). For adherence experiments, 4 µl of serial decimal dilutions were spotted on TBX agar with appropriate antibiotics and 100 µl of appropriate dilutions were spread plated in duplicates on TBX agar with antibiotics. For internalization experiments, 200 µl of the homogenate were spread plated in duplicates directly on TBX agar with appropriate antibiotics. The plates were incubated at 37 °C overnight and the cfu per gram of root were calculated the next day. For each strain three independent experiments were performed in triplicates.

2.12. Statistical analysis

Data were analyzed with Brown–Forsythe test for variance homogeneity, followed by either Welch's one-way analysis of variance (ANOVA) ($\alpha = 0.05$) and two-tailed Welch *t*-test with Bonferroni correction ($\alpha_{corrected} = 0.05$/number of comparisons), or by one-way ANOVA followed by two-tailed unpaired Student's *t*-test with Bonferroni correction. $p < \alpha_{corrected}$ was considered significant.

3. Results

3.1. Construction of isogenic deletion mutants

To investigate the influence of Iha and HcpA on adherence to and internalization into the roots of lamb's lettuce, isogenic deletion mutants in the respective genes were constructed as described. For this purpose, EHEC strain Sakai was transformed with plasmid pKD46, which promotes recombination of linear DNA as it carries an inducible λ Red recombinase system. PCR products for deleting the target genes carry the kanamycin resistance gene flanked by FRT sites and 40–50 nt homologous to the gene of interest. For the deletion of *hcpA*, the primer pair G70 hcpA-P1 and G71 hcpA-P2 was used as reported by Nuebling et al. (2017), and for *iha* primers deliha-for and deliha-rev were used (Table 2). In case of successful mutagenesis, application of these primer pairs led to deletion of 78% and 93% of *hcpA* and *iha*, respectively. Transformation of purified PCR products resulted in intermediate deletion mutants where the genes of interest were substituted by a kanamycin resistance cassette flanked by FRT sites. This antibiotic

resistance gene was removed by transformation of the plasmid pCP20 encoding for thermal inducible FLP recombinase. FLP targets the FRT sites and leads to double-crossover at those positions and thereby eliminates sequences between these two target sites. The resulting isogenic deletions mutants were verified by PCR applying primers that are specific for the corresponding genomic context (Table 2). Moreover, gene deletions were confirmed by sequencing the respective target sites (Table 2). Consequently, EHEC strain Sakai *Δiha* and EHEC strain Sakai *ΔhcpA* were successfully generated (Table 1).

3.2. Complementation of deletion mutants

In order to complement the single deletion mutants, plasmids were constructed that contain the functional genes under the control of their native promotors, which were knocked out in the chromosome. The native promotors were chosen since induction was not applicable considering the experimental set-up and as the aim was to investigate the influence of HcpA and Iha at natural conditions. Therefore, the genes *hcpA* and *iha* plus 400 bp and 378 bp upstream of the start codon, respectively, were amplified with the corresponding primers listed in Table 1, using genomic DNA as template. For plasmid-based complementation it was necessary to select a plasmid that is not incompatible with the RFP-encoding plasmid pWRG435 and the plasmids that EHEC strain Sakai carries naturally. Hence, the vector pOKD4 was used as backbone (Fig. 1A). After cloning, the resulting plasmids pKEC4, coding for Iha, and pKEC5, coding for HcpA (Fig. 1B), were verified by sequencing the target sites. Subsequently, the plasmids were transformed into the corresponding single deletion mutants, resulting in EHEC strain Sakai *Δiha*/pKEC4 and EHEC strain Sakai *ΔhcpA*/pKEC5 (Table 1).

3.3. Red fluorescence protein (RFP)-encoding plasmid pKEC2 is stable in EHEC strain Sakai grown soil

In the general experimental set-up, the microscopic and the numeric detection of the investigated EHEC strains relied on the stability of the RFP-encoding plasmid pKEC2. Therefore, it was important that this plasmid was not lost in EHEC O157:H7 strain Sakai throughout the time scale of the experiment. To analyze this, EHEC O157:H7 strain Sakai/pKEC2 was inoculated in soil, incubated for up to 14 days at room temperature and recovered therefrom. Samples were plated on TBX agar with and without 20 μg/ml chloramphenicol to determine the percentage of EHEC cells that lost plasmid pKEC2. Since bacterial colonies were not found on TBX agar without any antibiotics after recovery treatment of the uncontaminated soil, all detected colonies were considered as EHEC O157:H7 strain Sakai and/or EHEC O157:H7 strain Sakai/pKEC2. The total viable counts of EHEC O157:H7 strain Sakai declined over time, starting at 1.1×10^8 cfu/g soil and 9.6×10^7 cfu/g soil (Fig. 2), respectively. After 14 days, only 8.9×10^3 cfu/g soil and

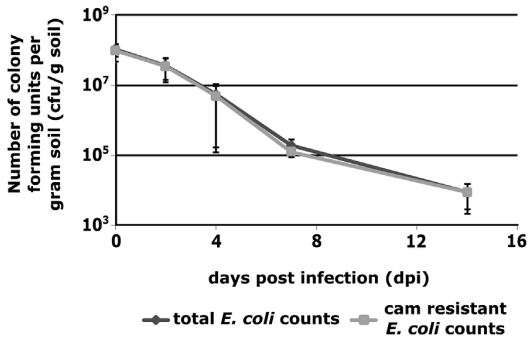


Fig. 2. Stability of EHEC O157:H7 strain Sakai/pKEC2 in soil. Number of bacteria in cfu per gram soil. Soil was inoculated with 1.0×10^8 cfu/g soil and incubated for up to 14 days. Samples were taken 0, 2, 4, 7 and 14 dpi, investigated concerning total counts of *E. coli* cfu, *i.e.* EHEC O157:H7 strain Sakai (dark grey), and chloramphenicol (cam) resistant *E. coli* counts, *i.e.* EHEC O157:H7 strain Sakai/pKEC2 (light grey). Data are means ± standard deviations of three independent experiments.

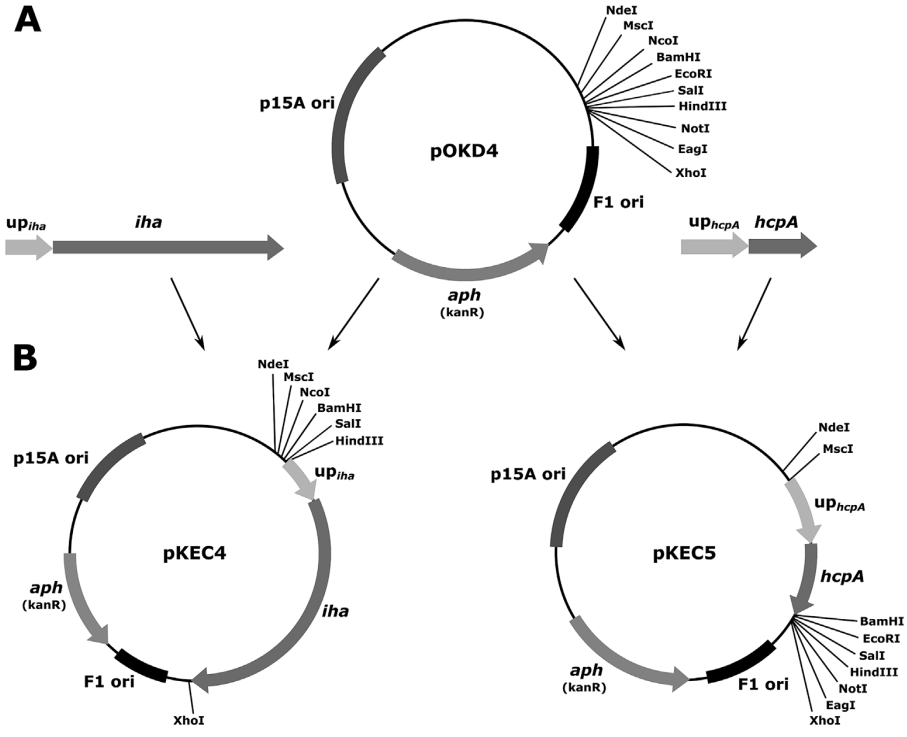


Fig. 1. Construction of plasmids for complementation. The genes iha and *hcpA* including 378 bp and 400 bp of the corresponding upstream region (up_{iha} and up_{hcpA}) were amplified by PCR using the primer pairs iha-HindIII-f/iha-XhoI-r and hcpA-MscI-f/hcpA-BamHI-r, respectively. (A) The resulting PCR products were cloned into pOKD4, resulting in (B) the respective complementation plasmid pKEC4 and pKEC5.

8.7×10^3 cfu/g soil could be detected (Fig. 2), respectively. This result is in line with findings that reported similar degression patterns for other EHEC O157:H7 strains and a variety of *Salmonella* strains (Fornefeld et al., 2017; Gurtler et al., 2013). The viable counts for chloramphenicol-resistant bacteria behaved the same as the total counts and were not significantly different (Fig. 2). These results indicate that pKEC2 is largely stable in EHEC O157:H7 strain Sakai in soil throughout the duration of the experiments.

3.4. EHEC O157:H7 strain Sakai and E. coli strain DH5α adhere both to the root surface of Valerianella locusta

Before conducting plant infection experiments with the EHEC O157:H7 strain Sakai, which is level 3** in Germany according to the German Ordinance on Biological Substances, a preliminary experiment was carried out with the RFP-labelled non-pathogenic *E. coli* laboratory strain DH5α/pWRG435 to test the overall experimental set-up. For this purpose, *Valerianella locusta* plants were inoculated with bacterial suspensions of different concentrations between 1.0×10^5 cfu/ml to 1.0×10^9 cfu/ml. Microscopic analysis showed that *E. coli* DH5α/pWRG435 could be detected upon inoculation with 10^8 cfu/ml or with 10^9 cfu/ml (Fig. S1). When inoculated with 10^8 cfu/ml, cells were mainly detected as single cells (Fig. S1A). In contrast, upon inoculation with 10^9 cfu/ml it was also possible to detect several bacterial cells in close proximity to each other and between the rhizodermal cells (Fig. S1B).

As this initial experiment showed that the overall set-up is applicable for plant infection experiments carried out with *E. coli*, experiments with EHEC O157:H7 strain Sakai were performed to determine suitable growth and infection parameters. Therefore, *Valerianella locusta* plants were first contaminated with two different inocula of EHEC O157:H7 strain Sakai and grown for up to four days post infection. Samples were investigated on days 2, 3, and 4, and EHEC strain Sakai was found to be root-associated in all cases (Fig. 3A). For both inocula the number of cfu per gram root decreased between day 2 and 3, and increased again at day 4. The highest numbers of adherent *E. coli* Sakai cells were detected at an inoculum level of 5.0×10^8 cfu/ml after 4 days of incubation (1.1×10^6 cfu/g root, Fig. 3A). Microscopic analysis verified adherence to roots (Fig. 3B). Hence, plants were incubated with 5.0×10^8 cfu/ml for 4 days for all subsequent experiments.

3.5. Deletion of iha and hcpA did not affect adherence to roots

Similar to other enteric bacteria, *E. coli* is known to possess a variety of molecular and physiological mechanisms that facilitate effective survival and colonization within the plant environment (Quilliam et al., 2012). In the current study, we investigated the influence of *iha* and *hcpA* deletions on the adherence to and internalization into lamb's lettuce roots using strains containing RFP-encoding plasmids for inoculation. Experiments with the wildtype strain and mutants were performed as described below and the viable counts were determined. As expected, investigating the native washed roots by fluorescence microscopy showed no red fluorescent bacteria (Fig. 4A–C). After contamination with *E. coli* O157:H7 Sakai, bacteria could be detected at the roots (Fig. 4D–F). Neither deletion of *iha* or *hcpA* nor deletion of both genes led to diminished appearance of EHEC at the roots (Fig. 4G–I, M-O, S-U). By using fluorescent microscopy no differences in adherence could be detected as all tested strains were found to be root-associated (Fig. 4 and Fig. S2). Formation of microcolonies was not observed for any tested strain.

For quantitative analysis, the root systems were separated from the plants after washing, homogenized and appropriate decimal dilutions of the homogenate were spread plated (see below). The viable counts per gram root varied slightly between the strains (Fig. 5). After inoculation with Sakai wildtype 1.3×10^6 cfu/g root could be found (Fig. 5). Wildtype strain Sakai/pOKD4 and Sakai *ΔhcpA* showed the same counts of adherent bacteria (1.4×10^6 cfu/g root and 1.5×10^6 cfu/g root, respectively, Fig. 5). A slightly higher capability in adherence was detected for the *hcpA* knock-out strain with the empty vector control and for the complementation strains of the single deletion mutants (2.4×10^6 cfu/g root for Sakai *ΔhcpA*/pOKD4, 2.5×10^6 cfu/g root for Sakai *ΔhcpA*/pKEC5, and 2.3×10^6 cfu/g root for Sakai *Δiha*/pKEC4, Fig. 5). The lowest counts were found for the double deletion mutant (8.9×10^5 cfu/g root, Fig. 5), while the *iha* deletion mutant (4.4×10^6 cfu/g root, Fig. 5) and the *iha* deletion mutant carrying the empty vector (5.8×10^6 cfu/g root, Fig. 5) were the most adherent. To test for significance, Welch's one-way analysis of variance (ANOVA) was applied as described below, which indicated significant differences between the strains. Further statistical analysis was performed assuming that if the strains that seem to differ from each other the most – in this case Sakai *Δiha*/pKEC4 and Sakai *ΔhcpAΔiha* – did not show significance, all differences observed between every strain were insignificant. Hence, the data from Sakai *Δiha*/pKEC4 and Sakai *ΔhcpAΔiha* were analyzed by two-tailed Welch *t*-test with Bonferroni correction ($\alpha_{corrected} = 0.00139$). The obtained *p* value was 0.00372 and therefore 2.6 fold higher than the corrected α value. Thence the observed differences can be considered as insignificant. Unlike results from cell culture experiments performed by other authors (Johnson et al., 2005; Ledesma et al., 2010; Tarr et al., 2000; Xicohtencatl-Cortes et al., 2007; Yin et al., 2009), Iha and HcpA do not seem to play a role in adherence to the roots of lamb's lettuce under the conditions tested.

3.6. Deletion of iha and hcpA leads to reduced internalization

As the absence of the genes *iha* or *hcpA* did not result in reduced adherence, it was further investigated whether these adherence factors are involved in the internalization of EHEC strain Sakai into the root tissues. Washing of the roots was followed by surface disinfection using gentamicin to eliminate all bacteria trapped on the root surface as described below. Disinfection was regarded successful as no viable counts were detected on the imprint plates. Analysis by fluorescence microscopy again did not reveal any differences between the strains concerning internalization (Fig. 6). After surface sterilization, all tested strains could be detected within the roots. However, bacteria did not localize in distinct patterns. They were found within the plant cells (Fig. 6D–F, M-O, S-U; Fig. S3G-I) as well as in the grooves between the cells (Fig. 6G–I, J-L, P-R; Fig. S3A-C, Fig. S3D-F). With this technique, it is difficult to define if there are less root-associated bacteria after surface disinfection compared to adherence experiments. Therefore, the roots were homogenized after gentamicin treatment and the homogenate was spread plated directly to determine the number of internalized bacteria. Quantitative analysis confirmed that internalized bacteria could be found for all tested strains (Fig. 7). In contrast to adherence assays, the differences between the strains were more prominent. For the wildtype strain 2.4×10^2 cfu/g root could be found, while only 1.8×10^1 cfu/g root were detected for the *hcpA* deletion mutant (Fig. 7). This reduction in internalization was almost restored to wildtype level by plasmid-based complementation (1.8×10^2 cfu/g root, Fig. 7). Decrease in internalization was even higher when *iha* was lacking (1.1×10^1 cfu/g root, Fig. 7). As shown for the *hcpA* knock-out mutant, the complemented *iha* deletion mutant displayed wildtype-like behavior (2.8×10^2 cfu/g root, Fig. 7). Interestingly, the double knock-out mutant showed slightly higher counts of internalized bacteria than the single deletion mutants (2.8×10^1 cfu/g root, Fig. 7). The highest number of internalized bacteria was found for Sakai/pOKD4 (3.6×10^2 cfu/g root, Fig. 7). In contrast, the lowest counts were detected for Sakai *Δiha*/pOKD4 (6.2×10^0 cfu/g root, Fig. 7).

Taken together, the *E. coli* O157:H7 Sakai wildtype, its derivative carrying the empty control vector and the complemented single deletion mutants, showed similar numbers of internalized bacteria between 1.8×10^2 and 3.6×10^2 cfu/g root, whereas the knock-out mutants were significantly ($p_{corrected} < 0.05$) hampered in internalization

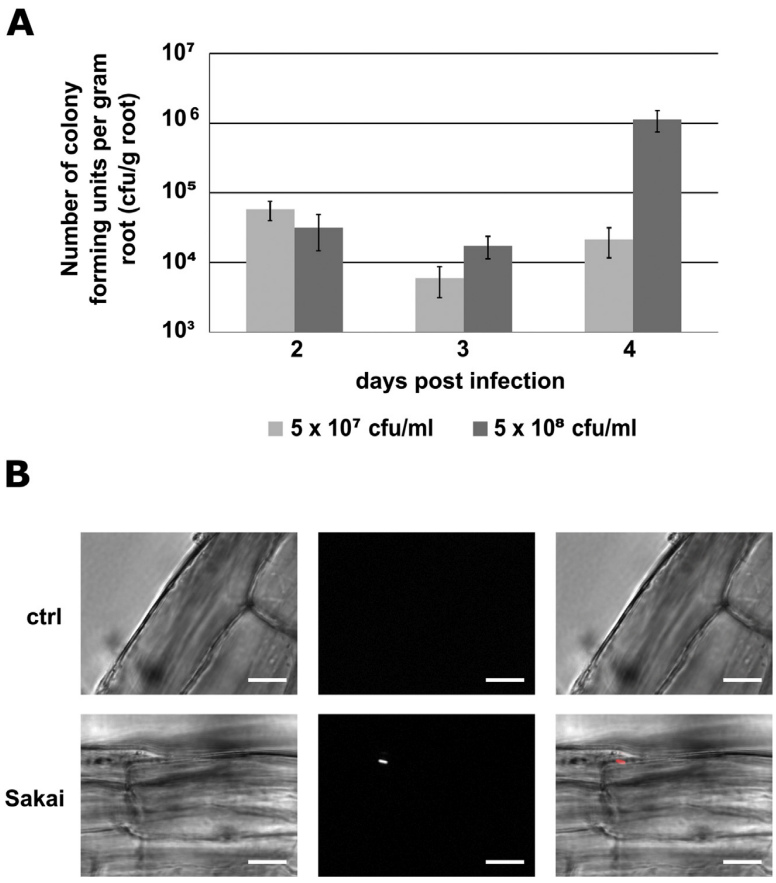


Fig. 3. Analysis of roots-association of EHEC. (A) Number of root-associated bacteria in cfu per gram root. Plants were inoculated with 5.0×10^7 cfu/ml (light grey) or 5.0×10^8 cfu/ml (dark grey) and incubated for 2, 3 and 4 days. Data are means ± standard errors of the experiment performed in duplicate. (B) Microscopic analysis of roots after 4 days of incubation performed with non-contaminated control roots (ctrl, upper panel) and roots contaminated with 5.0×10^8 cfu/ml of EHEC strain Sakai (lower panel). Shown are brightfield (left), and rfp signals (middle) and the overlays of both channels (right). Bars are 10 µm. Magnification is 100-fold.

(6.2×10^0 to 2.8×10^1 cfu/g root).

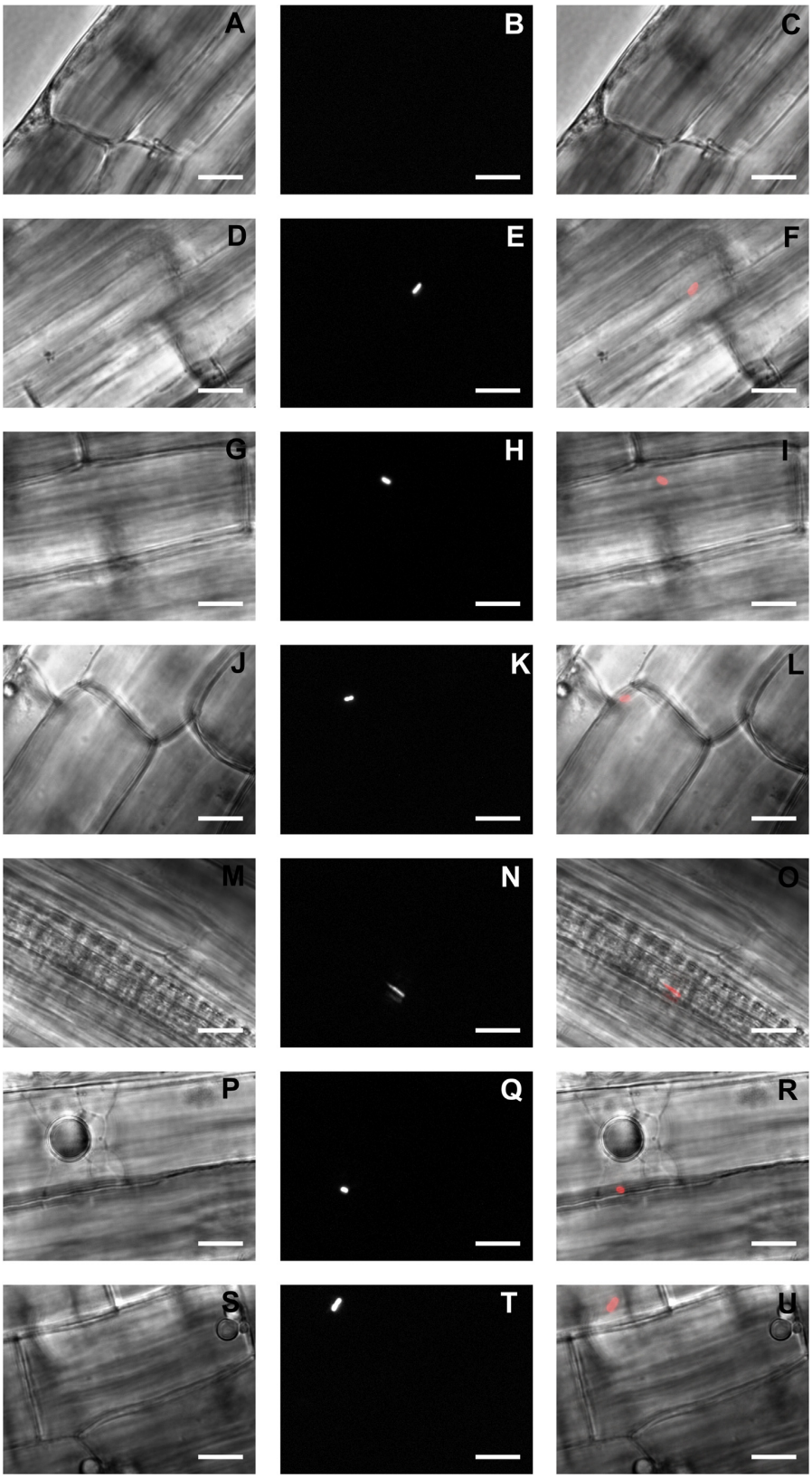
These results demonstrate that EHEC O157:H7 strain Sakai is able to internalize into root tissue resulting in viable counts similar or higher to the human infectious dose. Therefore, we conclude that both Iha and HcpA are involved in the internalization of root tissue during plant colonization.

4. Discussion

Cultivated plants can serve as host for EHEC bacteria and fresh produce generated from such plants is mainly consumed raw. Irrigation water, surface water and manure are some possible routes of pathogen contamination that can occur directly on the field. As a consequence, contamination of plant-based food represents a serious threat to public health. Recent studies on EHEC-plant interactions focused on the overall ability of EHEC to adhere, internalize, and persist in plant tissue (Sharma et al., 2009; Solomon et al., 2002; Wright et al., 2017) on laboratory scale. However, it remained unknown which intrinsic factors are important for EHEC in order to be able to internalize into the tissue of plants.

Conceivably, effective adherence is a prerequisite for successful internalization. In this study, we focused on two adherence factor genes, *hcpA* and *iha*, and their roles during colonization of roots of *Valerianella locusta*. In contrast to previous studies published for plant leaves, animal tissue and human cell lines (Johnson et al., 2005; Nuebling et al., 2017; Saldana et al., 2011; Tarr et al., 2000; Xicohtencatl-Cortes et al., 2007; Yin et al., 2009), the present study showed that deletion of *hcpA* or *iha* did not decrease the ability of *E. coli* O157:H7 strain Sakai to adhere to, in this case, plant roots but rather led to a slight increase in adherence. Also, the deletion of both genes did not significantly alter the strains' attachment behavior and resulted in only slightly reduced attachment. These results indicate that neither HcpA nor Iha did specifically interact with the roots of *Valerianella locusta,* which is different from results published for leaf surfaces of commercially available baby spinach (Saldana et al., 2011). In contrast to our study, in the mentioned study the object of investigation was leaves that were cut in equal pieces and contaminated by incubation in EHEC suspension resulting in a higher inoculation density. Moreover, plant leaves are covered by a waxy layer called cuticle which is not present at the roots. Hence, roots and leaves have different overall surface structures with distinct characteristics. As EHEC carries a variety of adherence-conferring factors, *e.g.* pili, flagella, intimin (Jaglic et al., 2014), it sounds reasonable that EHEC requires distinct adherence factors depending on its target host and its environment. Attachment to plant roots may follow different mechanisms than attachment to plant leaves or mammalian cell lines as their surface exhibit distinct properties.

We further investigated the involvement of *hcpA* and/or *iha* in internalization into the plant roots. Single deletion mutants as well as the double deletion mutant demonstrated significantly reduced levels of internalization. For *ΔhcpA*, the number of internalized EHEC per gram root decreased by more than a factor 10 from 2.4×10^2 cfu/g root (wildtype) to 1.8×10^1 cfu/g root. This finding is in accordance with *in vitro* cell culture studies conducted by Xicohtencatl-Cortes et al. (2009) demonstrating that the deletion of *hcpA* results in decreased internalization into HT-29 cells. The same study showed that HCP is involved in biofilm formation indicating a possible link between biofilm formation and internalization. The reduction of internalization was even more prominent upon deletion of the siderophore receptor gene *iha* by a factor > 30 (6.2×10^0 cfu/g root). For EHEC O157:H7 strain



(caption on next page)

Fig. 4. Microscopic analysis of fluorescent bacteria at the roots of lamb's lettuce after 4 days of incubation. Microscopy was performed after 10 min of washing in 0.5 × MS medium of untreated roots (A–C) and roots contaminated with several bacterial strains: EHEC Sakai wildtype (D–F), Sakai *Δiha* (G–I), Sakai *Δiha*/pKEC4 (J–L), Sakai *ΔhcpA* (M–O), Sakai *ΔhcpA*/pKEC5 (P–R) and Sakai *ΔhcpAΔiha* (S–U). Shown are brightfield (left), and rfp signals (middle) and the overlays of both channels (right). Bars are 10 µm. Magnification is 100-fold.

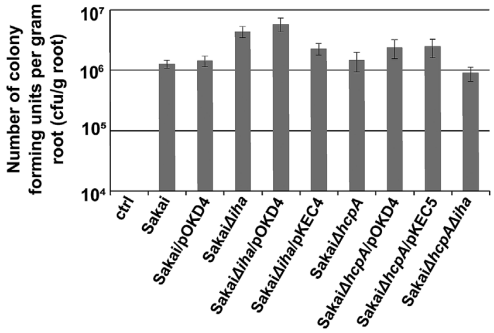


Fig. 5. Number of adherent bacteria at the roots of lamb's lettuce in cfu per gram root. Plants were inoculated with 5.0×10^8 cfu/ml of the strains indicated and incubated for 4 days. No bacteria could be detected at the non-contaminated roots (ctrl). Data are means ± standard errors of three independent experiments performed in triplicates.

86-24 and for *E. coli* strain UPEC76 it is known that the absence of Iha hampers successful colonization of pig ileal loops (Yin et al., 2009) and of the murine urinary tract (Johnson et al., 2005). It is currently not known how Iha mediates adherence and contributes to internalization. However, Iha participates in iron uptake (Leveille et al., 2006). Accordingly, there is a chance that Iha does not directly facilitate attachment and internalization, but rather acts indirectly by either inducing adhesive and/or invasive proteins or by repressing counteracting factors resulting in a better capability of adherence and internalization.

Surprisingly, deletion of both adherence-associated genes did not lead to further reduction of internalization and subsequently did not reveal synergistic effects as the double knock-out showed results similar to the *hcpA* single deletion mutant. If HcpA and Iha were the only proteins involved in internalization into roots, the *hcpA* and *iha* double negative mutant would not be able to internalize. Subsequently, no EHEC would be recovered from surface-disinfected roots. Hence, these results indicate that both investigated proteins are not the only factors involved in internalization and do not act synergistically. Presumably, the observed effects are not derived from specific interactions between these proteins and plant surface structures but are rather unspecific. Factors potentially contributing to these unspecific interactions are cell surface hydrophobicity and cell surface charge. Numerous studies investigated the effect of these two surface properties with respect to attachment. It was found that there is no general correlation between cell surface hydrophobicity and cell surface charge and adherence, as most studies gained contradicting results. Dickson and Koohmaraie (1989) as well as Li and McLandsborough (1999) investigated the relationship between surface hydrophobicity and attachment to beef. The former authors showed that hydrophobicity is involved in adherence in fat tissue whereas in lean beef muscle cell surface charge is more important (1989). On the contrary, the latter authors did not observe any correlation between surface charge, hydrophobicity and adhesion to beef muscle (Li and McLandsborough, 1999). Ukuku and Fett (2002) showed that adhesion of *E. coli*, *Salmonella* and *Listeria monocytogenes* to cantaloupe rind is influenced by cell surface hydrophobicity and charge. Zita and Hermansson (1997) demonstrated that cell surface hydrophobicity is important for attachment to activated sludge flocs. Boyer and colleagues (Boyer et al., 2007, 2011) detected that surface proteins such as curli fibers and the O-antigen of *E. coli* O157:H7 influence cell surface hydrophobicity and charge. However, deletion of the curli fibers did not alter attachment (Boyer et al., 2007) to lettuce leaves while reduced adherence to the same surface was observed in the absence of the O-antigen (Boyer et al., 2011). Notably, during these studies different *E. coli* O157:H7 strains were used and cultivation conditions were altered, which makes comparisons difficult. Thus, no generally valid statement can be made concerning adherence and cell surface properties. Conceivably the impact of cell surface charge and hydrophobicity to attachment depends on various factors like the bacterial strain, cultivation conditions, target host, and environment. It should be noted that none of the mentioned studies investigated the potential connection between internalization and cell surface properties. Hence, a possible role of surface properties in internalization cannot be excluded. Bacteria are supposed to use either natural plant openings for internalization, such as sites of lateral roots emergence, or plant lesions that either occurred naturally or are caused by plant pathogens (Deering et al., 2012). Contingently these potential plant entry points hold surface properties that are different from the residual root area and thus provide better access for the bacteria. Jozefaciuk et al. (2014) already observed that the surface charge density of roots may be more heterogeneous than expected and reported. This could explain why the adherence of *E. coli* O157:H7 strain Sakai is not affected upon deletion of the tested genes whereas its internalization is. For clarification of this question, experiments investigating the cell surface hydrophobicity as well as the cell surface charge of the used strains and further strains lacking additional surface proteins should be performed followed by attachment and internalization experiments.

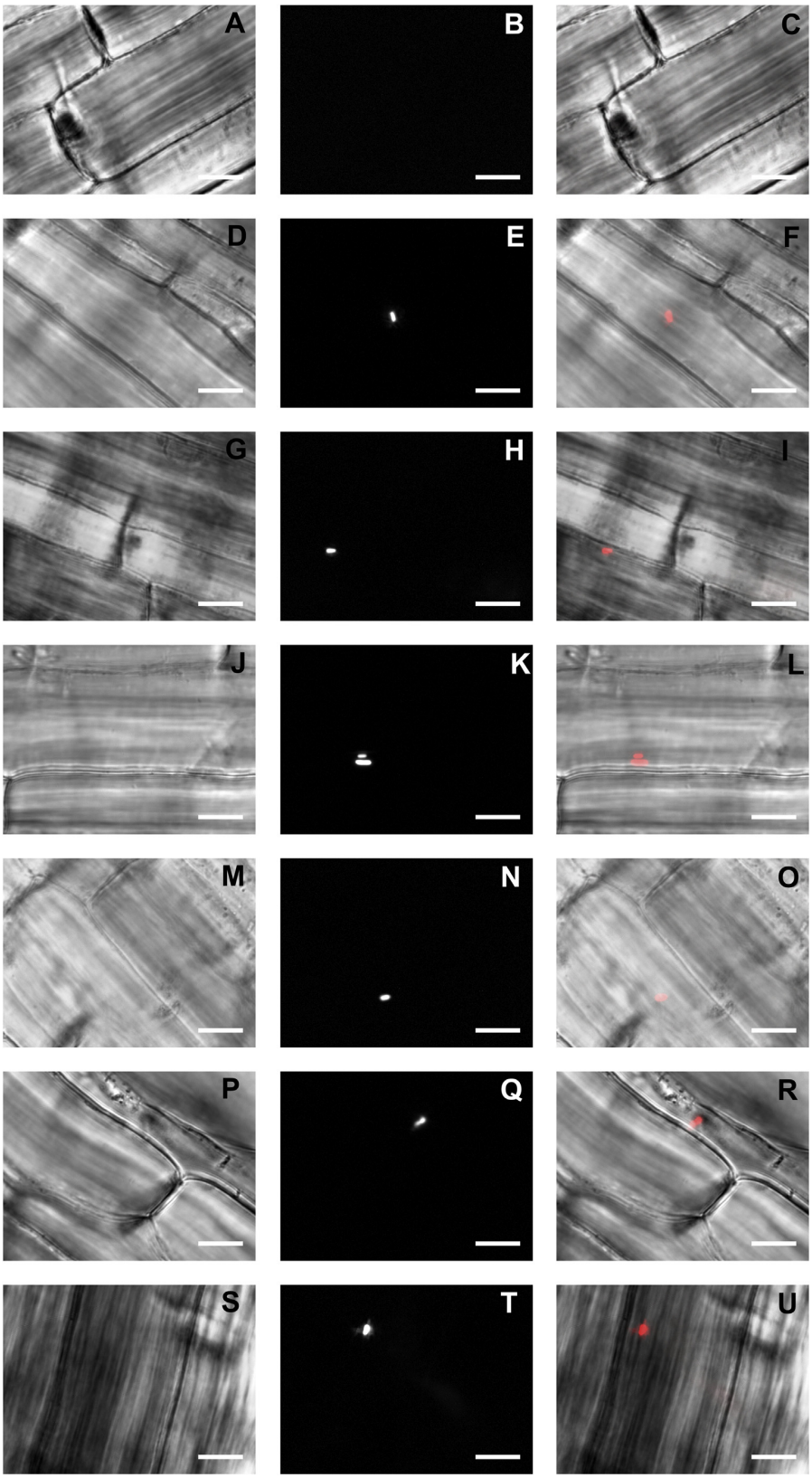
Taken together, the results of this study demonstrate that invasion of EHEC O157:H7 strain Sakai into root tissue of *Valerianella locusta* can occur under the tested conditions. The overall viable counts of EHEC O157:H7 strain Sakai found in the present study are comparable or even above the human infectious dose determined for ground beef. In this experimental set-up the so-called adherence factors HcpA and Iha do not act as adhesins during root colonization but are important internalization factors. Therefore, intrinsic factors which play a crucial role in the internalization of EHEC into plant roots were identified. In conclusion, further research is needed to fully understand the underlying mechanisms in order to develop countermeasures.

Funding information

This work was financially supported by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE), grant number 2813HS028. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We gratefully thank Dr. Daniel Rigling and Dr. Andreas Zurlinden (Swiss Federal Institute for Forest, Snow and Landscape (WSL) in Birmensdorf, Switzerland) for the opportunity to conduct these experiments in the new BSL3 greenhouse and laboratory containment, and Hélène Blauenstein (WSL) for assistance during the experimental periods. We thank Dr. Rita Grosch (Leibniz Institute of Vegetable and Ornamental Crops, Groβbeeren, Germany) for providing the soil, Prof. Hao Wu (Harvard Medical School, Boston, U.S.A.) for plasmid pOKD4, and Dr. Roman Gerlach (Robert Koch Institute, Wernigerode, Germany) for plasmid pWRG435. We thank Prof. Hans-Peter Piepho (University of Hohenheim, Stuttgart, Germany) for help in statistical analysis of the data. We are grateful to Dr. Fiona Walsh (Maynooth University,



(caption on next page)

Fig. 6. Microscopy of fluorescent bacteria after root surface disinfection. Analysis was conducted 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, with non-contaminated roots (A–C) and roots contaminated with several bacterial strains: EHEC Sakai wildtype (D–F), Sakai *Δiha* (G–I), Sakai *Δiha*/pKEC4 (J–L), Sakai *ΔhcpA* (M–O), Sakai *ΔhcpA*/pKEC5 (P–R) and Sakai *ΔhcpAΔiha* (S–U). Shown are brightfield (left), and rfp signals (middle) and the overlays of both channels (right). Bars are 10 µm. Magnification is 100-fold.

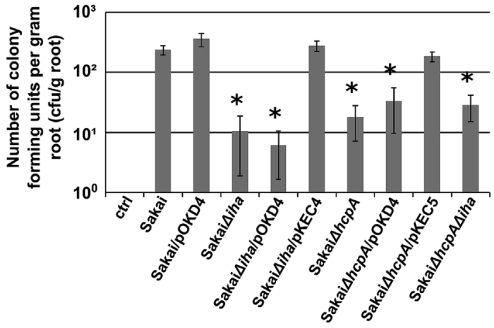


Fig. 7. Internalization of EHEC O157:H7 strain Sakai into the roots of lamb's lettuce. Number of internalized bacteria in cfu per gram root. Plants were inoculated with 5.0×10^8 cfu/ml of the strains indicated and incubated for 4 days. No bacteria could be detected at the non-contaminated roots (ctrl). Data are means ± standard errors of three independent experiments performed in triplicates. * $p_{corrected} < 0.05$ compared to wildtype-like strains (Welch's one way ANOVA followed by two-tailed Welch *t*-test with Bonferroni correction).

Maynooth, Ireland) for comments on the manuscript. The skillful technical assistance of Dipl. Ing. Jürgen Krauss and Carmela Total (Agroscope, Wädenswil, Switzerland) during plant propagation is greatly appreciated. We thank Markus Kranz (University of Hohenheim, Stuttgart, Germany) for skillful technical assistance during sequencing.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2018.05.016.

Conflicts of interest

The authors declare that there is no conflict of interest.

References

Bender, J.K., Wille, T., Blank, K., Lange, A., Gerlach, R.G., 2013. LPS structure and PhoQ activity are important for *Salmonella Typhimurium* virulence in the *Galleria mellonella* infection model [corrected]. PLoS One 8, e73287. https://doi.org/10.1371/journal.pone.0073287.

Boyer, R.R., Sumner, S.S., Williams, R.C., Kniel, K.E., McKinney, J.M., 2011. Role of O-antigen on the *Escherichia coli* O157:H7 cells hydrophobicity, charge and ability to attach to lettuce. Int. J. Food Microbiol. 147, 228–232. https://doi.org/10.1016/j.ijfoodmicro.2011.04.016.

Boyer, R.R., Sumner, S.S., Williams, R.C., Pierson, M.D., Popham, D.L., Kniel, K.E., 2007. Influence of curli expression by *Escherichia* coli O157:H7 on the cell's overall hydrophobicity, charge, and ability to attach to lettuce. J. Food Protect. 70, 1339–1345.

Cherepanov, P.P., Wackernagel, W., 1995. Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 158, 9–14.

Chitarra, W., Decastelli, L., Garibaldi, A., Gullino, M.L., 2014. Potential uptake of *Escherichia coli* O157:H7 and *Listeria monocytogenes* from growth substrate into leaves of salad plants and basil grown in soil irrigated with contaminated water. Int. J. Food Microbiol. 189, 139–145. https://doi.org/10.1016/j.ijfoodmicro.2014.08.003.

Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A 97, 6640–6645. https://doi.org/10.1073/pnas.120163297.

Deering, A.J., Mauer, L.J., Pruitt, R.E., 2012. Internalization of *E. coli* O157:H7 and *Salmonella* spp. in plants: a review. Food Res. Int. Salmonella Foods Evol. Strateg. Chall 45, 567–575. https://doi.org/10.1016/j.foodres.2011.06.058.

Dickson, J.S., Koohmaraie, M., 1989. Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. Appl. Environ. Microbiol. 55, 832–836.

Dzivenu, O.K., Park, H.H., Wu, H., 2004. General co-expression vectors for the over-expression of heterodimeric protein complexes in *Escherichia coli*. Protein Expr. Purif. 38, 1–8.

Erickson, M.C., Webb, C.C., Davey, L.E., Payton, A.S., Flitcroft, I.D., Doyle, M.P., 2014. Biotic and abiotic variables affecting internalization and fate of *Escherichia coli* O157:H7 isolates in leafy green roots. J. Food Protect. 77, 872–879. https://doi.org/10.4315/0362-028X.JFP-13-432.

Erickson, M.C., Webb, C.C., Diaz-Perez, J.C., Phatak, S.C., Silvoy, J.J., Davey, L., Payton, A.S., Liao, J., Ma, L., Doyle, M.P., 2010. Surface and internalized *Escherichia coli* O157:H7 on field-grown spinach and lettuce treated with spray-contaminated irrigation water. J. Food Protect. 73, 1023–1029.

Fletcher, M., Loeb, G.I., 1979. Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. Appl. Environ. Microbiol. 37, 67–72.

Fornefeld, E., Schierstaedt, J., Jechalke, S., Grosch, R., Schikora, A., Smalla, K., 2017. Persistence of *Salmonella Typhimurium* LT2 in soil enhanced after growth in lettuce medium. Front. Microbiol. 8 (757). https://doi.org/10.3389/fmicb.2017.00757.

Frank, J.F., 2001. Microbial attachment to food and food contact surfaces. Adv. Food Nutr. Res. 43, 319–370.

Franz, E., Visser, A.A., Van Diepeningen, A.D., Klerks, M.M., Termorshuizen, A.J., van Bruggen, A.H., 2007. Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar *Typhimurium*. Food Microbiol. 24, 106–112.

Greig, J.D., Ravel, A., 2009. Analysis of foodborne outbreak data reported internationally for source attribution. Int. J. Food Microbiol. 130, 77–87. https://doi.org/10.1016/j.ijfoodmicro.2008.12.031.

Gurtler, J.B., Douds Jr., D.D., Dirks, B.P., Quinlan, J.J., Nicholson, A.M., Phillips, J.G., Niemira, B.A., 2013. *Salmonella* and *Escherichia coli* O157:H7 survival in soil and translocation into leeks (*Allium porrum*) as influenced by an arbuscular mycorrhizal fungus (*Glomus intraradices*). Appl. Environ. Microbiol. 79, 1813–1820. https://doi.org/10.1128/AEM.02855-12.

Hanahan, D., 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166, 557–580.

Hayashi, T., Makino, K., Ohnishi, M., Kurokawa, K., Ishii, K., Yokoyama, K., Han, C.G., Ohtsubo, E., Nakayama, K., Murata, T., Tanaka, M., Tobe, T., Iida, T., Takami, H., Honda, T., Sasakawa, C., Ogasawara, N., Yasunaga, T., Kuhara, S., Shiba, T., Hattori, M., Shinagawa, H., 2001. Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. DNA Res. Int. J. Rapid Publ. Rep. Genes Genomes 8, 11–22.

Herold, S., Paton, J.C., Srimanote, P., Paton, A.W., 2009. Differential effects of short-chain fatty acids and iron on expression of *iha* in Shiga-toxigenic *Escherichia coli*. Microbiol. Read. Engl. 155, 3554–3563. https://doi.org/10.1099/mic.0.029454-0.

Hora, R., Warriner, K., Shelp, B.J., Griffiths, M.W., 2005. Internalization of *Escherichia coli* O157:H7 following biological and mechanical disruption of growing spinach plants. J. Food Protect. 68, 2506–2509.

Jaglic, Z., Desvaux, M., Weiss, A., Nesse, L.L., Meyer, R.L., Demnerova, K., Schmidt, H., Giaouris, E., Sipailiene, A., Teixeira, P., Kacaniova, M., Riedel, C.U., Knochel, S., 2014. Surface adhesins and exopolymers of selected foodborne pathogens. Microbiol. Read. Engl. 160, 2561–2582. https://doi.org/10.1099/mic.0.075887-0.

Johnson, J.R., Jelacic, S., Schoening, L.M., Clabots, C., Shaikh, N., Mobley, H.L., Tarr, P.I., 2005. The IrgA homologue adhesin Iha is an *Escherichia coli* virulence factor in murine urinary tract infection. Infect. Immun. 73, 965–971.

Johnson, J.R., Russo, T.A., Tarr, P.I., Carlino, U., Bilge, S.S., Vary Jr., J.C., Stell, A.L., 2000. Molecular epidemiological and phylogenetic associations of two novel putative virulence genes, *iha* and *iroN(E. coli)*, among *Escherichia coli* isolates from patients with urosepsis. Infect. Immun. 68, 3040–3047.

Jozefaciuk, G., Szatanik-Kloc, A., Lukowska, M., Szerement, J., 2014. Pitfalls and uncertainties of using potentiometric titration for estimation of plant roots surface charge and acid-base properties. Am. J. Plant Sci. 05 (13), 15.

Kaper, J.B., 1998. Enterohemorrhagic *Escherichia coli*. Curr. Opin. Microbiol. 1, 103–108.

Ledesma, M.A., Ochoa, S.A., Cruz, A., Rocha-Ramirez, L.M., Mas-Oliva, J., Eslava, C.A., Giron, J.A., Xicohtencatl-Cortes, J., 2010. The hemorrhagic coli pilus (HCP) of *Escherichia coli* O157:H7 is an inducer of proinflammatory cytokine secretion in intestinal epithelial cells. PLoS One 5, e12127. https://doi.org/10.1371/journal.pone.0012127.

Leveille, S., Caza, M., Johnson, J.R., Clabots, C., Sabri, M., Dozois, C.M., 2006. Iha from an *Escherichia coli* urinary tract infection outbreak clonal group A strain is expressed in vivo in the mouse urinary tract and functions as a catecholate siderophore receptor. Infect. Immun. 74, 3427–3436.

Li, J., McLandsborough, L.A., 1999. The effects of the surface charge and hydrophobicity of *Escherichia coli* on its adhesion to beef muscle. Int. J. Food Microbiol. 53, 185–193.

Macarisin, D., Patel, J., Bauchan, G., Giron, J.A., Sharma, V.K., 2012. Role of curli and cellulose expression in adherence of *Escherichia coli* O157:H7 to spinach leaves. Foodb. Pathog. Dis. 9, 160–167. https://doi.org/10.1089/fpd.2011.1020.

Macarisin, D., Patel, J., Sharma, V.K., 2014. Role of curli and plant cultivation conditions on *Escherichia coli* O157:H7 internalization into spinach grown on hydroponics and in soil. Int. J. Food Microbiol. 173, 48–53. https://doi.org/10.1016/j.ijfoodmicro.2013.12.004.

Maniatis, T., Fritsch, E.F., Sambrook, J., Engel, J., 1985. Molecular cloning-a laboratory manual. New York: cold spring harbor laboratory. 1982, 545 S., 42 $. Acta Biotechnol. 5, 104. https://doi.org/10.1002/abio.370050118.

Michino, H., Araki, K., Minami, S., Takaya, S., Sakai, N., Miyazaki, M., Ono, A.,

Yanagawa, H., 1999. Massive outbreak of *Escherichia coli* O157:H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. Am. J. Epidemiol. 150, 787–796.
Nuebling, S., Wohlt, D., Saile, N., Weiss, A., Schmidt, H., 2017. Antimicrobial effect of lauroyl arginate ethyl on *Escherichia coli* O157:H7 and *Listeria monocytogenes* on red oak leaf lettuce. Eur. Food Res. Technol. 243, 879–887. https://doi.org/10.1007/s00217-016-2802-1.
Quilliam, R.S., Williams, A.P., Jones, D.L., 2012. Lettuce cultivar mediates both phyllosphere and rhizosphere activity of *Escherichia coli* O157:H7. PLoS One 7, e33842. https://doi.org/10.1371/journal.pone.0033842.
Rashid, R.A., Tarr, P.I., Moseley, S.L., 2006. Expression of the *Escherichia coli* IrgA homolog adhesin is regulated by the ferric uptake regulation protein. Microb. Pathog. 41, 207–217.
Ruehlmann, J., Ruppel, S., 2005. Effects of organic amendments on soil carbon content and microbial biomass - results of the long-term box plot experiment in Grossbeeren. Arch. Agron Soil Sci. 51, 163–170. https://doi.org/10.1080/03650340400026651.
Saile, N., Voigt, A., Kessler, S., Stressler, T., Klumpp, J., Fischer, L., Schmidt, H., 2016. *Escherichia coli* O157:H7 strain EDL933 harbors multiple functional prophage-associated genes necessary for the utilization of 5- *N* -Acetyl-9- *O* -acetyl neuraminic acid as a growth substrate. Appl. Environ. Microbiol. 82, 5940–5950. https://doi.org/10.1128/AEM.01671-16.
Saldana, Z., Sanchez, E., Xicohtencatl-Cortes, J., Puente, J.L., Giron, J.A., 2011. Surface structures involved in plant stomata and leaf colonization by shiga-toxigenic *Escherichia coli* O157:h7. Front. Microbiol. 2 (119). https://doi.org/10.3389/fmicb.2011.00119.
Schikora, A., Virlogeux-Payant, I., Bueso, E., Garcia, A.V., Nilau, T., Charrier, A., Pelletier, S., Menanteau, P., Baccarini, M., Velge, P., Hirt, H., 2011. Conservation of *Salmonella* infection mechanisms in plants and animals. PLoS One 6, e24112. https://doi.org/10.1371/journal.pone.0024112.
Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Meth. 9, 676–682. https://doi.org/10.1038/nmeth.2019.
Schindelin, J., Rueden, C.T., Hiner, M.C., Eliceiri, K.W., 2015. The ImageJ ecosystem: an open platform for biomedical image analysis. Mol. Reprod. Dev. 82, 518–529. https://doi.org/10.1002/mrd.22489.
Schmidt, H., Zhang, W.L., Hemmrich, U., Jelacic, S., Brunder, W., Tarr, P.I., Dobrindt, U., Hacker, J., Karch, H., 2001. Identification and characterization of a novel genomic island integrated at *selC* in locus of enterocyte effacement-negative, Shiga toxin-producing *Escherichia coli*. Infect. Immun. 69, 6863–6873. https://doi.org/10.1128/IAI.69.11.6863-6873.2001.
Schreiter, S., Ding, G.C., Heuer, H., Neumann, G., Sandmann, M., Grosch, R., Kropf, S., Smalla, K., 2014. Effect of the soil type on the microbiome in the rhizosphere of field-grown lettuce. Front. Microbiol. 5 (144). https://doi.org/10.3389/fmicb.2014.00144.
Seo, K.H., Frank, J.F., 1999. Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. J. Food Protect. 62, 3–9.
Sharma, M., Ingram, D.T., Patel, J.R., Millner, P.D., Wang, X., Hull, A.E., Donnenberg, M.S., 2009. A novel approach to investigate the uptake and internalization of *Escherichia coli* O157:H7 in spinach cultivated in soil and hydroponic medium. J. Food Protect. 72, 1513–1520.
Slanec, T., Fruth, A., Creuzburg, K., Schmidt, H., 2009. Molecular analysis of virulence profiles and Shiga toxin genes in food-borne Shiga toxin-producing *Escherichia coli*. Appl. Environ. Microbiol. 75, 6187–6197. https://doi.org/10.1128/AEM.00874-09.
Solomon, E.B., Yaron, S., Matthews, K.R., 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Appl. Environ. Microbiol. 68, 397–400.
Tarr, P.I., Bilge, S.S., Vary Jr., J.C., Jelacic, S., Habeeb, R.L., Ward, T.R., Baylor, M.R., Besser, T.E., 2000. Iha: a novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. Infect. Immun. 68, 1400–1407.
Tuttle, J., Gomez, T., Doyle, M.P., Wells, J.G., Zhao, T., Tauxe, R.V., Griffin, P.M., 1999. Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. Epidemiol. Infect. 122, 185–192.
Ukuku, D.O., Fett, W.F., 2002. Relationship of cell surface charge and hydrophobicity to strength of attachment of bacteria to cantaloupe rind. J. Food Protect. 65, 1093–1099.
Wright, K.M., Crozier, L., Marshall, J., Merget, B., Holmes, A., Holden, N.J., 2017. Differences in internalization and growth of *Escherichia coli* O157:H7 within the apoplast of edible plants, spinach and lettuce, compared with the model species *Nicotiana benthamiana*. Microb. Biotechnol 10, 555–569. https://doi.org/10.1111/1751-7915.12596.
Xicohtencatl-Cortes, J., Monteiro-Neto, V., Ledesma, M.A., Jordan, D.M., Francetic, O., Kaper, J.B., Puente, J.L., Giron, J.A., 2007. Intestinal adherence associated with type IV pili of enterohemorrhagic *Escherichia coli* O157:H7. J. Clin. Invest. 117, 3519–3529. https://doi.org/10.1172/JCI30727.
Xicohtencatl-Cortes, J., Monteiro-Neto, V., Saldana, Z., Ledesma, M.A., Puente, J.L., Giron, J.A., 2009. The type 4 pili of enterohemorrhagic *Escherichia coli* O157:H7 are multipurpose structures with pathogenic attributes. J. Bacteriol. 191, 411–421. https://doi.org/10.1128/JB.01306-08.
Yin, X., Wheatcroft, R., Chambers, J.R., Liu, B., Zhu, J., Gyles, C.L., 2009. Contributions of O island 48 to adherence of enterohemorrhagic *Escherichia coli* O157:H7 to epithelial cells in vitro and in ligated pig ileal loops. Appl. Environ. Microbiol. 75, 5779–5786. https://doi.org/10.1128/AEM.00507-09.
Zita, A., Hermansson, M., 1997. Effects of bacterial cell surface structures and hydrophobicity on attachment to activated sludge flocs. Appl. Environ. Microbiol. 63, 1168–1170.

Chapter 3

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

Kristina Eißenberger[a], David Drissner[b,c,d], Fiona Walsh[e], Agnes Weiss[a], and Herbert Schmidt[a]

[a]Institute of Food Science and Biotechnology, Department of Food Microbiology and Hygiene, University of Hohenheim, Germany

[b]Microbiology of Plant Foods, Agroscope, Waedenswil, Switzerland

[c]Swiss Federal Institute for Forest, Snow, and Landscape Research WSL, Birmensdorf, Switzerland

[d]Department of Life Sciences, Albstadt-Sigmaringen University, Sigmaringen, Germany

[e]Department of Biology, Maynooth University, Maynooth, Ireland

Published in Food Microbiology Volume 86, Article 103316

DOI: 10.1016/j.fm.2019.103316

This manuscript contains an additional figure and supplementary on sequencing analysis which can be found at https://doi.org/10.1016/j.fm.2019.103316. Sequencing data are deposited at the European Nucleotide Archive (ENA) and can be found under the study accession number PRJEB30456.

Food Microbiology 86 (2020) 103316

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

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

Kristina Eissenberger[a], David Drissner[b,c,d], Fiona Walsh[e], Agnes Weiss[a], Herbert Schmidt[a,*]

[a] Institute of Food Science and Biotechnology, Department of Food Microbiology and Hygiene, University of Hohenheim, Stuttgart, Germany
[b] Microbiology of Plant Foods, Agroscope, Waedenswil, Switzerland
[c] Swiss Federal Institute for Forest, Snow, and Landscape Research WSL, Birmensdorf, Switzerland
[d] Department of Life Sciences, Albstadt-Sigmaringen University, Sigmaringen, Germany
[e] Department of Biology, Maynooth University, Maynooth, Ireland

ARTICLE INFO

Keywords:
EHEC/EAEC O104:H4 C227/11ϕcu
Lamb's lettuce
Lettuce
Internalization
Adherence
Soil microbiota

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

* Corresponding author. .
E-mail address: herbert.schmidt@uni-hohenheim.de (H. Schmidt).

https://doi.org/10.1016/j.fm.2019.103316
Received 28 February 2019; Received in revised form 19 July 2019; Accepted 31 August 2019
Available online 05 September 2019
0740-0020/ © 2019 Published by Elsevier Ltd.

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

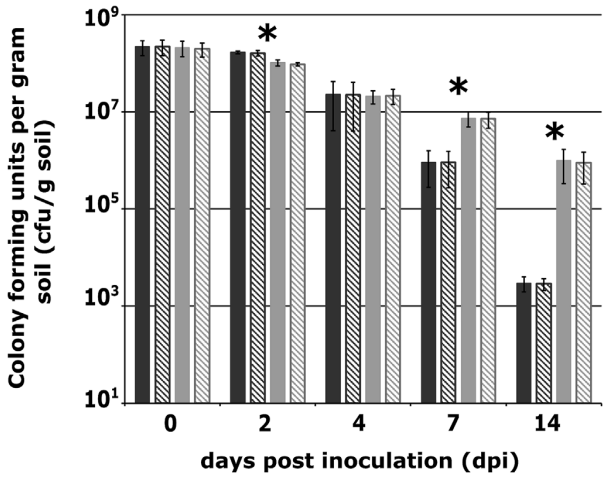


Fig. 1. Stability of plasmid pKEC2 in *E. coli* O104:H4 C227/11ϕcu/pKEC2. DS (dark grey) and AL (light grey) were inoculated with 1.0×10^8 cfu *E. coli* O104:H4 C227/11ϕcu/pKEC2 per gram soil and incubated for up to 14 days. Soil was sampled 0, 2, 4, 7, and 14 days post infection (dpi), and investigated 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-

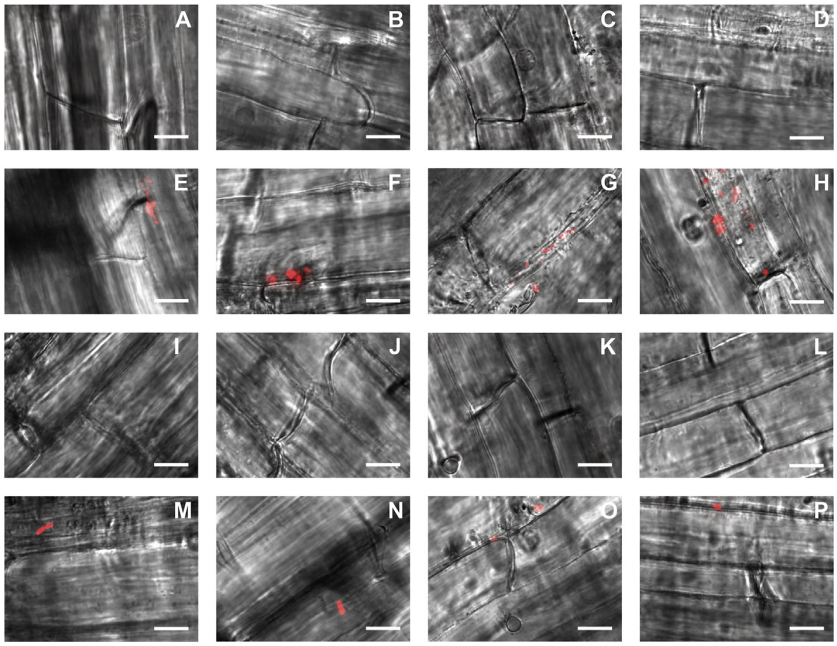


Fig. 2. Microscopic analysis of red fluorescent bacteria at and in the roots of *L. sativa* and *V. locusta* grown in different soils. Microscopy was performed either after 10 min of washing in 0.5 × MS medium to investigate adherence (A–H) or after 10 min of washing in 0.5 × MS medium and 20 min of 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.)

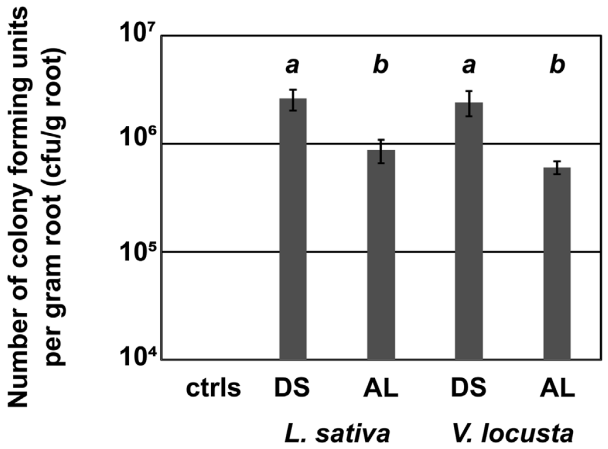


Fig. 3. Adherence of *E. coli* O104:H4 C227/11ϕcu/pKEC2 to the roots of *L. sativa* and *V. locusta* grown in different soil types. Viable counts of adherent bacteria at the roots in cfu/g root. Plants were grown in DS or AL, inoculated with 1.0×10^8 cfu/g soil and incubated for 4 days. Under all conditions 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 ($p < 0.05$).

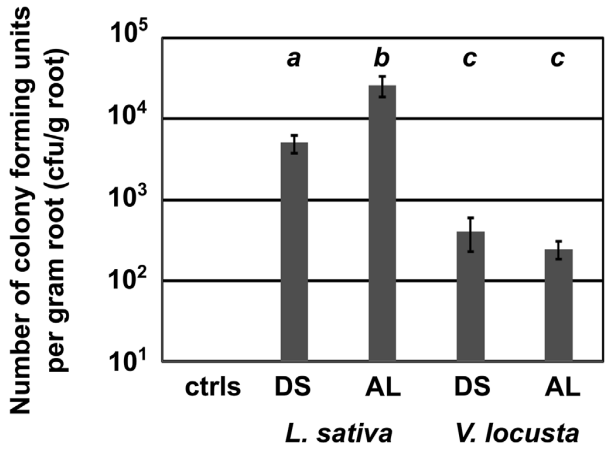


Fig. 4. Internalization of *E. coli* O104:H4 C227/11ϕcu/pKEC2 into the roots of *L. sativa* and *V. locusta* grown in different soil types. Viable counts of internalized bacteria at the roots in cfu/g root. Plants were grown in DS or AL, inoculated with 1.0×10^8 cfu/g soil and incubated for 4 days. No 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

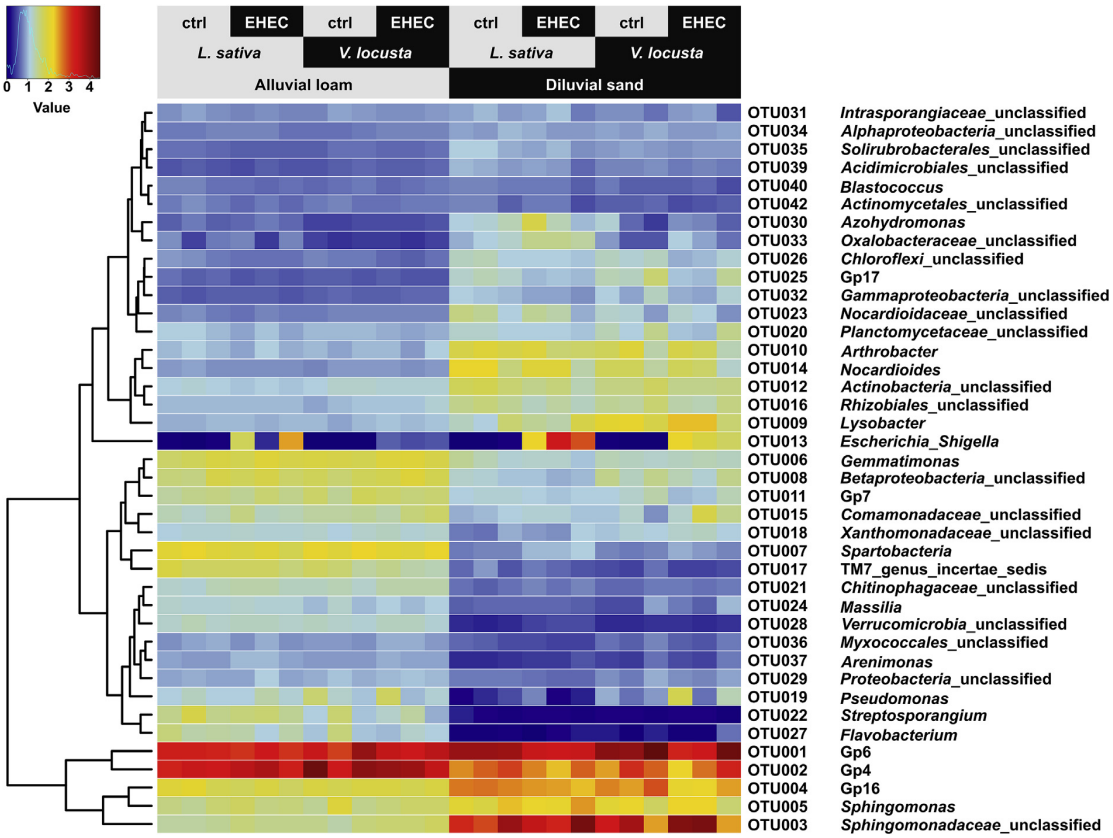


Fig. 5. Relative abundance of the 40 most dominant operational taxonomic units (OTUs). The heatmap indicates differences in the relative abundance of OTUs in the distinct soil type/lettuce variety combination. The individual samples are means of technical triplicates for each biological replicate. These 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.)

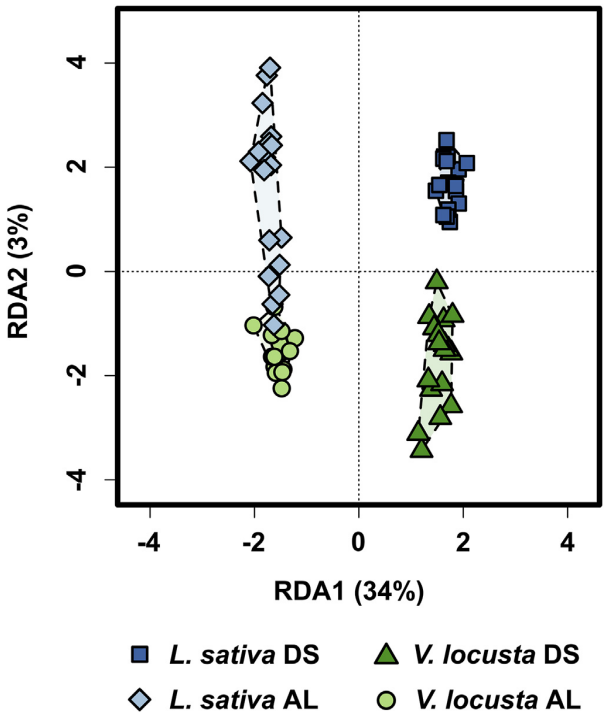


Fig. 6. Redundancy analysis (RDA) of the bacterial soil community composition. Multivariate RDA displays influence of soil type and lettuce variety on the microbial soil composition.

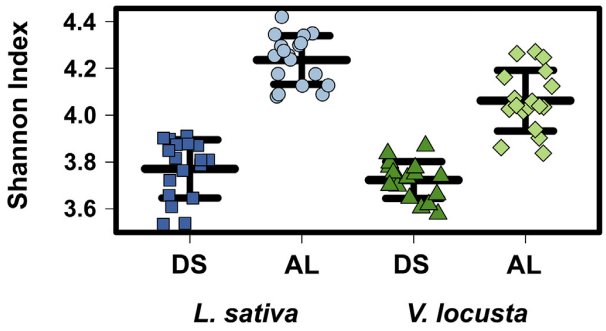


Fig. 7. Diversity within and between the soil type/lettuce variety combination. Alpha diversity given as Shannon index depicts differences in soil microbial community structure between the distinct combinations of soil type and lettuce variety.

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.

Funding information

This work was financially supported by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE), grant number 2813HS028. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgements

We gratefully thank Dr. Daniel Rigling and Dr. Andreas Zurlinden (Swiss Federal Institute for Forest, Snow and Landscape (WSL), Birmensdorf, Switzerland) for the opportunity to conduct these experiments in the new BSL3 greenhouse and laboratory containment, and Hélène Blauenstein (WSL) for assistance during the experimental periods. We thank Dr. Rita Grosch (Leibniz Institute of Vegetable and Ornamental Crops, Großbeeren, Germany) for providing the soil. We are grateful to Dr. Maike Krause (University of Hohenheim, Stuttgart, Germany) for critical discussion of the data. The skillful technical assistance of Dipl. Ing. Jürgen Krauss and Carmela Total (Agroscope, Wädenswil, Switzerland) during plant propagation is greatly appreciated. We thank Dr. Thuy Do and Aidan O'Flaherty (Maynooth University, Maynooth, Ireland) for helpful advice during sequencing data analysis.

Appendix A. Supplementary data

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

References

Abberton, C.L., Bereschenko, L., van der Wielen, P.W.J.J., Smith, C.J., 2016. Survival, biofilm formation, and growth potential of environmental and enteric *Escherichia coli* strains in drinking water microcosms. Appl. Environ. Microbiol. 82, 5320–5331. https://doi.org/10.1128/AEM.01569-16.

Berger, C.N., Shaw, R.K., Ruiz-Perez, F., Nataro, J.P., Henderson, I.R., Pallen, M.J., Frankel, G., 2009. Interaction of enteroaggregative *Escherichia coli* with salad leaves. Environ. Microbiol. Rep. 1, 234–239. https://doi.org/10.1111/j.1758-2229.2009.

00037.x.
Beuchat, L.R., 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. Microb. Infect. 4, 413–423.
Beutin, L., Hammerl, J.A., Reetz, J., Strauch, E., 2013. Shiga toxin-producing *Escherichia coli* strains from cattle as a source of the Stx2a bacteriophages present in enteroaggregative *Escherichia coli* O104:H4 strains. Int. J. Med. Microbiol. 303, 595–602. https://doi.org/10.1016/j.ijmm.2013.08.001.
Bielaszewska, M., Mellmann, A., Zhang, W., Köck, R., Fruth, A., Bauwens, A., Peters, G., Karch, H., 2011. Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. Lancet Infect. Dis. 11, 671–676. https://doi.org/10.1016/S1473-3099(11)70165-7.
Bolton, D.J., Monaghan, A., Byrne, B., Fanning, S., Sweeney, T., McDowell, D.A., 2011. Incidence and survival of non-O157 verocytotoxigenic *Escherichia coli* in soil: non-O157 VTEC in soil. J. Appl. Microbiol. 111, 484–490. https://doi.org/10.1111/j.1365-2672.2011.05057.x.
Brzuszkiewicz, E., Thürmer, A., Schuldes, J., Leimbach, A., Liesegang, H., Meyer, F.-D., Boelter, J., Petersen, H., Gottschalk, G., Daniel, R., 2011. Genome sequence analyses of two isolates from the recent *Escherichia coli* outbreak in Germany reveal the emergence of a new pathotype: entero-Aggregative-Haemorrhagic *Escherichia coli* (EAHEC). Arch. Microbiol. 193, 883–891. https://doi.org/10.1007/s00203-011-0725-6.
Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. 108, 4516–4522. https://doi.org/10.1073/pnas.1000080107.
Ceuppens, S., Johannessen, G., Allende, A., Tondo, E., El-Tahan, F., Sampers, I., Jacxsens, L., Uyttendaele, M., 2015. Risk factors for *Salmonella*, Shiga Toxin-Producing *Escherichia coli* and *Campylobacter* occurrence in primary production of leafy greens and strawberries. Int. J. Environ. Res. Public Health 12, 9809–9831. https://doi.org/10.3390/ijerph120809809.
Chitarra, W., Decastelli, L., Garibaldi, A., Gullino, M.L., 2014. Potential uptake of *Escherichia coli* O157:H7 and *Listeria monocytogenes* from growth substrate into leaves of salad plants and basil grown in soil irrigated with contaminated water. Int. J. Food Microbiol. 189, 139–145. https://doi.org/10.1016/j.ijfoodmicro.2014.08.003.
Cooley, M.B., Chao, D., Mandrell, R.E., 2006. *Escherichia coli* O157:H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. J. Food Prot. 69, 2329–2335.
D'Amore, R., Ijaz, U.Z., Schirmer, M., Kenny, J.G., Gregory, R., Darby, A.C., Shakya, M., Podar, M., Quince, C., Hall, N., 2016. A comprehensive benchmarking study of protocols and sequencing platforms for 16S rRNA community profiling. BMC Genomics 17. https://doi.org/10.1186/s12864-015-2194-9.
Deering, A., Jack, D., Pruitt, R., Mauer, L., 2015. Movement of *Salmonella* serovar Typhimurium and *E. coli* O157:H7 to ripe tomato fruit following various routes of contamination. Microorganisms 3, 809–825. https://doi.org/10.3390/microorganisms3040809.
Eißenberger, K., Moench, D., Drissner, D., Weiss, A., Schmidt, H., 2018. Adherence factors of enterohemorrhagic *Escherichia coli* O157:H7 strain Sakai influence its uptake into the roots of *Valerianella locusta* grown in soil. Food Microbiol. 76, 245–256. https://doi.org/10.1016/j.fm.2018.05.016.
Erickson, M.C., Webb, C.C., Diaz-Perez, J.C., Phatak, S.C., Silvoy, J.J., Davey, L., Payton, A.S., Liao, J., Ma, L., Doyle, M.P., 2010. Surface and internalized *Escherichia coli* O157:H7 on field-grown spinach and lettuce treated with spray-contaminated irrigation water. J. Food Prot. 73, 1023–1029.
Fremaux, B., Prigent-Combaret, C., Vernozy-Rozand, C., 2008. Long-term survival of Shiga toxin-producing *Escherichia coli* in cattle effluents and environment: an updated review. Vet. Microbiol. 132, 1–18. https://doi.org/10.1016/j.vetmic.2008.05.015.
Gagliardi, J.V., Karns, J.S., 2002. Persistence of *Escherichia coli* O157:H7 in soil and on plant roots. Environ. Microbiol. 4, 89–96. https://doi.org/10.1046/j.1462-2920.2002.00273.x.
Golberg, D., Kroupitski, Y., Belausov, E., Pinto, R., Sela, S., 2011. *Salmonella* Typhimurium internalization is variable in leafy vegetables and fresh herbs. Int. J. Food Microbiol. 145, 250–257. https://doi.org/10.1016/j.ijfoodmicro.2010.12.031.
Grant, J., Wendelboe, A.M., Wendel, A., Jepson, B., Torres, P., Smelser, C., Rolfs, R.T., 2008. Spinach-associated *Escherichia coli* O157:H7 outbreak, Utah and New Mexico, 2006. Emerg. Infect. Dis. 14, 1633–1636. https://doi.org/10.3201/eid1410.071341.
Greig, J.D., Ravel, A., 2009. Analysis of foodborne outbreak data reported internationally for source attribution. Int. J. Food Microbiol. 130, 77–87. https://doi.org/10.1016/j.ijfoodmicro.2008.12.031.
Heaton, J.C., Jones, K., 2008. Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. J. Appl. Microbiol. 104, 613–626. https://doi.org/10.1111/j.1365-2672.2007.03587.x.
Kaur, P., Chakraborti, A., Asea, A., 2010. Enteroaggregative *Escherichia coli*: an emerging enteric food borne pathogen. Interdiscip. Perspect. Infect. Dis. 1–10. 2010. https://doi.org/10.1155/2010/254159.
King, L.A., Nogareda, F., Weill, F.-X., Mariani-Kurkdjian, P., Loukiadis, E., Gault, G., Jourdan-DaSilva, N., Bingen, E., Mace, M., Thevenot, D., Ong, N., Castor, C., Noel, H., Van Cauteren, D., Charron, M., Vaillant, V., Aldabe, B., Goulet, V., Delmas, G., Couturier, E., Le Strat, Y., Combe, C., Delmas, Y., Terrier, F., Vendrely, B., Rolland, P., de Valk, H., 2012. Outbreak of Shiga toxin-producing *Escherichia coli* O104:H4 associated with organic fenugreek sprouts, France, June 2011. Clin. Infect. Dis. 54, 1588–1594. https://doi.org/10.1093/cid/cis255.
Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina Sequencing platform. Appl. Environ. Microbiol. 79, 5112–5120. https://doi.org/10.1128/AEM.01043-13.
Lee, J.G., Lee, B.Y., Lee, H.J., 2006. Accumulation of phytotoxic organic acids in reused nutrient solution during hydroponic cultivation of lettuce (*Lactuca sativa* L.). Sci. Hortic. 110, 119–128. https://doi.org/10.1016/j.scienta.2006.06.013.
Leveau, J., 2009. Life on leaves. Nature 461, 741.
Lindow, S.E., Brandl, M.T., 2003. Microbiology of the phyllosphere. Appl. Environ. Microbiol. 69, 1875–1883. https://doi.org/10.1128/AEM.69.4.1875-1883.2003.
Lopez-Velasco, G., Tydings, H.A., Boyer, R.R., Falkinham, J.O., Ponder, M.A., 2012. Characterization of interactions between *Escherichia coli* O157:H7 with bacteria *in vitro* and on spinach leaf surfaces. Int. J. Food Microbiol. 153, 351–357. https://doi.org/10.1016/j.ijfoodmicro.2011.11.026.
Marder, E.P., Garman, K.N., Ingram, L.A., Dunn, J.R., 2014. Multistate outbreak of *Escherichia coli* O157:H7 associated with bagged salad. Foodb. Pathog. Dis. 11, 593–595. https://doi.org/10.1089/fpd.2013.1726.
Matthews, K.R., 2013. Sources of enteric pathogen contamination of fruits and vegetables: future directions of research. Stewart Postharvest Rev. 9, 1–5. https://doi.org/10.2212/spr.2013.1.2.
Nagy, A., Xu, Y., Bauchan, G.R., Shelton, D.R., Nou, X., 2016. Aggregative adherence fimbriae I (AAF/I) mediate colonization of fresh produce and abiotic surface by Shiga toxigenic enteroaggregative *Escherichia coli* O104:H4. Int. J. Food Microbiol. 229, 44–51. https://doi.org/10.1016/j.ijfoodmicro.2016.04.007.
Nataro, J., 1998. Enteroaggregative *Escherichia coli*. Emerg. Infect. Dis. 4, 251–261. https://doi.org/10.3201/eid0402.980212.
Neumann, G., Bott, S., Ohler, M.A., Mock, H.-P., Lippmann, R., Grosch, R., Smalla, K., 2014. Root exudation and root development of lettuce (*Lactuca sativa* L. cv. Tizian) as affected by different soils. Front. Microbiol. 5. https://doi.org/10.3389/fmicb.2014.00002.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, D590–D596. https://doi.org/10.1093/nar/gks1219.
Quilliam, R.S., Williams, A.P., Jones, D.L., 2012. Lettuce cultivar mediates both phyllosphere and rhizosphere activity of *Escherichia coli* O157:H7. PLoS One 7, e33842. https://doi.org/10.1371/journal.pone.0033842.
Robert-Koch-Institute (Ed.), 2012. Infektionsepidemiologisches Jahrbuch meldepflichtiger Krankheiten für 2011, (Berlin).
Ruehlmann, J., Ruppel, S., 2005. Effects of organic amendments on soil carbon content and microbial biomass - results of the long-term box plot experiment in Grossbeeren. Arch. Agron Soil Sci. 51, 163–170. https://doi.org/10.1080/03650340400026651.
Schreiter, S., Ding, G.C., Heuer, H., Neumann, G., Sandmann, M., Grosch, R., Kropf, S., Smalla, K., 2014. Effect of the soil type on the microbiome in the rhizosphere of field-grown lettuce. Front. Microbiol. 5, 144. https://doi.org/10.3389/fmicb.2014.00144.
Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12, R60. https://doi.org/10.1186/gb-2011-12-6-r60.
Seo, K.H., Frank, J.F., 1999. Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. J. Food Prot. 62, 3–9.
Sharma, M., Ingram, D.T., Patel, J.R., Millner, P.D., Wang, X., Hull, A.E., Donnenberg, M.S., 2009. A novel approach to investigate the uptake and internalization of *Escherichia coli* O157:H7 in spinach cultivated in soil and hydroponic medium. J. Food Prot. 72, 1513–1520.
Solomon, E.B., Sharma, M., 2009. Microbial attachment and limitations of decontamination methodologies. In: The Produce Contamination Problem. Elsevier, pp. 21–45. https://doi.org/10.1016/B978-0-12-374186-8.00002-1.
Solomon, E.B., Yaron, S., Matthews, K.R., 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Appl. Environ. Microbiol. 68, 397–400.
Tangwatcharin, P., Khopaibool, P., 2012. Activity of virgin coconut oil, lauric acid or monolaurin in combination with lactic acid against *Staphylococcus aureus*. Southeast Asian J. Trop. Med. Public Health 43, 969–985.
Thurston-Enriquez, J.A., Gilley, J.E., Eghball, B., 2005. Microbial quality of runoff following land application of cattle manure and swine slurry. J. Water Health 3, 157–171.
Uber, A.P., Trabulsi, L.R., Irino, K., Beutin, L., Ghilardi, Ã.C.R., Gomes, T.A.T., Liberatore, A.M.A., Castro, A.F.P., Elias, W.P., 2006. Enteroaggregative *Escherichia coli* from humans and animals differ in major phenotypical traits and virulence genes: EAEC from humans and animals. FEMS Microbiol. Lett. 256, 251–257. https://doi.org/10.1111/j.1574-6968.2006.00124.x.
Vijay, D., Dhaka, P., Vergis, J., Negi, M., Mohan, V., Kumar, M., Doijad, S., Poharkar, K., Kumar, A., Malik, S.S., Barbuddhe, S.B., Rawool, D.B., 2015. Characterization and biofilm forming ability of diarrhoeagenic enteroaggregative *Escherichia coli* isolates recovered from human infants and young animals. Comp. Immunol. Microbiol. Infect. Dis. 38, 21–31. https://doi.org/10.1016/j.cimid.2014.11.004.
Walters, D.R., Walker, R.L., Walker, K.C., 2003. Lauric acid exhibits antifungal activity against plant pathogenic fungi: antifungal activity of lauric acid. J. Phytopathol. 151, 228–230. https://doi.org/10.1046/j.1439-0434.2003.00713.x.
Wang, H., Ibekwe, A.M., Ma, J., Wu, L., Lou, J., Wu, Z., Liu, R., Xu, J., Yates, S.R., 2014a. A glimpse of *Escherichia coli* O157:H7 survival in soils from eastern China. Sci. Total Environ. 476–477, 49–56. https://doi.org/10.1016/j.scitotenv.2014.01.004.
Wang, H., Zhang, T., Wei, G., Wu, L., Wu, J., Xu, J., 2014b. Survival of *Escherichia coli* O157:H7 in soils under different land use types. Environ. Sci. Pollut. Res. 21, 518–524. https://doi.org/10.1007/s11356-013-1938-9.
Wright, K.M., Crozier, L., Marshall, J., Merget, B., Holmes, A., Holden, N.J., 2017. Differences in internalization and growth of *Escherichia coli* O157:H7 within the apoplast of edible plants, spinach and lettuce, compared with the model species *Nicotiana benthamiana*. Microb. Biotechnol. 10, 555–569. https://doi.org/10.1111/

1751-7915.12596.
Yadav, R.K.P., Karamanoli, K., Vokou, D., 2005. Bacterial colonization of the phyllosphere of mediterranean perennial species as influenced by leaf structural and chemical features. Microb. Ecol. 50, 185–196. https://doi.org/10.1007/s00248-004-0171-y.
Yang, B., Wang, Y., Qian, P.Y., 2016. Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. BMC Bioinf. 17, 135. https://doi: 10.1186/s12859-016-0992-y.
Zakrzewski, M., Proietti, C., Ellis, J.J., Hasan, S., Brion, M.-J., Berger, B., Krause, L., 2016. Calypso: a user-friendly web-server for mining and visualizing microbiome–environment interactions. Bioinformatics btw725. https://doi.org/10.1093/bioinformatics/btw725.
Zangari, T., Melton-Celsa, A.R., Panda, A., Boisen, N., Smith, M.A., Tatarov, I., De Tolla, L.J., Nataro, J.P., O'Brien, A.D., 2013. Virulence of the Shiga toxin type 2-expressing *Escherichia coli* O104:H4 German outbreak isolate in two animal models. Infect. Immun. 81, 1562–1574. https://doi.org/10.1128/IAI.01310-12.
Zhang, T., Hu, S., Yang, W., 2017. Variations of *Escherichia coli* O157:H7 survival in purple soils. Int. J. Environ. Res. Public Health 14, 1246. https://doi.org/10.3390/ijerph14101246.

4. Discussion and concluding remarks

Contamination of lettuces such as *Lactuca sativa* and *Valerianella locusta* presents a serious threat to public health as they can serve as secondary hosts for human pathogens such as *E. coli* and *Salmonella*. Because of the raw consumption of lettuce, it is difficult to remove internal bacterial contamination, i.e. internalized bacteria. A variety of animals such as cattle, sheep, dogs, fox and rabbit can serve as reservoirs of pathogenic *E. coli* (41). Hence, contamination of lettuce via feces, irrigation water, manure, and surface water is an important issue. Several studies investigated the ability of EHEC, *S. enterica* and *Listeria monocytogenes* to adhere to and internalize into the leaves and roots of various plant host under different conditions, as reviewed by Brandl, Deering and Hirneisen (59, 64, 103). However, recent studies focused on EHEC-plant interactions regarding adherence, internalization, and persistence of EHEC within the plant tissue (56–58, 104). As a consequence, only little is known about a) which genetic factors drive EHEC adherence and internalization, and b) the ability of EAEC and EAEC/EHEC to colonize plants (105). These aspects were investigated within the scope of the present dissertation by genetic, microscopic and microbiological analyses. Moreover, the composition of the soil microbiota was addressed by bioinformatic methods.

The major finding of this thesis is that different *E. coli* strains are able to adhere to and to internalize into the roots of lettuce plants under greenhouse conditions (Chapter 2 and Chapter 3). These processes – adherence and internalization – are influenced by genetic factors as well as by environmental factors, especially soil type and lettuce variety.

Infection experiments presented the central experiments of the present thesis. Within the scope of these experiments, lettuce plants were inoculated with EHEC or EAEC/EHEC suspensions by dispensing the bacterial suspension into the soil surrounding the lettuce plants. After incubation in a biosafety level 3 greenhouse, the lettuce plants were freed from soil particles by washing for subsequent investigations of the roots regarding adherence and internalization by qualitative and quantitative analyses. For investigation of internalization, the washing step was followed by gentamicin-based surface disinfection of the plants to eliminate adherent bacteria. The effectiveness of the surface sterilization was monitored and confirmed. The roots were analyzed qualitatively by fluorescence microscopy and quantitatively by homogenization of the roots and spread plating of appropriate dilutions on TBX agar containing the corresponding antibiotics. Qualitative analysis by fluorescence microscopy was applied to investigate if fluorescently labeled *E. coli* cells were present at/in the surveyed roots or not. This approach aimed neither for quantifiable results nor for the exact localization of the detected bacteria within the root tissue. As depicted in Chapter 2 and Chapter 3, all of the used strains were detected at and in the roots of the inoculated lettuce plants.

It was demonstrated for *E. coli* O157:H7 strain Sakai that under greenhouse conditions this strain is able to adhere to and to internalize into the roots of DS grown *V. locusta* (Chapter 2). A central point of the investigation was to analyze the influence of two virulence factors which are encoded by *hcpA* and *iha*, respectively. These virulence factors were so far associated with adherence and therefore called "adherence factors". Assuming that adherence may be an important step towards internalization, these genes were chosen for further experiments. To investigate if *hcpA* and *iha* are involved, isogenic deletion mutants and complementation mutants were used for infection experiments.

The quantitative analysis of the infection experiments performed with *E. coli* O157:H7 strain Sakai and the mentioned deletion and complementation mutants showed that this specific strain and the tested mutants are able to adhere to and to internalized into the roots of *V. locusta* (Chapter 2). Interestingly, the extent of adherence was not statistically significant different between strain Sakai and the generated mutants (Chapter 2). These findings imply that *hcpA* and *iha* did not act as essential adherence factors under the tested conditions although they were described as adherence factors in other studies (22, 34–36). The contradicting observations of these studies raise the question if adherence to plant surfaces follow a certain pattern that is irrespective of the type of surface or if they follow distinct mechanisms which depend on the particular surface. Even though various studies report the contribution of certain *E. coli* genes and proteins (22, 23, 38, 106), adherence to plant surfaces – or even surfaces in general – may not be driven only by specific interactions between certain bacterial proteins and the surface. Instead, unspecific interactions, which are affected by cell surface hydrophobicity and cell surface charge, may also be important for adherence and even internalization.

Regarding internalization, it was demonstrated that upon deletion of *hcpA* and/or *iha* the viable counts of strain Sakai bacteria that entered the roots significantly decreased. This finding implies that both genes play an important role during root internalization. Unexpectedly, the viable counts did not significantly differ between the two single deletion mutants and the double deletion mutant. This means that *hcpA* and *iha* do not act in concert and that further genes are involved in root internalization. The role of *hcpA* and *iha* during adherence and internalization may result from both, specific differences between the distinct leaves and the roots, e.g. presence of cuticle, differences in surfaces roughness, and differences in surface hydrophobicity and/or surface charge. This is further suggested by findings from a recent study which surveyed the differential gene regulation of several *E. coli* strains in response to the exposure to *V. locusta* derived lettuce medium (107). In this study, neither *hcpA* nor *iha* was upregulated. Possibly, the two internalization factors were not needed as Bufe et al. (107) used *V. locusta* extracts, therefore lacking any plant surface. In roots, the surface charge density seems to be heterogeneously distributed along the roots (108). Meaning that, besides natural roots openings, like lesions or sites of lateral roots emergence, which are supposed to be the main entry points

for bacteria, the heterogeneous surface charge may facilitate the formation of foci that are more prone to bacterial invasion than other sites of the roots.

As the qualitative analysis was performed by fluorescence microscopy, the investigated strains were transformed with the plasmid pKEC2 which carries the gene coding for the red fluorescent protein (RFP) and a chloramphenicol resistance cassette. In the absence of selective pressure, plasmids may get lost within bacterial populations meaning that these plasmids are no longer inherited upon cytogenesis. To ensure that pKEC2 is stable throughout the time course of the experiments, plasmid stability tests were performed by incubating the strain of interest in the respective soil type for up to 14 days, i.e. strain Sakai/pKEC2 was incubated in DS and strain C227/11ϕcu/pKEC2 was incubated in DS as well as in AL. After 0, 2, 4, 7, and 14 days, the inoculated strains were recovered from soil and spread plated on TBX agar with and without chloramphenicol to distinguish between plasmid-carrier and bacteria that lost pKEC2. It was observed that in any case the viable counts declined over time (Chapter 2 and Chapter 3). Moreover, it was found that pKEC2 is largely stable in strain Sakai – tested in DS – as well as in strain C227/11ϕcu – tested in DS and AL – under the investigated conditions as the viable counts of the respective plasmid carrier and the total counts of the corresponding strain did not significantly differ at any tested time point. Moreover, it was observed that upon incubation in DS strain Sakai and strain C227/11ϕcu show similar patterns regarding the decline of the viable counts (Chapter 2 and Chapter 3). These findings indicate that the investigated strains behave similarly when incubated in the same soil type. On the contrary, when incubated in AL, the viable counts of strain C227/11ϕcu declined to a significantly lesser extent (Chapter 3) indicating that different soil types have distinct effects on bacterial strains. These observations were further corroborated by findings from another study that investigated the persistence of different *S. enterica* strains in the same soil types (109). It was demonstrated for all surveyed *S. enterica* strains that the persistence was enhanced in AL compared to DS (109).

Soil types may indeed vary in their physicochemical properties as well as in the composition of the soil microbial community. The physicochemical properties of soil are influenced, inter alia, by nutrient content, moisture level, particle size, and clay content. These factors are important for root colonization by pathogens, in particular human pathogens, as survival of the pathogen within the soil is a prerequisite. For this reason, in several studies the survival of different species and strains of human pathogens was investigated in distinct soil types (109–113). It was found that persistence and survival of *E. coli* were influenced by the clay content with four of the studies showing that a higher clay content resulted in longer persistence of *E. coli* within the soil (111–113). On the contrary, Ma et al. (110) demonstrated that a higher sand content resulted in prolonged soil persistence for several *E. coli* serotypes and different strains. Interestingly, Ma et al. (110) and Wang et al. (113) both conducted their

studies with *E. coli* O157:H7 strain EDL933 and gained different results. The former demonstrated for various serotypes that the survival time was prolonged in soil with low microbial biomass carbon, 9.4 % clay content on average, and approximately 76.3 % sand content. For *E. coli* O157:H7 strain EDL933, the survival time increased significantly by factor 1.7 to 2.1 to 40.4 days compared to soil types with higher clay and lower sand content (110). Moreover, this study showed that except for *E. coli* O157:H7 strain 4554 the survival time increased as well by factor 1.3 to 2.1 for all other serotypes (110). On the contrary, the latter found that the survival time to reach the detection limit of 100 cfu/g soil was longer (25.8 days) in an acidic soil with high content of microbial biomass carbon, 25.1 % clay content, and 6.5 % sand content (113). As the soil types AL and DS which were used in the present thesis also differ in clay and sand content (114), the results may indicate that survival of *E. coli* O104:H4 strain C227/11ϕcu is positively influenced by higher clay and lower sand content under the conditions tested. This was also proposed by (109) for *S. enterica*. As previously said, this study found results similar to those of the present thesis but for the persistence of different *S. enterica* strains (109).

These observations raised the question about the influence of environmental factors, such as soil type and plant variety, on the adherence and internalization behavior of *E. coli*. Therefore, infections experiments were performed with *V. locusta* and *L. sativa*, both grown in AL and DS each, as host and the pathogenic *E. coli* O104:H4 strain C227/11ϕcu (Chapter 3). Analyses of internalization showed that the extent of invasion of strain C227/11ϕcu significantly differed between the two lettuce varieties. However, for *V. locusta* similar viable counts of internalized bacteria were found for both soil types meaning that for this lettuce variety the soil type did not influence the internalization behavior of the investigated strain. The amount of internalized strain C227/11ϕcu was significantly higher when *L. sativa* served as host (Chapter 3). When grown in DS, the number of internalized bacteria increased by factor twelve for *L. sativa* compared to *V. locusta*. An even higher increase was observed when the lettuce plants were grown in AL. Compared to *V. locusta*, the viable counts of bacteria that internalized into the roots of *L. sativa* increased by factor 108. These results indicate that internalization is significantly influenced by the lettuce variety. Comparing the viable counts found for *L. sativa* grown in AL and *L. sativa* grown in DS showed that significantly more bacteria internalized into the roots of *L. sativa* when grown in AL (Chapter 3) indicating that the soil type also influenced the extent internalization in this case. This “soil type effect” was also observed during the analyses of adherence. In this case, the effect was even more pronounced. Significantly higher numbers of adherent bacteria where found when the lettuce plants were grown in DS compared to AL. When grown in the same soil type, the extent of adherence did not significantly differ between the two lettuce varieties (Chapter 3). These findings showed that adherence is significantly influenced by the soil type but not by the lettuce vari-

ety. Intriguingly, a study performed with different *S. enterica* strains but the same soil types and lettuce varieties also investigated the influence of these abiotic factors on the leaf colonization by *S. enterica* upon inoculation into the soil (109). This study showed that *S. enterica* can spread from the root to the leaves. Furthermore, it also demonstrated higher colonization of *L. sativa* compared to *V. locusta* (109). In contrast to the present thesis, leaf colonization by *S. enterica* was promoted upon plant growth in DS and also depended on the respective strain (109). In the present thesis, the amount of strain C227/11ϕcu cells (Chapter 3) which adhered to and internalized into the roots of *V. locusta* grown in DS was similar to the number of adherent and internalized strain Sakai from Chapter 2. These observations indicate that under these conditions different *E. coli* strains show a similar behavior regarding adherence and internalization.

The differences in colonization between different lettuce varieties may indicate variations in the plant immune response between plant varieties. Several studies demonstrated that distinct human pathogens and viruses differentially induce plant immune responses (61, 62, 115). Roy et al. (62) showed that upon leaf contamination *E. coli* O157:H7 strain 86-24 induces plant immunity stronger than *Salmonella enterica* serovar Typhimurium SL1344. Moreover, Jang and Matthews demonstrated that distinct strains of *E. coli* O157:H7 and of *E. coli* O104:H4 trigger the immune response of *Arabidopsis thaliana* to differing degrees (61). Apparently, plants can differentiate between human pathogens or at least recognize them differently, and therefore, the plant response varies depending on the pathogen. However, only little is known about potential differences regarding the immune response between plant varieties. Albeit, Markland et al. (115) were able to demonstrate that inoculation of *Arabidopsis thaliana* with murine norovirus or Tulane virus lead to a different immune response compared to treatment of *Lactuca sativa* cultivar Parris Island with the same viruses. To this point, it is not clear whether or not the immune systems of *L. sativa* and *V. locusta* respond differently to *E. coli* O104:H4 C227/11ϕcu. If this is the case, lower *E. coli* viable counts within the root tissue of *V. locusta* compared to *L. sativa* (Chapter 3) would imply that a higher number of *E. coli* is eliminated in *V. locusta* than in *L. sativa*. Admittedly, this aspect requires further research. However, the plant immune response is not restricted to plant tissue, but starts already in the rhizosphere. Plants release root exudates which contain various biologically active compounds and fulfill several functions regarding the interactions with the soil microbial community (116). Within its wide range of functions, root exudates are the first line of plant defense. Therefore, it is not surprising that, besides sugars and amino acids, the root exudates of *L. sativa* "Tizian" contain organic acids, and antimicrobial and antifungal agents like benzoic and lauric acid as well (117–120). Moreover, the release of exudates can help to modulate the root surrounding microbial community (116). For instance, phenolic metabolites can act as attractant for certain soil microbes whereas the amino acid canavanine serves bidirectional functions by

stimulating one group of microorganisms and suppressing other soil-borne bacteria, as reviewed by Baetz and Martinoia (116). Similarly, Schreiter et al. (114) showed for three different soil types that the composition of the soil microbial community differs between the bulk soils and the respective rhizospheres. The mixture of attractants, stimulants, repellents and inhibitors influences the composition of the soil microbiota within the rhizosphere.

As it was already described in literature that the two soil types differ in their composition of the soil microbiota (114), the composition of the soil microbial community was also investigated in Chapter 3 to analyze the connection between soil type, lettuce type, and the autochthonous microbiota. For this reason, during the infection experiments, soil samples were collected from the used plant pots after the lettuce plant were removed and prompted to further analyses. Amplification and Illumina MiSeq™ based sequencing of the hypervariable V4 region of the 16S rRNA gene from extracted total microbial community DNA was commissioned to the Center for Genomic Research at the University of Liverpool (Great Britain). The received sequences were further processed to operational taxonomic units (OTU) by the bioinformatic opensource software Mothur v1.40.5 as described in the MiSeq standard operating procedure (121). In total, 518 OTUs were identified which were analyzed statistically by Calypso v8.84 (122). These analyses detected 19 different phyla within the soil microbiota (Chapter 3).

By quantitative visualization and analyses of the 40 most abundant OTUs, it was shown that OTU013 (genus *Escherichia* or *Shigella*) was mainly found in soil samples which were taken from EHEC/EAEC contaminated soil (Fig. 5, Chapter 3). This finding suggested that OTU013 may be the applied *E. coli* O104:H4 strain C227/11ϕcu. Moreover, this approach indicated differences in the microbial composition between the soil types (Fig. 5, Chapter 3). To substantiate these findings, redundancy analysis (RDA) was performed which demonstrated that the samples clustered according to soil type / lettuce variety combination. Additionally, RDA identified that the soil microbial composition was statistically significantly influenced by soil type, lettuce variety, the lettuce / soil type combination, and the presence of strain C227/11ϕcu (Fig. 6, Chapter 3). However, the presence of EHEC/EAEC resulted only in comparatively minor changes in the microbial community composition in response to the soil type and its autochthonous microbiota.

Besides statistically significant differences in the microbial communities between bulk soil and rhizosphere within the same type of soil, Schreiter et al. (114) depicted variations in the rhizosphere of *L. sativa* grown in different soil types at different sampling time points. For instance, three weeks after planting, *Adhaeribacter* sp., *Bacillus* sp., *Nocardiodes* sp., *Pontibacter* sp. and *Rubrobacteridae* showed higher abundance in the rhizosphere of AL grown *L. sativa* plants compared to DS grown plants (114).

Whereas *Flavobacterium* sp., *Marmoricola* sp. and *Rhodococcus* sp. were more abundant in the rhizosphere of DS grown *L. sativa* plants compared to plants grown in AL (114). Intriguingly, these mentioned eight genera/subclasses were also found in the soil samples of the present dissertation (Chapter 3). However, *Bacillus* sp., *Flavobacterium* sp. and *Rhodococcus* sp. were more abundant in soil samples of AL grown *L. sativa* plants. *Adhaeribacter* sp., *Marmoricola* sp., *Nocardiodes* sp., *Pontibacter* sp. and *Rubrobacteridae* depicted higher abundance in soil samples of *L. sativa* plants which grew in DS (Chapter 3). Therefore, only two of them – namely, *Bacillus* sp. and *Marmoricola* sp. – showed similar tendencies in abundance compared to the results of the former study published by Schreiter et al. (114, Chapter 3). The remaining six genera/subclasses – *Adhaeribacter* sp., *Flavobacterium* sp., *Nocardiodes* sp., *Pontibacter* sp., *Rhodococcus* sp. and *Rubrobacteridae* – depicted the opposite (114, Chapter 3). Moreover, the present thesis demonstrated that soil type, lettuce variety, and the lettuce / soil type combination significantly influence the soil microbial composition. This finding is in line with other studies showing that the composition of the soil microbiota varies among plant species and soil types (114, 123, 124). It is not surprising that *E. coli* O104:H4 strain C227/11ϕcu showed differences in abundance between soil types and plant varieties (Chapter 3), as variations in the soil microbial composition may also result in differences in the abundance of *E. coli* antagonists. To the knowledge of the author, the presence of potentially *E. coli* inhibiting bacteria within soil microbial communities remains largely unknown. However, several studies investigated isolates gained from different kind of fresh produce and vegetables regarding their potential inhibitory activity against *E. coli* (125–128). Taken together, the mentioned studies identified a total of 22 bacterial genera that showed growth inhibition against *E. coli* HB101 (127) and *E. coli* O157:H7 (125, 126, 128). Upon investigation of soil samples after growth of *L. sativa* and *V. locusta* and subsequent soil contamination with *E. coli* O104:H4 strain C227/11ϕcu, 15 of these genera were also found within the soil microbiota of the different lettuce / soil type combinations (Chapter 3). In total, nine genera were found to be significantly associated with distinct lettuce / soil type combinations (Chapter 3). Interestingly, the number of associated potentially *E. coli* antagonistic genera were distributed more or less similarly among the particular lettuce / soil type combinations. Three genera were associated with AL grown *L. sativa* and two genera were associated with DS grown *L. sativa*, AL and DS grown *V. locusta*, each (Chapter 3). Notably, significant association of a certain genus with a particular lettuce / soil type combination does not imply that this genus was only found in the soil of the associated sample. In fact, the aforementioned nine genera were found in soil samples of all lettuce / soil type combinations investigated in Chapter 3. Because of this and because of the quite equal distribution of associated genera among the lettuce / soil type combinations, it is not possible to predict the number of viable counts of *E. coli* O104:H4 strain C227/11ϕcu within the rhizosphere based on the presence, abundance and number

of detected *E. coli* antagonistic genera. This kind of predictions is particularly difficult as soil is structured in micro- and macroaggregates as reviewed by Wilpiszeski et al. (129). Therefore, data from homogenized soil may not reflect the micrometer-scale spatial organization of soil communities.

Colonization of cultivated plants by human pathogenic bacteria depends on multiple factors. It is not only about the genetic equipment of the pathogen but also about the environmental conditions. Especially the natural environment may act as a highly dynamic matrix. For instance, when looking at colonization of plant roots in unsterile soil, there are multiple players involved as it is not only about the sole interaction between the plant root and the human pathogen. Before being able to colonize the roots, the pathogen has to find a way to survive within the soil and establish itself within the soil microbial community. Then the pathogen needs to overcome the plant immune response for successful invasion and colonization. In summary, human pathogens encounter multiple opponents, such as the abiotic composition of the soil, the soil microbial community, and the plant itself including its defense mechanisms. To conclude, the present thesis provides novel insights in adherence to and internalization into the root of lettuce plants by focusing on different aspects of these processes.

Further research is needed to unravel the highly complex interactions between soil, plant, soil microbial communities and pathogens. This thesis raised several questions. Does the plant immune response differ between distinct plant varieties, and between bacterial human pathogens? Is the extent of the immune reaction related to extent of internalized pathogens? Possibly, a weaker immune response causes a higher number of internalized bacteria. This would imply that some plant varieties are more prone to colonization by pathogens than others.

This consideration also raises the question if there are soil bacteria which are save for humans but antagonists of *E. coli*. These types of bacteria could act as biocontrol agents preventing human pathogenic *E. coli* from invading plant tissue. Potentially, they are already part of the natural soil microbial community. Infections experiments performed with sterile soil which is then inoculated with a microbial community of defined composition would help to address this question.

Moreover, the present dissertation focused only on roots. It is unclear if the investigated *E. coli* strains are able to systemically colonized lettuce plants from the root via the shoot into the leaves, i.e. the edible portion. This aspect is important for risk assessment. Therefore, experiments are needed during which whole lettuce plants are monitored. This means that after incubating the plants in the contaminated soil, roots, stem, and leaves are analyzed to track the movement of *E. coli* from soil to the leaves.

Taken together, the present thesis demonstrated that different human pathogenic *E. coli* strains are able to adhere to and to internalize into the roots. Adherence was mainly influenced by the soil type. Presumably, lower microbial alpha diversity poses a less stable community structure and therefore, favors establishment of *E. coli*. Moreover, it became evident that bacterial cell surface proteins and the plant host, i.e. the plant variety, play important roles during internalization. The results of this thesis and the thereby established methods provide conspicuous basis for future studies to gain better understanding of the interactions between human pathogens, plants, and soil microbiota.

References

The following references refer to chapter 1 and 4. Literature sources of chapter 2 and 3 are depicted in the corresponding chapter.

1. Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB. 2013. Recent advances in understanding enteric pathogenic *Escherichia coli*. Clin Microbiol Rev 26:822–880.

2. Kaper JB, Nataro JP, Mobley HLT. 2004. Pathogenic *Escherichia coli*. Nat Rev Microbiol 2:123–140.

3. Nataro J. 1998. Enteroaggregative *Escherichia coli*. Emerg Infect Dis 4:251–261.

4. Kaur P, Chakraborti A, Asea A. 2010. Enteroaggregative *Escherichia coli*: An emerging enteric food borne pathogen. Interdiscip Perspect Infect Dis 2010:1–10.

5. Levine MM. 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J Infect Dis 155:377–389.

6. Krause M, Barth H, Schmidt H. 2018. Toxins of locus of enterocyte effacement-negative Shiga Toxin-producing *Escherichia coli*. Toxins 10:241.

7. Scheutz F, Teel LD, Beutin L, Pierard D, Buvens G, Karch H, Mellmann A, Caprioli A, Tozzoli R, Morabito S, Strockbine NA, Melton-Celsa AR, Sanchez M, Persson S, O'Brien AD. 2012. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. J Clin Microbiol 50:2951–2963.

8. Unkmeir A, Schmidt H. 2000. Structural analysis of phage-borne stx genes and their flanking sequences in Shiga toxin-producing *Escherichia coli* and *Shigella dysenteriae* type 1 strains. Infect Immun 68:4856–4864.

9. Fraser ME, Chernaia MM, Kozlov YV, James MNG. 1994. Crystal structure of the holotoxino from *Shigella dysenteriae* at 2.5 Å resolution. Nat Struct Biol 1:59–64.

10. Lindberg AA, Brown JE, Strömberg N, Westling-Ryd M, Schultz JE, Karlsson KA. 1987. Identification of the carbohydrate receptor for Shiga toxin produced by *Shigella dysenteriae* type 1. J Biol Chem 262:1779–1785.

11. Stein PE, Boodhoo A, Tyrrell GJ, Brunton JL, Read RJ. 1992. Crystal structure of the cell-binding B oligomer of verotoxin-1 from *E. coli*. Nature 355:748–750.

12. Endo Y, Tsurugi K, Yutsudo T, Takeda Y, Ogasawara T, Igarashi K. 1988. Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. Eur J Biochem 171:45–50.

13. Furutani M, Kashiwagi K, Ito K, Endo Y, Igarashi K. 1992. Comparison of the modes of action of a vero toxin (a Shiga-like toxin) from *Escherichia coli*, of ricin, and of α-sarcin. Arch Biochem Biophys 293:140–146.

14. Obrig TG, Moran TP, Brown JE. 1987. The mode of action of Shiga toxin on peptide elongation of eukaryotic protein synthesis. Biochem J 244:287–294.

15. Sandvig K, Garred Ø, Prydz K, Kozlov JV, Hansen SH, van Deurs B. 1992. Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. Nature 358:510–512.

16. McDaniel TK, Jarvis KG, Donnenberg MS, Kaper JB. 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc Natl Acad Sci 92:1664–1668.

17. Jarvis KG, Giron JA, Jerse AE, McDaniel TK, Donnenberg MS, Kaper JB. 1995. Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. Proc Natl Acad Sci 92:7996–8000.

18. Jaglic Z, Desvaux M, Weiss A, Nesse LL, Meyer RL, Demnerova K, Schmidt H, Giaouris E, Sipailiene A, Teixeira P, Kacaniova M, Riedel CU, Knochel S. 2014. Surface adhesins and exopolymers of selected foodborne pathogens. Microbiol Read Engl 160:2561–2582.

19. Nataro JP, Kaper JB. 1998. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11:142–201.

20. Jerse AE, Yu J, Tall BD, Kaper JB. 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc Natl Acad Sci 87:7839–7843.

21. Kenny B, DeVinney R, Stein M, Reinscheid DJ, Frey EA, Finlay BB. 1997. Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell 91:511–520.

22. Saldana Z, Sanchez E, Xicohtencatl-Cortes J, Puente JL, Giron JA. 2011. Surface structures involved in plant stomata and leaf colonization by shiga-toxigenic *Escherichia coli* O157:H7. Front Microbiol 2:119.

23. Xicohtencatl-Cortes J, Sánchez Chacón E, Saldaña Z, Freer E, Girón JA. 2009. Interaction of *Escherichia coli* O157:H7 with leafy green produce. J Food Prot 72:1531–1537.

24. Schikora A, Virlogeux-Payant I, Bueso E, Garcia AV, Nilau T, Charrier A, Pelletier S, Menanteau P, Baccarini M, Velge P, Hirt H. 2011. Conservation of *Salmonella* infection mechanisms in plants and animals. PloS One 6:e24112.

25. Juergens D, Ozel M, Takaisi-Kikuni NB. 2002. Production and characterization of *Escherichia coli* enterohemolysin and its effects on the structure of erythrocyte membranes. Cell Biol Int 26:175–186.

26. Schmidt H, Beutin L, Karch H. 1995. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. Infect Immun 63:1055–1061.

27. Schmidt H, Karch H, Beutin L. 1994. The large-sized plasmids of enterohemorrhagic *Escherichia coli* O157 strains encode hemolysins which are presumably members of the *E. coli* Î±-hemolysin family. FEMS Microbiol Lett 117:189–196.

28. Aldick T, Bielaszewska M, Uhlin BE, Humpf H-U, Wai SN, Karch H. 2009. Vesicular stabilization and activity augmentation of enterohaemorrhagic *Escherichia coli* haemolysin. Mol Microbiol 71:1496–1508.

29. Bielaszewska M, Rüter C, Kunsmann L, Greune L, Bauwens A, Zhang W, Kuczius T, Kim KS, Mellmann A, Schmidt MA, Karch H. 2013. Enterohemorrhagic *Escherichia coli* hemolysin employs outer membrane vesicles to target mitochondria and cause endothelial and epithelial apoptosis. PLoS Pathog 9:e1003797.

30. Johnson JR, Russo TA, Tarr PI, Carlino U, Bilge SS, Vary JC Jr, Stell AL. 2000. Molecular epidemiological and phylogenetic associations of two novel putative virulence genes, *iha* and *iroN($_{E.\ coli}$)*, among *Escherichia coli* isolates from patients with urosepsis. Infect Immun 68:3040–3047.

31. Léveillé S, Caza M, Johnson JR, Clabots C, Sabri M, Dozois CM. 2006. Iha from an *Escherichia coli* urinary tract infection outbreak clonal group A strain is expressed *in vivo* in the mouse urinary tract and functions as a catecholate siderophore receptor. Infect Immun 74:3427–3436.

32. Schmidt H, Zhang WL, Hemmrich U, Jelacic S, Brunder W, Tarr PI, Dobrindt U, Hacker J, Karch H. 2001. Identification and characterization of a novel genomic island integrated at *selC* in locus of enterocyte effacement-negative, Shiga toxin-producing *Escherichia coli*. Infect Immun 69:6863–6873.

33. Toma C, Martinez Espinosa E, Song T, Miliwebsky E, Chinen I, Iyoda S, Iwanaga M, Rivas M. 2004. Distribution of putative adhesins in different seropathotypes of Shiga Toxin-producing *Escherichia coli*. J Clin Microbiol 42:4937–4946.

34. Johnson JR, Jelacic S, Schoening LM, Clabots C, Shaikh N, Mobley HL, Tarr PI. 2005. The IrgA homologue adhesin Iha is an *Escherichia coli* virulence factor in murine urinary tract infection. Infect Immun 73:965–971.

35. Tarr PI, Bilge SS, Vary JC Jr, Jelacic S, Habeeb RL, Ward TR, Baylor MR, Besser TE. 2000. Iha: a novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. Infect Immun 68:1400–1407.

36. Yin X, Wheatcroft R, Chambers JR, Liu B, Zhu J, Gyles CL. 2009. Contributions of O island 48 to adherence of enterohemorrhagic *Escherichia coli* O157:H7 to epithelial cells in vitro and in ligated pig ileal loops. Appl Environ Microbiol 75:5779–5786.

37. Ledesma MA, Ochoa SA, Cruz A, Rocha-Ramirez LM, Mas-Oliva J, Eslava CA, Giron JA, Xicohtencatl-Cortes J. 2010. The hemorrhagic coli pilus (HCP) of *Escherichia coli* O157:H7 is an inducer of proinflammatory cytokine secretion in intestinal epithelial cells. PloS One 5:e12127.

38. Nuebling S, Wohlt D, Saile N, Weiss A, Schmidt H. 2017. Antimicrobial effect of lauroyl arginate ethyl on *Escherichia coli* O157:H7 and *Listeria monocytogenes* on red oak leaf lettuce. Eur Food Res Technol 243:879–887.

39. Xicohtencatl-Cortes J, Monteiro-Neto V, Saldana Z, Ledesma MA, Puente JL, Giron JA. 2009. The type 4 pili of enterohemorrhagic *Escherichia coli* O157:H7 are multipurpose structures with pathogenic attributes. J Bacteriol 191:411–421.

40. Xicohtencatl-Cortes J, Monteiro-Neto V, Ledesma MA, Jordan DM, Francetic O, Kaper JB, Puente JL, Giron JA. 2007. Intestinal adherence associated with type IV pili of enterohemorrhagic *Escherichia coli* O157:H7. J Clin Invest 117:3519–3529.

41. Persad AK, LeJeune JT. 2014. Animal reservoirs of Shiga toxin-producing *Escherichia coli*. Microbiol Spectr 2.

42. Fegan N, Vanderlinde P, Higgs G, Desmarchelier P. 2004. The prevalence and concentration of *Escherichia coli* O157 in faeces of cattle from different production systems at slaughter. J Appl Microbiol 97:362–370.

43. McCabe E, Burgess CM, Lawal D, Whyte P, Duffy G. 2018. An investigation of shedding and super-shedding of Shiga toxigenic *Escherichia coli* O157 and *E. coli* O26 in cattle presented for slaughter in the Republic of Ireland. Zoonoses Public Health.

44. Thurston-Enriquez JA, Gilley JE, Eghball B. 2005. Microbial quality of runoff following land application of cattle manure and swine slurry. J Water Health 3:157–171.

45. Puri-Giri R, Ghosh A, Thomson JL, Zurek L. 2017. House flies in the confined cattle environment carry non-O157 Shiga Toxin-producing *Escherichia coli*. J Med Entomol 54:726–732.

46. Talley JL, Wayadande AC, Wasala LP, Gerry AC, Fletcher J, DeSilva U, Gilliland SE. 2009. Association of *Escherichia coli* O157:H7 with filth flies (*Muscidae* and *Calliphoridae*) captured in

leafy greens fields and experimental transmission of *E. coli O157:H7* to spinach leaves by house flies (*Diptera: Muscidae*). J Food Prot 72:1547–1552.

47. Wasala L, Talley JL, DeSilva U, Fletcher J, Wayadande A. 2013. Transfer of *Escherichia coli* O157:H7 to spinach by house flies, *Musca domestica* (*Diptera: Muscidae*). Phytopathology 103:373–380.

48. Kobayashi M, Sasaki T, Saito N, Tamura K, Suzuki K, Watanabe H, Agui N. 1999. Houseflies: not simple mechanical vectors of enterohemorrhagic *Escherichia coli* O157:H7. Am J Trop Med Hyg 61:625–629.

49. Franiek N, Orth D, Grif K, Ewers C, Wieler LH, Thalhammer JG, Würzner R. 2012. ESBL-producing *E. coli* and EHEC in dogs and cats in the Tyrol as possible source of human infection. Berl Munch Tierarztl Wochenschr 125:469–475.

50. Anonymous. 2011. Dog show sparks new Swedish EHEC outbreak. The Local. https://www.thelocal.se/20110616/34384

51. Kataoka Y, Irie Y, Sawada T, Nakazawa M. 2010. A 3-year epidemiological surveillance of *Escherichia coli* O157:H7 in dogs and cats in Japan. J Vet Med Sci 72:791–794.

52. Chitarra W, Decastelli L, Garibaldi A, Gullino ML. 2014. Potential uptake of *Escherichia coli* O157:H7 and *Listeria monocytogenes* from growth substrate into leaves of salad plants and basil grown in soil irrigated with contaminated water. Int J Food Microbiol 189:139–145.

53. Erickson MC, Webb CC, Davey LE, Payton AS, Flitcroft ID, Doyle MP. 2014. Biotic and abiotic variables affecting internalization and fate of *Escherichia coli* O157:H7 isolates in leafy green roots. J Food Prot 77:872–879.

54. Erickson MC, Webb CC, Diaz-Perez JC, Phatak SC, Silvoy JJ, Davey L, Payton AS, Liao J, Ma L, Doyle MP. 2010. Surface and internalized *Escherichia coli* O157:H7 on field-grown spinach and lettuce treated with spray-contaminated irrigation water. J Food Prot 73:1023–1029.

55. Seo KH, Frank JF. 1999. Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. J Food Prot 62:3–9.

56. Solomon EB, Yaron S, Matthews KR. 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Appl Environ Microbiol 68:397–400.

57. Wright KM, Crozier L, Marshall J, Merget B, Holmes A, Holden NJ. 2017. Differences in internalization and growth of *Escherichia coli* O157:H7 within the apoplast of edible plants, spinach and lettuce, compared with the model species *Nicotiana benthamiana*. Microb Biotechnol 10:555–569.

58. Wright KM, Holden NJ. 2018. Quantification and colonisation dynamics of *Escherichia coli* O157:H7 inoculation of microgreens species and plant growth substrates. Int J Food Microbiol 273:1–10.

59. Deering AJ, Mauer LJ, Pruitt RE. 2012. Internalization of *E. coli* O157:H7 and *Salmonella spp.* in plants: A review. Food Res Int Salmonella Foods Evol Strateg Chall 45:567–575.

60. Jang H, Matthews KR. 2018. Influence of surface polysaccharides of *Escherichia coli* O157:H7 on plant defense response and survival of the human enteric pathogen on *Arabidopsis thaliana* and lettuce (*Lactuca sativa*). Food Microbiol 70:254–261.

61. Jang H, Matthews KR. 2018. Survival and interaction of *Escherichia coli* O104:H4 on *Arabidopsis thaliana* and lettuce (*Lactuca sativa*) in comparison to *E. coli* O157:H7: Influence of plant defense response and bacterial capsular polysaccharide. Food Res Int 108:35–41.

62. Roy D, Panchal S, Rosa BA, Melotto M. 2013. *Escherichia coli* O157:H7 induces stronger plant immunity than *Salmonella enterica* Typhimurium SL1344. Phytopathology 103:326–332.

63. Erickson MC. 2012. Internalization of fresh produce by foodborne pathogens. Annu Rev Food Sci Technol 3:283–310.

64. Hirneisen KA, Sharma M, Kniel KE. 2012. Human enteric pathogen internalization by root uptake into food crops. Foodborne Pathog Dis 9:396–405.

65. Cooley MB, Miller WG, Mandrell RE. 2003. Colonization of *Arabidopsis thaliana* with *Salmonella enterica* and enterohemorrhagic *Escherichia coli* O157:H7 and competition by *Enterobacter asburiae*. Appl Environ Microbiol 69:4915–4926.

66. Kroupitski Y, Golberg D, Belausov E, Pinto R, Swartzberg D, Granot D, Sela S. 2009. Internalization of *Salmonella enterica* in leaves is induced by light and involves chemotaxis and penetration through open stomata. Appl Environ Microbiol 75:6076–6086.

67. Fremaux B, Prigent-Combaret C, Vernozy-Rozand C. 2008. Long-term survival of Shiga toxin-producing *Escherichia coli* in cattle effluents and environment: An updated review. Vet Microbiol 132:1–18.

68. Ceuppens S, Johannessen G, Allende A, Tondo E, El-Tahan F, Sampers I, Jacxsens L, Uyttendaele M. 2015. Risk Factors for *Salmonella*, Shiga Toxin-Producing *Escherichia col*i and *Campylobacter* occurrence in primary production of leafy greens and strawberries. Int J Environ Res Public Health 12:9809–9831.

69. Bolton DJ, Monaghan A, Byrne B, Fanning S, Sweeney T, McDowell DA. 2011. Incidence and survival of non-O157 verocytotoxigenic *Escherichia coli* in soil: Non-O157 VTEC in soil. J Appl Microbiol 111:484–490.

70. Tanaro JD, Pianciola LA, D'Astek BA, Piaggio MC, Mazzeo ML, Zolezzi G, Rivas M. 2018. Virulence profile of *Escherichia coli* O157 strains isolated from surface water in cattle breeding areas. Lett Appl Microbiol 66:484–490.

71. Probert WS, Miller GM, Ledin KE. 2017. Contaminated stream water as source for *Escherichia coli* O157 illness in children. Emerg Infect Dis 23:1216–1218.

72. Crampin M, Willshaw G, Hancock R, Djuretic T, Elstob C, Rouse A, Cheasty T, Stuart J. 1999. Outbreak of *Escherichia coli* O157 infection associated with a music festival. Eur J Clin Microbiol Infect Dis Off Publ Eur Soc Clin Microbiol 18:286–288.

73. Ogden ID, Hepburn NF, MacRae M, Strachan NJC, Fenlon DR, Rusbridge SM, Pennington TH. 2002. Long-term survival of *Escherichia coli* O157 on pasture following an outbreak associated with sheep at a scout camp. Lett Appl Microbiol 34:100–104.

74. Salvadori MI, Sontrop JM, Garg AX, Moist LM, Suri RS, Clark WF. 2009. Factors that led to the Walkerton tragedy. Kidney Int 75:S33–S34.

75. Abberton CL, Bereschenko L, van der Wielen PWJJ, Smith CJ. 2016. Survival, biofilm formation, and growth potential of environmental and enteric *Escherichia coli* strains in drinking water microcosms. Appl Environ Microbiol 82:5320–5331.

76. Thorpe CM. 2004. Shiga Toxin-Producing *Escherichia coli* infection. Clin Infect Dis 38:1298–1303.

77. Beutin L, Martin A. 2012. Outbreak of Shiga Toxin–producing *Escherichia coli* (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. J Food Prot 75:408–418.

78. Karch H, Tarr PI, Bielaszewska M. 2005. Enterohaemorrhagic *Escherichia coli* in human medicine. Int J Med Microbiol 295:405–418.

79. Miliwebsky E, Deza N, Chinen I, Martinez Espinosa E, Gomez D, Pedroni E, Caprile L, Bashckier A, Manfredi E, Leotta G, Rivas M. 2007. Prolonged fecal shedding of Shiga toxin-producing *Escherichia coli* among children attending day-care centers in Argentina. Rev Argent Microbiol 39:90–92.

80. Hong S, Song SE, Oh KH, Cho SH, Kim SH, Yoo S ju, Lim HS, Park MS. 2011. Prevalence of Farm and slaughterhouse workers carrying Shiga toxin-producing *Escherichia coli* in Korea. Osong Public Health Res Perspect 2:198–201.

81. Silvestro L, Caputo M, Blancato S, Decastelli L, Fioravanti A, Tozzoli R, Morabito S, Caprioli A. 2004. Asymptomatic carriage of verocytotoxin-producing *Escherichia coli* O157 in farm workers in Northern Italy. Epidemiol Infect 132:915–919.

82. Robert Koch-Institute. 2018. Infektionsepidemiologisches Jahrbuch meldepflichtiger Krankheiten für 2017. RKI-Bib1 Robert Koch-Inst.

83. Tarr PI, Gordon CA, Chandler WL. 2005. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. Lancet Lond Engl 365:1073–1086.

84. Tuttle J, Gomez T, Doyle MP, Wells JG, Zhao T, Tauxe RV, Griffin PM. 1999. Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. Epidemiol Infect 122:185–192.

85. Greig JD, Ravel A. 2009. Analysis of foodborne outbreak data reported internationally for source attribution. Int J Food Microbiol 130:77–87.

86. Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hargrett NT, Blake PA, Cohen ML. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med 308:681–685.

87. Michino H, Araki K, Minami S, Takaya S, Sakai N, Miyazaki M, Ono A, Yanagawa H. 1999. Massive outbreak of *Escherichia coli* O157:H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. Am J Epidemiol 150:787–796.

88. Robert Koch-Institute. 2012. Infektionsepidemiologisches Jahrbuch meldepflichtiger Krankheiten für 2011. Berlin.

89. Mellmann A, Harmsen D, Cummings CA, Zentz EB, Leopold SR, Rico A, Prior K, Szczepanowski R, Ji Y, Zhang W, McLaughlin SF, Henkhaus JK, Leopold B, Bielaszewska M, Prager R, Brzoska PM, Moore RL, Guenther S, Rothberg JM, Karch H. 2011. Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. PLoS ONE 6:e22751.

90. Scheutz F, Nielsen EM, Frimodt-Møller J, Boisen N, Morabito S, Tozzoli R, Nataro JP, Caprioli A. 2011. Characteristics of the enteroaggregative Shiga toxin/verotoxin-producing *Escherichia coli* O104:H4 strain causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011. Euro Surveill Bull Eur Sur Mal Transm Eur Commun Dis Bull 16.

91. Brzuszkiewicz E, Thürmer A, Schuldes J, Leimbach A, Liesegang H, Meyer F-D, Boelter J, Petersen H, Gottschalk G, Daniel R. 2011. Genome sequence analyses of two isolates from the recent *Escherichia coli* outbreak in Germany reveal the emergence of a new pathotype: Entero-Aggregative-Haemorrhagic *Escherichia coli* (EAHEC). Arch Microbiol 193:883–891.

92. Bielaszewska M, Mellmann A, Zhang W, Köck R, Fruth A, Bauwens A, Peters G, Karch H. 2011. Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: a microbiological study. Lancet Infect Dis 11:671–676.

93. Anonymous. 2019. Public Health Notice - Outbreak of *E. coli* infections linked to romaine lettuce. PHAC. https://www.canada.ca/en/public-health/services/public-health-notices/2018/outbreak-ecoli-infections-linked-romaine-lettuce.html

94. Anonymous. 2019. Outbreak of *E. coli* infections linked to romaine lettuce. CDC. https://www.cdc.gov/ecoli/2018/o157h7-11-18/index.html

95. Hayashi T, Makino K, Ohnishi M, Kurokawa K, Ishii K, Yokoyama K, Han CG, Ohtsubo E, Nakayama K, Murata T, Tanaka M, Tobe T, Iida T, Takami H, Honda T, Sasakawa C, Ogasawara N, Yasunaga T, Kuhara S, Shiba T, Hattori M, Shinagawa H. 2001. Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. DNA Res Int J Rapid Publ Rep Genes Genomes 8:11–22.

96. Alegbeleye OO, Singleton I, Sant'Ana AS. 2018. Sources and contamination routes of microbial pathogens to fresh produce during field cultivation: A review. Food Microbiol 73:177–208.

97. Anonymous. 2019. Reports of selected *E. coli* outbreak investigations. CDC. https://www.cdc.gov/ecoli/outbreaks.html

98. Pennington H. 2014. *E. coli* O157 outbreaks in the United Kingdom: past, present, and future. Infect Drug Resist 211.

99. Anonymous. 2018. *E. coli*. WHO. https://www.who.int/news-room/fact-sheets/detail/e-coli

100. Boisen N, Melton-Celsa AR, Scheutz F, O'Brien AD, Nataro JP. 2015. Shiga toxin 2a and enteroaggregative *Escherichia coli* – a deadly combination. Gut Microbes 6:272–278.

101. Boisen N, Hansen A-M, Melton-Celsa AR, Zangari T, Mortensen NP, Kaper JB, O'Brien AD, Nataro JP. 2014. The presence of the pAA plasmid in the German O104:H4 Shiga Toxin type 2a (Stx2a)–producing enteroaggregative *Escherichia coli* strain promotes the translocation of Stx2a across an epithelial cell monolayer. J Infect Dis 210:1909–1919.

102. Zangari T, Melton-Celsa AR, Panda A, Boisen N, Smith MA, Tatarov I, De Tolla LJ, Nataro JP, O'Brien AD. 2013. Virulence of the Shiga toxin type 2-expressing *Escherichia coli* O104:H4 German outbreak isolate in two animal models. Infect Immun 81:1562–1574.

103. Brandl MT. 2006. Fitness of human enteric pathogens on plants and implications for food safety. Annu Rev Phytopathol 44:367–392.

104. Sharma M, Ingram DT, Patel JR, Millner PD, Wang X, Hull AE, Donnenberg MS. 2009. A novel approach to investigate the uptake and internalization of *Escherichia coli* O157:H7 in spinach cultivated in soil and hydroponic medium. J Food Prot 72:1513–1520.

105. Berger CN, Shaw RK, Ruiz-Perez F, Nataro JP, Henderson IR, Pallen MJ, Frankel G. 2009. Interaction of enteroaggregative *Escherichia coli* with salad leaves. Environ Microbiol Rep 1:234–239.

106. Macarisin D, Patel J, Bauchan G, Giron JA, Sharma VK. 2012. Role of curli and cellulose expression in adherence of *Escherichia coli* O157:H7 to spinach leaves. Foodborne Pathog Dis 9:160–167.

107. Bufe T, Hennig A, Klumpp J, Weiss A, Nieselt K, Schmidt H. 2019. Differential transcriptome analysis of enterohemorrhagic *Escherichia coli* strains reveals differences in response to plant-derived compounds. BMC Microbiol 19:212.

108. Jozefaciuk G, Szatanik-Kloc A, Lukowska M, Szerement J. 2014. Pitfalls and uncertainties of using potentiometric titration for estimation of plant roots surface charge and acid-base properties. Am J Plant Sci Vol.05No.13:15.

109. Jechalke S, Schierstaedt J, Becker M, Flemer B, Grosch R, Smalla K, Schikora A. 2019. *Salmonella* establishment in agricultural Soil and colonization of crop plants depend on soil type and plant species. Front Microbiol 10.

110. Ma J, Mark Ibekwe A, Crowley DE, Yang C-H. 2014. Persistence of *Escherichia coli* O157 and non-O157 strains in agricultural soils. Sci Total Environ 490:822–829.

111. Ma J, Ibekwe AM, Yi X, Wang H, Yamazaki A, Crowley DE, Yang C-H. 2011. Persistence of *Escherichia coli* O157:H7 and its mutants in soils. PLoS ONE 6:e23191.

112. Wang H, Zhang T, Wei G, Wu L, Wu J, Xu J. 2014. Survival of *Escherichia coli* O157:H7 in soils under different land use types. Environ Sci Pollut Res 21:518–524.

113. Wang H, Ibekwe AM, Ma J, Wu L, Lou J, Wu Z, Liu R, Xu J, Yates SR. 2014. A glimpse of *Escherichia coli* O157:H7 survival in soils from eastern China. Sci Total Environ 476–477:49–56.

114. Schreiter S, Ding GC, Heuer H, Neumann G, Sandmann M, Grosch R, Kropf S, Smalla K. 2014. Effect of the soil type on the microbiome in the rhizosphere of field-grown lettuce. Front Microbiol 5:144.

115. Markland SM, Bais H, Kniel KE. 2017. Human norovirus and its surrogates induce plant immune response in *Arabidopsis thaliana* and *Lactuca sativa*. Foodborne Pathog Dis 14:432–439.

116. Baetz U, Martinoia E. 2014. Root exudates: the hidden part of plant defense. Trends Plant Sci 19:90–98.

117. Lee JG, Lee BY, Lee HJ. 2006. Accumulation of phytotoxic organic acids in reused nutrient solution during hydroponic cultivation of lettuce (*Lactuca sativa* L.). Sci Hortic 110:119–128.

118. Neumann G, Bott S, Ohler MA, Mock H-P, Lippmann R, Grosch R, Smalla K. 2014. Root exudation and root development of lettuce (*Lactuca sativa* L. cv. Tizian) as affected by different soils. Front Microbiol 5.

119. Tangwatcharin P, Khopaibool P. 2012. Activity of virgin coconut oil, lauric acid or monolaurin in combination with lactic acid against *Staphylococcus aureus*. Southeast Asian J Trop Med Public Health 43:969–985.

120. Walters DR, Walker RL, Walker KC. 2003. Lauric acid exhibits antifungal activity against plant pathogenic fungi: Antifungal activity of lauric acid. J Phytopathol 151:228–230.

121. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina Sequencing platform. Appl Environ Microbiol 79:5112–5120.

122. Zakrzewski M, Proietti C, Ellis JJ, Hasan S, Brion M-J, Berger B, Krause L. 2016. Calypso: a user-friendly web-server for mining and visualizing microbiome–environment interactions. Bioinformatics 782–783.

123. Kuramae EE, Yergeau E, Wong LC, Pijl AS, Veen JA, Kowalchuk GA. 2012. Soil characteristics more strongly influence soil bacterial communities than land-use type. FEMS Microbiol Ecol 79:12–24.

124. Ma S, De Frenne P, Vanhellemont M, Wasof S, Boeckx P, Brunet J, Cousins SAO, Decocq G, Kolb A, Lemke I, Liira J, Naaf T, Orczewska A, Plue J, Wulf M, Verheyen K. 2019. Local soil characteristics determine the microbial communities under forest understorey plants along a latitudinal gradient. Basic Appl Ecol 36:34–44.

125. Cooley MB, Chao D, Mandrell RE. 2006. *Escherichia coli* O157:H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. J Food Prot 69:2329–2335.

126. Johnston MA, Harrison MA, Morrow RA. 2009. Microbial antagonists of *Escherichia coli* O157:H7 on fresh-cut lettuce and spinach. J Food Prot 72:1569–1575.

127. Liao CH, Fett WF. 2001. Analysis of native microflora and selection of strains antagonistic to human pathogens on fresh produce. J Food Prot 64:1110–1115.

128. Schuenzel KM, Harrison MA. 2002. Microbial antagonists of foodborne pathogens on fresh, minimally processed vegetables. J Food Prot 65:1909–1915.

129. Wilpiszeski RL, Aufrecht JA, Retterer ST, Sullivan MB, Graham DE, Pierce EM, Zablocki OD, Palumbo AV, Elias DA. 2019. Soil aggregate microbial communities: Towards understanding microbiome interactions at biologically relevant scales. Appl Environ Microbiol 85.

Curriculum Vitae

Personal Details

Name:	Anna Kristina Eißenberger
Day of birth:	07. Dezember 1989 in Treuchtlingen
Resident in:	72488 Sigmaringen

Work Experience

11/2018 – 12/2019:	Researcher at Albstadt-Sigmaringen University, Department of Life Sciences, Group of Prof. Dr. David Drissner (Food Microbiology)
6/2015 – 12/2019:	Researcher and doctoral student at University of Hohenheim, Institute of Food Science and Biotechnology, Department of Food Microbiology and Hygiene, Group of Prof. Dr. Hebert Schmidt
4/2014 – 8/2014:	student apprentice at SuppreMol GmbH, division R&D
9/2013 – 12/2013:	student research assistant at LMU Department I Biology Group of Prof. Dr. Ute C. Vothknecht
6/2013 and 11/2013:	student tutor at LMU Department I Biology for the practical course “Signaltransduction in Plants”
11/2012 – 3/2013:	student research assistant at LMU Department I Biology Group of Prof. Dr. Ute C. Vothknecht

Education

6/2015 – 12/2019:	Researcher and doctoral student at University of Hohenheim, Institute of Food Science and Biotechnology, Department of Food Microbiology and Hygiene, Group of Prof. Dr. Hebert Schmidt Title of doctoral thesis: “Uptake of enterohemorrhagic *Escherichia coli* into the roots of lettuce plants"
10/2012 – 3/2015:	studies of biology (M.Sc.) at the Ludwig-Maximilians-Universität München Degree: M. Sc. Master of Science, grade: 1.40 (very good); Master Thesis “Characterisation of bacterial dynamin-like protein,

	DynA and a phage shock protein, PspA, in *Bacillus subtilis*" in the group of Prof. Dr. Marc Bramkamp (Bacterial Cell Biology); grade: 1.3 (very good)
10/2009 – 9/2012:	studies of biology (B.Sc.) at the Ludwig-Maximilians-Universität München Degree: B. Sc. Bachelor of Science; grade: 2.37 (good), Bachelor thesis "AtCML30-abhängige Calciumregulation in Mitochondrien" in the group of Ute Vothknecht (Molecular Cell Architecture and Trafficking); grade: 1.0 (very good)
9/2000 – 6/2009:	Werner-von-Siemens Gymnasium Weißenburg, Germany Degree: Abitur; grade: 2.2 (good)