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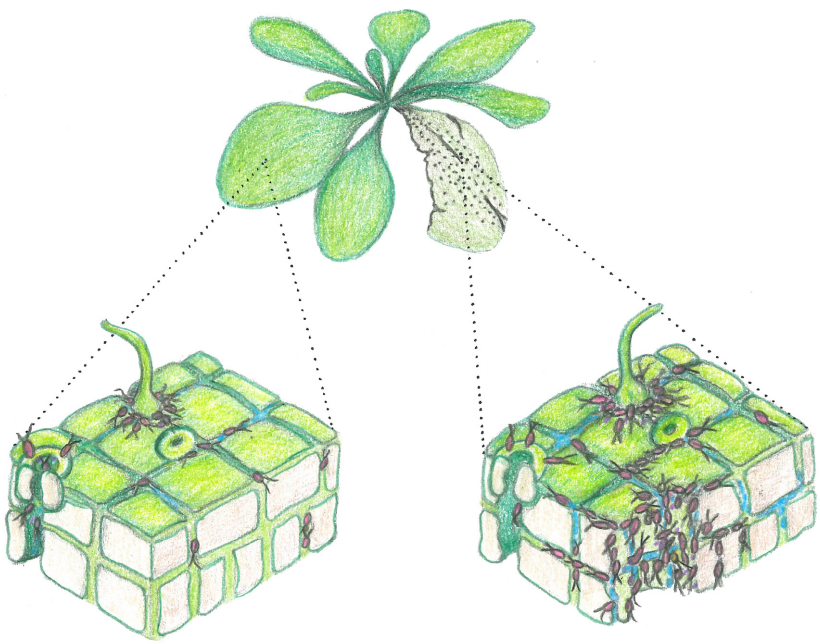
Emina Mulaosmanovic



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Department of Biosystems and Technology

Alnarp

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Abstract

Leafy vegetables (*baby leaves*) are considered an important source and vector for transmission of foodborne pathogens to humans. Contamination can occur from farm to fork. Although 'rare', contamination events have a substantial impact on public health. The shigatoxigenic bacterium, *Escherichia coli* O157:H7, can establish on the surface (epiphyte) and interior (endophyte) of leaves. Natural openings and leaf lesions serve as entry points, and internalised bacterial cells are shielded from rinse water and sanitisers. In this thesis, a new method for scrutinising leaf lesions in leafy vegetables was developed and used to link leaf damage to the dynamics of *E. coli* O157:H7*gfp*+ and the indigenous microbiota in the phyllosphere, and to evaluate use of calcium fortification of leafy vegetables for damage reduction. The new approach combines trypan blue dye staining of whole leaves with digital image analysis for detection and automated quantification of damage, enabling assessment of lesion size, shape and position. Number of lesions and relative lesion area were found to be crop-specific and increased along the production chain, while diversity of the leaf-associated microbial community decreased upon entry of baby leaves to the cold chain. The size of individual lesions and damaged leaf area affected the depth of invasion into plant tissue, dispersal to adjacent areas and number of culturable *E. coli* O157:H7*gfp*+ directly after inoculation. However, differences in culturable *E. coli* O157:H7*gfp*+ retrieved from leaf macerate evened out after 2 days post-inoculation (dpi). Leaf spraying with calcium decreased the number of lesions and damaged area on spinach leaves, lowering log CFU *E. coli* cm^{-2} detached at 0 and 1 dpi. Overall, the results in this thesis question the assumption that macroscopically intact leaves are free of lesions and safe. The method developed can assist in establishment of hurdles for preventing transmission of foodborne pathogens via baby leaves.

Keywords: calcium chloride, damage, *E. coli* O157:H7, Illumina, internalisation, microbial communities, metagenomics, phyllosphere, pre-harvest, post-harvest, spinach, Swiss chard, trypan blue

Author's address: Emina Mulaosmanovic, Swedish University of Agricultural Sciences, Department of Biosystems and Technology, Box 103, 230 53 Alnarp, Sweden

Interaktioner mellan bladskador och bladmiljöns mikroliv hos bladgrönsaker

Sammanfatning

Bladgrönsaker (*babyblad*) anses vara en viktig källa och vektor för överföring av livsmedelsburna patogener till människor. Kontaminering av bladen kan ske i alla led från jord till bord. Även om de är ”sällsynta” har dessa föroreningar en betydande inverkan på folkhälsan. Den shigatoxinbildande bakterien, *Escherichia coli* O157: H7, kan etablera sig på bladets yta (epifyt) eller i bladets inre (endofyt). Naturliga öppningar och bladskador fungerar som inkörsportar, och bakterier som tagit sig in blir skyddade från sköljvatten och desinfektionsmedel. I denna avhandling beskrivs en ny metod för granskning av bladskador på bladgrönsaker som kan användas för att koppla bladskador till samspelet mellan *E. coli* O157: H7*gfp*+ och det befintliga mikrolivet i bladmiljön samt för att utvärdera användningen av kalciumförstärkning av bladgrönsaker för att minska förekomsten av bladskador. Detta nya tillvägagångssätt kombinerar infärgning av hela blad med färgämnet trypanblått med digital bildanalys för detektion och automatiserad kvantifiering av skador, vilket möjliggör bedömning av mikroskopiska små skadors storlek, form och position. Antalet småskador och deras relativa area visade sig vara grödspecifika och ökade kontinuerligt i produktionskedjan, medan diversiteten hos det bladassocierade mikrobiella samhället minskade när småbladen inkorporerades i kylkedjan. Storleken på enskilda småskador och skadat bladområde påverkade hur långt in i vävnaden *E. coli* nådde, deras spridning till intilliggande områden samt det totala antalet odlingsbara *E. coli* O157: H7*gfp*+ direkt efter ympning. Emellertid utjämnades skillnaderna i mängd odlingsbara *E. coli* O157: H7*gfp*+ från krossade/mosade blad 2 dagar efter inympning. Att spraya bladen med kalciumsalt minskade antalet skador och storleken på skadade områden på spenatblad, vilket sänkte logg CFU av *E. coli* cm^{-2} hos blad som skördats 0 och 1 dag efter inympning. Sammantaget gör resultaten i denna avhandling att antagandet bör ifrågasättas att visuellt oskadade blad är fria från småskador och säkra att ätas. Den beskrivna metoden kan hjälpa till att utveckla metoder för att förhindra överföring av livsmedelsburna patogener via babyblad.

Nyckelord: *E. coli* O157:H7, efterskördshantering, Illumina, internalisering, kalciumklorid, mangold, metagenomik, mikrobiella samhällen, phyllosfär, skörd, spenat, skador, Trypanblått

Författarens adress: Emina Mulaosmanovic, Sveriges lantbruksuniversitet, Institutionen för Biosystem och teknologi, Box 103, 230 53 Alnarp, Sverige

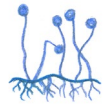
Interaktionen zwischen Blattläsionen und der Phyllosphärmikrobiota in Blattgemüse

Zusammenfassung

Blattgemüse gelten als eine wichtige Quelle und als Vektor zur Übertragung von lebensmittelbedingten Krankheitserregern auf den Mensch. Kontaminierung kann in der gesamten Lebensmittelkette von der Kultivierung bis zum Verzehr auftreten. Obwohl die Übertragung durch Blattgemüse als ein seltenes Ereignis ist, haben in dieser Weise ausgelöste Krankheitsausbrüche große Bedeutung für das Gesundheitswesen. Der shigatoxinproduzierende Keim *E. coli* O157:H7 kann sich sowohl auf der Oberfläche (epiphytisch) und im Gewebe (endophytisch) von Blättern etablieren. Ebenso wie natürliche Blattöffnungen dienen Blattwunden als Eintrittstellen in das Blattinnere, wo die bakteriellen Zellen vor dem Einfluss von Waschwasser und Desinfektionsmitteln geschützt sind. Das Ziel dieser Dissertation war a) eine Methode zur Einschätzung und Beurteilung der Möglichkeit von Blattschäden (Läsionen) auf Blattgemüse zu entwickeln, b) einen Zusammenhang zwischen Blattschäden und der Dynamik von *E. coli* O157:H7*gfp*+ sowie ambienten Mikrobiota darzustellen und c) den Effekt der Kalziumfortifikation von Blattgemüse auf das Vorkommen von Blattschäden auszuwerten. Durch die Kombination von Einfärbung der gesamten Blätter mit Hilfe von Trypan-blau und digitaler Bildanalyse wurde ein neuer Ansatz zur Detektion und automatisierten Quantifizierung von Blattschäden entwickelt, der die Bewertung der Größe, Form und Position von Schäden auf Blättern ermöglicht. Die Läsionenzahl und die relative Schadensfläche waren pflanzenabhängig und stiegen innerhalb der Produktverarbeitungskette an, während die Diversität der ambienten Mikrobiota nach Eintritt in die Kühlkette abnahm. Sowohl die Zahl individueller Läsionen als auch der Umfang der geschädigten Blattfläche waren entscheidend dafür, wie tief kultivierbare *E. coli* O157:H7*gfp*+ direkt nach der Inokulation in das Blattgewebe eindrangen und wie stark sie sich in das benachbarte Gewebe ausbreiteten. Zwei Tage nach Inokulation (dpi) glichen sich die ursprünglichen Unterschiede von *E. coli* O157:H7*gfp*+ zwischen Blättern mit unterschiedlichem Schadensumfang aus. Die Blattbehandlung mit Kalzium führte zu einer Reduktion von sowohl Läsionszahl als auch Schadensumfang an Spinatblättern. Dies spiegelte sich auch in dem geringeren Vorkommen von angehefteten *E. coli* O157:H7*gfp*+ (Log CFU cm^{-2}) innerhalb des ersten Tages nach der Inokulation (dpi 0, dpi 1). Die in dieser Arbeit vorgestellten Ergebnisse sind von Interesse für Strategien (hurdle approach), um die Übertragung von humanpathogenen Keimen durch Blattgemüse zu verhindern. Die vorliegende Arbeit stellt die allgemeine Annahme, dass makroskopisch intakte Blätter schadensfrei und damit aus lebensmittelhygienischer Perspektive sicher sind, in Frage.

Schlüsselwörter: Blattschaden, *Escherichia coli* O157: H7, Illumina, Internalisierung, Kalziumchlorid, Mangold, mikrobielle Gesellschaften, Metagenomik, Nachernte, Spinat, Trypanblau, Vorernte

Addresse des Autors: Emina Mulaosmanovic, Schwedische Landwirschaftsuniversität, Institut für Biosysteme und Technologie, Box 103, 230 53 Alnarp, Schweden

Dedication 

I dedicate this milestone to my beloved grandparents *Rabija* and *Muhamed*, with whom my scientific journey commenced, and to my son *Davud* and husband *Emir* for their immense support en route.

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List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Mulaosmanovic*†, E., T.U.T. Lindblom†, M. Bengtsson, S.T. Windstam, L. Mogren, S. Marttila, H. Stützel & B.W. Alsanius (2020). High-throughput method for detection and quantification of lesions on leaf scale based on trypan blue staining and digital image analysis. *Plant Methods* 16(1): 62.

II. Mulaosmanovic*, E., T.U.T. Lindblom, S.T. Windstam, M. Bengtsson, A.K. Rosberg, L. Mogren & B.W. Alsanius (2021). Processing of leafy vegetables matter: Damage and microbial community structure from field to bag. *Food Control* 107894.

III. Mulaosmanovic*, E., S.T. Windstam, I. Vågsholm & B.W. Alsanius (2021). Size matters: Biological and food safety relevance of leaf damage for colonization of *Escherichia coli* O157:H7. *Frontiers in Microbiology* 11:3458.

IV. Mulaosmanovic*, E., L. Mogren, H. Vogler, J. T. Burri, T. Lindblom, P. Lindqvist-Reis, S. Marttila, U. Grossniklaus & B.W. Alsanius. The power of barriers: Calcium leaf spraying as a candidate hurdle for foodborne illness risks from field to bag. Manuscript.

Papers I and III are open access. Paper II was reproduced with permission of the publisher.

*Corresponding author
†Equally contributing authors

The contribution of Emina Mulaosmanovic to the papers included in this thesis was as follows:

I. Conceptualisation and methodology development with the co-authors, investigation, formal analysis, validation, visualisation, writing original draft.

II. Conceptualisation with SW and BWA, investigation, formal analysis, data curation, visualisation, writing original draft with input from SW and BWA.

III. Conceptualisation with SW and BWA, investigation, formal analysis together with BWA and IV, visualisation, writing original draft together with BWA and SW.

IV. Conceptualisation with LM and BWA, investigation, formal analysis, data curation, visualisation, writing original draft with BWA and LM. Part funding acquisition.

Abbreviations

AB	Aniline blue
AFM	Atomic force microscopy
a_w	Water activity
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie
$CaCl_2$	Calcium chloride
CFM	Cellular force microscopy
CLSM	Confocal laser scanning microscopy
CUSPER	Reverse of REPSUC, for REProductive SUCcess
DAPI	4′,6-Diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
Dpi	Days post-inoculation
EFSA	European Food Safety Authority
EHEC	Enterohaemorrhagic *Escherichia coli*
FDA	Fluorescein diacetate
FISH	Fluorescence *in situ* hybridisation
GC-MS	Gas chromatography-mass spectrometry
GFP	Green fluorescent protein
HUS	Haemolytic uraemic syndrome
ITS	Internal transcribed spacer
LM	Light microscopy
MEMS	Micro-electro-mechanical systems
mRNA	Messenger ribonucleic acid
NGS	Next-generation sequencing
PR1	Pathogenesis-related protein 1
PI	Propidium iodide

QIIME	Quantitative insights into microbial ecology
RNA	Ribonucleic acid
ROI	Region of interest
rRNA	Ribosomal ribonucleic acid
SEM	Scanning electron microscopy
TB	Trypan blue
TEM	Transmission electron microscopy
T-RFLP	Terminal restriction fragment length polymorphism
UV	Ultraviolet
VBNC	Viable but not culturable
μ-XRF	Micro-energy-dispersive X-ray fluorescence

1. Introduction

1.1 Baby leaves: Convenience and food safety

Fresh leafy vegetables (baby leaves, leafy greens) eaten raw are wholesome and a good source of essential nutrients (especially minerals, vitamins and fibre), hence contributing to a healthy and balanced diet (Yahia *et al.*, 2019). Increased awareness of the relationship between diet and health and promotion of healthy lifestyles, including trends like the raw diet, have led to increased consumption of fresh produce (Betts, 2014). Baby leaf vegetables are not just nutritionally rich, but are also regarded as a convenience food and can be purchased washed, cut, as mixed-ingredient salads and packaged as ready-to eat commodities (Söderqvist, 2017). Convenience is an added value in today's society, with a busy lifestyle reducing the time available for meal preparation (Ragaert *et al.*, 2004).

However, a link between food illness outbreaks and increased consumption of raw fresh produce has been suggested (Aiyedun *et al.*, 2020; Carstens *et al.*, 2019). Fresh produce can be an appropriate substrate for growth of foodborne pathogens (Erhirhie *et al.*, 2020). *Escherichia coli* O157:H7, *Campylobacter* spp., *Listeria monocytogenes*, *Salmonella* spp. and *Staphylococcus aureus*, viral pathogens, *e.g.* norovirus, and parasites (*Cryptosporidium* spp., *Cyclospora* spp., *Giardia* spp.) pose risks to food safety, especially for leafy vegetables (Balali *et al.*, 2020; Wadamori *et al.*, 2017). From 1999 to 2019, a total of 44 outbreaks of microbial infection related to consumption of fresh produce were recorded in the European Union, with 22,236 individual cases, of which 34% were linked to consumption of contaminated vegetables and 30% were linked to

consumption of salads. The majority of the outbreaks caused by bacteria were ascribed to *E. coli* and *Salmonella* (Aiyedun *et al.*, 2020). *Escherichia coli* is normally found in high numbers in the gut of humans (up to 1,000,000 per g of faeces) and most strains are harmless (WHO, 2008). However, some *E. coli* strains, such as the group of enterohaemorrhagic *E. coli* (EHEC), are able to cause diverse symptoms at a very low infectious dose (10-100 cells) (EFSA, 2013). These symptoms range from abdominal cramps and haemorrhagic colitis to life-threatening diseases such as haemolytic uraemic syndrome (HUS; up to 10% of patients), a condition that can cause severe kidney damage or even death (3-5% of patients) (WHO, 2018; Palermo *et al.*, 2009; Griffin & Tauxe, 1991).

Plants can act as alternative hosts for human and animal enteric pathogens (Barak & Schroeder, 2012). Leafy vegetables can become contaminated with foodborne pathogens throughout cultivation in the field (contaminated irrigation water, soil, insects, wild and domestic animals, workers), by contact with manure and soil or harvesting equipment at harvest, or in post-harvest handling, washing and packaging (Gil *et al.*, 2015; Beuchat, 2002). While not as robust in the plant habitat as autochthonous (indigenous, native) bacteria, foodborne pathogens can successfully colonise the outer part (epiphyte) and the inner part (endophyte) of leaves (Gómez-López *et al.*, 2013; Deering *et al.*, 2012; Erickson, 2012; Brandl, 2006; Harris *et al.*, 2003). Internalized foodborne pathogens are protected from fluctuating environmental factors that may limit their survival on phyllosphere (Brandl & Mandrell, 2002), and from sanitizers (Takeuchi & Frank, 2001; Takeuchi & Frank, 2000).

Safe produce is critically important for growers and producers, and for distributors and consumers. As foodborne pathogens may enter the horticultural value network at any point, there is no unique solution for elimination of foodborne pathogens, and thus avoidance of contamination is crucial. Combining multiple mitigating approaches that are not sufficient individually could be an alternative for controlling foodborne pathogens on leafy vegetables from field to consumer (Mogren *et al.*, 2018).

1.2 Plants are never unaccompanied: Phytobiome and plant-microbe interactions beyond the holobiont

Plants host a remarkable diversity of microorganisms such as bacteria, yeasts and fungi, known collectively as the plant microbiota (Bulgarelli *et al.*, 2012; Knief *et al.*, 2012; Delmotte *et al.*, 2009). The microbiota is associated with different plants and plant spheres (Hardoim *et al.*, 2015; Reinhold-Hurek *et al.*, 2015; Vorholt, 2012), namely phyllosphere (aboveground surfaces of the plant, dominated by leaves), rhizosphere (belowground surfaces of the plant, dominated by roots) and endosphere (plant interior). The most numerous on plant surface are bacteria, with approximately 10^6-10^7 microbial cells/cm^2 leaf surface (Lindow & Brandl, 2003).

The relationship between the plant as a host and microorganisms as inhabitants can be pathogenic (parasitic or amensal) or non-pathogenic (neutral, beneficial, mutualistic, commensal) (Jones *et al.*, 2019; Vorholt, 2012). The plant as a habitat, together with the associated microorganisms, is called the holobiont (Simon *et al.*, 2019; Margulis & Fester, 1991). The complex system comprising interactions between plants, microscopic and macroscopic organisms, and the environment in which these interactions occur, is called the phytobiome (Jones *et al.*, 2019; Leach *et al.*, 2017). The phytobiome goes beyond the plant and considers both its biotic interactions and environmental conditions (Leach *et al.*, 2017). To understand plant-microbe interactions, it is not sufficient to consider only the holobiont level, because both plants and microorganisms are affected by the environment. Therefore the phytobiome needs to be considered, especially with respect to abiotic stresses (environmental factors) that impact microbial community formation, plant health and productivity (Copeland *et al.*, 2015; Bogino *et al.*, 2013).

1.2.1 Phyllosphere microbiota

The phyllosphere is dominated by leaves. The leaf as a microbial habitat is characterised by severe fluctuations in temperature, humidity (water stress) and ultraviolet (UV) radiation and, more importantly, is carbon-limited (Leveau & Lindow, 2001a; Hirano & Upper, 2000) (for details on the leaf as a habitat, see 1.3.1). The distribution of nutrients (Leveau & Lindow, 2001a; Leveau & Lindow, 2001b), including diffusion of carbohydrates to the phylloplane (leaf surface), is uneven (Remus-Emsermann *et al.*, 2011), as is the availability of water (hotspots) (Axtell & Beattie, 2002). For this reason,

the phyllosphere is predominantly inhabited by generalist bacteria, adapted to environmental heterogeneity (Massoni *et al.*, 2020).

Studies using fluorescence-labelled model microorganisms have revealed heterogeneous colonisation of the phylloplane, with denser microbial distribution in hotspots (Remus-Emsermann *et al.*, 2012; Monier & Lindow, 2005a; Monier & Lindow, 2004). The majority of the phyllosphere bacteria occur in large clusters or aggregates of up to 1,000 cells or more (Monier & Lindow, 2003; Morris & Monier, 2003). The size of these aggregates is correlated with surface water availability (Leveau, 2015). Using a multi-labelled combinatorial fluorescence *in situ* hybridisation (FISH) approach, Remus-Emsermann *et al.* (2014) found that about 5% of the abaxial side of *Arabidopsis thaliana* leaves is covered with bacteria, whereas hardly any bacterial cells reside on the adaxial side, with leaf edges being more densely populated than other parts of the leaf. Using FISH labelling and spatial statistics, it has been shown that different taxa exhibit short-distance (up to 7 μm) and intraspecific taxa aggregation (up to 10 μm) (Remus-Emsermann *et al.*, 2014).

Existing colonisation of an environment by bacterial aggregates affects survival of bacterial immigrants (alien, invaders, migrants, non-native, transient) on plants (Monier & Lindow, 2003), either enhancing or hindering immigration of bacteria onto leaf surfaces (Monier & Lindow, 2005b). Once established on the leaf, alien microorganisms interact with their host and native microorganisms, which may include competition, parasitism, predation, mutualism, commensalism and amensalism (Faust & Raes, 2012). It has been shown that competition for nutrients is a prominent form of interaction and that the reproductive success of alien microbial species is lower when the density of microbial cells already present is higher (Remus-Emsermann *et al.*, 2013). Using the REProductive SUCcess (CUSPER) bacterial bioreporter, it has been revealed that individual bacterial immigrants do not contribute equally to population sizes in a heterogeneous environment such as the phyllosphere (Remus-Emsermann & Leveau, 2010). These findings have been corroborated by individual-based assessments of leaf colonisation at increasing inoculum densities (Remus-Emsermann *et al.*, 2012). Other studies using bioreporters have revealed that the aptitude of secondary immigrants for establishment is negatively correlated with the level of primary colonisers in the phylloplane (Remus-Emsermann *et al.*,

2013), and that interactions among bacteria are confined to spatial scales up to 5-20 µm (Esser *et al.*, 2015).

Microbiota can provide benefits for plants by supporting plant growth and health and by modifying plant responses to abiotic and biotic stress (heat, drought, limited resources, insects and pathogens) (Brader *et al.*, 2017; Lemanceau *et al.*, 2017; Hardoim *et al.*, 2015; Bulgarelli *et al.*, 2013; Vorholt, 2012). Previous studies have suggested that plants can actively manage the overall microbiome composition (Jones *et al.* (2019) and references therein). Thus, apart from being attributable to differences in nutrient availability, variations in the establishment and survival of foodborne pathogens can be explained by diverse factors, such as environmental (electromagnetic radiation (Alsanius *et al.*, 2019; Allende *et al.*, 2017) and desiccation (López-Gálvez *et al.*, 2018)), biological (native microbiota (Klerks *et al.*, 2007)), chemical (induction of plant-defence response (Jang & Matthews, 2018; Roy *et al.*, 2013; Seo & Matthews, 2012) and antimicrobial (Ruppel *et al.*, 2008)), and physical factors (hydrophobicity, roughness, venation, flatness of the surface) (Doan *et al.*, 2020a; Van der Linden *et al.*, 2016; Beattie, 2011; Beattie & Lindow, 1995). Therefore, disentangling the complexity and the interplay between plants and their associated microbiota (including foodborne pathogens) (Figure 1), and factors that drive the microbiome composition is not a trivial task. However, extensive research on the microbial communities in different plant spheres is beginning to reveal the nature of interactions between microbiota and their plant hosts.

1.2.2 Interactions between *E. coli* O157:H7 and host plant: Alien invader, but not plant pathogen

Escherichia coli O157:H7 is not considered part of the native microbiota on leafy vegetables, and its presence is a result of contamination (Buck *et al.*, 2003) (Figure 1). (See 1.3.3 for details of the invasion concept.) Ruminants appear to be the main reservoir for *E. coli* O157:H7 (WHO, 2018; Caprioli *et al.*, 2005). Contamination of leafy vegetables with *E. coli* O157:H7 is influenced by diverse pre- and post-harvest factors (Julien-Javaux *et al.*, 2019; Mogren *et al.*, 2018; Castro-Ibáñez *et al.*, 2017; Gil *et al.*, 2015). There is substantial evidence to support the hypothesis that plants are used as alternative hosts by foodborne pathogens like *E. coli* O157:H7 (Lim *et al.* (2014); Holden *et al.* (2009) and references therein), and as vehicles for their

transmission to a more suitable host, such as animals or humans (Barak & Schroeder, 2012). The prevalence of most foodborne pathogens associated with leafy vegetables is reported to be low (<1%) (Holvoet *et al.*, 2015; Holvoet *et al.*, 2014; EFSA, 2013). Thus contamination of leafy vegetables can be considered a "rare" event (EFSA, 2013), but it is associated with a considerable impact.

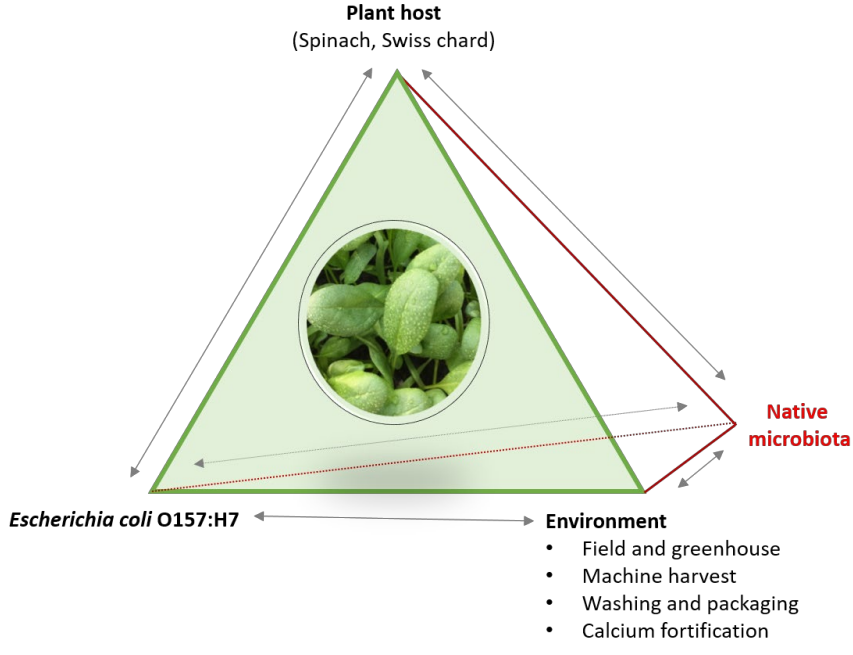


Figure 1. Interactions of human pathogens in the phytobiome. Illustration based on diagrams in Agrios (2005) and Leveau (2019).

Foodborne pathogens such as *E. coli* O157:H7 are not known as good leaf colonisers (Brandl & Mandrell, 2002), because unlike common plant epiphytes, their origin is enteric. Nevertheless, *E. coli* O157:H7 can establish as a leaf epiphyte, capable of living on the leaf exterior (phyllosphere) (Hirano & Upper, 1983), and as an endophyte capable of living in leaf intercellular spaces and sub-stomatal cavities (endosphere) (Deering *et al.* (2012) and references therein). To colonise plants, *E. coli* O157:H7 can employ different attachment mechanisms (pili, filamentous type III secretion system, flagella) (Berger *et al.* (2010) and references therein).

The fate of *E. coli* O157:H7 on a leaf surface is determined by extrinsic factors (environment) and by intrinsic (phylloplane) factors such as diversity of native microbiota, tissue pH and presence of antimicrobials and pectinolitic soft-rot bacteria (Beuchat, 2002; Carmichael *et al.*, 1998). Declines observed in artificially introduced human pathogens may be partly explained by the less favourable conditions that prevail on 'intact' leaves (see 1.3.1), resulting in nutrient stress due to transfer from nutrient-rich propagation conditions to the nutrient-deprived phylloplane, and to the protective nature of cuticle and cell walls that act as plant natural barriers and prevent access to nutrient-rich niches (Alam *et al.*, 2014; Oliveira *et al.*, 2012; Harris *et al.*, 2003; Dickinson, 1986). To cope with the conditions of the phyllosphere, microbial colonisers are equipped with traits which allow adaptation and enable microbial survival (Doan *et al.*, 2020b; Remus-Emsermann & Schlechter, 2018; Doan & Leveau, 2015; Vorholt, 2012). One such trait commonly displayed by microbial colonisers is biofilm formation (Morris *et al.*, 1997; Leigh & Coplin, 1992).

Colonisation by foodborne pathogens extends beyond the outer leaf surface, and hence leaf damage may aid *E. coli* persistence and internalisation (Hartmann *et al.*, 2017; Scott *et al.*, 2017; Simko *et al.*, 2015; Aruscavage *et al.*, 2010; Barker-Reid *et al.*, 2009; Aruscavage *et al.*, 2008; Brandl, 2008; Seo & Frank, 1999). *Escherichia coli* O157:H7 internalisation within the tissue of leafy vegetables has frequently been detected (Erickson *et al.*, 2019; Merget *et al.*, 2019; Hartmann *et al.*, 2017; Wright *et al.*, 2017; Macarisin *et al.*, 2014; Gómez-López *et al.*, 2013; Deering *et al.*, 2012; Erickson, 2012; Erickson *et al.*, 2010). Once internalised, bacteria are sheltered from environmental factors that might otherwise limit their survival, proliferation or biofilm formation on plants, *e.g.* desiccation and UV light (Brandl & Mandrell, 2002), but also rinse water and sanitisers (Davidson *et al.*, 2013; Niemira & Cooke, 2010; Allende *et al.*, 2009; López-Gálvez *et al.*, 2009; Takeuchi & Frank, 2000). Due to their low infectious dose, internalisation of *E. coli* O157:H7 like human pathogens poses a high risk to consumers, as internalised bacteria can utilise nutrients and grow inside leaves (Deering *et al.* (2012) and references therein; Paper III).

There is no evidence that *E. coli* is a plant pathogen, *e.g.* leaves contaminated or infiltrated with *E. coli* O157:H7 do not show visible effects (Thilmony *et al.*, 2006). However, *E. coli* O157:H7 flagella are recognised by plants (Seo & Matthews, 2012). When plants detect flagellated bacteria,

they try to prevent these bacteria from entering into their internal tissues by closing their stomata. Stomatal closure as an innate immunity response has been reported for *E. coli* O157:H7, where the bacteria could not reverse the stomatal defence (Melotto *et al.*, 2006). *Escherichia coli* O157:H7 induces stronger plant immunity than another common human pathogen, *Salmonella* Typhimurium, as suggested by substantially higher expression of the pathogenesis-related protein 1 (PR1) gene (Roy *et al.*, 2013). *Salmonella enterica*, but not *E. coli* O157:H7, interacts with plants and is considered an endopathogen (Schikora *et al.*, 2008).

1.3 Leaf as a microbial habitat

1.3.1 The 'simplicity' of the 'intact' leaf

In the context of food safety, leaves in the holobiont are considered intact unless they show macroscopic deviations. Leaf morphology, chemistry and physiology differ between plant species (Vacher *et al.*, 2016). Topography of the leaf surface is an important factor for colonisation (Doan *et al.*, 2020b). Leaching sites such as stomata, veins, trichomes and hydathodes, and cuticle thickness on the intact phylloplane, affect availability of resources (Vorholt, 2012; Remus-Emsermann *et al.*, 2011; Leveau & Lindow, 2001a). The phyllosphere harbours high species richness, but bacterial community diversity is lower than in the more environmentally stable rhizosphere (Knief *et al.*, 2012; Delmotte *et al.*, 2009).

Understanding surface topography is relevant for understanding the fate of enteropathogens on leafy vegetables (Doan *et al.*, 2020a). The leaf surface is covered by a plant cuticle, a thin extracellular hydrophobic layer mitigating non-stomatal water loss and leaching from apoplast to leaf surface (Tukey, 1970). The hydrophobicity of the cuticle makes the phyllosphere water-repellent and dry, and thus an oligotrophic environment (Vorholt, 2012). The ability of microorganisms to establish and thrive on the leaf surface depends also on functional traits, such as ability to attach to the cuticle and to use available nutrients. Leaf roughness and vein density are important factors for establishment of invading microorganisms (Doan *et al.*, 2020a; Doan *et al.*, 2020b; Macarisin *et al.*, 2013). Leaf surfaces with more venation (pronounced topography) retain higher numbers of *E. coli* cells than flatter surfaces (Doan *et al.*, 2020b). Pronounced leaf topography has been

shown to contribute to enhanced survival of *E. coli* following chlorine treatment (Doan *et al.*, 2020a).

In order to sustain or grow populations, phyllosphere bacteria need access to resources, with carbohydrates and water being most important (Beattie, 2011; Aruscavage *et al.*, 2010; Leveau & Lindow, 2001a). Photosynthates such as glucose, fructose and sucrose can be utilised by phyllosphere microorganisms (van der Wal & Leveau, 2011; Leveau & Lindow, 2001a; Mercier & Lindow, 2000; Tukey, 1970). The origin of the phylloplane nutrients is the leaf interior, and they are deposited on the leaf surface through passive leakage across the cuticle (Remus-Emsermann *et al.*, 2011; Leveau & Lindow, 2001a; Morgan & Tukey, 1964; Tukey & Mecklenburg, 1964) or guttation (Singh, 2014). Photosynthesis determines the availability of nutrients (Vacher *et al.*, 2016). The phyllosphere is characterised by substantial environmental heterogeneity, and thus only a limited number of sites on intact leaves may offer conditions favourable for bacterial growth (Remus-Emsermann *et al.*, 2012). Stomata, trichome bases, epidermal cell wall junctions (depressions) and grooves along the veins and near hydathodes are the preferred sites for epiphytes (Vorholt, 2012; Lindow & Brandl, 2003; Beattie & Lindow, 1995). Therefore, on the phylloplane of an 'intact' leaf, resources are characterised by low abundance and heterogeneous (patchy) distribution (Axtell & Beattie, 2002; Leveau & Lindow, 2001a; Joyner & Lindow, 2000). Water plays an important role in the ability of microorganisms to access nutrients and to relocate and communicate with other microorganisms (Beattie, 2011), and nutrient leaching is affected by and/or related to cuticle wettability. Survival of epiphytic bacteria is greater with increased water availability (Hirano & Upper, 2000; Beattie & Lindow, 1995).

Leaf surface heterogeneity in terms of location and the amount of available nutrients might partly explain the distinctive phyllosphere communities that establish (Rastogi *et al.*, 2012; Hunter *et al.*, 2010; Delmotte *et al.*, 2009). Patchy distribution of nutrients, and subsequently of the indigenous bacterial populations on leaves, is associated with major nutrient leakage sites (Monier & Lindow, 2004; Morris *et al.*, 2002; Morris *et al.*, 1997) at the micro-scale (Vos *et al.*, 2013), leaving many prospective habitats empty (see 1.3.3 for 'empty niche concept'). The native spinach microbiota is dominated by Proteobacteria, Firmicutes and Actinobacteria (Rosberg *et al.*, 2020; Lopez-Velasco *et al.*, 2013). On arrival on the leaf

surface, establishment of non-native microbiota is limited by nutrient and water availability, UV radiation and competition with native microbiota (Vacher *et al.*, 2016; Hunter *et al.*, 2010; Monier & Lindow, 2005b). However, niches empty of native microbiota can ease establishment of immigrating bacteria. Immigrant *E. coli* O157:H7 can form colonies around and inside stomata and bases of trichomes, as those are the sites with the highest nutrient abundance (Jeter & Matthysse, 2005).

1.3.2 The 'complexity' of wounds: Wound-mediated establishment of *E. coli* O157:H7 and a dangerous passage to the leaf interior

Baby leaves intended for direct human consumption are exposed to stress factors causing wounding from field to fork. Those factors can be biotic (plant pathogens and insects) and abiotic (wind, hail, drought, temperature, farming practices, harvest and post-harvest handling). Damage to baby leaves is unavoidable, especially for fresh-cut, ready-to-eat products, and can take the form of crushing, cutting or bruising of leaf tissue. At harvest, the leaf transitions from an autotrophic to a heterotrophic state, and thus from anabolic to catabolic processes (Mogren *et al.*, 2018). Damage as a perturbation has impacts on the plant as a host, the native microbiota and the establishment of non-native microbiota, such as enteric pathogen *E. coli* O157:H7. Damage induces great change in the phylloplane, and these damage-induced changes in the host impair the physical quality (leaf landscape) and chemical characteristics (nutrient and water leakage), and enhance deterioration of baby leaves (Ariffin *et al.*, 2017). This affects the nature of the relationship between plant host and native microbes, between native microbes and *E. coli* O157:H7, and between plant host and *E. coli* O157:H7 (Figure 1).

Injury causes breaching of the natural barrier of cuticle and epidermal layer. Due to local removal of the hydrophobic cuticle and damage to the epidermis or even deeper leaf tissue layers, more contaminants can adhere to the surface. Damage to the epidermis modifies leaf roughness and the three-dimensional landscape of the leaf surface, increases surface area and disrupts internal leaf structure, causing water loss (Aruscavage *et al.*, 2008; Tukey & Morgan, 1963); Papers III-IV). This affects the wetness of the leaf surface and induces local cell death (Iakimova & Woltering, 2018). Injury sites provide new microbial colonisation sites in the wound area.

As an outcome of damage, nutrient pools not typically available on the phylloplane are released (Aruscavage *et al.*, 2008; Brandl, 2008), temporarily alleviating local resource scarcity. Through new opportunities created in the form of hotspots or microsites (Mallon *et al.*, 2015a; Mallon *et al.*, 2015b), leached carbon sources can support survival of invading microorganisms and enhance fast-growing and spoilage microorganisms (Ariffin *et al.*, 2017; Aruscavage *et al.*, 2010; Aruscavage *et al.*, 2008; Brandl, 2008). However, the change in nutrient availability may not necessarily result in increased carrying capacity of a leaf as a habitat (‚acroscale), *e.g.* Remus-Emsermann *et al.* (2012) suggested that local nutrient availability (microscale) determines the local carrying capacity on leaves. From this perspective, processing practices that inflict wounds or injuries are relevant (Brandl, 2008).

Enteric pathogens can exploit lesion sites occurring due to mechanical damage and biotic lesions that develop as a result of tissue degradation by bacterial and fungal pathogens (Simko *et al.*, 2015; Aruscavage *et al.*, 2008; Brandl, 2008). However, this requires pathogens to cope with the physicochemical stress imposed by leakage of osmolytes in the wounded area and plant innate immunity response to injury (Kyle *et al.*, 2010). Notable adaptability of enteric pathogens to a range of physicochemical stresses by using choline to synthesise glycine betaine for osmo-adaptation at the wound site has been demonstrated (Scott *et al.*, 2017).

In general, plant lesions can promote high *E. coli* proliferation rates (Brandl, 2008). Takeuchi and Frank (2000) showed that *E. coli* O157:H7 preferably colonises cut edges compared with the intact leaf surface. Wounds can permit internalisation by opportunistic bacteria that lack the ability to break down pectin and would be unable to internalise the tissue otherwise (Deering *et al.*, 2012; Aruscavage *et al.*, 2010; Aruscavage *et al.*, 2008; Brandl, 2008). Compared with the environment found on the leaf exterior, the leaf interior is a sanctuary for internalised microorganisms, as it gives access to a continuous nutrient supply (Mercado-Blanco, 2015). *Escherichia coli* thrives in tip burn lesions (Brandl, 2008), while sites infected with downy mildew support greater multiplication rates of *E. coli* O157:H7 than observed on healthy lettuce leaves (Simko *et al.*, 2015). Moreover, *E. coli* O157:H7 has been shown to internalise via cut edges on lettuce and its presence has been detected after chlorine sanitisation treatment (Seo & Frank, 1999). Numbers of *E. coli* O157:H7 cells decrease more rapidly on

intact leaves compared with biotically and abiotically damaged leaves (Aruscavage *et al.*, 2008). Studies have shown that plant species (Hartmann *et al.*, 2017) and cultivar (Erickson *et al.*, 2019) are significant variables for internalisation of enteric pathogens in plants. This could be partly due to the plant defence system and constituent antimicrobials (Ruppel *et al.*, 2008). Apart from providing access to nutrients, internalisation within plant tissue limits the number of competing species (Holden *et al.*, 2009). Likewise, using wounds for internal colonisation does not require the bacteria to avoid or suppress the plant immune response, therefore making invasion 'effortless' from that perspective.

1.3.3 Ecological concepts relevant to perturbations

Ecological concepts, such as microbial invasion and niche theories, may improve understanding of the role of perturbation events, such as damage-mediated nutrient and water release, in the fate and establishment of enteric pathogen as immigrants or invading microorganisms in the phyllosphere. Better understanding of these interactions can lead to better solutions to keep plants and plant consumers safe. Definitions of ecological concepts relevant for this work can be found in Appendix 1. For further details and ecological concepts translated in non-technical terms, please consult Hawkes and Connor (2017), Enders *et al.* (2020) and Mallon *et al.* (2015b).

Microbial invasions represent entry of non-native microorganisms, in the present case the enteric pathogen *E. coli* O157:H7, into a resident community of microbes. Microbial invasions are prevalent in nature and, according to Mallon *et al.* (2015b), comprise four stages: (i) introduction, (ii) establishment, (iii) growth and spread, and (iv) impact on residential community and host. The likelihood of a non-native microorganism completing all four stages depends on many factors that dictate its establishment in the phylloplane. According to Thakur *et al.* (2019), an "alien species is invasive when it has reached the spread stage (and is detectable) and alters the dynamics of host and non-host species leading to losses in biodiversity and ecosystem functions". Invasion success depends on the adaptability of the non-native microorganism and the ability of the native community to maintain function during perturbation (resistance), and to regain function after a perturbation (resilience) (Pimm, 1984).

The phyllosphere is a microbially diverse environment, where population sizes on macro scale may vary considerably due to large fluctuations in the

physical and nutritional conditions and in leaf age (Lindow & Brandl, 2003). Immigration is a gradual process, and cells arrive in the phylloplane at different times and normally in low amounts (Upper & Hirano, 2002). Early colonisers of the phylloplane determine the fate of subsequent colonisers, in what is known as the "priority effects concept" (Fukami, 2015; Maignien *et al.*, 2014; Gleason, 1926). Further, community diversity mediates invasion resistance (Mallon *et al.*, 2015b), where more diverse microbial communities are better able to exploit available resources than less diverse communities. This limits invading populations and eventually leads to their exclusion (Mallon *et al.*, 2015b). Artificially added *E. coli* O157:H7 has been shown to change the composition of lettuce bacterial biota (Williams & Marco, 2014). Impacts of invading *E. coli* on *e.g.* native soil microbiota have also been demonstrated (Yao *et al.*, 2014; Van Elsas *et al.*, 2012). However, impact imposed by *E. coli* upon invasion is not always the case, possibly due to higher diversity of native microbiota (Xing *et al.*, 2020).

A wound is an important ecological perturbation and a niche that provides increased resource availability (nutrients, water, surface). The success of invasion increases with the availability of resources, in what is known as the "increased resource availability" hypothesis (Sher & Hyatt, 1999). According to Monier and Lindow (2005b), the fate of an immigrant bacterium depends on the nature of the leaf structure to which it immigrates, and indirectly on the amount of available water and nutrients at the immigration site, to support the bacterial aggregates. Sites that do not provide carbon sources in high abundance are unlikely to support reproduction of bacterial immigrants (Remus-Emsermann *et al.*, 2012) (habitat filtering). Lesion-mediated addition of resources to a highly diverse community can provisionally alleviate the diversity-invasion relationship by creating more opportunities for immigrants to prosper (diversity-invasion effect) (Mallon *et al.*, 2015a; Mallon *et al.*, 2015b), resulting in their establishment, growth and spread (Enders *et al.* (2020) and references therein).

Niche is defined as the range of requirements of a species for survival and reproduction in a given environment and impacts on the environment (Chase & Leibold, 2003). The establishment of a particular microorganism on a plant organ (niche colonisation) depends on environmental heterogeneity, increased resource availability and windows of opportunity, among other factors. To adapt better and withstand environmental conditions found on the leaf after introduction, foodborne pathogens employ different strategies,

such as conversion to viable but not culturable cell (VBNC) state and biofilm formation (adaptation).

Overall, damage as a perturbation provides more opportunities for *E. coli* O157:H7 to establish and even invade (impact) the native microbial community. A limiting factor for establishment is presence of similar native species, suggesting similar nutrient utilisation profiles and competition for the same resources (exploitative competition) (Schlechter *et al.*, 2019), thus outcome of high species resource overlap index (Wilson & Lindow, 1994). Therefore, the invasion success of non-native species is higher in areas that have less closely related species (Darwin, 1859), where they are less likely to compete for the same resources (lower niche overlap) (MacArthur & Levins, 1967). Niche overlap of the invading microorganism with the niche of residential microbes and niche partitioning affect co-existence (Schlechter *et al.*, 2019; Hawkes & Connor, 2017). A study by Cooley *et al.* (2006) showed that presence of *Enterobacter asburiae* decreased *E. coli* O157:H7 numbers by 20-30 fold on leaves, probably due to competition for the same carbon and nitrogen sources, while presence of *Wausteria paucula* enhanced *E. coli* survival by six-fold. In that study, *E. coli* utilised 23 of the 27 substrates tested, *E. asburiae* used 25 of the substrates (high overlap in nutrient use) and *W. paucula* utilised only four of the same substrates that *E. coli* used (low overlap in nutrient use). In another study, co-inoculation with *Enterobacter cloacae* reduced the population of *E. coli* O157:H7 and *Listeria monocytogenes* by one log, but *Salmonella enterica* was not affected (Jablasone *et al.*, 2005).

Niche-based hypotheses are based on differences between species that would allow invading species to access unused resources (empty niche hypothesis) or to remain more competitive in exploiting resources (niche replacement hypothesis) (Ricciardi *et al.*, 2013). Due to high resource heterogeneity, the phylloplane may provide competition-free resources (empty niche). A niche opportunity is the potential provided by a native community for non-native organisms, and may occur because of a resource opportunity or favourable physical environmental conditions (Shea & Chesson, 2002). Lesion-provided conditions may be viewed as leaf opportunities or hotspots with unique and highly localised nutritional conditions depending on lesion size, depth and position. From this perspective, higher numbers of lesions in a homogenous distribution across the leaf surface might contribute to initial adaptation and establishment of *E.*

coli O157:H7. The effect of lesion distribution establishment of *E. coli* O157:H7 was not studied in this thesis, but it should be considered in future studies. On the other hand, low levels of niche opportunities lead to invasion resistance of a native community (Shea & Chesson, 2002). Stochasticity also determines the fate of individual cells upon arrival at random sites in the phylloplane, which may or may not be in their favour (Remus-Emsermann *et al.*, 2012; Hubbell, 2001).

1.4 Methods to display perturbations and hurdles

Studying microbial life on leaf surfaces and translating the findings into function is challenging. The culture-dependent approach in studying microbial life on leaf surfaces has dominated the field for a long time. Recent important advances in the field of plant-microbe interactions can be attributed to the emergence of new technologies, such as DNA- and RNA-based molecular methods (deep sequencing and -omics technology), and microscopy-based visualisation techniques (Lugtenberg, 2016). To characterise the whole microbiome, molecular methods can be employed. However, these methods lack spatial information, a gap that can be bridged by microscopy visualisation techniques. Studying bacteria at single cell resolution has been enabled with the introduction of green fluorescent protein (GFP). Fluorescent dyes are often used to visualise bacteria on leaves by microscopy. Microscopy methods such as electron microscopy (SEM, TEM), confocal laser scanning microscopy (CLSM), Raman spectroscopy, super resolution and atomic force microscopy (AFM) are suitable for visualisation of plant-microbe interactions in the phyllosphere (Cardinale & Berg, 2015). The advantage of such direct visualisation is that it delivers spatial, qualitative and quantitative information on microorganisms and their variation at micro-scale, which is essential (Cardinale & Berg, 2015). The challenges are that multiple samples cannot be run in parallel at the same time and that using a statistical approach is problematic (Cardinale & Berg, 2015). Avoiding artefacts in sample preparation and finding suitable sampling spots due to limited sample area in the microscope are additional challenges.

For these reasons, integrating direct microscopy observations with culture-dependent methods and indirect molecular characterisation of microbiomes is often appropriate to draw more reliable conclusions. This

combined approach can be coupled with additional complementary methods such as volatile collection and gas chromatography-mass spectrometry (GC-MS) to capture airborne signals of plant-microbe communication. The challenge lies in disentangling information on the spatial distribution of microbial diversity on leaves obtained using these combined methods to identify interplay between plants and microbes (culture-based; DNA-based), the mechanisms involved (function; RNA-based), the timing (environmental factors) and the location on the phylloplane (spatial information; microscopy). Ultimately, it could be possible to exploit these insights to ensure food safety at all times.

1.4.1 Visualisation of plant-microbe interactions on leaves

There are several tools and approaches for visualising leaf damage and phyllosphere microorganisms. Such visualisation is an important complement to other commonly employed approaches. A summary of appropriate visualisation techniques is provided below.

Visualisation of damage

It is necessary to discriminate between damage that generates visible symptoms (*e.g.* plant disease), and damage that is either too small to be seen with the naked eye (microscopic damage) or when there is no change in colour or texture between damaged and intact tissue. Much research has been devoted to detection and automated quantification and classification of damage that generates symptoms in the visible spectrum. Solutions based on artificial neural networks and deep learning as state-of-the-art in digital image analysis have been presented (as summarised in Paper I). However, when the contrast between damaged and intact tissue (colour separation) is weak, the accuracy of damage detection algorithms is reduced, resulting in false positives and false negatives (Barbedo, 2014). This means that for microscopic and mechanical damage where colour contrast is not generated, such approaches will not provide satisfactory results. However, contrast enhancement can be employed as an intermediate step to enable discrimination of tissue viability (live/dead tissue staining). Alternative methods for damage detection are microscopy-based (detection of microscopic damage; not adequate for leaf scale) or hyperspectral imaging (not suitable for microscopic damage) (Thomas *et al.*, 2018).

A common characteristic of cell damage or cell death is that the cell membrane ceases to function as a selective barrier (Truernit & Haseloff, 2008). As a consequence, dyes that cannot penetrate through the plasma membrane of living cells, such as trypan blue (TB), will traverse damaged membranes and stain internal components of such cells (viability exclusion) (Fernández-Bautista *et al.*, 2016; van Wees, 2008). A range of fluorescent DNA binding dyes for visualising live and dead plant cells by means of fluorescent microscopy has been developed, such as SYTOX dyes (Truernit & Haseloff, 2008), propidium iodide (PI) (Chen *et al.*, 2006), and fluorescein diacetate (FDA) (Saruyama *et al.*, 2013; Celenza *et al.*, 1995). Simultaneous staining with PI and FDA is often appropriate for fluorescence imaging of plant cell death dynamics under pathogen attack (Jones *et al.*, 2016). In the experiments described in this thesis (Papers I-IV), TB dye provided enough contrast between damaged and intact tissue and a digital image analysis approach could be applied with high accuracy, so there was no need to use fluorescent dyes for detection of lesions on leaf-scale.

Visualisation of microorganisms

Studying bacteria at single-cell resolution requires visualisation, and this can be achieved by microscopy in combination with different fluorescent markers such as labelling bacteria with fluorescent proteins (Ledermann *et al.*, 2015), DNA introducing dyes (Berney *et al.*, 2007) or fluorescence *in situ* hybridisation (FISH) (Wagner *et al.*, 2003).

Fluorescent microscopy has become another important tool for studying labelled model bacterial strains and their colonisation at micrometer or single-cell scale. Labelling of bacterial strains with fluorescent proteins has been used extensively (Remus-Emsermann & Schlechter, 2018; Remus-Emsermann *et al.*, 2013; Monier & Lindow, 2005a; Brandl & Mandrell, 2002). Fluorescent proteins are used under the control of inducible promoters as a bioreporter. Bioreporters contain two essential genetic elements, a promoter gene and a reporter gene, introduced into a microbial host either on a plasmid or as a chromosomal insertion (Leveau & Lindow, 2002). Plasmids can easily be delivered into a host cell, but to overcome the disadvantage of plasmid loss during cell division, chromosomal insertion of fluorescent markers is favoured (Schlechter *et al.*, 2018). The most commonly used reporter genes are those which code for GFP. It is a powerful tool for *in situ* (microscopy) and *ex situ* (viable count) abundance assessment of a bioreporter microorganism (Leveau *et al.*, 2007). It is also easily detected at

single-cell resolution using confocal laser scanning microscopy (Phillips, 2001). However, heterogeneity in the activity of bioreporters recovered from habitats has been observed (Leveau & Lindow, 2002). A fluorescent protein toolbox suitable for simultaneous fluorescent tagging and tracking of multiple bacterial strains, and for facilitating studies on biofilm, host-microbe and microbe-microbe interactions, has been developed (Schlechter *et al.*, 2018).

Different general DNA intercalating dyes, such as acridine orange, DAPI, Hoechsts 33342 and 33258 and Syto dyes, can be used to visualise bacterial cells on leaves. These dyes pass through the cell membrane and enable microscopy of microorganisms on leaves (Remus-Emsermann & Schlechter, 2018; Vacher *et al.*, 2016). Alternatively, live/dead staining approaches can be applied simultaneously with DNA intercalating red and green fluorescent dyes (*i.e.* Backlight Kit), with PI for dead cells staining (red) and Syto 9 dye for all cells (green) (Remus-Emsermann & Schlechter, 2018; Boulos *et al.*, 1999).

Further, FISH is a robust and widely employed method for cultivation- and transformation-independent *in situ* identification of bacteria in mixed microbial communities (Wagner *et al.*, 2003), and has been used to study bacterial colonisation of plants (Remus-Emsermann *et al.*, 2014; Bulgarelli *et al.*, 2012; Bisha & Brehm-Stecher, 2009). FISH is based on ribosomal RNA-targeted oligonucleotide probes designed for different phylogenetic groups, and evaluated using epifluorescence microscopy or flow cytometry. Double labelling of oligonucleotide probes (DOPE)-FISH (5′- and 3′-double-labelled probes instead of single-labelled probes for FISH) as an improved method of FISH with stronger fluorescent signal has been introduced (Compant & Mathieu, 2013; Compant *et al.*, 2013; Stoecker *et al.*, 2010). Natural epiphytic communities can be studied using a multi-labelled combinatorial FISH approach. Using probes for different taxonomic groups, FISH allows investigation of phylloplane colonisation patterns (distribution of bacterial groups) and relationships with different taxa (random, non-random) (Remus-Emsermann *et al.*, 2014). The downside of FISH is that quantitative abundance data are mostly obtained by counting, so the accuracy of this quantification approach is relatively low in densely colonised biofilms (Wagner *et al.*, 2003). For example, one study employing a multi-labelling combinatorial FISH approach found that Alphaproteobacteria, Betaproteobacteria and Actinobacteria dominated in the *Arabidopsis*

thaliana phylloplane, Gammaproteobacteria was rare and detected as single cells, while about 31% of bacteria did not hybridise (Remus-Emsermann *et al.*, 2014).

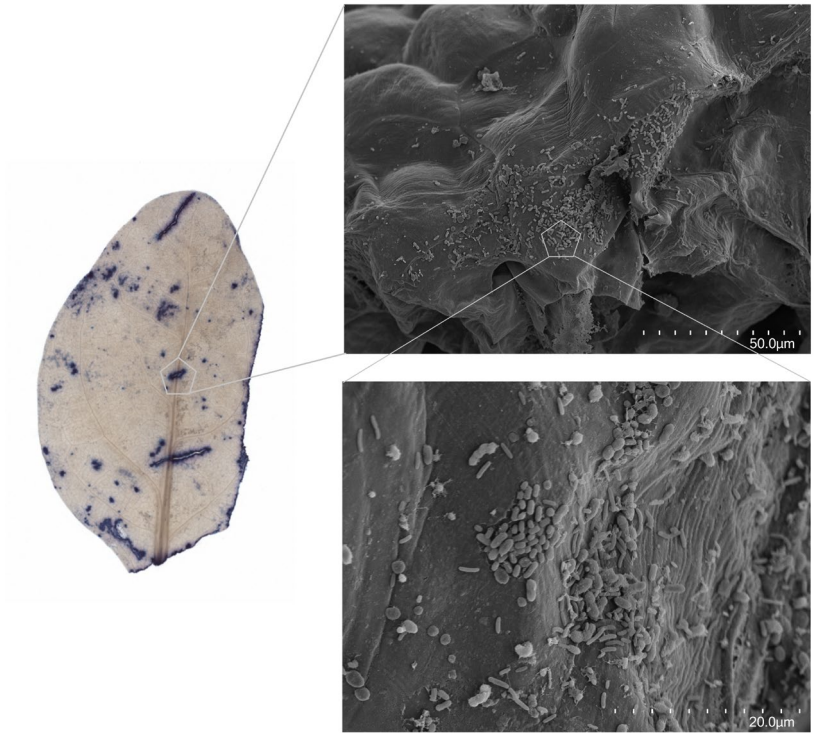


Figure 2. (Left) Visualisation of leaf damage on spinach leaf, using trypan blue dye, and (right) visualisation of bacteria colonising a lesion edge using scanning electron microscopy. Scale bars 50 µm and 20 µm.

Light microscopy (LM), scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM), have been used to visualise plant-microbe interactions. Both SEM (López-Gálvez *et al.*, 2010; Wang *et al.*, 2010; Keskinen *et al.*, 2009; Kroupitski *et al.*, 2009) (Figure 2) and CLSM (Hartmann *et al.*, 2017; Wright *et al.*, 2017; Mitra *et al.*, 2009; Brandl, 2008; Solomon *et al.*, 2002; Takeuchi & Frank, 2000) have been applied to visualise enteric pathogens in the phyllosphere. Although SEM micrographs reveal presence of bacteria on the leaf surface, with this approach it is not possible to distinguish between artificially introduced and residential bacteria. CLSM is the most common approach for corroborating presence of

the particular fluorescent protein-tagged microorganism, and identifying internalised bacteria, where images can be taken at different microscopic depths. It has been used for visualisation of internalised *E. coli* cells and colonies and to determine depth of *E. coli* penetration into tissue of leafy vegetables (Hartmann *et al.*, 2017; Wright *et al.*, 2017; Brandl, 2008).

1.4.2 Methods to study phyllosphere microbiota: From the great plate count to next-generation sequencing

Microorganisms associated with plants can be studied using culture-dependent (based on growing microorganisms on medium) and culture-independent (target nucleic acids DNA and RNA) methods.

Culture-dependent approaches have limited capacity as only a small proportion of microorganisms, estimated to be about 1% of the environmental bacterial diversity, are cultivable. This known as "the great plate count anomaly" (Staley & Konopka, 1985), although the 1% estimate has been debated (Rothschild, 2006). Microorganisms can be non-culturable for different reasons, such as lack of specific growth requirements (nutritional, temperature, aeration *etc.*), out-competed in the presence of fast-growing microorganisms or stressful conditions imposed by cultivation (Vaz-Moreira *et al.*, 2011).

Culture-independent methods enable high-throughput identification of entire microbial communities, through metagenomics ("Study of the collective genomes recovered from environmental samples without prior cultivation" (van Elsas *et al.*, 2008)). Conventional culture-independent approaches, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) analysis or Sanger sequencing of 16S rRNA gene clone libraries, are being replaced with next-generation sequencing (NGS) platforms such as the SOLiD system and Illumina (Logares *et al.*, 2014; Mardis, 2008). In sequencing, fluorescent nucleotides are added one by one to an elongating DNA template strand by DNA polymerase, with each incorporated nucleotide identified by its fluorescent label (Illumina, 2020). The greatest advantages of NGS over conventional culture-independent approaches are high throughput (massive parallel sequencing) and lower costs.

There are two main approaches for phylogenetic microbial community profiling, amplicon sequencing (short marker genes; genus-level taxonomic classification) and shotgun sequencing (captures complete range of sample

DNA; species- or strain-level classification) (Morgan & Huttenhower, 2012). A systematic comparison of amplicon and shotgun sequencing can be found in Rausch *et al.* (2019). Characterisation of microbial community composition and structure using Illumina sequencing, namely MiSeq and HiSeq platforms, is enabled through in-depth, paired-end sequencing of amplified fragments of the 16S rRNA gene, the internal transcribed spacer (ITS) region and other marker genes (Holm *et al.*, 2019). Sequencing on the Illumina HiSeq platform results in a greater number of reads per sample, of significantly higher quality, compared with sequencing on the Illumina MiSeq platform (Holm *et al.*, 2019). In Papers II and IV in this thesis, bacterial and fungal communities were analysed with Illumina sequencing.

Metagenomics (DNA), metatranscriptomics (RNA), metaproteomics (proteins) and metabolomics (metabolites) provide sufficient input on the functional dynamics of microbial communities (Simon & Daniel, 2011). The use of RNA as a molecular target for living and active microbes in microbial communities is on the rise (Emerson *et al.*, 2017). However, given the shorter average half-life of RNA (half-life of rRNA days, mRNA minutes), processing of RNA is more challenging than processing of DNA (Johnson *et al.*, 2005). Losses of up to 80% of the total mRNA have been reported during sample preparation (Johnson *et al.*, 2005).

Microbial diversity obtained from sequencing can have biases introduced during the amplification and sequencing procedures (Lundberg *et al.*, 2013), and data analyses (Edgar, 2013). Microbiota samples are thus often 'contaminated' with host DNA, RNA or proteins (Knight *et al.*, 2012), *i.e.* meta-approaches cannot be the only tool used for plant-microbiome research (Bulgarelli *et al.*, 2013). The advantage of the culture-dependent approach is that it takes into account only viable cells, whereas DNA-based methods take into account DNA from both viable and non-viable cells. Therefore, to minimise the bias caused by using only one method to evaluate the microbial communities in samples, combining DNA- and RNA-based meta-approaches with culture-dependent methods (viable count, isolates) is recommended (Schlaeppi & Bulgarelli, 2015).

Popular high-throughput microbial community sequence analysis pipelines are QIIME (Caporaso *et al.*, 2010) and mothur (Schloss *et al.*, 2009). Data produced with high-throughput sequencing are compositional, and therefore many standard methods of analysis are not applicable. Handling such large datasets and choice of appropriate methodology for

ecological and statistical interpretation pose challenges (Weiss *et al.*, 2017). The relevance of the total number of reads obtained per sample (library size; sometimes referred to as depth of coverage) is debated, because the sequencing instrument can deliver reads only up to the capacity of the instrument (Gloor *et al.*, 2017). Moreover, there is great variation between samples, especially with respect to number of sequences, and thus reproducibility of results is difficult to achieve (McMurdie & Holmes, 2013). Therefore, to enable accurate comparisons, sequence data first need to be normalised prior to downstream analysis, which can be achieved by transformation via scaling (base analyses on the proportional abundance of each species) or by rarefaction (subsampling read counts of each sample to a common read depth) (Weiss *et al.* (2017) and references therein). Due to suboptimality and limitations, use of rarefaction is not recommended (McMurdie & Holmes, 2014). There is thus obviously a need for establishment of a gold standard for metagenomics data analyses that would enable objective comparisons of communities and increase the reproducibility of results.

Microbial diversity, namely alpha- and beta-diversity, are commonly used approaches for comparing microbial communities between two or more groups (Whittaker, 1960). Alpha-diversity represents within-sample diversity and comprises species richness (number of unique taxa in community) and species evenness (how evenly taxa are distributed). Alpha-diversity of a community is affected by perturbations and can be assessed using the Shannon, Chao1 and Simpson indices (Willis, 2019). Beta-diversity, also known as species turnover, is a measure of similarity (or dissimilarity) between samples, or the rate of change in species composition between samples (Ma, 2018; Palmer, 2004; Whittaker, 1972). Typically employed beta-diversity indices are UniFrac, Sorensen, Bray-Curtis and Jaccard (Schroeder & Jenkins, 2018; Lozupone & Knight, 2005).

2. Aims of the thesis

The focus of this thesis was leaf tissue damage as a means for proliferation and internalisation of *Escherichia coli* O157:H7 bacteria in leafy vegetables. Invasion by *E. coli* O157:H7 through double perturbation dimensions caused by leaf damage, namely landscape modification and nutrient pulses, was considered. The general aim was to identify the relationship between lesion size or leaf-scale damage and establishment and internalisation rate of *E. coli* O157:H7.

Specific objectives were to:

- Develop a robust method for detection of multiple and single-cell leaf tissue damage, and automated damage quantification and classification of lesion parameters (size, shape and location), combining image processing together with TB dye staining (Paper I).
- Quantify damage and changes in microbiota on leaves of leafy vegetables at different steps from field to bag (cultivation, machine harvest, commercial washing and packaging), using the method developed in Paper I and Illumina MiSeq sequencing (Paper II).
- Assess whether level of leaf damage has biological relevance for adhesion and internalisation of *E. coli* O157:H7 and determine the concomitant food safety consequences (Paper III).
- Investigate calcium-aided leaf fortification and its use as a candidate hurdle for foodborne illnesses risks from field to bag, by assessing whether toughness of baby spinach leaves can be increased with calcium leaf spray and damage occurrence can thus be reduced, resulting in reduced establishment and internalisation rate of *E. coli* O157:H7 on baby spinach leaves (Paper IV).

Damage caused by biotic and abiotic factors during cultivation and further mechanical damage throughout harvest and post-harvest handling are unavoidable repercussions of processing in the baby leaf value chain. It is impossible to replace these damage-causing steps without significant changes to current logistics, so it is important to gather knowledge on the current system and identify ways to enhance food safety. Treatments that can mitigate leaf damage or impede post-damage deterioration are a plausible solution.

Novel aspects of the work reported in this thesis are: (i) development of a method for leaf-scale detection and automated quantification of damage on leaf-scale; (ii) quantification of damage and changes in the leaf microbiota of leafy vegetables from field to bag; (iii) assessment of different levels of standardised artificial leaf damage, and associated changes to niche factors (*i.e*. nutrient availability, hydrophobicity, and leaf landscape) affect proliferation and internalisation of *E. coli* O157:H7 in leafy vegetables; and (iv) use of calcium fortification of baby leaves as a plausible solution for damage reduction.

New knowledge gathered in this thesis is intended for use in establishment of hurdles and measures preventing transmission of foodborne pathogens within the horticultural value network.

Table 1 lists the goals and hypotheses tested within the framework of this thesis.

Table 1. *Goals (Paper 1) and hypotheses tested (Papers II-IV)*

Paper	Goals/hypotheses
I	○ Design a method for leaf-scale detection and automated quantification of damage. ○ Select a suitable dye and dye concentration for leaf-scale damage visualisation. ○ Select a suitable set of image-processing techniques to enable adequate segmentation of damaged areas including microlesions (single cell level). ○ Validate the method.
II	○ Intact baby leaves rarely occur. ○ Baby leaf damage accumulates from field into a bag. ○ Level of leaf damage pre- and post-harvest is affected by crop species. ○ Damage morphometric and location parameters are specific to steps in the leafy vegetables value chain. ○ Post-harvest processing modifies the microbial community structure of baby leaves.
III	○ Adhesion of *E. coli* O157:H7*gfp*+ on spinach leaf surfaces is enhanced by increasing lesion area. ○ Any size of leaf wound enables *E. coli* O157:H7*gfp*+ to internalise into spinach leaf. ○ Number of internalised *E. coli* O157:H7*gfp*+ cells increases with increasing wound size and relative leaf damage, increasing consumer infection risk.
IV	○ Calcium spray enhances the structural integrity and toughness of spinach leaves. ○ Relative damage to greenhouse-grown spinach is lower on calcium-fortified leaves. ○ Calcium fortification increases resistance of leaves to artificial damage. ○ Calcium fortification indirectly results in reduced establishment and internalisation rate of *E. coli* O157:H7*gfp*+ on baby spinach leaves. ○ Water activity is lower in calcium-fortified leaves than in control leaves. ○ Microbial community structure of spinach baby leaves is not directly affected by calcium spraying.

3. Material and methods

The experimental work in this thesis focused on the four constituents outlined in Figure 1:

- Host plants: (*Spinacia oleracea*) (Papers I-IV) and Swiss chard (*Beta vulgaris* subsp. *cicla*) (Papers I-II).
- Target microorganism: *Escherichia coli* O157:H7*gfp*+ (Papers III-IV).
- Indigenous microbiota (Papers II and IV).
- Environment, from micro-scale (scrutinising interactions on plant leaves or leaf sites with and without damage) to macro-scale (considering different events pre- and post-harvest).

The experiments were performed in both laboratory and greenhouse settings and on samples from commercial field production. A tool for visualisation and quantification of leaf damage developed in Paper I was then applied in all subsequent studies in the thesis.

Table 2 displays how the different elements were investigated in Papers I-IV.

Table 2. *Experimental design and aims at glance (SE = Sweden, IT = Italy)*

Paper	Sample origin	Plant species	Treatment	Aims at glance
I	Field (SE)	Spinach Swiss chard	Manual harvest	Design of leaf-scale damage detection and quantification method.
II	Field (SE)	Spinach Swiss chard	Manual harvest Machine harvest Washing Packaging	Field to bag damage detection and quantification on leaf-scale. Field to bag leaf microbiota assessment.
III	Imported (IT)	Spinach	Artificial damage *E. coli* O157:H7*gfp*+ inoculation	Interaction between leaf damage and *E. coli* O157:H7*gfp*+ growth.
IV	Greenhouse (SE)	Spinach	Calcium leaf spray Artificial damage *E. coli* O157:H7*gfp*+ inoculation	Assessment of calcium chloride leaf spray as a candidate hurdle for foodborne illness risks. Impact of calcium spray on phyllosphere microbiota.

3.1 Experimental design

3.1.1 Field experiment (Papers I-III)

Swedish-grown leafy vegetables (Paper I-II)

Spinach and Swiss chard were grown in open fields in southern Sweden, under commercial farming practices, for four weeks. Samples from the same field were collected at baby-leaf stage (BBCH stage 13), (i) manually (manual harvest was used as a proxy for damage accumulated in the field), and after (ii) machine harvesting, (iii) washing and (iv) packaging in a commercial setting. This plant material was used for development and validation of a method for detection and quantification of leaf-scale damage

(Paper I), and assessment of leaf and lesion morphometric parameters and microbial communities from field to bag (Paper II).

Imported field-grown leafy vegetables (Paper III)

Spinach grown in Italy under commercial farming practices and harvested at baby leaf stage was used in assessments of *E. coli* O157:H7*gfp*+ proliferation on leaves exposed to different levels of artificial damage (January - May 2020). Spinach was imported by the Swedish company Vidinge Grönt AB as unwashed (raw) material. Only leaves without visible (macroscopic) symptoms of damage were selected for the experiments.

3.1.2 Greenhouse experiments with calcium (Paper IV)

Greenhouse experiments (2018-2020) were conducted on spinach plants grown in trays, in peat-based growing medium, with a 16-h photoperiod, day and night temperature of 21 °C and 16 °C, respectively, and 70% relative humidity. In initial experiments two different calcium salts were tested, namely calcium chloride dihydrate ($CaCl_2 \cdot 2\ H_2O$) and calcium sulphate dihydrate ($CaSO_4 \cdot 2\ H_2O$), with six different concentrations of spraying solution (see Table 1 in Paper IV). Leaf spray was prepared by dissolving the salts in de-ionised water (diH_2O), with diH_2O spraying as a control. In the following experiments, only 5 mM and 50 mM concentrations of $CaCl_2$ salt spray were used. The $CaCl_2$ spray was applied early in spinach development, starting from unfolding of first true leaves, and then on four subsequent occasions until baby leaf stage. For detailed spraying scheduling and applied volumes, see Table 2 in Paper IV.

Calcium foliar supplementation was tested as a pro-active measure to improve mechanical properties of growing cells/leaves. Leaves were harvested at baby leaf stage, and only first true leaves were considered in all analyses except for yield measurement, which considered the whole tray. The experiments investigated whether calcium leaf spraying increased the toughness of spinach leaves, reduced relative damage during cultivation and increased resistance of the leaves to artificial damage. Establishment and internalisation in leaves of *Escherichia coli* O157:H7*gfp*+, alpha- and beta-diversity, relative taxa abundance and core spinach microbiota, as affected by calcium spray concentration, were also examined.

3.1.3 Development of a method for automated lesion quantification (Paper I)

For accurate quantification of damage, reliable differentiation between healthy and damaged tissue is essential. However, depending on the type of damage, colour change in the wounded area does not always occur, especially with mechanical damage. The novel approach developed in Paper I consists of clearing and staining steps for damage detection (visualisation), leaf image collection, and a pipeline based on LiMu image analysis for automated lesion quantification and data export. Leaf area, damage area and number of lesions per leaf were determined. Lesions identified were classified based upon size, shape and position (Paper II).

Staining: To highlight damaged areas, whole leaves were stained with different concentrations of TB staining solution. Trypan blue dye is known as a viability exclusion dye that stains damaged and dead cells, but is not taken up by viable cells. Staining solution with 0.01% TB showed the best staining results. Detailed descriptions of staining and the preceding clearing step can be found in Paper I. The proposed staining approach enables quantification of lesions at any stage of development and without requiring symptoms in the visible spectrum due to viability exclusion staining.

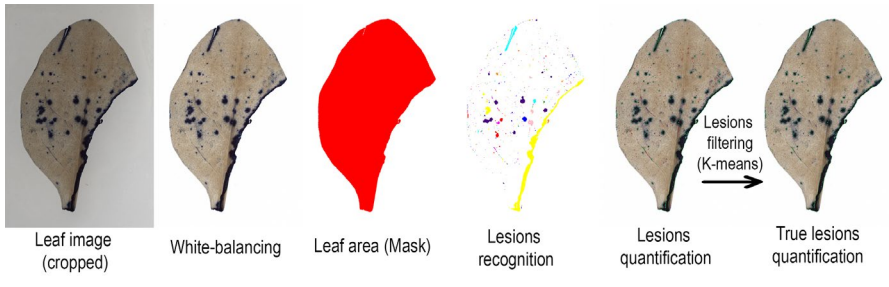


Figure 3. Example of damage quantification in the LiMu pipeline.

LiMu pipeline: LiMu image analysis was developed for the purpose of automated measurement of leaf and lesions and their size, shape and position (Figure 3). The output of LiMu image analysis is a data file containing information on: leaf area, number of lesions per leaf, individual lesion (stained) areas, height and width, eccentricity (shape), and distance from closest point to the leaf edge and midrib. The pipeline consists of five steps: (i) image pre-processing, *i.e.* finding leaf image, (ii) processing, *i.e.* finding

and segmenting regions of interest (ROIs), (iii) quantification of ROIs, (iv) data export and (v) post-filtering of segments to remove false positive lesions (Figure 4).

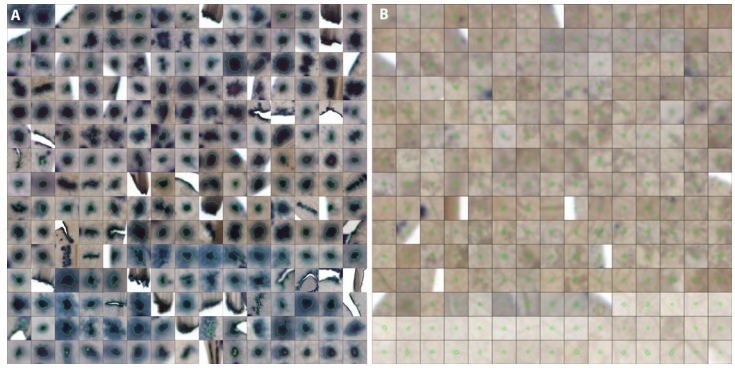


Figure 4. Classification of lesions based on parameters described in Paper I. True positive (A) and false positive (B) lesions are shown.

Size-based classification: Individual lesions were classified based on their respective size into: microlesions (1 px; single cell lesions), mesolesions (2-200 px) or macrolesions (>200 px).
Shape-based classification: Shape was characterised based on the eccentricity value of individual lesions. Lesions with eccentricity 0-0.9 were classified as round lesions and lesions with eccentricity >0.9 as cuts.
Position-based classification: Lesion spatial patterns and their relative position on leaf scale (distance from the midrib and the leaf edge) were determined. Based on this classification, lesions were classified as edge lesions, leaf blade lesions or midrib lesions.
Combined size- and shape-based classification: Eccentricity and lesion area values were combined into two size-based categories, small (<200 px; microlesions and mesolesions) and large (≥200 px; macrolesions). All lesions were then grouped into four categories considering size and shape: small round, large round, small cut, and large cut lesions.
Combined position- and shape-based classification: Position and shape parameters were combined and all lesions were divided into six categories: leaf-blade round, edge round, midrib round, leaf blade cut, edge cut, and midrib cut lesions.

Method validation: As a control for both staining and LiMu-based lesion quantification, a set of artificial damaged leaves was created (see 3.1.5). To highlight callose, which is normally found deposited in wounded areas, TB staining was coupled with aniline blue (AB) staining for microscopy-based validation. Success of staining was verified by observing damaged and stained areas macroscopically, coupled with microscopy (Figure 5). While damaged tissue was stained blue, intact tissue did not take up TB dye. Performance of the LiMu software was validated by comparison with IMAGEJ software and manual assessment. Comparisons encompassed leaf and lesion morphometric variables using artificially damaged leaf samples (positive and negative control images; see 3.1.5), and randomly selected experimental images. A detailed description of the staining and damage quantification can be found in Paper I.

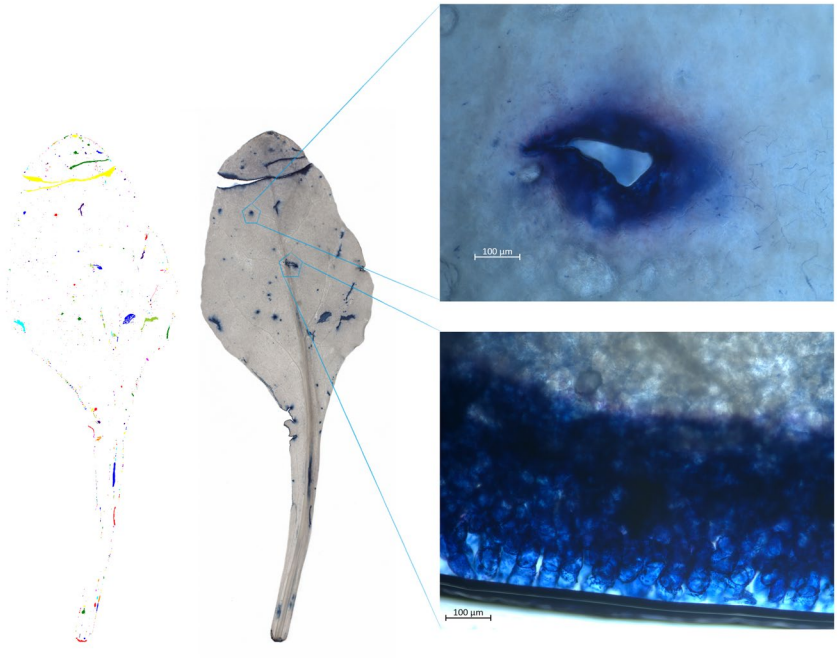


Figure 5. (Left) Example of whole-leaf staining of Swiss chard, and lesions labelling in the LiMu pipeline. (Right) Verification of lesion staining with trypan blue dye, using compound Bright Field inverted microscope. Scale-bars 100 µm.

Complete leaf clearing is critical in achieving accurate feature segmentation in the LiMu pipeline, since incompletely cleared areas will be recognised as lesions, *i.e.* false positives. To achieve complete clearing, the amount of clearing solution needs to be optimised for the amount of plant material. Some common types of mis-segmentation in LiMu are: (i) false negative lesions, (ii) false positive lesions, (iii) under-segmentation and (iv) over-segmentation.

3.1.4 Effect of production chain and foliar calcium application on baby leaf microbiota (Papers II & IV)

Microbiota of spinach and Swiss chard baby leaves grown in the open field and sampled during summer 2017 (Paper II) and spinach grown in greenhouse in the spring 2020 and sprayed with calcium spray (Paper IV) was analysed. For assessment of bacterial and fungal communities in field-to-bag experiments (Paper II), samples collected at different steps were taken from the same production lot (n=6). In the calcium fortification experiments (Paper IV), only first true leaves were used for assessment of microbial communities (n=8).

In Paper II, manually harvested leaves were sampled from 1 m^2 plots. Machine-harvested, commercially washed and packaged samples considered the entire field, and only technical replicates were made (one 10 L plastic box filled with machine-harvested or washed sample, or 200 g of packaged sample, was considered one replicate). For more information on the sequence of events related to leafy green primary production and processing, leaf matrix and environmental conditions, see Figure 1 in Paper II.

For the greenhouse-grown spinach in Paper IV, each tray was considered a replicate, and leaves were processed immediately post-harvest.

3.1.5 Experiments with artificial leaf damage (Papers I-IV)

Different levels of standardised artificial damage of known patterns were inflicted upon spinach leaves with a Derma stamp or Derma roller, mimicking dot-like lesions, or a scalpel, mimicking cut-like lesions (Papers I and III). The extent of the artificial damage was varied to provide verification for TB staining (see Additional File 6 in Paper I), and positive and negative control images for image analysis software development and verification (Figures 8 & 10 in Paper I). Four levels of damage severity were

created (undamaged, low, moderate and high damage) to assess lesion-mediated establishment success of *E. coli* O157:H7*gfp*+ (Figures 1 & 3-5 in Paper III). Standardised artificial damage in the form of scratches made using a dishwashing sponge with a handle was used to assess whether calcium spraying increased the resistance of spinach leaves to artificial damage and thus reduced establishment success of *E. coli* O157:H7*gfp*+ (Figures 5 & 6 in Paper IV).

3.2 Methodology

3.2.1 Detection and quantification of damage on leaf-scale

In Paper I, a method for leaf-scale detection, quantification and description (classification) of lesions on leaves of leafy vegetables was developed (see also 3.1.3). This method was used thereafter as the damage quantification approach for both naturally occurring damage (Papers I, II & IV) and artificial damage (Papers I, III & IV). A detailed step-wise description of the method can be found in Paper I, while Figure 6 provides an illustration of the method steps.

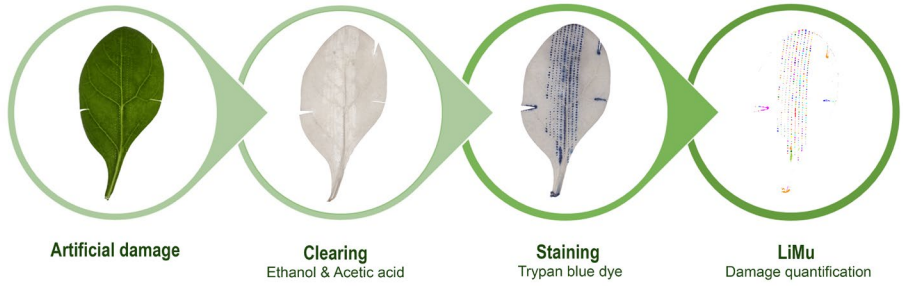


Figure 6. Steps in the method for detection and quantification of damage on leaf scale.

3.2.2 Microscopy and micro X-ray fluorescence spectroscopy

Verification of TB staining, and dual TB and AB staining, was done using an inverted bright field microscope (Carl Zeiss Microscopy) (Paper I). Visualisation of epiphytically residing bacteria, including artificially added *E. coli* O157:H7, on the leaf surface and lesion edges was done using a high-resolution scanning electron microscope (SEM; HITACHI SU3500) (Paper

III). To visualise GFP-tagged *E. coli* O157:H7 internalised within artificial lesions (Paper III), a Zeiss LSM880 confocal laser scanning microscope (Carl Zeiss Microscopy) was used. For this, GFP and chlorophyll were excited at 488 nm and 561 nm, and the emissions were collected using 493-531 nm and 661-701 nm filters, respectively. A cellular force microscope (CFM) set-up, consisting of a MEMS-based capacitive force sensor (FTS1000; FemtoTools AG) to which a micro-indenter is attached, was used to evaluate the impact of calcium spraying on apparent stiffness of baby spinach leaves (Paper IV). Two-dimensional elemental distribution maps were obtained with a Bruker M4 Tornado benchtop micro-energy-dispersive X-ray fluorescence (μ-XRF) spectrometer (Bruker Nano GmbH) visualising differences in calcium and other elements (elemental mapping) between calcium-treated and control leaves (Paper IV). Full details of sample preparation and imaging protocols can be found in Papers I, III and IV.

3.2.3 Preparation of *Escherichia coli* O157:H7*gfp*+ inoculum, and inoculation and extraction technique

An *E. coli* O157:H7 strain lacking virulence factors verotoxin-1 and -2, but positive for intimin (adherence) encoding gene (*eae*), was used for the experimental work in Papers III and IV. This strain hosts the pGLO plasmid, which encodes for ampicillin resistance and GFP, coupled with an arabinose responsive promoter. The *E. coli* O157:H7*gfp*+ inoculum preparation protocol used was that devised by El-Mogy and Alsanius (2012), as described in Papers III and IV. In brief, the bacteria were grown in lysogeny broth supplemented with ampicillin (100 μg mL^{-1}) and arabinose (0.2%) for about 18 h at 37 °C. The final concentration of inoculum solution was log 6 CFU *E. coli* O157:H7*gfp*+ × mL^{-1} in 0.085% NaCl.

Spinach leaves without (Paper III) and with (Paper IV) calcium fortification and artificial damage were individually dip-inoculated by 15 s immersion in the inoculum solution using forceps. The inoculated strain was re-extracted from individual spinach leaves directly post-inoculation at 0 dpi, and after incubation in sealed plastic bags at room temperature for 24 h (1 dpi) and 48 h (2 dpi) (Papers III and IV).

Re-extraction of *E. coli* O157:H7*gfp*+ comprised five steps, namely (i) detachment, (ii) washing, (iii) surface sanitation, (iv) leaf imprint and (v) maceration. Details of the successive extraction steps can be found in Papers III and IV. To measure the total *E. coli* O157:H7*gfp*+ counts from the

different extraction steps, serial dilutions of supernatant or leaf macerate from each sample were plated in triplicate on Luria Bertani agar, supplemented with ampicillin (100 μg mL^{-1}) and arabinose (0.2%). Plates were incubated at 37 °C for approximately 18 h, and colonies were enumerated under UV-light. Leaf imprint plates (Paper III) were incubated and enumerated in the same manner.

3.2.4 Culture-independent analyses of baby leaf microbiota as affected by field-to-bag steps and foliar calcium application

The phyllosphere microbial community of manually harvested unwashed leaves (control), machine-harvested, commercially washed leaves and packaged leaves (24 h post-packaging) (Paper II), and calcium-sprayed leaves (Paper IV) was assessed. Microbial DNA was extracted with the ZymoBIOMICS™ DNA Miniprep kit, as described in Papers II and IV.

Bacterial and fungal communities were analysed with 300 bp paired-end read Illumina MiSeq V3 at LGC Genomics GmbH (Berlin, Germany). For bacterial communities, the 16S ribosomal gene was targeted using 799F and 1115R primers. To evaluate the fungal communities, the ITS region was targeted with ITS7F and ITS4R primers. The data generated were quality checked and pre-processed by the bioinformatics service of LGC Genomics GmbH (Berlin, Germany).

4. Selected results and discussion

Wound-aided establishment of the foodborne pathogen *E. coli* O157:H7*gfp*+ as a leaf (i) epiphyte and (ii) endophyte (endosphere) was studied in this thesis. Specifically, interactions between leaf damage size and invasion success of *E. coli* O157:H7, for which plants are not considered to be a primary habitat, were examined. The strength of the nutrient pulse caused by wounding was not determined. The information obtained on leaf-scale damage quantification (Paper I) along the leafy vegetable value chain (Paper II), and on the relationship between amount of damage and numbers of *E. coli* O157:H7 (Paper III), is valuable input when assessing related risks of consumer infection with human pathogens. As contamination with human pathogens in pre-harvest steps cannot be completely avoided (Mogren *et al.*, 2018), leaf integrity is imperative for food safety. Hence treatments applied throughout cultivation that can mitigate damage or impede deterioration are a plausible way to lower risks of consumer infection with human pathogens (Paper IV). This thesis considered changes in native microbiota and *E. coli* O157:H7 invasion as a result of dual perturbation dimensions caused by leaf damage, namely nutrient pulse and landscape modification, and examined new hurdles for establishment of *E. coli* on baby leaf surfaces.

4.1 The power of perturbations

4.1.1 Wounds as a source of nutrients (Papers II-IV)

Damage as a perturbation in the leaf habitat affects the plant host, the native microbiota and the success of invasion. A damaged leaf experiences changes in topography and surface area, and increases in nutrient and water availability on the leaf surface (Simko *et al.*, 2015; Aruscavage *et al.*, 2010; Brandl, 2008; Lindow & Brandl, 2003; Leveau & Lindow, 2001a; Mercier & Lindow, 2000; Beattie & Lindow, 1995; Tukey & Morgan, 1963). These disturbances modify the habitat, impacting the nature of relationships within

the holobiont with respect to resource competition, resilience and diversity-invasion suppressiveness of native microbiota.

Damaged leaves have a greater surface area that can be colonised at the damaged site and a pool of readily available nutrients that determines leaf carrying capacity (Remus-Emsermann *et al.*, 2012). Damage to leaves thus improves conditions for native and non-native microorganisms to explore and establish on and in the leaf (Figure 7). However, the relationship between accumulated damage and nutrient concentration may not be linear. Thus it is reasonable to assume that the extent of a leaf-scale nutrient pulse depends on the number of lesions and their size, depth and position and time since wounding. Midrib damage can be assumed to result in more prominent nutrient pulses than equivalent damage to other parts of the leaf blade (Paper II). The amount of leachate will also depend on whether the lesion is induced pre- or post-harvest and on the age of the leaf, due to alterations in the physicochemical phyllosphere environment (anabolism *versus* catabolism) (Mogren *et al.*, 2018).

Damaged leaves comprise both damaged and intact areas, as the distribution of wounds and their size and position on macro-scale (leaf) are heterogeneous and unpredictable (Papers I and II). Thus the amount of released nutrients on micro-scale (lesion) is also unpredictable. Intact and injured tissue heterogeneity on macro scale (leaf containing both injured and intact areas) (Paper II) and micro scale (some microorganisms on the same injured leaf have access to released water and nutrients at microsites on leaf scale, while others do not) may affect proportional loss/establishment success rate of the contaminant (or inoculated population in experiments). In dip-inoculation at high inoculation densities (mode of inoculation in Papers III and IV), all leaf sites could be expected to be colonised, so the nutrient pool will determine the carrying capacity (Remus-Emsermann *et al.*, 2012). At low inoculation densities, some sites at macro scale will remain uncolonised, creating empty niches, and carrying capacity will not be reached (Remus-Emsermann *et al.*, 2012). At higher inoculation density, more bacterial cells will be available per unit area and reproductive success will decline (Remus-Emsermann *et al.*, 2012), but this is again a function of micro-scale nutrient availability.

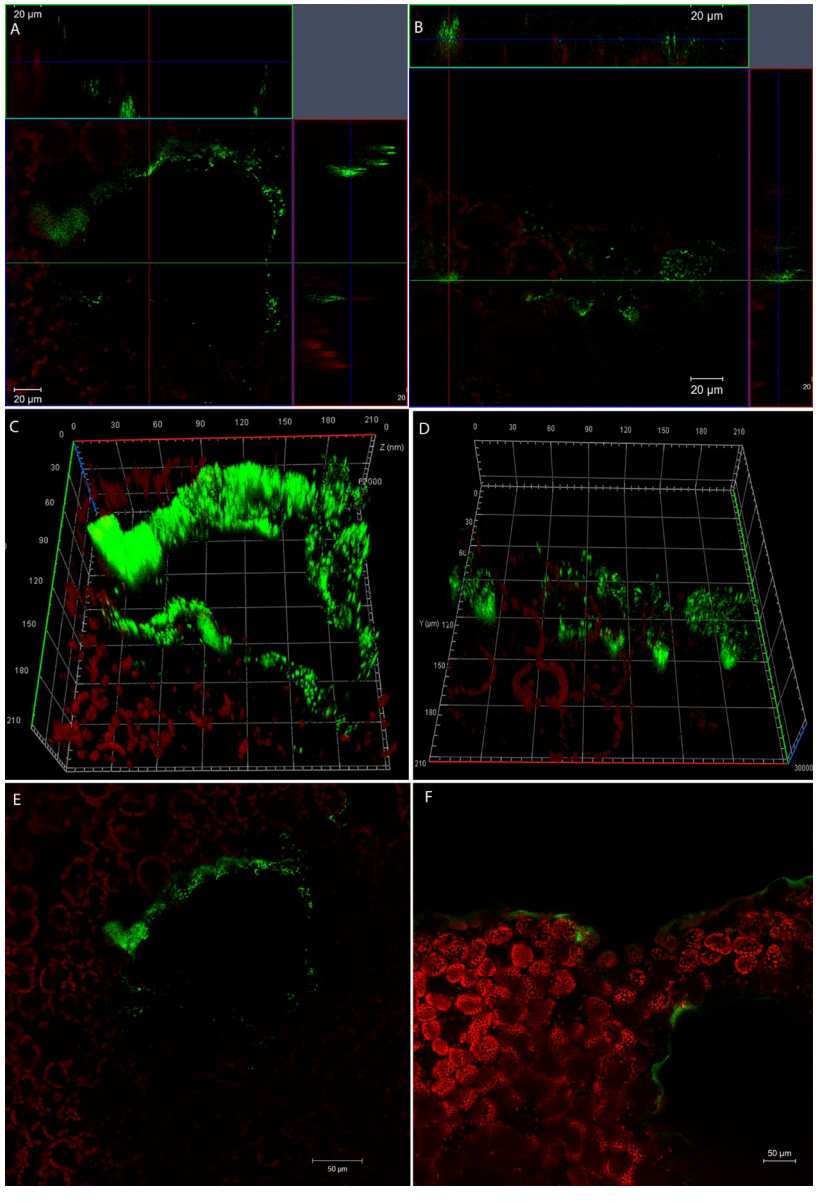


Figure 7. Colonisation of artificially damaged spinach (*Spinacia oleracea* L.) leaves by *E. coli* O157:H7 bacteria labelled with GFP. Artificially damaged leaves were dip-inoculated with 10^6 CFU × mL^{-1} *E. coli* O157:H7 bacteria and observed at 2 dpi. Z-stack orthogonal projections of lesions and adjacent tissue (A-B), 3D z-stack projections (C-D) and tile scans (E-F) are shown. Scale bars indicate 20 µm (A-B) and 50 µm (E-F). Red colour indicates autofluorescence of the chlorophyll within chloroplasts, green fluorescence indicates cells of *E. coli* O157:H7*gfp*+ bacteria.

Previous studies have demonstrated that biotic and abiotic damage supports persistence, growth and internalisation of human pathogens within tissues of leafy vegetables (Hartmann *et al.*, 2017; Scott *et al.*, 2017; Simko *et al.*, 2015; Aruscavage *et al.*, 2010; Aruscavage *et al.*, 2008; Brandl, 2008; Seo & Frank, 1999). Bacterial invasion on plants has been studied in detail (Melotto *et al.*, 2014; Van Elsas *et al.*, 2012; Barak *et al.*, 2011; Underwood *et al.*, 2007; Melotto *et al.*, 2006), and several studies have compared altered nutrient availability (Berg & Koskella, 2018; Mallon *et al.*, 2015a; Mallon *et al.*, 2015b), revealing contrasting invasion success. In Paper III, *E. coli* O157:H7*gfp*+ proliferated two- to three-fold more on moderate- and high-damage leaves than on undamaged and low-damage spinach. This can be attributed to nutrient release from damaged tissue (see 1.3.1). However, no attempts were made in this thesis to quantify released nutrients or changed nutritional conditions. Such information would be needed to fully evaluate the interactions between leaf damage and foodborne pathogens in leafy vegetables, for hurdle development and food safety assessment (see 4.2).

The first colonisers of the phyllosphere determine the fate of subsequent colonisers (Maignien *et al.*, 2014). From this perspective, the fate of artificially added *E. coli* O157:H7*gfp*+ on baby spinach leaves is determined by the native microbiota. This is probably true for 'intact' leaves, but is unlikely to apply when high-density inoculum is used and when the leaf is damaged. More importantly, damage changes the conditions for 'competitors', as nutrients and water released from damaged sites create opportunities for the invader (Enders *et al.*, 2020), since according to increased resource availability hypothesis the success of invasion increases with increasing availability of resources (Sher & Hyatt, 1999). It is worth noting that high inoculum densities of *E. coli* O157:H7*gfp*+ used in all studies in this thesis (Papers III and IV) corresponded to that created by severe shedders under natural conditions (Chase-Topping *et al.*, 2007; Omisakin *et al.*, 2003). High inoculum densities were necessary to conduct the experiments under controlled conditions and to follow the inoculated strain in the various niches.

Leaf damage is a disturbance. Erlacher *et al.* (2014) demonstrated that, particularly after intermediate disturbances to plants such as disease attacks, Enterobacteriaceae are enhanced. Even in the case of a highly diverse native community, a wound-mediated nutrient pulse creates more opportunities for invading species to thrive. Thus microbial diversity-facilitated invasion

resistance of the native community (Carlström *et al.*, 2019; Mallon *et al.*, 2015a; Mallon *et al.*, 2015b) would not be relevant for an invading microorganism when perturbation events occur. The increased resource availability hypothesis then applies and conditions are in favour of establishment of enteric pathogens, thereby increasing food safety risks. However, native communities will also be able to utilise the nutrients leached at the wound site and, depending on the number of windows of opportunity and level of niche overlap/niche partitioning and resilience, post-perturbation co-existence of native species will be decided (see section 1.3.3).

In the experiments described in Papers III and IV, *E. coli* O157:H7*gfp*+ established well on dip-inoculated detached spinach baby leaves. Its growth and spreading within the tissue depended on the damage level, but the impact of an artificially added strain on the resident community was not assessed (Paper III). Driven by the favourable conditions, artificially added *E. coli* O157:H7*gfp*+ preferentially colonised the edges of fresh wounds (Figure 5 in Paper III). Leaf damage significantly increased the numbers of *E. coli* O157:H7*gfp*+ retrieved from macerate at 1 and 2 dpi, following dip inoculation of entire single leaves. However, the proliferation pattern varied depending on relative leaf area damaged and size of individual lesions. The larger the lesion, the more *E. coli* O157:H7*gfp*+ colonised lesion edges in three dimensions. However, *E. coli* O157:H7*gfp*+ internalised into spinach leaves irrespective of the wound size, and even undamaged spinach leaves allowed internalisation, probably via natural openings or microscopic damage. Higher damage levels led to deeper *E. coli* O157:H7*gfp*+ invasion and dispersal to adjacent undamaged areas (Figure S7 and Video S2 in Paper III).

Incubation time also played a role in the numbers of *E. coli* O157:H7*gfp*+ retrieved from spinach surface (washed-off fractions), but not in the macerate, with a slight decrease from 1 to 2 dpi, possibly as a result of nutrient exhaustion on the phylloplane. However, it is possible that the decline in *E. coli* O157:H7 numbers retrieved at 2 dpi could be a result of plasmid loss in a non-selective leaf surface, or lack of plasmid expression due to absence of arabinose as a promoter. At single cell resolution, Remus-Emsermann and Leveau (2010) showed that reproductive information is lost beyond reproductive success after five doublings, due to dilution of GFP, so any contribution of immigrants that have replicated more than five times will be underestimated. The decline noted in Paper III did not have biological

significance, however, and thus did not affect the overall conclusions, especially due to very low infectious dose of *E. coli* O157:H7. Future studies should seek to verify these findings with a more suitable system for detection *in planta*, such as constitutively expressed fluorescent protein genes that are chromosomally inserted.

4.1.2 Environmental perturbations (Papers II and IV)

Leaves of leafy vegetables are continually fluctuating entities exposed to diverse biotic and abiotic factors that affect the integrity of the plant matrix and the fate of associated microorganisms along the value chain. Under the adverse conditions found in the phyllosphere (see 1.3.1), microorganisms are driven to survive and, if conditions are more affluent, available nutrients are used for proliferation (Lugtenberg *et al.*, 2002). Therefore, microorganisms need to have a level of plasticity and adapt to the phyllosphere environment (Leveau, 2006; Beattie & Lindow, 1999). The environment acts as a filter that is decisive for the fate of microorganisms (Leveau (2019) and references therein).

In Sweden, leafy vegetables, such as spinach and Swiss chard, are grown in the open field from April to September, with a crop cycle length of 30 days. They are machine-harvested at baby leaf stage, and then washed and packaged in processing plants, distributed and displayed in retail outlets. Critical steps in the value chain, relevant for damage and evaluation of dynamics in the microbiota investigated in this thesis (Paper II) were: primary production (manual harvest), machine harvesting, washing and packaging. During cultivation of leafy vegetables (pre-harvest) anabolic processes occur (plant growth, photosynthesis, evapotranspiration, nutrient uptake). These processes are interrupted at harvest, followed by ageing and catabolic processes post-harvest (deterioration, respiration, transpiration) (Mogren *et al.*, 2018). Such changes result in physicochemical alterations in the phylloplane. The pre-harvest stage is characterised by diurnal fluctuations in temperature (5-27 °C), moisture, exposure to UV-light and relatively static physicochemistry of the phylloplane (Schlechter *et al.*, 2019). For greenhouse-grown produce (Paper IV), environmental factors are less variable compared with field growing, but diurnal fluctuations are still present. Immediately post-harvest, leafy vegetables enter the cold chain, where temperature fluctuations are minimal (2-7 °C), surface moisture increases and the level of CO_2 in bagged produce increases, while O_2 is

consumed by respiration in packaged leaves. Cold chain conditions are maintained until the produce reaches the consumer (Mogren *et al.*, 2018). Washing and subsequent drying results in altered conditions of the leaf surface due to cooling (4 °C), and drying by exposing washed plant material for a short time to high (35 °C) and low (2.5 °C) temperature (Grudén *et al.*, 2016) (Figure 1 in Paper II). In this thesis, bacterial, but not fungal, diversity was found to be higher in field-grown spinach (Figure 10 in Paper II) than in greenhouse-grown spinach (Figure 8 in Paper IV). This difference was expected, because field-grown plants are exposed to diverse environments, aerosols and dust (including airborne bacteria), and are thus more likely to harbour different bacterial species than indoor-grown plants (Maignien *et al.*, 2014; Williams & Marco, 2014). Williams and Marco (2014) demonstrated 10- to 100-fold higher numbers of bacteria and significantly higher bacterial diversity on field-grown, compared with laboratory-grown, lettuce plants. Diversity of microbial phyllosphere communities at field level is higher than in cold-chain associated steps post-harvest (Rosberg *et al.*, 2020). Entry into the cold chain results in changes in population dynamics (decrease in alpha-diversity and taxa-relative abundance), and Pseudomonadaceae and Enterobacteriaceae are favoured (Rosberg *et al.*, 2020). No drastic change in alpha-diversity of washed produce was observed in this thesis, but relative abundance of Pseudomonadaceae and Enterobacteriaceae increased, while proportional Moraxellaceae abundance declined, on washed and packaged samples (Figures 10 and 12 in Paper II).

Although not simultaneously investigated in this thesis, species richness, nutrient availability and use are important variables for the diversity-invasion hypothesis (see 1.3.3). Reduced diversity of a community may ease establishment of invading microorganisms (Mallon *et al.*, 2015b). Due to contact with soil and harvesting equipment and mixing with leaves from other parts of the same field, or from other fields, during washing, opportunities for cross-contamination increase (Luna-Guevara *et al.* (2019); Gil *et al.* (2015) and references therein). With increasing damage at different steps of the leafy vegetable value chain (Figures 3 & 4 in Paper II), success of invasion may increase as a result of increased availability of resources (Sher & Hyatt, 1999). This will weaken the biodiversity-invasion relationship in microbial communities (Mallon *et al.*, 2015a) and favour proliferation of opportunistic, fast-growing, psychrophilic (grow at more rapid rate at, or below 7.2 °C) microorganisms (Witter, 1961).

The optimum temperature range for *E. coli* O157:H7 is 21-37 °C, with a minimum temperature of 15 °C and a maximum of 45 °C (Mogren *et al.* (2018), and references therein). Temperature affects the growth kinetics of *E. coli* O157:H7, namely lag phase and growth rate (Lee *et al.*, 2019). For example, *E. coli* O157:H7 grown in pure culture at 15 °C has a substantially prolonged lag phase and slower growth rate than *E. coli* grown at 37 °C (Duffy *et al.*, 1999). In a study where final *E. coli* O157:H7 cell numbers were not significantly different, the length of the lag phase increased with decreasing temperature, with an observed incubation time until the stationary phase was reached of 6 h (at 37 °C), 12 h (25 °C) and 42 h (15 °C) (Lee *et al.*, 2019).

The amounts of nutrients released in different steps of the leafy vegetable value chain was not investigated in Paper II. However, as discussed above, factors other than nutrient availability also have an impact on *E. coli* O157:H7 establishment, such as temperature, pH, water activity (a_w) and diversity and abundance of native microbiota (see 1.3.1-1.3.3). Different probabilistic models for *E. coli* growth have been proposed. For example, Presser *et al.* (1998) proposed a model with a_w limiting the growth of *E. coli* in synergy with pH and temperature. The ability of enteric pathogens to colonise and persist in lesions during cold-chain handling (washing, packaging, distribution, retail, display) needs further investigation. Given that contamination with human pathogens cannot be abolished, maintenance of cold chain conditions is essential to prevent the growth of *E. coli* O157:H7.

4.1.3 Processing and food safety (Papers I-III)

Plant-microbe interactions are complex and changes in the abundance of individual community members can result in non-linear changes to the entire microbiome composition, and ultimately to negative effects on plants and humans as consumers (Erlacher *et al.*, 2014). Changes in tissue integrity in leafy vegetables may yield successional shifts in the leaf microbiome, in addition to creating entry points for human pathogens to invade the leaf interior.

Considering ecological concepts, the main modifiers of establishment of *E. coli* O157:H7 on plants are the resources available at the time of invasion (Enders *et al.*, 2020), and the structure and diversity of the resident microbiota (Klerks *et al.*, 2007). Contamination of leafy vegetables is

unpredictable and there are different critical steps within the value chain from primary production to consumer (Mogren *et al.*, 2018; Allende *et al.*, 2017; Castro-Ibáñez *et al.*, 2017; Gil *et al.*, 2015; Gómez-López *et al.*, 2013; Medina *et al.*, 2012; López-Gálvez *et al.*, 2010; Gil *et al.*, 2009; López-Gálvez *et al.*, 2009). However, *E. coli* O157:H7 cannot actively invade plant tissue but relies on natural openings and damaged sites for internalisation (Erickson *et al.*, 2019; Hartmann *et al.*, 2017; Wright *et al.*, 2017; Deering *et al.*, 2012; Aruscavage *et al.*, 2008; Brandl, 2008). Given the low infectious dose of *E. coli* O157:H7, in order to accurately assess food safety risks in the value chain, all potential invasion sites need to be detected and quantified.

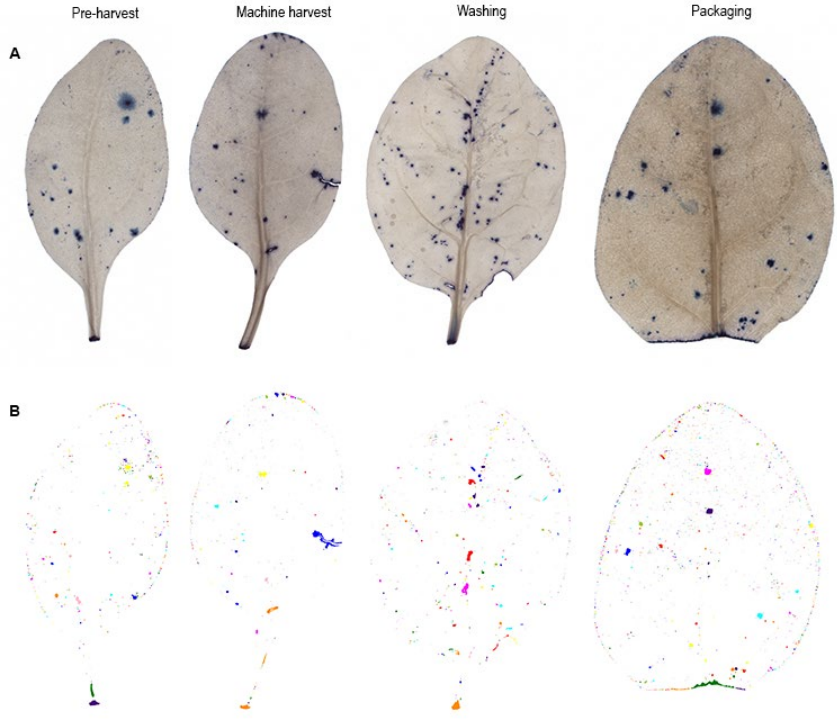
With only few exceptions, all baby leaves grown outdoors are already damaged during cultivation and harbour a range of lesions in sizes ranging from microscopic (single cell lesions) to shredded leaves (Papers I-II). Baby leaves accrue additional damage during machine harvesting and post-harvest processing. Lesion area increases from field to bag, at rates depending on plant species and leaf age. Most lesions are round and medium-sized, but severe cuts to leaf edge and midrib are inflicted during harvesting and post-harvest processing (Figures 1-4 in Paper II). Directly in the wounded area, conditions change from hydrophobic to hydrophilic, allowing microorganisms to adhere *e.g.* in the field or during harvesting, washing or drying.

Contamination with human pathogens can occur at various steps from field to table (Machado-Moreira *et al.*, 2019; Matthews, 2013). Harvesting and processing operations provide opportunities for cross-contamination, increasing the likelihood of produce coming into contact with contaminants (Grudén *et al.*, 2016; Buchholz *et al.*, 2012; Fallon *et al.*, 2011). Results regarding the effect of washing on reduction of overall microbial load are somewhat conflicting (Rosberg *et al.*, 2020; Gil *et al.*, 2009), indicating that standard baby leaf processing practices post-harvest are not sufficient for complete elimination of enteric pathogens (Rosberg *et al.*, 2020). Hence accumulation of lesions during processing increases food safety risks (Table 1 in Paper III). In this context, it is relevant to consider the incidence of viable but not culturable cells (VBNC), which are incapable of cell division. VBNC is used as a survival strategy by microorganisms in response to unfavourable environmental conditions, such as environmental stress or starvation (Xu *et al.*, 1982), which can occur in the phylloplane. Their presence introduces uncertainty in risk assessments for food-borne pathogens, since it is unknown

whether such cells will emerge from their VBNC state on entering the human body and gaining access to a more suitable environment (Cocolin *et al.*, 2013).

Processing-induced damage causes changes on macro scale (leaf) and micro scale (lesion), and thus not all parts of a leaf are affected in the same manner. Even for two leaves with the same relative damage, there can be differences in the number, size and distribution of lesions across the leaf blade and the lesion area (Figure 8). Knowledge of how these differences affect food safety is emerging. The results obtained in this thesis suggest that the size of a lesion is a relevant variable for colonisation and internalisation, and that adhesion of *E. coli* O157:H7 to the spinach leaf surface is enhanced by increasing total lesion area (Paper III). This was confirmed by higher washed-off fraction of cells at 0 dpi from moderately and highly damaged leaves compared with undamaged and low-damage leaves (Figure S3 in Paper III). This is potentially a result of increased surface area and breaches to the hydrophobic cuticle layer, allowing the damaged area to become more conductive to inoculum attachment. The level of damage applied corresponded to damage normally occurring in packaged spinach leaves (Paper III). A limitation of the study in Paper III was that it was performed on whole leaves (macro-scale) and not individual lesions (micro-scale), and that leaf size varied within and between treatments. However, the results of the risk assessment revealed that at high inoculum densities, level of leaf damage was not relevant, as even macroscopically intact leaves represented an infection risk due to the low infectious dose of *E. coli* O157:H7 (Table 1 in Paper III).

Species richness (Mallon *et al.*, 2015a; Van Elsas *et al.*, 2012) and evenness (Vivant *et al.*, 2013) are negatively correlated with the abundance of invading microorganisms. In Paper II, abundance of Pseudomonadaceae, especially the genus *Pseudomonas* comprising diverse spoilage microorganisms and plant pathogens, increased during washing, while Enterobacteriaceae showed a similar trend (Paper II). Damage introduced during processing could provide empty niches (competition-free nutrients) that support establishment of an invading microorganism, even in a well-established and diverse microbial community (Carlström *et al.*, 2019).



C

Image number	Treatment	Leaf age	Leaf area (cm^2)	Lesions area (cm^2)	Damage (%)	Lesions (n)
447	Pre-harvest damage	2nd true leaf	12	0.16	1.35	220
831	Machine harvest	1st true leaf	26	0.35	1.36	303
1319	Washing	1st true leaf	26.5	0.37	1.39	443
1509	Packaging	1st true leaf	30	0.41	1.37	564

Figure 8. Damage to spinach leaves from field to bag (A), quantified using the LiMu image analysis pipeline (B). Basic leaf and lesion parameters were obtained as LiMu analysis output (C).

In future studies, controlled experiments with single lesions of different sizes inoculated with increasing *E. coli* inoculum strength should be performed. Changes in niche at the micro and macro scale and number and area of micro-niche perturbations (lesions) are factors that need to be considered for *E. coli* O157:H7 invasion success as an epiphyte and endophyte. Thus, knowledge about quantity of lesions associated with different steps in the value chain, and lesion-mediated fluctuations in

resource availability on leaf scale, may help predict foodborne illness risks along the leafy vegetable value chain.

4.2 The power of hurdles

There is no unique solution that ensures elimination of human pathogens in horticultural value chains, but a hurdle approach combining several mitigation approaches with synergistic effects could be an alternative for controlling human pathogens on leafy vegetables from field to consumer (Mogren *et al.*, 2018).

4.2.1 Intact leaf as a hurdle (Papers I-III)

Leaf micro-scale heterogeneity in terms of conditions that a bacterium can experience (topography, nutrient and water availability) is an established fact (Doan *et al.*, 2020a; Doan *et al.*, 2020b; Esser *et al.*, 2015; Leveau, 2015; Remus-Emsermann *et al.*, 2012; Beattie, 2011; Leveau & Lindow, 2001a; Beattie & Lindow, 1995). Leaves have a unique local carrying capacity (Remus-Emsermann *et al.*, 2012), *i.e.* a combination of different biotic and abiotic factors and local nutrient availability that determines the probability of colonisation success of leaf immigrants (Remus-Emsermann & Schlechter, 2018). The epidermis is covered with a thin hydrophobic layer of cuticle, which makes leaves water-repellent (Tukey, 1970). At micrometre scale, the leaf has a heterogeneous topography with two main abundant features, elevations in the form of epidermal cells and grooves at the junctions between those cells (Remus-Emsermann & Schlechter, 2018). Leaves have a myriad of microsites with unique local conditions (Remus-Emsermann *et al.*, 2012), and it has been discussed whether individual plant cells should be considered as individual entities (Libault *et al.*, 2017). Leaf veins, stomata, trichomes and hydathodes are recognised as microhabitats for microorganisms (Remus-Emsermann & Schlechter, 2018).

Phyllosphere bacteria require access to resources to sustain or grow populations. Phyllosphere nutrients are products of photosynthesis and are deposited by leakage and guttation across the cuticle (Singh, 2014; Leveau & Lindow, 2001a; Morgan & Tukey, 1964; Tukey & Mecklenburg, 1964). However, on the surface of an 'intact' leaf, nutrients and water are characterised by low abundance and heterogeneous distribution (Remus-Emsermann & Schlechter, 2018; Axtell & Beattie, 2002; Leveau & Lindow,

2001a; Joyner & Lindow, 2000; Mercier & Lindow, 2000). Microscopy studies have revealed that bacteria are less prevalent on the elevated epidermal cells and more established in epidermal cell grooves, at the base of trichomes and in stomata due to increased resources (Esser *et al.*, 2015).

However, intact leaves are a rare occurrence according to the findings in this thesis (Paper I) showing that all leaves are damaged, although they may look macroscopically intact. Microlesions were detected after staining with TB dye even in control leaves (Paper I). From this perspective, it would be more accurate to use the term 'intact leaf part' or 'macroscopically intact leaf', instead of 'intact leaf'. Retention of *E. coli* O157:H7*gfp*+ inoculum was lower on leaves without artificial damage than on artificially damaged leaves at 0 dpi, as shown by the lower number of *E. coli* cells retrieved at 1 and 2 dpi (Figures 5 & S3 in Paper III). This could be due to the hydrophobic nature of intact cuticles making them less conductive for inoculum adherence than damaged leaf surfaces, which have a greater exposed surface area and more nutrients to support microbial adaptation and growth. In Paper III, artificially introduced *E. coli* O157:H7 was located around stomata and epidermal cell wall junctions on non-damaged leaves at 2 dpi (Figure S5 in Paper III). Those sites are reported to be preferred colonisation sites for epiphytes (Vorholt, 2012; Lindow & Brandl, 2003; Beattie & Lindow, 1995). This is probably due to topography-driven lateral leaf moisture distribution (Doan *et al.*, 2020b), with longer water retention and subsequently increased nutrient availability at such sites. Artificially added *E. coli* O157:H7*gfp*+ was also found internalised in sub-stomatal cavities on undamaged leaf areas, at about 5 µm depth, while *E. coli* was found at 40-45 µm depth in wounded areas (Figures S5 & S7 in Paper III).

It is known that *E. coli* does not form the pectinases necessary to break down pectin in epidermal cell walls, and thus its survival and proliferation depend on it accessing sites with high nutrient abundance (Jeter & Matthysse, 2005). Therefore, the fate of individual plant cells during colonisation of leaves is determined by stochasticity, namely pathogen cell location on the phylloplane and with how many other cells arrive in the vicinity (Remus-Emsermann *et al.*, 2012). Intact leaf areas act as natural barriers, or hurdles, for organisms that cannot overcome the plant defence system. However, due to the tenderness of baby leaves and the different biotic and abiotic factors to which leafy vegetables are exposed along the value chain, tissue integrity cannot be guaranteed.

4.2.2 Calcium fortification as a hurdle (Paper IV)

Calcium is a macronutrient that strengthens plant cell walls (Alandes *et al.*, 2006; White & Broadley, 2003) and improves cellular structural integrity (Matoh & Kobayashi, 1998; García *et al.*, 1996; Demarty *et al.*, 1984). Calcium ions form cross-links between free carboxyl groups on pectin chains, resulting in strengthened cell wall and increased tissue stability and structural integrity (Cybulska *et al.*, 2011; Matoh & Kobayashi, 1998; García *et al.*, 1996; Demarty *et al.*, 1984; Van Buren, 1979). Considering that nutrient leaching, proliferation and internalisation of enteric pathogens are enhanced at injury sites on leaves (Hartmann *et al.*, 2017; Scott *et al.*, 2017; Simko *et al.*, 2015; Aruscavage *et al.*, 2010; Aruscavage *et al.*, 2008; Brandl, 2008), implementation of horticultural production procedures that increase the resilience of plant material to wounding could be a plausible solution in controlling the human health risks.

The work in Paper IV assessed whether calcium-based pre-harvest fortification can reduce damage to leafy vegetables and thus act as a candidate hurdle in managing foodborne illness risks. The results showed that foliar application of 50 mM $CaCl_2$ spray throughout cultivation significantly increased leaf calcium content in spinach foliage, while biomass formation, yield, leaf area and chlorophyll fluorescence were not affected. At concentrations higher than 50 mM, however, $CaCl_2$ spraying damaged the foliage of spinach (Figure S1 in Paper IV). Leaf tissue toughness increased significantly as a result of calcium fortification, irrespective of the calcium spray concentration. The calcium-induced strengthening can be explained by the influence of calcium on primary cell walls and middle lamella (Morris, 1980). Leaf tissue strength and stiffness increase in plants grown with added calcium (Newman *et al.*, 2005), and thus greater firmness and resistance to mechanical damage can be achieved when foliar fertilisers contain $CaCl_2$ (Ochmian, 2012). Whether the resulting increase in leaf firmness affects the consumer's experience of texture and taste has yet to be investigated.

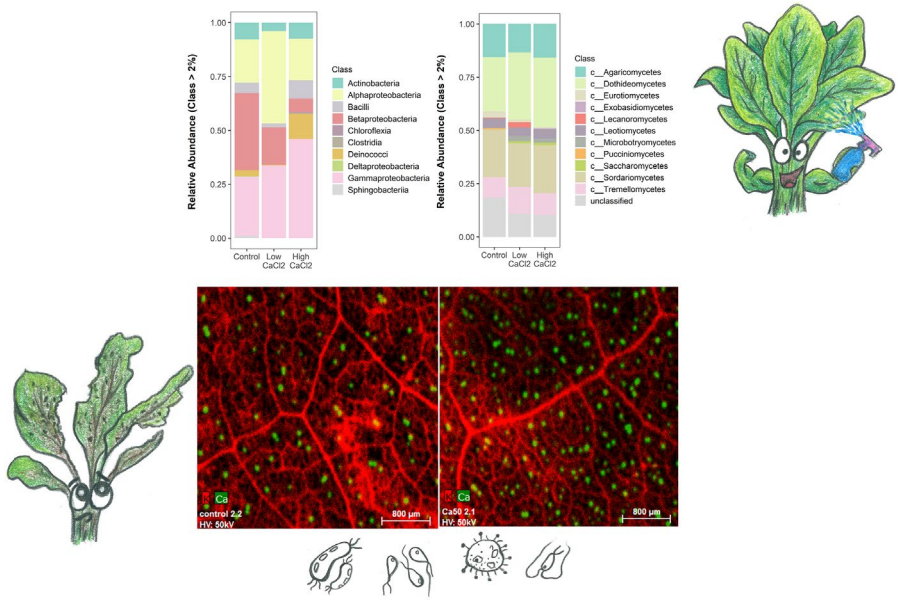


Figure 9. Baby spinach leaves sprayed with (left) de-ionised water (control) and (right) 50 mM calcium chloride ($CaCl_2$) spray. $CaCl_2$-sprayed leaves have fewer lesions and higher numbers of calcium particles (heatmaps; green calcium; red potassium). Stacked bar plots show relative abundance of (left) bacterial and (right) fungal classes, as affected by foliar $CaCl_2$ supply.

Microbial growth is reported to be restricted by leaf water activity (Troller, 1980), with decreased water activity prolonging the lag phase and decreasing growth rate and cell yield (Scott, 1957). At water activity values below a minimum threshold, bacteria either stay dormant or die (Sperber, 1983). Minimum a_w value for growth of *E. coli* is 0.95 (Fontana Jr, 2007). The calcium foliar treatments in Paper IV led to a reduction in a_w on the leaf surface, possibly explained by an effect of calcium on membrane stability and permeability (Hepler (2005). This observed reduction in a_w on the leaf surface needs further attention when considering leaf spraying with calcium as a candidate hurdle for foodborne illnesses risks from field to bag. The results need to be assessed for a crop stand or the packaged material. It would be useful to investigate whether exudation of organic compounds over the cuticle is affected by calcium fortification.

Increase in calcium content strengthened the leaves in Paper IV (Figure 9), making them less vulnerable to damage. The results suggested that damage during cultivation was reduced and resilience to artificial

(mechanical) damage was significantly increased with calcium spraying. The naturally occurring damage that accumulates during cultivation was significantly decreased as a result of calcium spraying compared with a water-sprayed control. The greater resistance of calcium-treated leaves to artificial damage led to significant decreases in relative damage and lower number of lesions as potential entry points for human pathogens (Figure 5 in Paper IV). Interestingly, calcium fortification did not impact microbial community composition or the alpha- (Chao1 and Shannon) or beta-diversity of the leaf-associated microbiota (Figures 8 & S8 in Paper IV). Thus the core microbiota dominated the treatment-specific OTUs, indicating that the spinach microbiota is resilient to $CaCl_2$ treatment (Figure 8 in Paper IV). However, as the method used for assessment of microbial communities comprised both viable and non-viable cells, it is possible that certain taxa may have been affected both directly by the calcium foliar fertilisation and indirectly via reduced water activity. Lowered a_w might also have resulted in reduced nutrient availability in the phylloplane, thus affecting the composition and abundance of phylloepiphytes, although that aspect was not investigated in Paper IV.

Overall, calcium spraying of leaves may be viewed as a certain perturbation that can modify the habitat, thus resulting in habitat filtering of phyllosphere microbiota via preservation of tissue integrity and modification of the abundance of available water and nutrients (see 1.3.3). Based on species *phenotypic plasticity* (West-Eberhard, 1989) and their tolerance to $CaCl_2$ direct and indirect effects, species sorting (Leibold *et al.*, 2004) by environmental tolerances may occur as a result of changes in local environmental characteristics mediated by spatial niche partitioning. This needs to be investigated in future experiments. There were significant differences between water-sprayed control and calcium-sprayed leaves in terms of log CFU *E. coli* cm^{-2} detached at 0 and 1 dpi. Calcium fortification did not directly protect already damaged spinach leaves from internalisation of *E. coli* O157:H7. Rather, it had an indirect effect on *E. coli* establishment by reducing leaf tissue damage, thereby affecting expression of the main niche modifiers, *i.e.* a_w, nutrient availability and hydrophobicity (Figure 9 in Paper IV).

5. Concluding remarks

The main findings in this thesis were:

- The new approach developed for detection and automated quantification of lesions allows investigation of the size, shape and location of individual lesions on leaf scale.
- Intact baby leaves rarely occur in nature.
- Leaf damage increases with harvesting and post-harvest processing, but at different rates for different leafy vegetable species.
- Round-shaped, medium-sized lesions located on leaf blade and edges are prominent lesion classes found from field to bag.
- Post-harvest processing changes the alpha- and beta-diversity and relative taxa abundance of baby leaf microbiota.
- Any size of leaf wound enables *E. coli* O157:H7*gfp*+ internalisation into spinach leaf.
- Adhesion of *E. coli* O157:H7*gfp*+ on spinach leaf surfaces is enhanced by artificial lesions, with the level of pathogen proliferation depending on damaged area on the leaf.
- Leaf integrity is fundamental to food safety, but even macroscopically intact spinach leaves can allow internalisation of *E. coli* O157:H7.
- Spraying spinach leaves with calcium throughout cultivation reduces the number of naturally occurring lesions and damaged area, and increases resistance of the leaves to artificial damage.
- Calcium fortification indirectly lowers log CFU *E. coli* cm^{-2} detached at 0 and 1 days post-inoculation.

6. Future perspectives

While conducting the experiments described in Papers I-IV and reviewing the extensive literature on leaf microbiota and plant-microbe interactions in the phyllosphere, new interesting questions arose. A deeper understanding of plant-microbe interactions and the environment in a field to fork perspective is needed. Future work in this regard could improve the food safety of leafy vegetables. Additional research is also needed in the following areas:

- Development of the LiMu software described in Paper I to make it more user-friendly, *i.e.* not requiring the user to have good knowledge of Python programming.
- It was shown that *E. coli* can internalise within lesion neighbouring (intact) tissue, using lesion edges as entry points, but the spatial scale or reach of bacteria into intact tissue from the edge of a lesion remains to be defined. Spatial statistics should be used to assess the penetration radius of *E. coli* O157:H7 from the centre of a lesion, and the impact of lesion size.
- Effects on bacterial reach of inoculum density, shape, depth and location of lesion on leaf blade should also be determined.
- The role of lesion size in the plant-*E. coli* O157:H7 relationship at single lesion level under increasing inoculum densities and different environmental factors needs further attention. Temperatures relevant to the farm-to-fork value chain should be taken into account when designing such experiments.
- The finding that damage to greenhouse-grown spinach can be reduced by spraying the leaves with calcium is of interest for growers and has commercial applications, provided that research results acquired *in vitro* can be translated into practice. Therefore the results need to be validated in relevant field studies and on

different spinach cultivars and on other species of leafy vegetables used as baby leaves.

- It was found that leaf damage increased at harvest and post-harvest processing and that there are differences in size, shape and position of lesions at different steps from field to bag. Further investigations are needed on the susceptibility of calcium-treated leaves to mechanical damage at harvest and during washing and packaging in commercial settings. Potential differences between calcium-fortified and control baby leaves with respect to changes in microbial community composition during storage and effects on spoilage microorganisms need to be identified.
- Interactions between bacteria, lesions and intact tissue need further attention, considering both the abaxial and adaxial sides of the leaf.
- Further investigation is needed of how increasing levels of damage affect numbers of autochthonous and artificially introduced bacteria at increasing inoculum densities, to help understand invasion-diversity relationship at the wound site. In particular, it would be interesting to use FISH probes to assess the spatial distribution of autochthonous bacteria and their proliferation and abundance (competition) at the wound site. Amount of leaked carbohydrates and time as factors in proliferation/nutrient depletion also need further evaluation.

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Popular science summary

Baby leaves, leafy greens, salad mixes and salad kits are packaged, ready-to-eat products made from fresh leafy vegetables and intended for raw consumption. In addition to convenience, ready-to-eat salads made from fresh leafy vegetables such as spinach, Swiss chard and lettuce are healthy and nutritious. Through the minerals, vitamins and fibre they contain, they can contribute to a balanced diet and overall healthy lifestyle. During field growth, baby leaves live in association with microorganisms, macroorganisms and the surrounding environment. The exterior of baby leaves is home to a remarkable variety of bacteria, fungi and yeasts, and some are also known to populate the leaf interior. Most of these microorganisms are not harmful to baby leaves or human consumers. Some can even be beneficial by stimulating plant growth and helping the plant cope with the environment. However, baby leaves can sometimes become contaminated with bacteria that cause food poisoning, such as *Escherichia coli* or *Salmonella*, compromising their food safety. The food safety of baby leaves can be compromised on farm, during harvesting, washing and packaging, in storage and in the consumer's home. Thus food safety is important in all phases of baby leaf production and is an important variable for growers, producers and consumers. The most commonly used measure to remove impurities and harmful microorganisms from baby leaves in commercial settings is washing with tap-water, while in some countries a sanitising product such as chlorine can be added to the wash water. Intact leaf tissue is necessary for ensuring food safety, but baby leaves are tender and their tissue can easily break from farm to fork. Damaged tissue can be used by foodborne pathogens as entry points and such internalised bacteria are sheltered from washing water, increasing the risk of food poisoning. Leaf damage also increases growth of other bacteria, changing their relationships and

dynamics. In this thesis, a new method for measuring damage to baby leaves was developed. The method was then used to measure damage build-up on baby leaves from field to bag, and resulting changes in bacteria and fungi present on leaves. The results showed that the amount of damage differed for different species of leafy vegetables (spinach, Swiss chard), with some leaves being more sensitive to damage than others. For both spinach and Swiss chard, damage increased from field to bag and number of spoilage bacteria increased after harvest. Assessments of interactions between leaf damage and growth of *E. coli* on baby leaves showed that success of *E. coli* cells in colonising the leaf exterior and the depth to which they internalised in the leaf interior depended on the size of leaf lesions. Since it is not possible to avoid contamination of leafy vegetables, an attempt was made to protect baby leaves from damage by spraying the leaves with $CaCl_2$ spray. Encouragingly, preliminary results showed that spraying spinach plants with calcium during growth reduced damage, so reducing adhesion and growth of *E. coli* cells. These findings are of interest to growers, baby leaf processers and consumers, and can contribute to ensuring safe produce.

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Appendix

Appendix 1: Definitions of ecological concepts relevant for the work in this thesis. For definitions of concepts translated in non-technical terms, please consult Hawkes and Connor (2017), Enders et al. (2020) and Mallon et al. (2015b).

Concept	Definition	Reference
Carrying capacity	A maximum number of individuals that a certain environment can support.	(McArthur, 2006)
Competitive exclusion	"When competition between species results in the elimination of one species from a given habitat or region."	(Hibbing *et al.*, 2010)
Disturbance	"Any relatively discrete event in time that disrupts ecosystem, community or population structure and changes resources, substrate availability, or the physical environment." According to Hobbs and Huenneke (1992), any change in type of disturbance, or increase in disturbance frequency "may alter species composition by reducing the importance of native species, by creating opportunities for invasive species, or both."	(White, 1985)
Diversity-invasion effect	"A common relationship witnessed between microbial community diversity (often measured in species richness or evenness) and the survival of an invading microbe, where more diverse communities better resist invasion than less diverse communities."	(Mallon *et al.*, 2015b)
Empty niche	Empty, also known as vacant, unoccupied, free, or unfilled niche represents unused, but potentially usable resources.	(Lekevičius, 2009)
Exploitation competition	"Competition in which one competitor deprives another of a resource (such as a nutrient or habitable space) by depleting that resource."	(Hibbing *et al.*, 2010)

Microbial invasion	"A four-step process consisting of the (i) introduction, (ii) establishment, (iii) growth and spread, and (iv) impact of a microbial invader. While a successful invasion passes all four steps, reference to the term 'microbial invasion' could imply any one of the phases."	(Mallon *et al.*, 2015b)
Niche	"The environmental conditions that allow a species to satisfy its minimum requirements so that the birth rate of a local population is equal to or greater than its death rate along with the set of per capita effects of that species on these environmental conditions"	(Chase & Leibold, 2003)
Niche overlap	"Partial or complete sharing of resources or other ecological factors (predators, foraging space, soil type, and so on) by two or more species." Two species cannot co-exist if they use the same resources and/or environments.	(Cornell, 2012)
Priority effects	"The effect of species on one another depends on the order in which they arrive at a site. Species that arrive early reduce the amount of resources (nutrients, space, light, etc.) available to other species and, in doing so, limit the local abundance that can be attained by late-arriving species that need these resources to survive and reproduce."	(Fukami, 2015)
Resilience	"How fast the variables return towards their equilibrium (initial value) following a perturbation."	(Pimm, 1984)
Persistence	"The time a variable lasts before it is changed to a new value."	(Pimm, 1984)
Resistance	"The degree to which a variable is changed, following a perturbation."	(Pimm, 1984)
Stochastic processes	"Change in population structure over time due to random factors, i.e., neutral evolution not due to selective pressure."	(Shafquat *et al.*, 2014)

Appendix 2: List of the most relevant publications for the framework of this thesis

	Article
1	Brandl, M.T. (2008). **Plant lesions promote the rapid multiplication of *Escherichia coli* O157: H7 on postharvest lettuce**. Applied and Environmental Microbiology, 74(17), pp. 5285-5289.
2	Remus-Emsermann, M.N., Tecon, R., Kowalchuk, G.A. & Leveau, J.H. (2012). **Variation in local carrying capacity and the individual fate of bacterial colonizers in the phyllosphere**. The ISME Journal, 6(4), pp. 756-765.
3	Aruscavage, D., Miller, S.A., Lewis Ivey, M.L., Lee, K. & LeJeune, J.T. (2008). **Survival and dissemination of *Escherichia coli* O157: H7 on physically and biologically damaged lettuce plants**. Journal of Food Protection, 71(12), pp. 2384-2388.
4	Leveau, J.H. & Lindow, S.E. (2001a). **Appetite of an epiphyte: quantitative monitoring of bacterial sugar consumption in the phyllosphere.** Proceedings of the National Academy of Sciences, 98(6), pp. 3446-3453.
5	Mogren, L., Windstam, S., Boqvist, S., Vågsholm, I., Söderqvist, K., Rosberg, A.K., Lindén, J., Mulaosmanovic, E., Karlsson, M. & Uhlig, E. (2018). **The Hurdle Approach–A Holistic Concept for Controlling Food Safety Risks Associated With Pathogenic Bacterial Contamination of Leafy Green Vegetables. A Review.** Frontiers in Microbiology, 9.
6	Mallon, C.A., Van Elsas, J.D. & Salles, J.F. (2015b). **Microbial invasions: the process, patterns, and mechanisms**. Trends in Microbiology, 23(11), pp. 719-729.
7	Remus-Emsermann, M.N. & Schlechter, R.O. (2018). **Phyllosphere microbiology: At the interface between microbial individuals and the plant host**. New Phytologist, 218(4), pp. 1327-1333.
8	Hartmann, R., Fricke, A., Stützel, H., Mansourian, S., Dekker, T., Wohanka, W. & Alsanius, B. (2017). **Internalization of *Escherichia coli* O157: H7 gfp+ in rocket and Swiss chard baby leaves as affected by abiotic and biotic damage**. Letters in Applied Microbiology, 65(1), pp. 35-41.
9	Vorholt, J.A. (2012). **Microbial life in the phyllosphere**. Nature Reviews Microbiology, 10(12), pp. 828-840.
10	Remus-Emsermann, M.N., Lücker, S., Müller, D.B., Potthoff, E., Daims, H. & Vorholt, J.A. (2014). **Spatial distribution analyses of natural phyllosphere-colonizing bacteria on A rabidopsis thaliana revealed by fluorescence in situ hybridization**. Environmental Microbiology, 16(7), pp. 2329-2340.

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12 Aruscavage, D., Phelan, P.L., Lee, K. & LeJeune, J.T. (2010). **Impact of changes in sugar exudate created by biological damage to tomato plants on the persistence of *Escherichia coli* O157: H7**. Journal of Food Science, 75(4), pp. M187-M192.

13 Simko, I., Zhou, Y. & Brandl, M.T. (2015). **Downy mildew disease promotes the colonization of romaine lettuce by *Escherichia coli* O157: H7 and *Salmonella enterica***. BMC Microbiology, 15(1), pp. 1-9.

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20 Rosberg, A.K., Darlison, J., Mogren, L. & Alsanius, W.B. (2021). **Commercial wash of leafy vegetables do not significantly decrease bacterial load but leads to shifts in bacterial species composition**. Food Microbiology, 94, 103667.

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Leaf damage causes changes in niche factors, e.g. nutrient availability, hydrophobicity and leaf landscape. In this thesis, a novel method for detection and automated quantification of leaf-scale damage was developed, and field-to-bag changes in damage and leaf microbiota in leafy vegetables were evaluated. The results revealed that, irrespective of size, leaf tissue damage supports proliferation and internalisation of *Escherichia coli* O157:H7 bacteria, and that calcium fortification of baby leaves can be a plausible solution for damage reduction.

Emina Mulaosmanovic received her doctoral education at the Department of Biosystems and Technology, SLU, Alnarp, Sweden. She obtained her BSc and MSc in Plant Production from the Faculty of Agriculture and Food Sciences at the University of Sarajevo, Bosnia and Herzegovina.

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