Environmental and agronomic effects on phyllosphere microbiology and the persistence of *E. coli* O157:H7 during the cultivation of leafy vegetables

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Dedicated to my Dad

Abstract

During the past decades, reported outbreaks related to fresh produce are augmenting. Causative agents with the highest incidents are pathogenic *Escherichia* (*E.*) *coli* and *Salmonella* spp., while *Listeria* (*L.*) *monocytogenes* is not linked to produce related outbreaks that frequently, but is of interest due to its fatal character. Contamination can occur during the whole production chain, including, pre- and postharvest activities as well as by the consumer. The aim of this thesis was to evaluate different production factors during the cultivation of leafy greens with regard to their impact on the contamination risk.

Iceberg lettuce, Swiss chard and wild rocket seeds from different origins all harbored considerable numbers of heterotrophic bacteria (up to more than 9 Log CFU * g^{-1}). Enteric bacteria (but no *E. coli*) and *Listeria* spp. were detected in all seed lots, while some seeds even harbored *L. monocytogenes*.

The microbial colonization of iceberg lettuce, Swiss chard and wild rocket leaves was monitored during growth in the field with respect to different input factors. The treatments, namely organic fertilization (chicken manure and pig hair pellets), irrigation water sources (pond, well and tape water), irrigation methods (overhead and drip) and mulch coverage (with and without), mimicked different levels of contamination hazards. Microbial colonization varied between sampling occasion and crops, but no consistent pattern was seen for the treatments. Lowest numbers for general colonization were detected on iceberg lettuce, indicator organisms *E. coli* and *L. monocytogenes* showed the highest incidents on Swiss chard.

Further, the establishment of an artificially introduced *E. coli* O157:H7 strain on greenhouse grown Swiss chard and wild rocket baby leaves was analyzed in dependency of the plants nitrogen source (chicken manure and pig hair pellets), when the bacterium was spray inoculated. Again, treatments did not result in significant differences in microbial colonization and leaf nitrogen content did not affect the abundance of *E. coli* O157:H7. Some micronutrients, playing a role in photosynthetic activity, and plant physiological factors were predictors for numbers of *E. coli* O157:H7.

Clearest differences were found between the crops: Significantly more *E. coli* O157:H7 were reisolated from Swiss chard as compared to wild rocket leaves when employing a smasher. Interestingly, when the establishment of the bacterium was evaluated by the use of a laser scanning confocal microscope, cells were more abundant on wild rocket leaves.

Salmonella spp. and *Listeria* spp. were occasionally detected on seeds and leaves in all trials when applying culture-dependent methods. Further identification of isolated bacteria (from greenhouse grown wild rocket leaves) by sequencing of the 16S rRNA gene failed to confirm the results.

Keywords: food safety, leafy vegetables, phyllosphere

Kurzfassung

Die Anzahl der durch den Verzehr von Gemüse und Obst verursachten Krankheitsausbrüche hat in den letzten Jahrzehnten zugenommen. Während pathogene *E. coli* und *Salmonellen* die bakteriellen Krankheitserreger mit der höchsten Inzidenz darstellen, ist *L. monocytogenes* von Interesse, da das Bakterium schwere Krankheitsverläufe auslösen kann. Gemüse und Früchte können während der gesamten Produktionskette kontaminiert werden, dies bedeutet während der Kultivierung, bei der Ernte oder der Aufbereitung, aber auch durch den Konsumenten selbst. Ziel dieser Arbeit ist die Evaluierung verschiedener Einflüsse auf die Kontamination von Blattgemüse mit humanpathogenen Bakterien während der Kultivierung.

Eisbergsalat-, Mangold- und Rucolasamen verschiedener Herkunft wiesen beträchtliche Gesamtbakterienzahlen von bis zu mehr als 9 Log KbE * g^{-1} auf. Enterische Bakterien (jedoch keine *E. coli*) und *Listeria* spp. wurden in allen Chargen gefunden, wobei mache Samen sogar *L. monocytogenes* beherbergten.

Die mikrobielle Besiedelung von Eissalat-, Mangold und Rucolablättern wurde während der Freilandkultivierung in Abhängigkeit verschiedener Maßnahmen analysiert (Probennahme alle zwei Wochen). Unterschiedliche Behandlungen in Bezug auf organische Düngemittel (Hühnertrockenkot, Schweinehaarmehlpellets), Beregnungswasser (Teich-, Brunnen-, Leitungswasser), Beregnungsmethode (Überkopf-, Tropfbewässerung) und Mulchfolie (mit und ohne Abdeckung) stellten verschiedene Gefährdungsstufen in Bezug auf eine bakterielle Kontamination dar. Die mikrobielle Besiedelung unterschied sich zwischen den Probennahmezeitpunkten sowie zwischen den Pflanzen, ein konsistenter Einfluss der verschiedenen Kultivierungsmaßnahmen wurde jedoch nicht offensichtlich. Die niedrigste Gesamtbakterienzahl wurde für Eisbergsalat gefunden, die Indikatorbakterien *E. coli* und *L. monocytogenes* wurden am häufigsten von Mangoldblättern isoliert.

Des Weiteren wurde die Etablierung eines künstlichen, via Spray applizierten *E. coli* O157:H7-Stammes auf Mangold und Rucola Baby-Leaves, in Abhängigkeit der pflanzlichen Stickstoffquelle (Hühnertrockenkot, Schweinehaarmehlpellets), analysiert. Auch hier konnte kein statistisch verifizierter Unterschied in der mikrobiellen Besiedelung der Düngervarianten gefunden werden, gleichermaßen erwies sich der Nitratgehalt der Blätter nicht als Prädiktor für die Höhe der koloniebildenden Einheiten von *E. coli* O157:H7. Einige Mikronährstoffe, welche im Photosynthesestoffwechsel essentiell sind, sowie pflanzenphysiologische Faktoren zeigten Korrelationen mit der Anzahl an *E. coli* O157:H7.

Die deutlichsten Unterschiede bestanden jedoch zwischen den Pflanzen: Signifikant mehr *E. coli* O157:H7 wurden unter Zuhilfenahme eines Smashers von Mangold im Vergleich zu Rucola isoliert. Bei Betrachtung der Etablierung des gleichen Bakteriums unter dem Konfokalmikroskop war dieser Sachverhalt interessanterweise umgekehrt. Hier erwiesen sich die *E. coli* O157:H7 Zellen auf Rucolablättern als persistenter. Neben der Pflanzenart zeigte sich der Gesundheitszustand der Blätter als ausschlaggebend für die Etablierung des potenziell humanpathogenen Bakteriums. Biotische oder

abiotische Schädigung eines Blattes führte zu einer höheren Anzahl an *E. coli* O157:H7 in einem Blattausschnitt gegenüber unbeschädigten Blättern (Visualisierung mittels Konfokalmikroskop).
Salmonella spp. und *Listeria* spp. wurden zumindest gelegentlich auf Samen und Blättern in allen Versuchen mit Hilfe von kulturabhängigen Methoden gefunden. Eine weitere Identifizierung einiger dieser isolierten Bakterien (von im Gewächshaus kultivierten Rucola) durch die Sequenzierung des 16S rRNS Gens konnte das Ergebnis jedoch nicht bestätigen.

Keywords: Blattgemüse, Lebensmittelsicherheit, Phyllosphäre

Contents

Abstract II
Kurzfassung III
Contents V
Abbreviations IX
List of tables XI
List of figures XIII

Chapter 1: General introduction 1
Background 1
Human pathogens on vegetables 2
EHEC 2
Salmonella spp. 3
L. monocytogenes 4
Indicator organisms 5
Contamination sources/routes 6
Contamination of seeds 6
Contamination during cultivation 7
Special case: organic vs. conventional farming 9
Persistence in phyllosphere 9
Environmental factors influencing the persistence in the phyllosphere 10
Biological factors influencing the persistence in the phyllosphere 11
Internalization 12
Microbial analysis 13
Objectives 15

Chapter 2: Microbial colonization of vegetable seeds 17
Abstract 17
Background 18
Material and methods 19
Plant material 19
Analysis 19
Calculations and statistical analysis 20
Results 21
Iceberg lettuce 21
Swiss chard 21
Wild rocket 22
Discussion 26

Chapter 3: Season, plant species and age, but not cultural management, govern microbial colonization of organically grown leafy vegetables **29**
Abstract **29**
Introduction **30**
Material and methods **31**
Experimental set-up 31
Analysis 32
Statistical analyses 34
Results **35**
Plant assessment 35
Climate data 36
Microbial colonization 37
Interactions between weather conditions and phyllosphere bacteria 43
Interactions between yield gain and phyllosphere bacteria 47
Discussion **47**
Acknowledgement **51**
Chapter 4: Impact of the source of organic manure on persistence of *E. coli* O157:H7 *gfp*+ in rocket (*Diplotaxis tenuifolia*) and Swiss chard (*Beta vulgaris* cicla) **52**
Highlights **52**
Abstract **52**
Introduction **54**
Materials and Methods **55**
Inoculum preparation 55
Greenhouse experiment 56
Nitrogen analysis 56
Microbial analysis 56
Identification of dominant *Enterobacteriaceae* 57
Dual culture test 57
Verification of *Listeria* spp. and *Salmonella* 57
16S DNA sequencing of isolated bacterial cells 58
Risk assessment 59
Statistics 59
Results **59**
Nitrogen content in leaves 59
Nitrogen content in growth medium 59
Microbial colonization 61
Characterization of bacteria grown on media selective for *Enterobacteriaceae* 62

Interactions between nitrogen content in leaves and microbial colonization 66
Risk assessment 67
Discussion 68
Conclusion 70
Acknowledgements 71

Chapter 5: Impact of organic fertilizers on the microbial phyllosphere structure and prevalence of *E. coli* O157:H7 *gfp*+ in rocket (*Diplotaxis tenuifolia*) and Swiss chard (*Beta vulgaris* cicla) 72
Highlights 72
Abstract 72
Introduction 74
Material and methods 74
Inoculum preparation 74
Greenhouse experiment 75
Analysis 76
Calculation and statistical analyses 78
Results 78
Plant parameters 78
Leaf colonization 80
Culture-independent analysis 82
Interactions between plant parameters and *E. coli* O157:H7 *gfp*+ 85
Interactions between plant parameters and the microbial community structure 86
Interactions between plant parameters, microbial community structure and the presence of *E. coli* O157:H7 *gfp*+ 88
Discussion 89
Conclusions 91
Supplementary material 92

Chapter 6: Internalisation of *Escherichia coli* O157:H7 *gfp*+ in rocket and Swiss chard baby leaves as affected by abiotic and biotic damage 94
Significance and impact of the study 94
Abstract 94
Introduction 96
Material and methods 97
Plant material and treatments 97
Microorganisms 98
Analysis 98
Re-isolation 99

Inhibition test 99
Results and Discussion **99**
Acknowledgements **103**
Chapter 7: General discussion **104**
Impacts on phyllosphere colonization **104**
Climatic factors 104
Crops 105
Treatments 106
Epi- and endophytic colonization of vegetable leaves **107**
Conclusion and future perspective **108**
Literature **110**
Curriculum Vitae
Acknowledgements

Abbreviations

µg	microgram
ANOVA	Analysis of variance
bp	Base pair
CFU	Colony forming units
CM	Chicken manure
ddNTP	dideoxynucleotides
DNA	Deoxyribonucleic acid
dNTP	dideoxynucleotides
E.	*Escherichia*
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic *E. coli*
h	Hour
H_2O	Water
HPC	Heterotrophic bacterial plate count
HUS	Hemolytic-uremic syndrome
L.	*Listeria*
LB	lysogenic broth
min	Minutes
ml	milliliters
mm	millimeters
$MgCl_2$	Magnesium chloride
MPN	Most probable number
NaCl	Sodium chloride
nm	nanometers
OD	Optical density
OTU	Operational taxonomic unit

PBS	Phosphate buffered saline solution
PCA	Principal component analysis
PCR	Polymerase chain reaction
PHP	Pig hair pellets
R2A	Reasoner’s 2A
rDNA	Ribosomal deoxyribonucleic acid
rpm	Rounds per minute
rRNA	Ribosomal ribonucleic acid
S.	*Salmonella*
TBE	Tris/Borate/EDTA
TN	Total nitrogen
TRIS	Tris(hydroxymethyl)aminomethane
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VRBD	Violet red bile dextrose agar

List of tables

Table 1-1. Favorable conditions for the growth of *E. coli, Salmonella* spp. and *L. monocytogenes*. 5

Table 2-1. Cultivar, breeder and thousand seed weight (TSW) [g] of iceberg lettuce, Swiss chard and wild rocket seeds. 19

Table 2-2. Used media and supplements as well as incubation conditions (temperature [°C], length [h]) for the microbial analysis of seeds. 20

Table 3-1. Cultivation methods associated with different microbial hazards that were applied in a field trial with iceberg lettuce, wild rocket and Swiss chard, wild rocket and Swiss chard were not exposed to drip irrigation. 32

Table 3-2. Semi-selective media and supplements and incubation conditions used for microbial analysis of leaves, manure and soil samples. 34

Table 3-3. Comparison between different bacterial loads [log CFU g^{-1}] on iceberg lettuce (IB), Swiss chard (SC), and wild rocket (WR) during two growing seasons (2013 and 2014) in different treatments. Sampling of leaves (S1-S4) for microbial analysis with culture-dependent methods was conducted at two-week intervals. Values for each treatment, crop, and harvest marked with different letters are significant different according to ANOVA (Tukey-test, $p<0.05$)........ 42

Table 3-4. Microbial loads in water from different sources applied to field-grown leafy vegetables in 2013 and 2014 at different sampling occasions (S). Heterotrophic plate count (HPC) was assessed as colony-forming units [log CFU mL^{-1}] and coliform bacteria, E. coli, and enterococci as most probable number [MPN 100 mL^{-1}]. 43

Table 3-5. Pearson correlation ($p<0.05$) between yield gain and bacterial groups on iceberg lettuce (IB), Swiss chard (SC), and wild rocket (WR) over two growing seasons. Bacterial loads were determined at two-week intervals and correlated to yield gained between sampling occasions. HPC = heterotrophic bacterial plate count. 47

Table 4-1. Semi-selective media and supplements, and incubation conditions used in culture-dependent analysis. 57

Table 4-2. Total nitrogen (N_{tot}), nitrate (NO_3^-) and ammonium (NH_4^+) content in organic fertilizers (pig hair pellets: PHP, chicken manure: CM) applied to rocket and Swiss chard. DM = dry matter. 60

Table 4-3. Risk measured as probability of infection with *E. coli* O157:H7 assuming a 30 g portion of rocket and Swiss chard is consumed. Crops were fertilized with pig hair pellets (PHP) or chicken manure (CM), or left untreated (control). Leaves were spray-inoculated with *E. coli* O157:H7. Risks are given as averages. 68

Table 5-1. Incubation conditions (temperature, °C; length, h) as well as targeted groups of organisms for the different semi-selective media (TSA: Tryptic Soy Agar; MA: Maltextract Agar; LB: Lysogenic Broth; VRBD: Violet-Red Bile Dextrose Agar). 77

Table 5-2. Performance (fresh biomass, g m^{-2}; dry matter content, leaf area, cm^2 $leaf^{-1}$; average leaf temperature T_{av}, °C; minimum leaf temperature $T_{min,}$ °C) of Swiss chard and rocket exposed to a

low risk and high risk fertilization scenario involving pig hair pellets (PHP, low risk) and raw chicken manure (CM, high risk). Plants in the control plots did not receive any additional fertilizers apart from the basis fertilizers in the growing medium. Leaf temperature values display average values for each tray. 79

Table 5-3. Macro- (% dry leaf weight) and micronutrient (mg (kg leaf dry weight)$^{-1}$) content in leaves of greenhouse grown Swiss chard and rocket, fertilized either with pig hair pellets (PHP) or fresh chicken manure (CM). The control treatment was not supplied with any additional fertilizer. Values followed within the same line, followed by different letters are significantly different according to ANOVA followed by Tukey test ($p<0.05$). N=12. 79

Table 5-4. Microbial colonization (Log CFU g^{-1}) of Swiss chard and rocket leaves grown in the presence of *E. coli* O157:H7 *gfp*+ under greenhouse conditions. Organic fertilizers were supplied to the growing medium during sowing bed preparation, before sowing and the fertilization strategies displayed a high risk (fresh chicken manure, CM) and a low risk scenario (pig hair pellets, PHP) and a non-fertilized control plot. *E. coli* O157:H7 *gfp*+ was sprayed two times a week with an interval of 3 and 4 days, respectively, with three and five inoculations for Swiss chard and rocket. The experiment was repeated twice with six individual replicates per trial. (n=12). 80

Table 5-5. Numbers of 16S rDNA sequences (excluding chloroplast and mitochondria reads) in the phyllosphere samples from Swiss chard and rocket exposed to different fertilizer regimes accounting for a low and high risk scenario (Control=no additional fertilizer supplied; organic fertilizers: PHP=pig hair pellets, CM=fresh chicken manure). 82

Table 5-6. Biodiversity of the bacterial phyllosphere biome of greenhouse grown Swiss chard and rocket estimated on genus level by Shannon-H and Chao1. The crops were fertilized with either pig hair pellets (PHP) or fresh chicken manure (CM). The control did not receive any additional fertilization. (N=12). Values within the same column followed by different letters are significantly different according to Anova followed by Tukey-test ($p<0.05$). 83

Table 5-7. Pearson correlations between leaf dry matter nutrient content and relative abundance of bacterial families, inhabiting the phyllosphere of rocket. The rocket plants had been fertilized with either organic fertilizers (raw chicken manure or pig hair pellets or remained without additional fertilization). Probabilities are displayed. 87

Table 5-8. Pearson correlations between leaf dry matter content of manganese (Mn), iron (Fe) and boron (B) and the relative abundance of bacterial families, inhabiting the phyllosphere of rocket. The rocket plants had been fertilized with either organic fertilizers (raw chicken manure or pig hair pellets or remained without additional fertilization). Probabilities are displayed. 92

List of figures

Figure 1-1. Overview of possible contamination routes (arrows) and factors affecting the persistence of human pathogens in the phyllosphere of leafy vegetables. Circles display topics discussed in the different chapters of this thesis. 16

Figure 2-1. Heterotrophic bacterial plate counts (HPC), enterobacteria, Listeria, enterococci and coliform bacteria determined by culture-dependent methods on iceberg lettuce IB (A), Swiss chard SC (B) and wild rocket WR (C) seeds intended for organic production (lot 1: org_1, lot 2: org_2), direct use by home gardeners (hg) and for conventional production (tr). No analyses were conducted for treated iceberg lettuce seeds. Bars within one microbial group that do not share a letter are significantly different as revealed by ANOVA (Tukey-test, $p<0.05$). No statistical analysis was performed for coliform bacteria on iceberg lettuce, where both organic batches were below the detection limit. 24

Figure 2-2. Similarity (average linklage, Euclidian distance) of different seed lots (seeds intended for organic production, batch 1 (org_1) and 2 (org_2), for direct use by home gardener (hg) and treated seeds (tr)) as revealed by microbial contamination with heterotrophic bacterial plate counts, enterobacteria, Listeria, enterococci and coliform bacteria (culture-dependent analysis) on iceberg lettuce IB (A), Swiss chard SC (B) and wild rocket WR (C) seeds. No analyses were conducted for treated iceberg lettuce seeds. Clustering is stated by different colors.................. 25

Figure 3-1. Fresh weight (FW) [g] as a function of temperature sum (T sum, degree-days) [°Cd] for: field-grown iceberg lettuce (2013. A and 2014: B), Swiss chard (2013: C and 2014: D), and wild rocket (2013: E and 2014: F). No data were available for the last sampling of Swiss chard in 2014 and the curve was thus extrapolated to a temperature sum of 844°Cd, when the final sampling took place. Due to a shorter vegetative phase of wild rocket, the final sampling in 2014 took place at 638°C. Fast growth in 2013 resulted in fewer sampling occasions and an additional sampling (generative phase) for wild rocket was performed to obtain enough data for creating a curve. . 36

Figure 3-2. Daily mean temperature (T, broken line) and daily precipitation (P, bars) during the growing season in 2013 (A) and 2014 (B). Measurements represent the interval between planting and final harvest of the crops. Arrows indicate sampling occasions. 37

Figure 3-3. Comparison between different bacterial loads (A-E) on: iceberg lettuce, Swiss chard, and wild rocket during two growing seasons (2013 and 2104). Sampling of leaves (S1-S4) for microbial analysis with culture-dependent methods was conducted at two-week intervals. Bars for each harvest with different letters are significantly different according to ANOVA (Tukey-test, $p<0.05$). HPC = heterotrophic bacterial plate count; na = no data available. □: Iceberg lettuce, ▨: Swiss chard, ■: wild rocket 38

Figure 3-4. Samples testing positive for *E. coli* (A) and *L. monocytogenes* (B) [%] on iceberg lettuce, Swiss chard, and wild rocket during two growing seasons (2013 and 2014). Sampling of leaves (2013: S1-S3; 2014: S1-S4) for microbial analysis with culture-dependent methods (lower

detection limit: ~1.7 log CFU g^{-1} fresh weight) was conducted at two-week intervals. na = no data available. □: Iceberg lettuce, ▨: Swiss chard, ■: wild rocket.. 40
Figure 3-5. Scatterplots of bacterial counts [log CFU g^{-1}] and temperature [°C]/precipitation [mm] on leaves of iceberg lettuce (A), Swiss chard (B, D), and wild rocket (C). Bacterial loads were determined at two-week intervals and correlated to weather data averaged over five days prior to analysis.. 44
Figure 3-6. Scatterplots for bacterial counts [log CFU g^{-1}] on leaves of iceberg lettuce (A, B), Swiss chard (C), and wild rocket (D, E). Bacterial loads were determined at two-week intervals....... 45
Figure 3-7. Principal component analysis based on temperature (TEMP), precipitation (PCPN), and microbial loads on leaves of iceberg lettuce (A, B), Swiss chard (C, D), and wild rocket (D, E) during cultivation. Samples were taken at two-week intervals after planting (□ = sampling 1, ○ = sampling 2, ◊ = sampling 3, Δ = sampling 4) in two growing seasons (filled symbols = 2013, open symbols = 2014). No data were obtained for wild rocket on the last sampling event in 2013 and 2014. Microbial loads [log CFU g^{-1}] were determined for heterotrophic bacterial plate count (HPC), *Enterobacteriaceae* (ENT), *Listeria* spp. (LIS), *Enterococcus* spp. (ECC), and coliform bacteria (COL) by culture-dependent methods. Temperature [°C] and precipitation [mm] values were averaged over five days before each sampling.. 46
Figure 4-1. Comparison of total nitrogen content (N_{tot}), nitrate (NO_3-N) and ammonia (NH_4-N) on the day of harvest in growth medium of rocket baby leaves (A, B, C) and Swiss chard baby leaves (D, E, F) for different organic fertilizer treatments. Growth medium was supplemented with pig hair pellets (PHP) or chicken manure (CM) to an amount of 81 kg readily available N ha^{-1}, while the control did not receive any fertilizer. Bars within diagrams marked with different letters are significantly different according to ANOVA (Tukey-test, $p<0.05$)... 61
Figure 4-2. Comparison of bacterial loads (TSA: Heterotrophic bacterial plate count, LB: *E. coli* O157:H7 gfp+, VRBD: *Enterobacteriaceae*) on rocket baby leaves (A) and Swiss chard baby leaves (B) for different organic fertilizer treatments. Plants received pig hair pellets (PHP) or chicken manure (CM) to an amount of 81 kg readily available N ha^{-1}, while the control did not receive any fertilizer. Bars for each medium with different letters are significantly different according to ANOVA (Tukey-test, $p<0.05$). 62
Figure 4-3. Phylogenetic tree based on analysis of 16S rRNA gene sequences retrieved from isolates from the leaf surface of rocket cultured in the greenhouse and fertilized with chicken manure (CM) or pig hair pellets (PHP). Control treatments did not receive any fertilizer. The isolates were collected from nutrient agar for selective for *Enterobacteriaceae* (violet red bile dextrose agar). 64
Figure 4-4. Phylogenetic tree based on analysis of 16S rRNA gene sequences retrieved from isolates from the leaf surface of Swiss chard cultured in the greenhouse and fertilized with chicken manure

(CM) or pig hair pellets (PHP). Control treatments did not receive any fertilizer. The isolates were collected from nutrient agar selective for *Enterobacteriaceae* (violet red bile dextrose agar)... 65

Figure 4-5. Principal component (PC) analysis based on nitrogen content on the day of harvest in leaves and microbial loads on leaves (determined by culture-dependent methods for heterotrophic bacterial plate count, *E. coli* O157:H7 gfp+, *Enterobacteriaceae*, presumptive *Salmonella* spp., and presumptive *Listeria* spp.) of baby leaf rocket (filled symbols) and Swiss chard (open symbols) exposed to different fertilizer regimes: ○ = control, □ = pig hair pellets, ◊ = chicken manure). 66

Figure 4-6. Scatterplots for total nitrogen (TN) content in Swiss chard (■) and rocket (●) leaves [mg g^{-1} fresh weight (FW)] and epiphytic bacteria [log CFU g^{-1} FW]. Plants were spray-inoculated with E. coli O157:H7. HPC: heterotrophic bacterial plate count. 67

Figure 5-1. Principal component analysis of all plant parameters (yield m^{-2}; leaf fresh and dry weight, dry matter content; leaf area; minimum, maximum and average leaf temperature; chlorophyll fluorescence; leaf macro- and micronutrient content) and log values of enumerated microbial groups on semi selective media (culturable heterotrophic bacteria; culturable fungi; Enterobacteriaceae; inoculated *E. coli* O157:H7 *gfp*+; intestinal enterococci). Greenhouse grown rocket (A) and Swiss chard (B) plants were subjected to three fertilizer strategies (no additional fertilizer (●); pig hair pellets (■); fresh chicken manure (♦)). Filled and open symbols denote the first and second experiment, respectively. 81

Figure 5-2. Relative abundance of bacterial phyla on greenhouse grown Swiss chard (A) and rocket (B) leaves which had been spray inoculated with E. coli O157:H7 gfp+. The Swiss chard and rocket crops were sown in growing medium and exposed to different fertilizer strategies mimicking a high (CM: fresh chicken manure) or low (PHP: pig hair pellets) risk scenario for transmission of enteric diseases (Control: no additional fertilizer). 83

Figure 5-3. Relative abundance of bacterial orders within Proteobacteria on rocket (A-C) and Swiss chard (D-F) leaves that had been spray-inoculated by *E. coli* O157:H7 *gfp*+. The crops were grown under greenhouse conditions in growing medium without additional fertilizer (A, D) or fertilized with different organic fertilizers mimicking a low (B, E: pig hair pellets) or a high (C, F: fresh chicken manure) risk scenario for transmission of enteric diseases. 85

Figure 5-4. Principal component analysis of all plant parameters (yield m^{-2}; leaf fresh and dry weight, dry matter content; leaf area; minimum, maximum and average leaf temperature; chlorophyll fluorescence; leaf macro- and micronutrient content) and relative abundance of bacterial phyla present in greenhouse grown rocket and Swiss chard leaves. The plants were subjected to three fertilizer strategies (Control: no additional fertilizer; PHP: pig hair pellets; CM: fresh chicken manure). 88

Figure 6-1. Laser scanning confocal microscope images of intact (A, D), abiotically damaged (B, E) and biotically damaged (C, F) leaf tissue of Swiss chard (A, B, C) and wild rocket (D, E, F)

contaminated with *E. coli* O157:H7 cells (green fluorescence). Wild rocket leaves were surface-sanitized (20 s in 25% sodium hypochlorite), while Swiss chard leaves were not sanitized before microscopy. The images show an xy section (90 x 90 µm) with corresponding cross-sections to the red and green lines. 100
Figure 6-2. Number of *gfp*-tagged *E. coli* O157:H7 cells that invaded into 90 x 90 µm2 portions of leaf tissue as determined by laser scanning confocal microscopy. Wild rocket leaves were surface-sanitized (20 s in 25% sodium hypochlorite), while Swiss chard leaves were not sanitized before microscopy. Numbers are not representative of the entire leaf, as contaminated spots were selected.. 102
Figure 6-3. Leaves of Swiss chard (A-C) and wild rocket (D-F). A and D show intact leaves; B and E show leaves abiotically immediately after damage by silicon carbide; and C and F show biotic damage caused by Pseudomonas syringae pv. aptata and Xanthomonas campestris pv. campestris, respectively. 97

Chapter 1: General introduction

Background

Considered as an essential part of a healthy diet, the consumption of fresh produce increased during the last decades (Olaimat & Holley, 2012; Subhasree *et al.*, 2009). Yet, also the cases of reported outbreaks related to the consumption of raw eaten produce is augmenting (European Food Safety Authority (EFSA), 2013; Warriner *et al.*, 2009). A higher consumption alone does not explain the rising number of human infections. The demand for all kinds of produce during the whole year has led to increased import from counties with less pronounced hygiene surveillance and hence microbial quality of the imported produce might be inferior to produce cultivated in industrial nations (Islam *et al.*, 2005). Shopping habits also changed over time: nowadays a majority of people purchase their food in big retail markets. Consequently, if infections occur, more people are affected and thus the outbreak will be more likely recognized as compared to sporadic cases (Heaton & Jones, 2008). Furthermore, improved medical care and demographic change, encompassing more elderly and other less immunocompetent people, contribute to an increase in numbers of infections (Carrasco *et al.*, 2010). Also the bacteria themselves evolve over time, adopting to new conditions which gives them a higher persistence in the environment or makes them more infectious (Theron & Cloete, 2002). In addition, improved surveillance results in enhanced detection (Heaton & Jones, 2008).

With respect to the causative agent of an outbreak, *Salmonella* serovars account for the majority of fresh produce in total, whereas *Escherichia coli* (*E. coli*) pathovars have been most frequently linked to leafy greens associated outbreaks (Interagency Food Safety Analytics Collaboration, 2015). The U.S. Food and Drug Administration (2009) describes leafy greens to be the overall riskiest food in terms of microbial outbreaks.

Identifying the source of an outbreak, the ideal case would request the genetically match of pathogens, isolated from contaminated produce with pathogens received from infected individuals. This requirement most often cannot be followed, due to variabilities in laboratory procedures and the fact that food with a short shelf life might not be on hand to be tested any longer. If a product is identified to harbor the causative agent, trace back to the grower can be difficult in case products from different farmers are mixed. Therefore, the identification of the source and cause of an outbreak is often not accomplished (Sapers & Doyle, 2014). The source of an outbreak, however, is of great interest as suitable control points can be set regarding high risk sections (Harris *et al.*, 2003).

Due to scarce outbreaks and the difficulties in identifying the source, evaluation of outbreaks alone is not sufficient to determine strategies for the production of minimal risk vegetables. Further research aims to achieve greater insight into conditions of high microbial risk during the production chain and the behavior of human pathogens in the phyllosphere of fresh produce.

Human pathogens on vegetables

Leaves harbor a large community of microorganisms, containing yeasts, filamentous fungi and bacteria (Lindow & Brandl, 2003), which will be discussed in more detail below (see section ”Persistence in the phyllosphere”). Although the largest part of the microorganisms persisting in the phyllosphere is indifferent or even beneficial to human health, they can also comprise potential pathogens (Berg *et al.*, 2014; Erlacher *et al.*, 2014; Vitali *et al.*, 2012). Among the most relevant pathogenic bacteria on leafy vegetables are human pathogenic *Escherichia* (*E.*) *coli* such as enterohemorrhagic *E. coli* (EHEC), *Salmonella* spp. and *Listeria* (*L.*) *monocytogenes* (Carrasco *et al.*, 2010; Brandl, 2006), which will be discussed below in more detail. An overview on optimal growth conditions is given in Table 1-1.

EHEC

E. coli are Gram negative rods, that are motile due to their peritrichous flagella and a majority of the strains own fimbria (Madigan *et al.*, 2012; Rheinbaben, 2011). *E. coli* are tolerant to acidic conditions (Armstrong *et al.*, 1996) and can therefore survive the passage through the stomach (Freter *et al.*, 1983). Bacteria comprised by the species *E. coli* belong to the order of *Enterobacteriales,* the family *Enterobacteriaceae* and the genus *Escherichia* (Madigan *et al.*, 2012). Different serotypes are classified through cell surface antigens, namely “O” which stands for a part of the lipopolysaccharide layer and “H” which refers to flagellum (Orskov *et al.*, 1977). *E. coli* are commonly belonging to symbionts of the gut flora in mammals (Bell, 2002). Integration of pathogenicity islands via plasmids or bacteriophages make this usually non-pathogenic bacterium threatening for human health. There are six virulence factors that apply for pathogenicity, namely the production of shiga-like toxins (also called vero-toxin), *eae*-gene (attaching and effacing *E. coli*), intimine for adhesion, haemolysin, serin protease and a heat-stable enterotoxin. Here, the former three count as the most crucial ones (Bell, 2002). Pathogenic strains are called shiga-toxin producing *E. coli* (STEC) or vero-toxin producing *E. coli* (VTEC). Infections can already occur at low doses of less than 100 cells (Rheinbaben, 2011) and evoke symptoms such as diarrhea and hemorrhagic colitis. More severe courses can manifest in hemolytic uremic syndrome (HUS) and thrombotic thrombocytopaenic purpura (TTP) (Smith *et al.*, 2002). Beside the serotype of the causative agent (Tarr *et al.*, 2005), the age of the infected person is decisive for the severity of an illness (Bell, 2002). STEC-strains that potentially lead to severe courses are also referred to as enterohemorrhagic *E. coli* (EHEC) (Rheinbaben, 2011). Among these, *E. coli* O157:H7 causes worldwide the majority of outbreaks (Ferens & Hovde, 2011), but also strains belonging to serogroups O26, O103, O111, O118 and O145 have been identified to be the causative agent for severe diseases (Tzschoppe *et al.*, 2012).

Cattle and other ruminants are thought to be the main reservoir for EHEC (Stevens *et al.*, 2002). Pruimboom-Brees *et al.* (2000) showed that cattle lack a receptive receptor for shiga-toxins in their intestinal tract and therefore function as carrier animals without getting infected (Smith *et al.*, 2002; Pruimboom-Brees *et al.*, 2000). Meat, in particular ground beef was found to be the source in a majority

of outbreaks (Armstrong *et al.*, 1996), but recently alertness has also been drawn to vegetables (Soon *et al.*, 2013). In this scope, leafy greens are most often associated with serious outbreaks (Heaton & Jones, 2008). In 2005 an outbreak in Sweden with 135 cases in total and 11 confirmed HUS was most likely linked to the consumption of iceberg lettuce (Söderström *et al.*, 2008). One year later, spinach seemed to be the source for an *E. coli* O157:H7 outbreak in the USA with about 200 cases of which 32 resulted in HUS and 3 ended fatal. Of these, 71% of the infected people were women (Centers for Disease Control and Prevention (CDC), 2006a). In 2010 another outbreak in the USA could be traced back to roman lettuce (Centers for Disease Control and Prevention (CDC), 2010). In Germany, there was a huge outbreak in 2011 which comprised about 4000 cases of which at least 56 ended fatal. The infections were mediated through the consumption of organically grown sprouts that were contaminated with *E. coli* O104:H4, a highly virulent strain that lacks the *eae*-gene (Bundesinstitut für Risikobewertung, 2011; European Centre for Disease Prevention and Control (ECDC), 2011). Shortly thereafter, the same strain was responsible for an epidemic in France (Buchholz *et al.*, 2011; European Centre for Disease Prevention and Control (ECDC), 2011).

Salmonella spp.

Salmonella spp. are Gram negative, rod shaped bacteria (Francis *et al.*, 1999), which are motile owing to their flagella (Partridge & Harshey, 2013). *Salmonella* exhibit optimal growth between 35 and 43 °C. Most strains are not capable of multiplying below 5 °C (Francis *et al.*, 1999). They have low requirements in terms of nutrients and can survive at low oxygen concentrations (Xu *et al.*, 2010). Like *E. coli* they belong to the family of *Enterobacteriaceae* (Tajkarimi, 2006; Francis *et al.*, 1999). The genus *Salmonella* comprises two species - *S. enterica* and *S. bongori* - (Madigan *et al.*, 2012) and more than 2500 serovars (=serotypes) (Trevejo *et al.*, 2003). *S. enterica,* which itself can be divided into 7 subspecies, includes more than 1400 serovars that are known to have the ability to cause infections in humans (Madigan *et al.*, 2012), with serovars Typhimurium and Enteritidis being the most frequently identified causative agents in foodborne outbreaks (Lim *et al.*, 2003). Minimal infectious doses are dependent on the serovar, but in most cases rather high with 10^5 to 10^{10} cells (Kothary & Babu, 2001). Manifestation arises in symptoms such as gastroenteritis and septicemia and to a lesser extend pneumonia and meningitis (Trevejo *et al.*, 2003).

Salmonella have been detected in a wide range of different animals, namely poultry, birds, reptiles, amphibians, cattle and many other mammals (Francis *et al.*, 1999). Animals as well as humans can carry the pathogen in their intestinal tract without showing any symptoms, disseminating disease (Gopinath *et al.*, 2012). The bulk of salmonellosis outbreaks is caused by these carrier animals, particularly poultry, pigs and to some minor impact ruminants. Meat may be contaminated during slaughter and eggs either transovarial or externally by fecal staining (Oosterom, 1991). Furthermore, *Salmonella* is prevailing when it comes to outbreaks related to produce (Brandl, 2006). A wide range of vegetables like pepper, tomato, alfalfa and bean sprouts have been reported to act as transmitter in outbreaks (Mody *et al.*, 2011; Centers for Disease Control and Prevention (CDC), 2009; Rohekar *et al.*, 2008; Centers for Disease

Control and Prevention (CDC), 2006b) and also lettuce could be identified as vector for salmonellosis (Oliveira *et al.*, 2010).

L. monocytogenes

Bacteria belonging to the genus *Listeria* are Gram positive rods that are motile due to their peritrichous flagella (Kim *et al.*, 2005). Higher ranking, this genus is assigned to the class of *Bacilli* and the family of *Listeriaceae* (Madigan *et al.*, 2012) and is divided into six species of which one can cause infections in humans, namely *L. monocytogenes* (Jemmi & Stephan, 2006). Altogether thirteen strains, possessing a variable virulence, are known (Wiedmann *et al.*, 1997), which can be classified into three genetic lineages. Lineage I contains serotypes 1/2b, 3b, 3c and 4b, lineage II 1/2a, 1/2c as well as 3a and lineage III 4a and 4c (Chen & Knabel, 2007). Strains isolated from human infections mostly belong to the serotypes 1/2a, 1/2b and 4b, the latter being associated most frequently with large outbreaks (Wiedmann *et al.*, 1997; Farber & Peterkin, 1991). Infections caused by *Listeria* are called listeriosis and lead to meningitis, sepsis and miscarriage in pregnant women. Susceptibility varies within human population, whereat pregnant women, children, elderly and immunosuppressed people are at highest risk (Farber & Peterkin, 1991). Mortality rates during an outbreak can be as high as 40% (Carrasco *et al.*, 2010). The minimal infectious dose for *L. monocytogenes* in humans is not known, but is thought to be strongly dependent on the susceptibility of the individual as well as the causative strain (Farber & Peterkin, 1991). The infectious dose is thought to be rather high in healthy people, but in susceptible people low numbers are sufficient to cause an infection (Todd & Notermans, 2011; Franciosa *et al.*, 2005). Animal models revealed infectious doses to be 10^2 cells for mice or chicken and 10^9 cells for Cynomologous monkeys. However, such results have only a limited relevance for comparison with humans (McLauchlin *et al.*, 2004).

L. monocytogenes can survive under unfavorable conditions such as starvation and in acidic or salty environments (Madigan *et al.*, 2012). Special attention is drawn to its ability to multiply at low temperatures around the freezing point of 0 °C (Todd & Notermans, 2011; Carlin *et al.*, 1995). *L. moncytogenes* have a ubiquitous occurrence and have been isolated from a wide range of habitats such as soil, water, feces (Arvanitidou *et al.*, 1997; MacGowan *et al.*, 1994) and plants (Schuchat *et al.*, 1991). Cattle can act as carrier animals and milk, raw dairy as well as meat are thought to be the main source for listeriosis (Todd & Notermans, 2011), but also vegetables have been identified as transmitter. The first outbreak that could be traced back to the consumption of raw vegetables occurred in the year 1981 in Canada (Schlech *et al.*, 1993) and demanded 5 abortions and 4 stillbirths (Schuchat *et al.*, 1991). More recently, an outbreak in the USA (2010) related to celery reveled 10 cases of which 5 ended fatal (U.S. Food and Drug Administration (FDA), 2010). In 2011, another outbreak in the USA, where cantaloupe could be determined to be the source, reported 146 cases comprising 30 deaths and one miscarriage (Centers for Disease Control and Prevention (CDC), 2011). Altogether, listeriosis outbreaks are seldom, but nevertheless relevant due to their fatal character and wide occurrence in foods (Carrasco *et al.*, 2010). Furthermore, the number of reported cases in Europe increased during the past years

(European Food Safety Authority (EFSA) & European Centre for Disease Prevention and Control (ECDC), 2014).

Table 1-1. Favorable conditions for the growth of *E. coli*, *Salmonella* spp. and *L. monocytogenes.*

	E. coli	***Salmonella* spp.**	***L. monocytogenes***
pH optimum	6.0-7.0[1]	6.5-7.5[2]	7.0[4]
pH range	4.4-9.0[1]	3.7-9.0[3]	4.4-9.4[4]
Temperature optimum	37°C[1]	35-37°C[3]	37°C[4]
Temperature range	7-46°C[1]	5-45°C[3]	-1.5-45°C[4]
Water availability a_W	0.995[1]	0.960-0.999[3]	0.99[5]
Metabolism	facultative anaerobe[1]	facultative anaerobe[3]	facultative anaerobe[4]

[1] ESR (2001a)
[2] Richard (2013)
[3] Tajkarimi (2006)
[4] ESR (2001b)
[5] Zarei *et al.* (2012)

Indicator organisms

The surveillance and quantification of all potential human pathogens in foods is difficult to perform and as well time as cost consuming. Therefore the microbial status of food is evaluated by the means of indicator organisms (Savichtcheva & Okabe, 2006). An indicator can be defined as “a variable that describes the state of a system” (Walz, 2000). These microorganisms are not necessarily pathogenic to humans (Scott *et al.*, 2002), but should fulfill similar criteria as the pathogen they represent. Indicators should have a high correlation to the occurrence of the pathogen(s) it stands for, should be at least as imperishable to decontamination agents and environmental influences as its substituent and easily and fast to detect (Savichtcheva & Okabe, 2006).

In Germany required quality of water used for irrigation of raw eaten vegetables is regulated in DIN DIN 19650 (1999). According to this standard, 100 ml should be free of *Salmonella*, *streptococci* should be below 100 MPN and *E. coli* below 200 MPN. The safe use of manure is regulated by the “Biostoffverordnung” (BioStoffV, 2013), which suggests treatments that reduce the number of pathogens. To date no governmental defined measurements for indicator organisms are available.

The German Society for Hygiene and Microbiology (Deutsche Gesellschaft für Hygiene und Mikrobiologie, 2010) published guidance and alert values for salads in Germany. Hereafter, the heterotrophic bacterial plate count is noncritical up to numbers of $5x10^7$ CFU g^{-1}. To assure safety of the product, *E. coli* and *L. monocytogenes* may not exceed values of 10^3 and 10^2 CFU g^{-1}, respectively, and *Salmonella* should not be detected in a sample with the weight of 25 g. *L. monocytogenes* and

Salmonella have already been discussed in this thesis and it is only left to add, that *Listeria* are good hygiene indicators due to their ubiquitous character (Jemmi & Stephan, 2006). The heterotrophic bacterial plate count can give a first impression on the general hygienic status of fresh produce (Ragaert *et al.*, 2007). *E. coli* is the most frequently used indicator for fecal contamination (Gómez-Aldapa *et al.*, 2013) as its primary habitat is limited to the intestinal tract of higher animals (Winfield & Groisman, 2003). Further advantages are, that most *E. coli* strains are not pathogenic to humans and are usually present in higher numbers as compared to pathogens (Scott *et al.*, 2002).
Other indicator organisms that are consulted to assess microbial quality are total coliform bacteria and *Enterococci*. As coliform bacteria not only include bacteria of fecal origin (Geldreich *et al.*, 1964), they represent rather an indication for bad hygiene as for fecal contamination (Gómez-Aldapa *et al.*, 2013). *Enterococci* are habitants of the human intestine and therefore indicate fecal contamination. A drawback is posed by their ability to persist in the environment for a long time once introduced (Scott *et al.*, 2002).

Contamination sources/routes

Contamination of produce can occur along the whole production chain, starting from contaminated seeds, over the cultivation to processing and by the consumer himself. As the focus in this studies lies on the pre-harvest activities, the contamination risks coming from handling the produce after the cultivation will not be discussed.

Contamination of seeds

The hazard posed by the use of contaminated seeds is of great concern in the sprout production, as cultivation parameters are favorable for human pathogens. High humidity, darkness and high temperatures promote the multiplication of these microorganisms (Buck *et al.*, 2003). Until now, only little attention has been drawn to the evolvement of human pathogenic bacteria on seeds that are cultivated for vegetable production other than sprouts. These bacteria are not optimal adapted to lower temperatures like common in the soil. Nevertheless, Jablasone *et al.* (2005) found *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* to establish well in the phyllosphere of different vegetable seeds when inoculated before germination and an initial temperature of 15°C. During germination, nutrients are set free when the stored energy is mobilized to supply the seed with energy for growth. These seed exudates represent also an energy resource for bacterial multiplication (Nelson, 2004). Further, Jablasone *et al.* (2005) showed the invasion of *E. coli* O157:H7 and *S.* Typhimurium into tissue of several crops, among others lettuce. Besides, bacteria will more likely internalize leaves when they are introduced to the plant at an early stage (Warriner *et al.*, 2003a).
Root exudates contain nutrients like monosaccharides, amino as well as organic acids and these exudates are considered to be advised to attract beneficial microorganisms (Lugtenberg *et al.*, 2001). The attracting compounds, however, could also be an allurement for human pathogenic bacteria (Aruscavage *et al.*, 2006). Klerks *et al.* (2007) showed that several *Salmonella enterica* serovars actively moved to root exudates of lettuce.

Contamination during cultivation

Cultivation methods and herewith associated commodities as well as extrinsic factors can have an impact on the contamination of fresh produce. The latter is described by the risk posed by feces of animals, namely wild rodents and larger mammals, birds, snails, flies and dogs which misemploy fields during their walks as a toilet (Beuchat, 2006; Sproston *et al.*, 2006; Janisiewicz *et al.*, 1999). Also insects have been shown to function as carrier of human pathogens. Remedy can be found in fences which can restrain large mammals to a certain extent (Local Government Management Association of British Columbia, 2013), but this will not affect flying birds or small rodents. Also, air borne transmission, e.g. from nearby animal husbandry is of concern (Berry *et al.*, 2015). Even humans can introduce pathogens to the crop and hygiene of field workers should be taken into account (Brackett, 1999).
Among production input means, two major areas can be distinguished that have drawn attention in concerns of produce safety, namely organic fertilizer of animal origin and the irrigation water source and mode of administration.

Manure

Soil used for farming is frequently amended with manure, in organic as well as in conventional production (Paulsen *et al.*, 2013), to enhance soil fertility by increasing the organic matter content (Xie *et al.*, 2014). But in organic farming it is applied more often and to a greater extent as it is an important nitrogen provider due to the prohibition of the usage of synthetic fertilizers (McMahon & Wilson, 2001). Manure can harbor microorganisms that can be threatening to human health. Preventive measures have to be employed (Nicholson *et al.*, 2005). In Germany the safe use of manure is regulated by the Biostoffverordnung (2013) and has to be ensured by treatments prior to application like pasteurization, aerobic composting or anaerobic fermentation. Composting is a widely used traditional method to control pathogens in manure (Bernal *et al.*, 2009), but its efficiency is dependent on several factors like moisture content, time of incubation, temperature and the location in the heap (Shepherd Jr *et al.*, 2010; Wichuk & McCartney, 2007; Turner, 2002) and the desired quality may not be achieved in case of incorrect processing (Termorshuizen & Alsanius, 2016). Shepherd Jr *et al.* (2010) tracked the inactivation of *E. coli* O157:H7 and *S.* Typhimurium in cattle manure with regular waste during composting in winter and in summer. Compost heaps were turned four times during each trial. In summer, both *E. coli* O157:H7 and *S.* Typhimurium could not be detected after 14 days, whereas culture-dependent methods with preenrichment were employed. Samples taken from the heap during winter were still tested positive for both bacteria at the end of the experiment after 60 days. The highest temperature achieved in winter and summer was 40 °C or 60 °C, respectively. For effective composting, it is important that after the initial mesophilic phase (30 – 45 °C), temperatures of 45 to 75 °C are reached in the thermophilic phase and that the heaps are turned on a regular basis (Shepherd Jr *et al.*, 2010; Shepherd *et al.*, 2007). Human pathogens can hence be transmitted to the soil in which field crops are grown, if manure is composted wrong (Guan & Holley, 2003). It has been shown, that pathogens can survive in the soil when applied to the field together with manure (Islam *et al.*, 2005). Nyberg *et al.*

(2010) analyzed the survival time of *E. coli* O157:H7 and *S.* Typhimurium in soil that was amended with inoculated chicken or cattle manure. To detect the introduced bacteria, culture-dependent methods were employed, with and without previous enrichment. Both bacterial strains could be reisolated at the end of the trial after 6 months in chicken as well as in cattle manure with enrichment. Differences were visible between the two manures when no enrichment was used (detection limit 2 Log CFU (g soil)$^{-1}$). *E. coli* O157:H7 and *S.* Typhimurium were enumerated up to 90 days in soil amended with chicken manure and 60 days in soil amended with cattle manure, respectively. Also Islam *et al.* (2005) found better survival rates of *E. coli* O157:H7 on vegetables when soil was amended with chicken instead of cattle manure.
Other possibilities to provide nitrogen of animal origin in organic farming can be horn products or grinded swine kemp pressed to pellets. As these materials are heated to a high temperature during production, they represent a safer alternative with respect to microbial contamination.

Water and irrigation

There are several sources of water with varying microbial quality that can be used for vegetable irrigation. Rain, tap and ground/well water apply to be of minor concern compared to surface and waste water regarding contamination with pathogenic microorganisms (Alsanius, 2014; Kirby *et al.*, 2003). It is prohibited to use waste water for irrigation due to the great risk of transmitting human pathogens (Gerba, 2009). However, this is still used as a measure in areas facing water scarcity (Decol *et al.*, 2017; Norton-Brandão *et al.*, 2013).
Contamination of surface water, including reservoirs for water storage, can occur for example through runoff from nearby animal husbandry, wild animals, leaking sanitary facilities and incorrect discharge of industrial and household waste (Kirby *et al.*, 2003). Once introduced to the water, the survival of a bacterium is dependent on factors like temperature, organic matter content, available nutrients and the presence of other microorganisms (Pachepsky *et al.*, 2011). It has been shown that *E. coli* O157:H7 can survive in different surface waters for up to two months and more (Avery *et al.*, 2008), whereas *Salmonella* are thought to endure even better in aquatic environments (Winfield & Groisman, 2003). Beside *E. coli* O157:H7 and *Salmonella* also *L. monocytogenes* has been isolated from surface waters (Wilkes *et al.*, 2009) and hence untreated irrigation water can act as vectors for all three pathogens.
The application method of irrigation water can have an impact on the contamination of the irrigated crops (Pachepsky *et al.*, 2011). Application close to the soil surface reduces the contact with edible parts of leafy vegetables and therefore has the potential to minimize transmission (Qadir *et al.*, 2010). Song *et al.* (2006) gained lower *E. coli* counts for lettuce watered with drip irrigation compared to furrow irrigation when water was contaminated with *E. coli*. However, this effect could not be observed in all trials. Moyne *et al.* (2011) compared drip and overhead irrigation with *E. coli* O157:H7 contaminated water of lettuce in two consecutive years. While they found a significant difference in 2008, one year later the two irrigation methods resulted in the same load of *E. coli* O157:H7 on lettuce plants.

Beside the direct contamination of vegetables with contaminated water, the irrigation method can also have an impact on the transmission of pathogens from soil to the leaves. Splashing of water droplets increases the splashing of soil particles carrying pathogens which potentially land on the leaves (Fischer-Arndt *et al.*, 2010; Abu-Hamdeh *et al.*, 2006). Protection can be given by using ground cover like mulch foil, which reduces the contact surface of soil and leaves (Song *et al.*, 2006). Its effect, however, is controversial (Fischer-Arndt *et al.*, 2010).
Not to be neglected is the hygiene of systems for irrigation. Many bacteria are harbored in these tubes, including human pathogens (Pachepsky *et al.*, 2011). The ability to form biofilms on the walls and hence be protected from water flow and sanitizers presents a reservoir for continuous contamination (Pachepsky *et al.*, 2012; LeChevallier *et al.*, 1987).

Special case: organic vs. conventional farming

The growing concern for a healthy diet not only led to an increase in overall vegetable consumption, also the demand for organic produce is rising. During the last years, the turnover gained with organic products as well as the area used for organic vegetable production increased steadily in Germany (Statista, 2014; Hauschild *et al.*, 2013). In a public view, advantages of organic vegetables are seen in a higher nutrient content, less pesticide residues and fewer harmful organisms as compared to conventional crops (Hoefkens *et al.*, 2009). However, no scientific proof has been provided that supports these presumptions (Forman & Silverstein, 2012; Williams, 2002) and the fundamental idea behind organic farming is rather an environmental friendly, sustainable production than generating healthy products. In contrast to the broad belief, the professional point of view on the microbial safety of organic produce is more critical. As already mentioned, organic farming relies on manure for fertilization, which is likely to harbor zoonotic pathogens that can be transmitted during cultivation (Shepherd Jr *et al.*, 2010). In addition, the use of sanitizing agents to reduce microbial loads is restricted (Ponce *et al.*, 2003). Evaluation of produce from different origins and the reckoning of vectors in food related outbreaks do not suggest a higher microbial hazard of organic produce (Oliveira *et al.*, 2010; Bourn & Prescott, 2002). Others, however, see an increasing risk for foodborne outbreaks with a growing organic sector (Adl *et al.*, 2011).

Persistence in phyllosphere

Above-ground parts of plants that function as habitat for microorganisms are called phyllosphere and include stem, buds, flowers fruits and leaves (Whipps *et al.*, 2008). To address the latter more specifically, the term phylloplane can be used (Inácio *et al.*, 2002). The phyllosphere applies to be a rather hostile environment for microorganisms with regard to low moisture content, temperature, UV-radiation and nutrient availability, whereupon these conditions can be inconsistent in time and space (Monier & Lindow, 2005; Thompson *et al.*, 1993). Nevertheless, the phyllosphere is inhabited by bacteria, archaea, filamentous fungi and yeasts, algae and sometimes protozoa and nematodes (Whipps *et al.*, 2008). The first are most abundant with counts from 10^2 to 10^{12} CFU g^{-1} leaf of culturable bacteria

followed by filamentous fungi and yeasts which show numbers of 10^1 to 10^{10} CFU g^{-1} leaf (Whipps *et al.*, 2008). To defy stress, between 30 – 80% of the microorganisms on a leaf surface gather in biofilms (microbial assembly) which protects them from desiccation. In addition, biofilms have the ability to extract nutrients from leaf veins and trichomes (Heaton & Jones, 2008; Morris & Monier, 2003). Biofilms consist of exopolysaccharides which are secreted by bacteria (Carmichael *et al.*, 1998) and different bacterial and fungal species can live together in one aggregate (Morris & Monier, 2003).
The bacterial phyllosphere biota of different plants including lettuce consists of species belonging to the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* (Bulgarelli *et al.*, 2013; Rastogi *et al.*, 2012). Lopez-Velasco *et al.* (2011) analyzed the microbiome of spinach by means of 16S rRNA gene pyrosequencing and found *Proteobacteria* to be dominating, representing about 80% of the sequences. But also the phyla *Firmicutes, Chlamydiae, Gemmatimonadetes, Verrucomicrobia, TM7, Deinococcus-Thermus, Planctomycetes, Actinobacteria, Acidobacteria* and *Bacteroidetes* were present. On lower classification levels, *Enterobacteria* and *Pseudomonas* were the most abundant, like also revealed for several lettuce (Hunter *et al.*, 2010).
Human pathogens, although some of them belonging to *Enterobacteriaceae*, are not considered common members of the leaf microbiota (Chitarra *et al.*, 2014). However, *E. coli*, *Salmonella* and *L. monocytogenes* have been isolated from fresh produce from retail markets (Viswanathan & Kaur, 2001; Beuchat, 1996) and it has been shown, that *S.* Typhi as well as *E. coli* were able to persist in the phyllosphere of lettuce until harvest when inoculated on young plants (Ercolani, 1979). Islam *et al.* (2004b) found *S.* Typhimurium to survive on lettuce and parsley for 63 and 231 days, respectively. But not only have human pathogens been shown to invade this harsh environment, if the conditions are favorable, they even can multiply on lettuce leaves (Brandl & Amundson, 2008).
Beside intrinsic factors of the bacteria themselves (e.g. chemotaxis, motility) (Yaron, 2014), several aspects have been identified to alter the competence to establish in the phyllosphere.
Influences of environmental (climate) and biological (leaf surface, nutrient availability, microbial community) factors as well as the ability of some bacteria to internalize leaf tissue will be discussed below.

Environmental factors influencing the persistence in the phyllosphere

Climate

Several studies found varying contamination levels of the phyllosphere in different seasons or years, *ceteris paribus* (Caponigro *et al.*, 2010; Fischer-Arndt *et al.*, 2010; Ailes *et al.*, 2008), indicating the influence of weather based factors. Warm temperatures and free water promote the survival of human pathogens on leaves (Brandl & Amundson, 2008), whereas UV-radiation leads to a decline in bacterial cells (Dreux *et al.*, 2007). Some microorganisms, however, protect themselves from this hazard by pigmentation or repair of damaged DNA (Brandl, 2006; Sundin & Jacobs, 1999). Thus, UV radiation also leads to a shift in the microbial community (Heaton & Jones, 2008).

Comparison between *E. coli* and *Pseudomonas (P.) syringae*, a species incorporating many plant epiphytes, revealed differences in establishing in the phyllosphere of different vegetable crops depending on extrinsic conditions. At favorable conditions, namely high humidity, survival of both microorganisms was similar, at non optimal conditions (low humidity) *P. syringae* showed significantly higher numbers than *E. coli* (O'brien & Lindow, 1989).

Biological factors influencing the persistence in the phyllosphere

Leaf surface

The leaf surface consists of a cuticle layer, which is highly hydrophobic (Frank, 2001). Pathogenic bacteria showing hydrophobic components on their cell surface can attach to the cuticle by means of molecular interactions (Patel & Sharma, 2010; Hassan & Frank, 2003). The topography of a leaf displays an important factor for bacterial adhesion and is dependent on plant species and leaf age (Yaron, 2014). Epiphytic bacteria as well as alien bacteria like *E. coli* O157:H7 if introduced to the phyllosphere, are not homogeneously distributed on the leaf surface. Preferred sites for attachment have been identified to be stomata, trichomes, epidermal cell wall junctions, veins and hydathodes (Hunter *et al.*, 2010; Seo & Frank, 1999), where they are protected and nutrients are available (Yaron, 2014). Differences in abundance of stomata and trichomes (Frank, 2010) could provide explanation approaches for different attachment abilities of human pathogens to different plant species (Patel & Sharma, 2010).

Nutrient availability

Plants exudate or leak nutrients through exudates or guttation fluid from the inside of the leaf to the surface (Brandl & Amundson, 2008; Wilson & Lindow, 1994). These liquids contain carbohydrates, amino acids, organic acids and inorganic nutrients (Tukey Jr, 1970). The amount of leakage is dependent on several factors including plant species, leaf characteristics and the presence of water on the leaf surface (Leveau & Lindow, 2001). The distribution on the leaf surface of these substrates is not homogeneously (Leveau & Lindow, 2001). Veins, trichomes, cavities and other sites that can retain fluids harbor more nutrients, as water promotes the leaking (Leveau & Lindow, 2001; Tukey Jr, 1970). However, changes in the moisture content on the leaf influence the nutrient concentration (Hirano & Upper, 2000).
Nutrients account for the limiting factor for microbial abundance on leaves, confirmed by studies that found higher microbial counts when additional nutrients were applied (Wilson & Lindow, 1994). If a community is more carbon or nitrogen deprived is depending on the C/N ratio of the available nutrients (Wilson & Lindow, 1994).
Conditions with enhanced leakage of nutrients have been shown to promote the persistence of human pathogens in the phyllosphere. Brandl and Amundson (2008) found the multiplication rate of *E. coli* O157:H7 and *S.* Thompson to be higher in young lettuce leaves as compared to middle old leaves. At the same time, the exudates of the young plants contained more nitrogen. Amendment with nitrogen resulted in higher *E. coli* O157:H7 counts whereas the application of glucose did not affect the count,

suggesting that nitrogen was the limiting resource. In addition, young leaves have an increased metabolic activity and a denser cover with trichomes (Brandl & Amundson, 2008). Brandl (2008) compared the persistence of *E. coli* O157:H7 in lettuce phyllosphere for healthy, physically damaged and soft rot infected leaves. Damaged tissue, irrespective the origin, showed significantly higher *E. coli* O157:H7 counts after four as well as 22 hours as compared to healthy leaves (Brandl, 2008). Also *Salmonella* have been shown to have a higher abundance in vegetables affected by soft rot compared to healthy plants (Wells & Butterfield, 1997). Abiotic lesions of plant tissue as well as biotic damage caused e.g. by soft rot injure the leaf tissue and hence leakage from plant cells is enhanced, providing more nutrients which stimulate bacterial growth (Aruscavage *et al.*, 2010; Brandl, 2008).

Microbial community

The native microbial community on a leaf can have a suppressive, promoting or no effect on human pathogens in the phyllosphere (Rastogi *et al.*, 2013; Lopez-Velasco *et al.*, 2011). Suppressive effects between bacteria known from biocontrol in the rhizosphere are characterized by competition for nutrients and/or space, siderophore production and antibiosis (Whipps, 2001). Commensalism between microorganisms can occur by making nutrients accessible (e.g. via degradation of present source or by releasing plant nutrients) or by altering the microenvironment (modifying tissue, production of polysaccharides as protection from desiccation) (Deering *et al.*, 2012; Cooley *et al.*, 2006).
Several studies identified bacterial strains from the indigenous microbiota of leafy vegetables to have an effect of human pathogens in the phyllosphere. Cooley *et al.* (2006) found *Wausteria paucula* to enhance the survival of *E. coli* O157:H7 on lettuce seedlings, whereas *Enterobacter asburiae* reduced its survival 30- to 40-fold. Competition for nutrients was most likely the suppressing effect. *E. asburiae* has also been shown to reduce the number of *Salmonella* on tomato plants (Ye *et al.*, 2009). Another species of the *Enterobacter* genus, *E. cloaceae*, was able to reduce the numbers of *E. coli* O157:H7 and *L. monocytogenes* cells on lettuce seeds by approximately 1 Log CFU g^{-1} (Jablasone *et al.*, 2005). Lopez-Velasco *et al.* (2012) analyzed the influence of bacteria from the spinach phyllosphere on *E. coli* O157:H7. They found different strains, mainly belonging to *Firmicutes* and *Enterobacteriaceae*, to suppress the growth of *E. coli* O157:H7 *in vitro*. Several strains, e.g. *Erwinia perscina*, *Pantoea agglomerans*, *Pseudomonas* spp. and *Bacillus* spp., were also able to reduce the pathogen on mature spinach leaves. *In vitro* studies identified nutrient competition and acid production to be the growth inhibiting factor. In the same study, *Flavobacterium* sp. enhanced the survival of *E. coli* O157:H7 in the spinach phyllosphere. Yet, the previously as promoting identified bacterium *W. paucula* (Cooley *et al.*, 2006) did not have an effect. A possible explanation can be differences in leaf age chosen in the two studies, which result in an altering nutrient abundance in plant exudates (Lopez-Velasco *et al.*, 2012).

Internalization

Several studies revealed human pathogens not only to persist on the surface of vegetable plants, but also to invade the tissue (Chitarra *et al.*, 2014; Brandl, 2008; Franz *et al.*, 2007; Jablasone *et al.*, 2005;

Warriner *et al.*, 2003f). If a bacterium will internalize plant tissue, however, is dependent upon different factors. A bacterium will more likely internalizes plant tissue at lower temperatures (Chitarra *et al.*, 2014; Takeuchi & Frank, 2000), at ambient compared to lowered oxygen levels (Takeuchi & Frank, 2000), at suboptimal water supply (Ge *et al.*, 2012) and by light (Kroupitski *et al.*, 2009).
Plant species as well as plant age have been identified to play a role (Deering *et al.*, 2012). Vegetables producing essential oils have a miner risk to be invaded by human pathogens (Chitarra *et al.*, 2014; Friedman *et al.*, 2004), but also different salads show to have varying potential of being penetrated (Chitarra *et al.*, 2014). Contradictory results have been found considering the susceptibility of young and mature leafy greens (Mootian *et al.*, 2009; Warriner *et al.*, 2003a).
Also bacterial strain (Klerks *et al.*, 2007; Dong *et al.*, 2003) and species have an impact on internalization of leafy vegetables. Comparing the three pathogens that are discussed in this thesis, *E. coli* O157:H7 is most prone to invade tissue, followed by *Salmonella* spp., whereas *L. monocytogenes* was only very rarely able to overcome the barrier (Chitarra *et al.*, 2014; Franz *et al.*, 2007; Jablasone *et al.*, 2005).
There are two main routes of internalization, described by either active or passive invasion. Pathogens like EHEC or *Salmonella* are not known to be able to metabolize the plant cell wall constituent pectin (Teplitski *et al.*, 2012), hence the former route is characterized by openings in the surface of the plant, e.g. stomata, lateral root emergence sites and damages in the plant material, which can be either abiotic or biotic. Secondly, the bacteria could be transported into the tissue along with water, e.g. during irrigation or when soaking seeds (Deering *et al.*, 2012). Several studies found evidence, that the pathogens invaded the plants actively. Kroupitski *et al.* (2009) examinated the impact of light regime (light, darkness) on iceberg lettuce inoculated with *S. enterica* and found that the bacterium agglomerates and invades stomatal openings under light but not dark conditions, suggesting active movement when attracted by photosynthetic borne nutrients. Klerks *et al.* (2007) monitored the same species moving actively to root exudates of lettuce and it was presumed that flagella assists *Salmonella* and *E. coli* O157:H7 cells by invading plant tissue (Cooley *et al.*, 2003). A contrary result was obtained by a study that compared the invasion of *E. coli* O157:H7 and fluorescent microparticles. Both were found at similar numbers on the interior of lettuce plants, indicating a passive transport (Solomon & Matthews, 2005).
Internalization of human pathogens into vegetables is of special concern as they are protected from washing and sanitizing treatments (Takeuchi & Frank, 2001). There is an indication, however, that the number of pathogens decline faster after invading plant tissue compared to cells on the leaf surface (Li *et al.*, 2008). This could be due to a higher nutritional/physiological stress upon the inside of a vegetable or efficient plant defense (Yaron, 2014).

Microbial analysis

To assess microorganisms, two main approaches can be distinguished, namely culture-dependent and culture-independent methods. To study microorganisms by means of culture-dependent methods, they

are commonly, if not already in a liquid environment, brought to suspension (Yang *et al.*, 2001). This suspension is then plated on culture media, providing essential nutrients for bacterial growth. There are many different media available, which can be non-selective, selective and/or differential. Selective media inhibit the growth of certain microorganisms, whereas differential media exhibit an indicator, allowing the identification of a certain group of microorganisms (Madigan *et al.*, 2012). Further study of single colonies can be done by description of the phenotype (Hugenholtz & Pace, 1996) or by genotype sequencing (Yarza *et al.*, 2014; Johnsen & Nielsen, 1999). Quantification of the microorganisms in the suspension can be achieved by counting the Colony Forming Units (CFU) on a plate inoculated at an appropriate dilution and calculating the CFU g^{-1} or ml^{-1} of the analyzed sample (Madigan *et al.*, 2012). Assembly of microorganisms in biofilms and other elevated attachments on the sample, however, prevent these microbes from being washed off and therefore underestimation of the viable count can occur (Hunter *et al.*, 2010). Another drawback of culture-dependent methods is that more than 99% of the microorganisms are not culturable (Hugenholtz & Pace, 1996) and therefore diversity in the analyzed environment is underestimated (Griffiths *et al.*, 2000). Remedy can be found in culture-independent methods which address both, culturable and unculturable microorganisms (Ward *et al.*, 1990). Metagenomics by means of high throughput sequencing describes methods that allow the study of diversity and relative abundance of microorganisms in a certain environment (Huse *et al.*, 2008). For reliable characterization of microbial groups, the sequenced genomic fragments should be at least 100 bp long (Jones, 2010). To date, two high throughput applications are available, namely Solexa/Illumina and 454-pyrosequencing by Roche (Bacci *et al.*, 2015; Mardis, 2008) of which the former was employed within the study of this thesis and is thus discussed in more detail. The sequencing preceded is a solid-phase PCR. Here, ssDNA is washed across a flow cell, hybridizing randomly to primers fixed on the surface. Hereafter, the strands are elongated and the obtained dsDNA is denatured, leaving the newly synthesized strand covalently bound to the surface. Reduced temperatures motivate the single strands to bend over and hybridize to the adjacent primer on the surface (single stranded bridge). The hybridized primer is elongated by polymerase, resulting in a double stranded bridge, which is subsequently denaturized. This procedure is repeated until a cluster of identical ssDNA is bound to the surface of the flow cell. After cleaving the reverse strands, the actual sequencing is carried out. Polymerase that is equivalent to the upper end of the strands as well as fluorescent labeled, terminated nucleotides are added. After the binding of one nucleotide to each strand, surplus material is washed off. Different coloring of the nucleotides reveal the just incorporated molecules. After removal of terminator and dye, the procedure is repeated, exhibiting the sequence of the bound DNA (Metzker, 2010; Mardis, 2008).

One drawback of methods deploying PCR when active microbial members of a biotope should be monitored is that also dead cells are analyzed (Josephson *et al.*, 1993). To get the best idea of a microbial community it is reasonable to combine culture-dependent and –independent methods (Shade *et al.*, 2012).

Beside metagenomics, sequencing is also applicable for other purposes. One approach is the further qualification of single colonies obtained by culture-dependent methods (Weisburg *et al.*, 1991). As the analysis of pure cultures does not require a throughput as high as for diversity rich samples, the advantages of low error rates and long read lengths gained by the first generation Sanger sequencing can be used (Thomas *et al.*, 2012). With the Sanger sequencing, DNA is amplified by PCR using among normal dideoxynucleotides (dNTP) dideoxynucleotides (ddNTP), which terminate strand elongation when incorporated at a random space. Each of the four ddNTPs is labeled with a different fluorescent dye and hence the nucleotide sequence can be determined after separating the unequally long, amplified fragments by capillary electrophoresis (Siqueira Jr *et al.*, 2012).
For the identification of bacterial groups or isolates by sequencing, without employing the expensive reading of the whole genome, targeting the gene of the 16S rRNA gives a good insight into taxonomic relationships (Weisburg *et al.*, 1991). The 16S rRNA is highly conserved and rarely underlies horizontal gene transfer (Yarza *et al.*, 2014), but comprises highly variable regions which can give information about the linage of a bacterium (Hamp *et al.*, 2009).

Another culture-independent method, which has been used and developed for centuries, is microscopy (Madigan *et al.*, 2012). In microbiology, it can be used for e.g. (live and dead) cell counts or description of microbial morphology. A special approach is applied by Laser Scanning Confocal Microscopes (LSCM) which raster the picture with a focused laser beam, illuminating only one point of the preparation at once. Thus the background will not be imaged and a higher resolution compared to conventional microscopy as well as the possibility to scan different layers of a sample are obtained (Hellmuth, 1993).

Objectives

The goal of this thesis was to evaluate different contamination routes of human pathogens on leafy vegetables and to investigate influences on the establishment in the phyllosphere during cultivation. Figure 1-1 displays an overview of possible routes and impacts on the persistence on vegetable leaves.

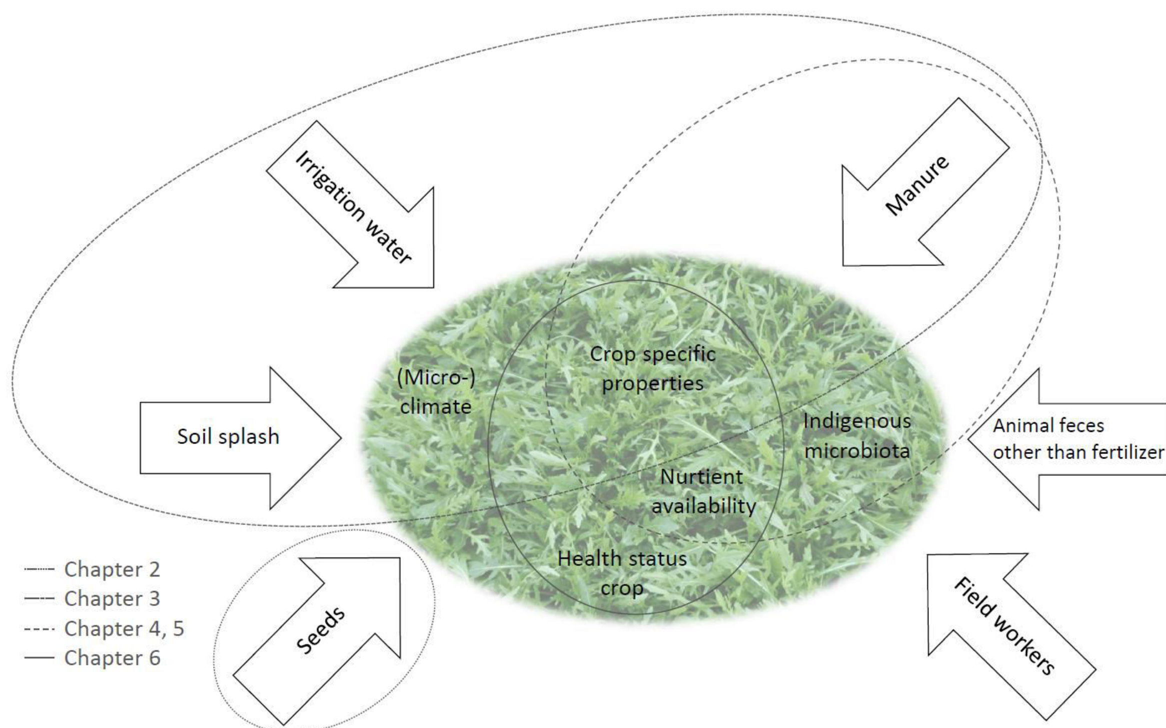

Figure 1-1. Overview of possible contamination routes (arrows) and factors affecting the persistence of human pathogens in the phyllosphere of leafy vegetables. Circles display topics discussed in the different chapters of this thesis.

The specific objectives were as follows:

(i) To describe the microbial quality of vegetable seeds, as they pose a possible transmission point for pathogens (chapter 2)

(ii) To simulate high/low risk treatments, regarding fertilizer, irrigation water, irrigation method and mulch coverage, of organic leafy vegetables under field conditions (chapter 3)

(iii) To understand the establishment of *gfp*-labeled *E. coli* O157:H7 on greenhouse grown baby leaves in dependence of the administered organic nitrogen source, with special emphasis on crop species and indigenous leaf microbiota (chapter 4 and 5)

(iv) To verify internalization of a *gfp*-labeled *E. coli* O157:H7 in biotically/abiotically damaged rocket and Swiss chard baby leaves (chapter 6).

The thesis aims to provide insights into phyllosphere microbiology, which helps in appointing critical control points in the cultivation of safe produce.

Chapter 2: Microbial colonization of vegetable seeds

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Abstract

Microbial quality of vegetables has been of increasing concern during the last decades. While most attention is drawn to microbial safety during cultivation in open fields and during processing, little is known about possible microbial contaminations of seeds as the basic material of plant production. The objective of this study was to analyze lettuce, wild rocket and Swiss chard seeds from different origins (home gardener packed, treated (not lettuce) and two batches of organically produced seeds) with respect to their initial microbial colonization by means of culture-dependent methods. In general, Swiss chard seeds harbored more culturable bacteria (8.0 to 9.9 Log CFU seed^{-1}) than iceberg lettuce (4.6 to 6.3 Log CFU seed^{-1}) and wild rocket seeds (3.6 to 6.8 Log CFU seed^{-1}). While *Enterobacteriaceae* were detected in all lots except for one batch of organic lettuce seeds, no *E. coli* was found in any of the seed stocks. *Enterococcus* spp. and *Listeria* spp. could be isolated from all tested seed lots and *L. monocytogenes* was detected in all iceberg lots as well as on home gardener packed wild rocket seeds, but not on Swiss chard seeds. Organic seeds harbored considerably less coliform bacteria than home gardener packed and treated seeds. Similarity analysis revealed grouping patterns for different lots, but failed to distinguish between all origins.

Keywords: enteric bacteria, food safety, iceberg lettuce, Swiss chard, viable counts, wild rocket,

Background

During the last years, there has been an increase in foodborne disease outbreaks linked to leafy greens (European Food Safety Authority (EFSA), 2013; Warriner *et al.*, 2009). The main causative agents are bacteria and *Salmonella* and enterohemorrhagic *Escherichia* (*E.*) *coli* are accountable for the majority of infections (Brandl, 2006). While most research focuses on contamination during cultivation and postharvest, only little attention has been drawn to microbial seed quality for crop production (Warriner *et al.*, 2005). It is well known, that sprout production offers good conditions for multiplication of bacteria (high temperatures, darkness and high humidity) (Buck *et al.*, 2003). The same applies for germinating seeds at lower temperatures which is in general due to the nutrient rich conditions in the spermosphere (Jablasone *et al.*, 2005). When different vegetable seeds were inoculated with human pathogens (*E. coli* O157:H7, *Salmonella* Typhimurium and *Listeria (L.) monocytogenes*), more of these bacteria could be isolated from young than from mature plants (Jablasone *et al.*, 2005). Due to low infectious doses for enterohemorrhagic *E. coli* (>100 cells) and *L. monocytogenes* (100 cells) also small numbers are of concern (Rheinbaben, 2011; Schlech *et al.*, 1993).

Warriner *et al.* (2003a) compared microbial loads of spinach plants grown in soil, where either the seeds or the growing medium was inoculated with *E. coli* P36. For the latter, the microbial load increased during cultivation and bacteria could be isolated from the leaf surface but not from the inner plant tissue. When *E. coli* P36 was introduced to the seeds before germination, the bacterium was also able to proliferate and cells could be isolated from the leaf surface as well as the inner plant tissue (Warriner *et al.*, 2003a). Invasion of bacteria into plant tissue is of special concern, as they are then protected from postharvest sanitizing treatments (Yaron, 2014).

Contamination of seeds may occur during seed production or handling (National Advisory Committee on Microbiological Criteria for Foods, 1999). Here the same transmission routes as for produce production intended for direct consumption are applicable (National Advisory Committee on Microbiological Criteria for Foods, 1999), namely through irrigation water, manure, soil, wild and domestic animals and workers (Heaton & Jones, 2008; Brackett, 1999). In organic cultivation manure is commonly used as a source of nitrogen supply since the application of synthetic fertilizers is prohibited (McMahon & Wilson, 2001). Therefore, organic produce might more likely be colonized by enteric bacteria.

Postharvest treatment to reduce pathogenic loads usually consists of washing the fresh produce either solemnly with water or the addition of antimicrobial agents like chlorine or citric acid (Francis *et al.*, 1999). As these treatments are never totally effective (Brackett, 1992), it is of high importance to assure a high microbial quality during the entire production chain. The need for seeds with a minimal pathogenic contamination is hence indispensable.

To date, little is known about the microbial quality, besides plant pathogens, of seeds used for vegetable production other than sprouts. In this paper, seeds from different origins, namely for direct use by the home gardener as well as for conventional and organic farming, were evaluated according to their

microbial colonization to give a first view on microbial colonization of vegetable seeds. Seeds from iceberg lettuce, wild rocket and Swiss chard were analyzed and compared with regard to their load with total heterotrophic bacteria, overall *Enterobacteriaceae* as well as *Salmonella* spp. and *E. coli* in particular, *Listeria* spp., *L. monocytogenes*, *Enterococcus* spp. and total coliform bacteria by means of culture-dependent methods. We assume (I) that the microbial colonization is characteristic for each seed lot and (II) that organically produced seeds harbor more bacteria than conventionally produced.

Material and methods

Plant material

Seeds of three crop species, namely iceberg lettuce (*Lactuca sativa* L.), Swiss chard (*Beta vulgaris* subsp. *cicla*) and wild rocket (*Diplotaxis tenuifolia)* were used for analysis. For these crops, the microbial quality of miscellaneous lots was compared. The analyzed lots comprised two batches of seeds intended for organic production, obtained from the same producer in two successive years, seeds for hobby growers and treated seeds. The latter was not analyzed for iceberg lettuce. Organic iceberg lettuce seeds were coated by the producer. The studied seeds are presented in Table 2-1.

Table 2-1. Cultivar, breeder and thousand seed weight (TSW) [g] of iceberg lettuce, Swiss chard and wild rocket seeds.

	Iceberg lettuce		Swiss chard			Wild rocket		
	organic	home gardener	organic	bated	home gardener	organic	bated	home gardener
Cultivar, breeder	Diamantinas (45-92 RZ), Rijk Zwaan	Saladin (15312), Johnsons	Bright lights (H397), HILD	Red Swiss chard (SCR 107), Advan-Seed	Bright lights (14460), Johnsons	Grazia,	Tricia, Enzo	Organic rocket (12519), Johnsons
TSW	41.90	1.27	21.98	14.63	14.30	0.32	0.27	1.63

Analysis

Six batches of twenty seeds for every lot were germinated on autoclaved filter paper soaked with sterile, deionized water at 19°C in the dark. When the seed sprouts reached approximately 1.5 to 2.5 cm, they were macerated with a mortar under the addition of 5 ml detergent (0.1% proteose peptone (Merck, Darmstadt, Germany, product no. 1.07229), 0.2% Na-hexametaphosphate (Fluka, Neu-Ulm, Germany, product no. 71600)) in a sterilized bowl. The suspension was filled into a sterile tube and the bowl was rinsed with another 5 ml detergent, which was subsequently also added into the tube. Agar plates with media for quantification of different bacteria were inoculated with 100 µl of the suspension and incubated as listed in Table 2-2. Plates with Colony Forming Units (CFU) ≤ 300 were enumerated and considered for the analysis. Serial dilutions were carried out using 0.85% NaCl (VWR, Leuven; Belgium, product no. 27810.295). Plating was performed in triplicates under sterile conditions. The microbial load was calculated as CFU per seed.

Table 2-2. Used media and supplements as well as incubation conditions (temperature [°C], length [h]) for the microbial analysis of seeds.

Medium		Supplement	Selective for	Incubation	
				Temperature	Length
0.1 x Tryptic soy agar	Difco 236950	1.2% Bacto agar Difco 214010	Total aerobic count	25	72
Crystal-violet neutral-red bile glucose agar	Merck 1.10275.0500	-	*Enterobacteriaceae*	37	18
Salmonella Chromogenic agar base	Oxoid CM1007	Salmonella selective supplement Oxoid SR0194E	*Salmonella* spp.	37	24
Brillance E. coli/coliform selective medium	Oxoid CM1046	-	Coliform bacteria, *E. coli*	37	24
Bile aesculin agar	LAB LAB207	-	*Enterococcus* spp.	37	48
Harlequin Listeria chromogenic agar	LAB HAL010	HAL010 Listeria selective diagnostic supplement with cyclohexamide LAB X010; Polymyxin B, ceftazidime LAB X072	*Listeria* spp., *L. monocytogenes*	37	24

Calculations and statistical analysis

Plate counts were log-transformed (x'=log (x+1)) and Minitab statistical software (version 16) was used for analysis. Comparison between lots within one crop was conducted employing ANOVA followed by a Tukey-test (α=0.05). Normal distribution of residuals was tested by a Ryan-Joiner-test (α=0.05).
Using average linkage between data for the different lots within each crop, similarity analysis was conducted by dendrochronology, using average linkage between data for the different lots within each crop, according to their heterotrophic bacterial plate count (HPC) and counts of *Enterobacteriaceae*, *Listeria* spp., *Enterococcus* spp. and coliform bacteria.

Results

Heterotrophic bacterial plate counts revealed germinated Swiss chard seeds to harbor considerably more bacteria than iceberg lettuce or wild rocket seeds. Except for one organic iceberg lettuce batch, all seed lots harbored *Enterobacteriaceae,* but no *E. coli* was detected. Organic seeds were less colonized with coliform bacteria than home gardener packed and treated seeds. *Enterococcus* spp. as well as *Listeria* spp. were isolated from all lots, while *L. monocytogenes* was detected in all iceberg lettuce lots and home gardener packed wild rocket seeds, but not on Swiss chard seeds.

Iceberg lettuce

Heterotrophic bacterial plate counts on iceberg seeds for organic production were significantly different between the two studied batches whereas the ones on seeds intended for hobby growers were in the same range as the organic batch 2 (Figure 2-1A).

Likewise, the highest average value was found for organic seeds batch 1 for *Enterobacteriaceae*, at which all organic replicates were positive but only half of the ones for direct use by home gardeners.

Presumptive *Salmonella* were detected on seeds from batch 1 of seeds for organic production and on seeds for direct use by home gardeners.

Iceberg lettuce seeds from all lots were contaminated with *Listeria* spp. *L. monocytogenes* was detected in both organic batches as well as on seeds for direct use by home gardeners. Two and four out of six replicates of the former ones and one out of six samples of the latter ones tested positive.

Also culturable enterococci followed the same colonization pattern as the heterotrophic bacterial plate counts, with significantly ($p<0.001$) lower values for home gardener packages.

Coliform bacteria were detected on two out of six replicates from seeds for direct use by home gardeners, showing both a bacterial load of 0.7 Log CFU seed^{-1}. Three of the coliform-negative replicates hosted less *Enterobacteriaceae* than the detection limit. None of the batches intended for organic production revealed measurable contamination with coliform bacteria.

On iceberg lettuce seeds, the heterotrophic bacterial plate counts correlated with the abundance of *Listeria* spp. (Pearson correlation coefficient $r=0.572$, $p=0.013$) and *Enterococcus* spp. (Pearson correlation coefficient $r=0.963$, $p<0.001$). Also the contamination with *Listeria* spp. and *Enterococcus* spp. were linked (Pearson correlation coefficient $r=0.642$, $p=0.004$), as well as the load of coliform bacteria and *Enterobacteriaceae* (Pearson correlation coefficient $r=0.628$, $p=0.005$).

Similarity dendrochronology, considering the analyzed microbial loads, revealed a higher Euclidian distance between both organic batches than between seeds from organic batch 2 and the majority of the seeds for direct use by the home gardeners (Figure 2-2A).

Swiss chard

Heterotrophic bacterial plate counts on organic Swiss chard seeds were on the same level. Insignificantly higher counts were found on seeds for direct use by home gardeners and treated seeds (Figure 2-1B). Lowest values for *Enterobacteriaceae* were determined for seeds for organic production, followed by

home gardener packed seeds and treated seeds. Significant differences were observed between both batches intended for organic production and treated seeds as well as organic seeds batch 1 and seeds for direct home gardener use ($p<0.001$).
Presumptive *Salmonella* were present in all seed lots except batch 2 of seeds for organic production, whereas all replicates for batch 1, all but one for home gardener packages and half of the treated showed to be contaminated.
The microbial load with *Listeria* spp. of Swiss chard seeds for organic production was significantly ($p<0.001$) different for batch 1 and 2, respectively, the low average value of batch 2 being a result of only one positive replicate out of six. The mean value of seeds for direct use by home gardeners was significantly different ($p<0.001$) to the organic seeds from both batches and the treated seeds. *L. monocytogenes* was not detected in any of the Swiss chard samples.
No differences were stated for culturable *Enterococcus* spp. for the tested Swiss chard seed lots. Enterococci counts on half of the replicates from treated seeds as well as two from batch 1 revealed to be below the detection limit and one of the latter also tested negative for *Listeria* spp. The same replicate showed to have 3-4 Log CFU seed^{-1} higher counts for *Enterobacteriaceae* and coliform bacteria compared to the other replicates.
Examining the contamination of coliform bacteria on Swiss chard seeds, organic batches showed significantly lower loads as compared to seeds for direct use by home gardeners and treated seeds ($p<0.001$). Regarding batch 2 of seeds intended for organic production, only two out of six replicates showed counts above the detection limit, whereas all replicates within the other lots were positive.
Correlations between the analyzed bacterial groups were significant for heterotrophic bacterial plate counts and *Enterobacteriaceae* (Pearson correlation coefficient $r=0.431$, $p=0.036$) as well as coliform bacteria (Pearson correlation coefficient $r=0.497$, $p=0.014$). Also, *Enterobacteriaceae* and coliform bacteria showed to be interdependent (Pearson correlation coefficient $r=0.826$, $p<0.001$).
Similarity analysis discriminated between two groups, (i) including samples from both organic batches and (ii) mainly consisting of treated seeds and seeds from home gardener packages (Figure 2-2B).

Wild rocket

Heterotrophic bacterial plate counts on wild rocket seeds were lowest for seeds for direct use by home gardeners, followed with a significant difference by the organic batch 2 (Figure 2-1C). Treated seeds and seeds for organic production from batch 1 showed to have the significantly highest bacterial loads ($p<0.001$).
Regarding *Enterobacteriaceae*, treated seeds had the highest contamination. Seeds for direct use by home gardeners took an intermediate position and significantly lowest *Enterobacteriaceae* counts were obtained in lots with seeds for organic production, whereas only one replicate exceeded the detection limit in batch 2 ($p<0.001$). Presumptive *Salmonella* were detected in all lots except for treated seeds, organic batch 1 tested positive for all replicates and batch 2 as well as home gardener packages embodying two out of six replicates above the detection limit.

Only one and three out of six replicates with seeds from batches 1 and 2 for organic production were contaminated with *Listeria* spp. Higher counts were found for seeds for direct use by home gardeners. Significantly higher counts ($p<0.001$) as compared to seed lots for organic production, but not as compared to seeds for direct use by home gardeners, were revealed on treated seeds. Among wild rocket seeds, only the home gardener packed seeds carried *L. monocytogenes* above the detection limit with loads between 0.7 and 1.7 Log CFU $seed^{-1}$.
Treated seeds hosted highest values for *Enterococcus* spp. Seed lots intended for organic production showed to be significantly less colonized ($p=0.002$); while no significant difference was found for seeds for direct use by home gardeners, either to the lowest or the highest load.
Among all replicates from wild rocket seeds for organic production, only one tested positive for coliform bacteria, belonging to batch 2. As the only sample in batch 2, the same replicate was also tested positive for *Enterobacteriaceae*. Significant differences between values were apparent for organically produced seeds and both other lots, the lowest in seeds for organic production, followed by seeds for direct use by home gardeners and treated seeds ($p<0.001$).
There was a positive correlation between *Enterobacteriaceae*, *Listeria* spp. and coliform bacteria in all constellations ($p<0.001$), the highest being between *Enterobacteriaceae* and coliform bacteria with a Pearson correlation coefficient r of 0.979, the others ranging between 0.699 and 0.739.
Similarity analysis considering the analyzed bacteria classified all treated seed replicates, all organic seed replicates except for one (belonging to batch 2) and all from home gardener package together with one organic replicate in three different groups (Figure 2-2C).

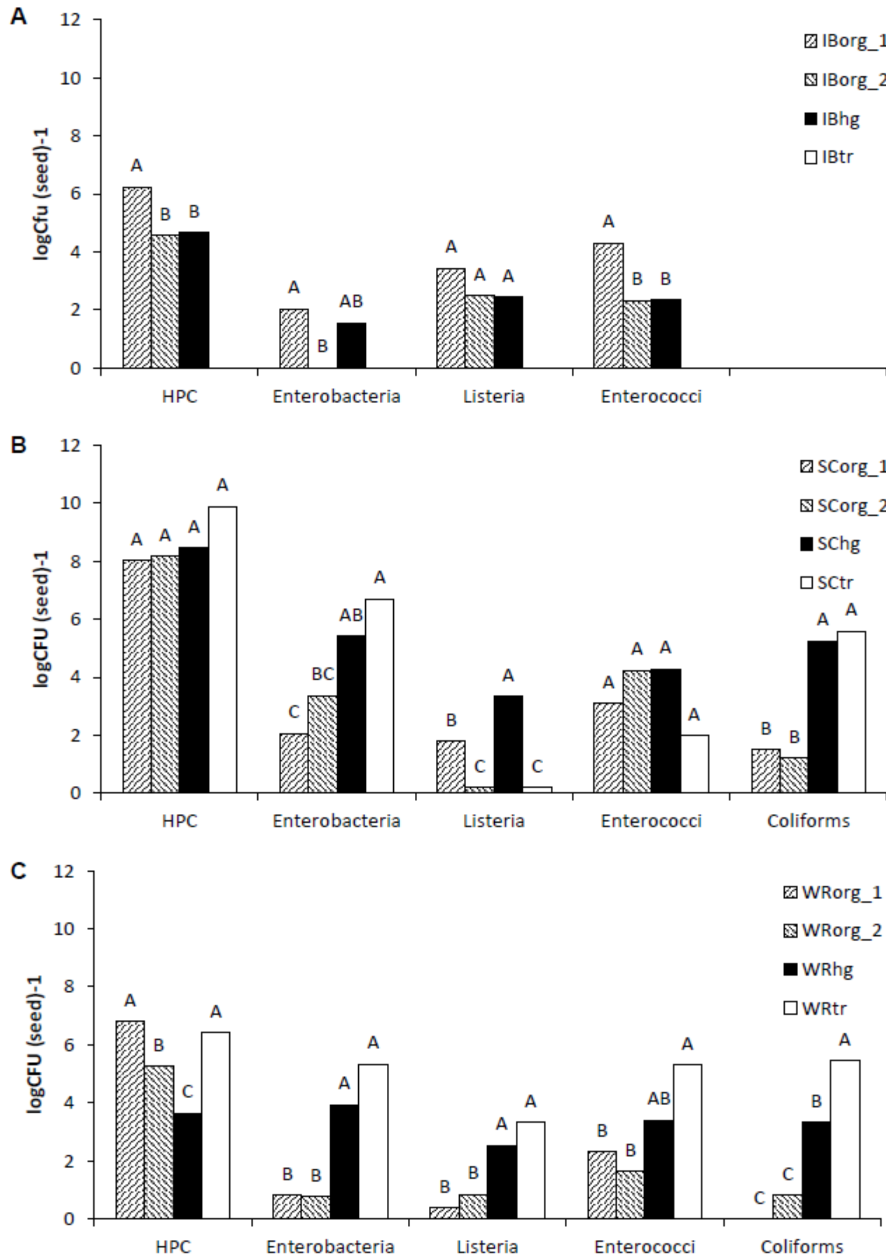

Figure 2-1. Heterotrophic bacterial plate counts (HPC), enterobacteria, *Listeria*, enterococci and coliform bacteria determined by culture-dependent methods on iceberg lettuce IB (A), Swiss chard SC (B) and wild rocket WR (C) seeds intended for organic production (lot 1: org_1, lot 2: org_2), direct use by home gardeners (hg) and for conventional production (tr). No analyses were conducted for treated iceberg lettuce seeds. Bars within one microbial group that do not share a letter are significantly different as revealed by ANOVA (Tukey-test, $p<0.05$). No statistical analysis was performed for coliform bacteria on iceberg lettuce, where both organic batches were below the detection limit.

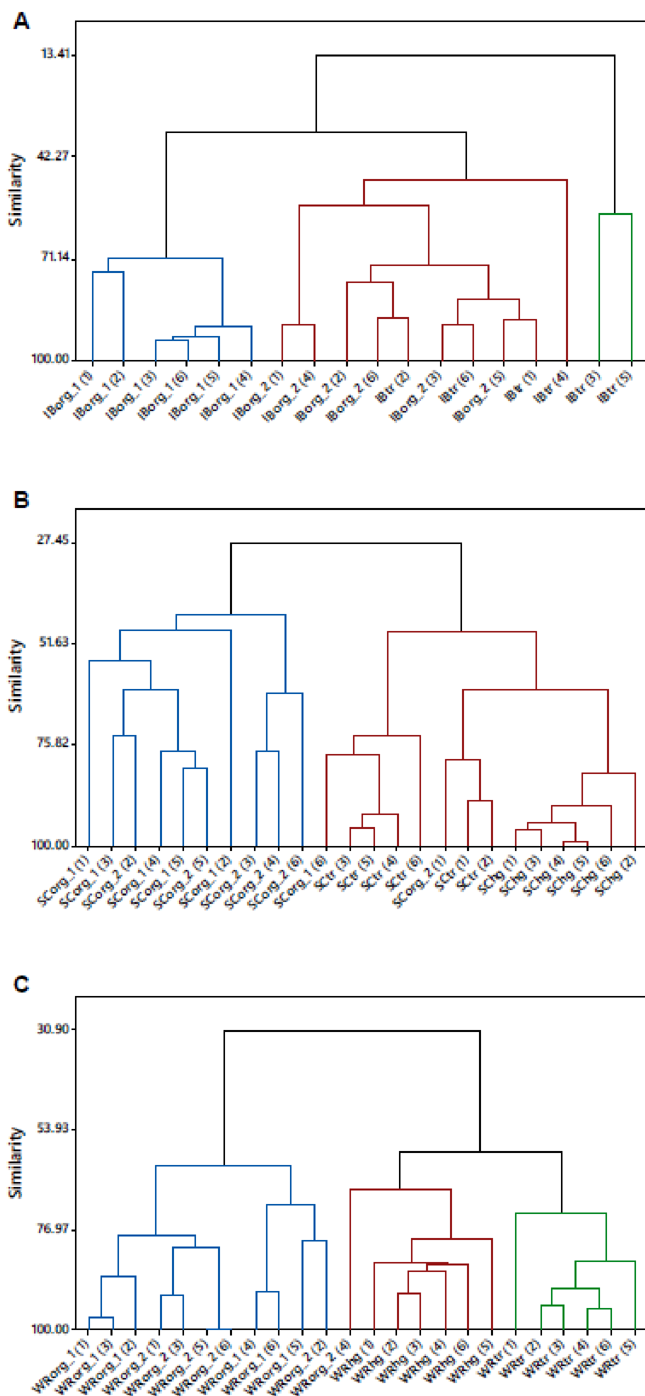

Figure 2-2. Similarity (average linklage, Euclidian distance) of different seed lots (seeds intended for organic production, batch 1 (org_1) and 2 (org_2), for direct use by home gardener (hg) and treated seeds (tr)) as revealed by microbial contamination with heterotrophic bacterial plate counts, enterobacteria, *Listeria*, enterococci and coliform bacteria (culture-dependent analysis) on iceberg lettuce IB (A), Swiss chard SC (B) and wild rocket WR (C) seeds. No analyses were conducted for treated iceberg lettuce seeds. Clustering is stated by different colors.

Discussion

The first phase of seed germination is induced with an intensive uptake of water, resulting in a rapid swelling. The established pressure within the seed is released by a rupture of the seed coat, leading to death of the outer cells of the cotyledon and the leakage of cellular and vascular substances (Powell & Matthews, 1978). Nutrients are set free again in a later phase of germination by breaking down high molecular structures to mobilize stored energy to initiate the active growth of the seedling. These seed exudates represent a good energy source for microbial growth (Nelson, 2004). During germination, bacterial numbers associated with the seed rise until they reach a steady plateau (Van der Linden *et al.*, 2013; Piernas & Guiraud, 1997), which is denoted with 8 to 9 Log CFU g^{-1} for the heterotrophic bacterial plate count (Piernas & Guiraud, 1997). Analysis of sprouted seeds thus mirrors the microbial colonization including the potential multiplication from available nutrients. Isolation of human pathogenic bacteria from the interior of seeds is not possible until germination (personal communication, Beutin, 2016). This is why we analyzed sprouted seeds in this study. Further, bacterial colonization is stated as CFU $seed^{-1}$ because differences in weight due to seed coatings would have made the results incomparable.

Counts of *Enterobacteriaceae* and coliform bacteria of the analyzed sprouted seeds showed a high variation of 6 and 5 Log CFU $seed^{-1}$, respectively. Seeds of alfalfa and rapeseed analyzed by Kim *et al.* (2005) showed a considerable variation (3.5 Log CFU g^{-1}) of coliform bacteria at the seed stage, but on sprouts the range was within 1 Log level. Numbers of coliform bacteria on alfalfa, rape and rice sprouts as determined by others showed values of 7 to 8 Log CFU g^{-1} (Kim *et al.*, 2013; Piernas & Guiraud, 1997). Goñi *et al.* (2013) found lettuce to harbor already 3.5 Log CFU g^{-1} on non-germinated seeds. Likewise the HPC, numbers of coliform bacteria rise dramatically during germination, which was shown by Kim *et al.* (2013) who found coliform bacteria to increase by up to more than 5 Log CFU g^{-1} on alfalfa and rapeseeds. *Enterobacteriaceae* and coliform bacteria, showing an overlapping contingent of bacterial genera, comprising indigenous members of the vegetable microbiome and their presence is thus no dependable proof for a fecal contamination per se. Identification of specific species like the indicator organisms *E. coli* and *Salmonella* spp. are more suitable for evaluating the microbial quality of produce and production input. In this study, no *E. coli* were detected, but colonies presumptive to be *Salmonella* were found in different lots posing a potential risk. Yet, these results should be viewed under the consideration that resent analysis questions the specificity of the employed medium for *Salmonella* (Hartmann *et al.*, submitted) and that these colonies were not further verified.

The genus *Enterococcus* comprises species indicating fecal contamination but also others that have a natural occurrence in plants environment. In general, colonization of sprouts with enterococci is common (Mesak, 2012), which could also be seen in our trial where all seed lots were tested positive. Enterococci loads found on rice seeds by Piernas and Guiraud (1997) showed numbers of 6.5 Log CFU g^{-1}. *Listeria* spp. are known for their ubiquitous occurrence and their presence on the analyzed sprouted seeds is not considered alarming. Nevertheless, apparent contamination of organic lettuce seeds as well

as lettuce and wild rocket seeds for home gardeners demand with the species *L. monocytogenes* poses a potential health risk as it is known to cause severe diseases especially in immunosuppressed people and (unborn) children.

To enhance the microbial quality of seeds, several physical and chemical treatments are available, mainly applied in sprout production (Warriner *et al.*, 2005). The effectiveness of a treatment is dependent on its intensity, for instance the exposure time, the concentration of the agent or the temperature (Scouten & Beuchat, 2002) and a major problem is to eliminate epiphytic as well as endophytic microorganisms without affecting the viability of the seed (Buck *et al.*, 2003). To date, no universal implementation to eliminate the microbial load of seeds and instantaneously conserve the viability of the seeds is available (Warriner *et al.*, 2005; Scouten & Beuchat, 2002; Taormina & Beuchat, 1999). As already small numbers of pathogenic bacteria can proliferate to health threatening counts during germination, a reduction alone is not satisfactory (Buck *et al.*, 2003). Therefore, other implementations like the use of biocontrol agents that contain the growth of potential pathogens are of interest and prior research has illustrated its potential (Palmai & Buchanan, 2002). Nevertheless, prevention of contamination is the most suitable way to produce safe produce.

Organic seeds surveyed in this study did not reveal a higher colonization with bacterial genera that comprise species with enteric origin, thus does not support our hypothesis. On the contrary, treated seeds showed highest counts for *Enterobacteriaceae* and coliform bacteria, suggesting that fungicidal coating had a direct or indirect effect on bacterial establishment during germination. In agreement with grouping by similarity analysis between some of the analyzed seed lots, previous studies not only revealed that the phyllosphere microbiota on leaves varies between plant species (Kinkel *et al.*, 2000), but also between different cultivars within one species (Hunter *et al.*, 2010). Our findings indicate that this is not only true for the phylloplane, but can be extrapolated to the spermosphere.

As similarity was occasionally lower between identical as compared to different cultivars (iceberg lettuce seeds), other influences also play a role in the microbial colonization of the germinating seed. Rastogi *et al.* (2012) and Williams *et al.* (2013) found the field site to explain shifts in microbial communities on lettuce leaves as well as an impact of the season on the phyllosphere microbiota. As seeds from different batches were probably not cultivated at the same time nor on the same side, changes in microbial colonization could be a result.

Outliers of several lots that singly grouped within other lots display an uneven colonization within seed batches, which also applies for pathogens as demonstrated in our study for *L. monocytogenes*. Hence, the sampling procedure and the number of replicates for microbial validation of seeds is of high importance.

In conclusion, the study gives a first overview on the microbial colonization of sprouted seeds, which has to be confirmed in further studies, addressing a greater number of samples. Differences in microbial colonization were apparent between seeds from different lots, but from the analyzed origins none seemed to be of a poor microbial quality in terms of enteric contamination. Nevertheless, considerable

numbers of *L. monocytogenes* found on iceberg lettuce seeds in all tested lots and in one lot of wild rocket seeds are of concern.

Chapter 3: Season, plant species and age, but not cultural management, govern microbial colonization of organically grown leafy vegetables

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Abstract

Aim:
This study analyzed microbial colonization of field-grown leafy vegetables under different cultivation treatments posing different levels of microbial contamination hazard.
Method and Results:
Iceberg lettuce, Swiss chard, and wild rocket were grown in an open field in a two-year trial. Samples were taken every two weeks after planting and analyzed for epiphytic loads of heterotrophic bacteria (HPC), *Enterobacteriaceae*, *Escherichia coli*, *Salmonella*, coliform bacteria, *Enterococcus*, *Listeria*, and *L. monocytogenes*. Compared with Swiss chard and occasionally wild rocket, bacterial counts on iceberg lettuce tended to be lower for HPC, *Enterobacteriaceae*, and coliform bacteria. No consistent effect was seen for *Bacilli*. *Escherichia coli* and *L. monocytogenes* were randomly found, with the highest incidence on Swiss chard. Principal component analysis clustered samples according to sampling occasion, with average temperature and precipitation showing a moderate impact in some cases on culturable microbial leaf colonization.
Different cultivation methods were not reflected in microbial load.
Significance and Impact:
The study considered several factors, including cultivation method (organic fertilizer, irrigation water quality, irrigation method, mulch), climate factors, plant species, and age, that affect the food safety of vegetables eaten raw.
Keywords: Agriculture, *E. coli* (all potentially pathogenic types), Enterobacteria, Environmental, Food safety

Introduction

Vegetables eaten raw are an increasing concern, as no efficient decontamination step precedes consumption (Islam *et al.*, 2005) and reports of associated disease outbreaks are increasing (European Food Safety Authority (EFSA), 2013; Warriner *et al.*, 2009). *Salmonella* spp. is the causative agent with the highest incidence in fresh produce, while pathogenic serovars of *E. coli* account for a majority of outbreaks related to lettuce (Interagency Food Safety Analytics Collaboration, 2015). *Listeria monocytogenes* plays a minor role in terms of frequency, but is a serious pathogen due to its fatal character (Carrasco *et al.*, 2010).
Several studies have been conducted in recent years to evaluate possible contamination sources. Manure and irrigation water are principal factors for transmission of microorganisms in open field crop production (Guan & Holley, 2003; Kirby *et al.*, 2003). Manure is used as a fertilizer (Bernal *et al.*, 2009) and to enhance physical soil properties (Xie *et al.*, 2014; Paulsen *et al.*, 2013). Alsanius *et al.* (2017b) examined links between manure of animal origin and produce in the One Health concept, an approach that addresses the transmission of zoonoses, but lacks a view on vegetable foodstuffs. However, farm animals can shed human pathogenic bacteria with their feces and improperly treated manure can act as a vector (Shepherd Jr *et al.*, 2010). Thus, use of manure is regarded as a critical control point (Alsanius *et al.*, 2017b). To minimize the risk, manure should be treated before application, e.g., through anaerobic digestion or aerobic composting (van der Wurff *et al.*, 2016). However, complete decontamination is not feasible and pathogens may be transmitted to the soil together with the organic fertilizer (Leifert *et al.*, 2008). *Escherichia coli* O157:H7 and *Salmonella* Typhimurium can be detected in soil amended with inoculated chicken and cattle manure for at least six months after application (Nyberg *et al.*, 2010). Direct analysis has revealed better survival in chicken manure for both pathogens (Nyberg *et al.*, 2010). Islam *et al.* (2004a) found *E. coli* O157:H7 on lettuce and parsley leaves when seedlings were planted in soil amended with inoculated manure (7 log CFU g^{-1}) until 63 and 231 days after transplantation, respectively. A similar experimental setup with *Salmonella* Typhimurium also resulted in contamination of edible plant parts, where the bacterium was detectable for 63 and 161 days on lettuce and parsley, respectively (Islam *et al.*, 2004b). Alternative nutrient resources of animal origin, e.g., hair pellets or bone meal, can be considered less risky regarding microbial contamination (Alsanius *et al.*, 2017b; Möller & Schultheiss, 2014).
Transmission of pathogens from soil to edible parts of leafy vegetables can occur either by an intrinsic route through internalization via the roots into the leaves (Hirneisen *et al.*, 2012) or by direct contact through soil or water splashing (Fischer-Arndt *et al.*, 2010; Abu-Hamdeh *et al.*, 2006). Minimizing soil contact with edible parts of leafy greens has the potential to avoid contamination and can be accomplished by employing ground coverage with mulch materials. Irrigation methods that avoid soil splashing can also potentially reduce transmission of pathogens from soil to above-ground part of plants. Municipal water used for irrigation is considered safe (Alsanius, 2014), whereas groundwater, often thought to be hygienically safe, can be infiltrated with pathogens, as can river, lake or pond water (Gerba,

2009). Surface water itself can be contaminated through runoff from nearby animal rearing facilities, wild animals, leaking sanitary facilities, and incorrect discharge of industrial and household waste (Kirby *et al.*, 2003). Once introduced, the survival of a pathogen in water is dependent on different factors, namely temperature, organic matter content, available nutrients, and the presence of other microorganisms (Pachepsky *et al.*, 2011). Likewise, for transmission of pathogens from soil to leaves, drip irrigation near the ground reduces contact between contingent pathogens and edible parts of leafy greens (Song *et al.*, 2006). In a two-year trial applying *E. coli* O157:H7-contaminated water to lettuce by either drip or overhead irrigation, Moyne *et al.* (2011) found significantly lower numbers of bacterial cells on drip-irrigated lettuce in the first year, but this difference could not be observed in the following season.

In addition to these two input factors, other sources can also function as vectors for human pathogens on field-grown crops and their control may be even more difficult. The soil itself harbors a great number of harmless microorganisms (Souza *et al.*, 2013), but pathogens like *L. monocytogenes* are also common inhabitants of this biosphere (Francis *et al.*, 1999). Another risk is posed by wild animals, which can contaminate fresh produce with their feces (Beuchat & Ryu, 1997). Fences around fields can restrain larger mammals (Local Government Management Association of British Columbia, 2013), but fail to prevent smaller animals like rodents and birds from invading crop production units.

Tracing an outbreak back to its source is often difficult, due to complex distribution systems. Identification of the assumed source is also often uncertain, as the contaminated produce is no longer available. Potential contamination sources are of great interest, as their control is fundamental for the reduction of risks (Harris *et al.*, 2003).

In this study, indicator organisms on leafy greens were monitored during the growing season in different treatments. The aim was to evaluate the microbial quality of the leaves and to identify factors influencing the microbial safety of fresh produce while growing in the field. The hypothesis tested was that the bacterial load on organically grown leafy vegetables is influenced by crop management, plant species and age, and climate conditions.

Material and methods

Experimental set-up

Iceberg lettuce (*Lactuca sativa* L., cultivar Diamantinas, 45-92 RZ, Rijk Zwaan Welver GmbH, Welver, Germany), wild rocket (*Diplotaxis tenuifolia*, cultivar Grazia), and Swiss chard (*Beta vulgaris* subsp. *cicla*, cultivar ‘Bright lights’, H397, HILD Samen GmbH, Marbach am Neckar, Germany) were grown in the field. The crops were exposed to different cultural management methods representing different microbial contamination hazards by either supplying different initial microbial loads or by potentially preventing or enhancing transmission of bacteria to the leaves. The factors varied in the methods were

fertilizer (pig hair pellets, chicken manure), surface mulching, water source (pond, well, tap), and irrigation method (drip, overhead; only tested for iceberg lettuce) (Table 3-1).

Table 3-1. Cultivation methods associated with different microbial hazards that were applied in a field trial with iceberg lettuce, wild rocket and Swiss chard, wild rocket and Swiss chard were not exposed to drip irrigation.

<table>
<tr><th>Factor</th><th colspan="2">Low risk</th><th colspan="2">High risk</th></tr>
<tr><td>Fertilizer</td><td colspan="2">Pig hair pellets (PHP)</td><td colspan="2">Chicken manure (CM)</td></tr>
<tr><td>Mulching</td><td colspan="2">Yes</td><td colspan="2">No</td></tr>
<tr><td>Water source</td><td>Tap</td><td colspan="2">Well</td><td>Pond</td></tr>
<tr><td>Irrigation method</td><td colspan="2">Drip</td><td colspan="2">Overhead</td></tr>
</table>

The trial was organized as a multifactorial split-plot design with three replicates, resulting in 144 plots. Whole plots were organized according to water quality and divided into irrigation method/crop. These subplots were split by fertilizer and mulch. Plot size was 1.5 m x 2.7 m for iceberg lettuce and Swiss chard and 1.5 m x 0.9 m for wild rocket. The space between the plots was 1.0 m, while the planting space between seedlings within a plot was 0.3 m for iceberg lettuce and Swiss chard and 0.15 m for wild rocket.

The experiment was conducted on an organic vegetable farm on a field with sandy soil near Hanover in Northern Germany. Seedlings were cultivated in an unheated greenhouse and planted on 25 June 2013 for trial 1 and 5 May 2014 for trial 2. Soil was sampled (0-30 cm) and analyzed for mineral nitrogen (N). Fertilizer was added to the soil, to a total amount of 150 kg ha^{-1} readily available nitrogen (assumption: PHP = 14% N; CM = 30 kg N (t fresh weight)$^{-1}$) one (2013) and two weeks (2014) before planting the crops, respectively. The field was kept weed-free by hoeing manually. Irrigation was carried out three days before harvesting, if the soil was dry, in an amount of 4.7 mm. Due to heavy rainfall, no irrigation was necessary until 19 July in 2013 (sampling event 2) and 13 June in 2014 (sampling event 3).

Analysis

Sampling

Water samples collected before irrigation were analyzed for every irrigation event. Soil samples were taken before planting and during cultivation, at the same sampling events as the plants, in 2014. For each treatment, iceberg lettuce plots of all three replicates were sampled to 0-30 cm soil depth with a soil auger. This resulted in four samples when no irrigation was possible and 24 samples when irrigation was performed, per sampling occasion. Samples of organic fertilizers were collected before application.

Crop samples were collected at planting (seedlings) and every two weeks until harvest at maturity, resulting in three sampling events in 2013 (S1-S3) and four sampling events in 2014 (S1-S4). Plants were harvested from the inner rows of each plot, with two samples each for determination of fresh weight and for microbial analysis.
All samples were chilled directly after harvest and kept under cool conditions during transport to the laboratory until microbial analysis.

Climate data

Data on air temperature [°C] and precipitation [mm] were obtained from a meteorological station (Hanover airport) approximately 14 km away from the experimental site.

Plant assessment

Plant fresh weight was determined by weighing two plants for each plot and sampling event. Growth curves for both cultivation periods were calculated by including all treatments.

Microbial analyses

Fertilizer and soil samples were prepared for microbial analysis by shaking about 10 g of sample with 100 ml 0.2% $Na_2P_2O_7$ at 110 rpm for 30 min under sterile conditions. The suspension was placed in an ultrasonification bath for 5 min and soil particles were allowed to settle for another 5 min. The supernatant was poured through a sterile strainer (mesh size: 1 mm).
For plant analysis, about 10 g of leaves were transferred into a sterile smasher bag together with 50 mL autoclaved 0.1 M TRIS buffer and smashed for 20 s at normal speed in a Stomacher (Smasher, Chemunex, Bruz, France). The suspension was then separated from the leaves. For the analysis of iceberg lettuce (after head formation), the outer layers of the heads were used for microbial qualification to cover the greatest extrinsic influences.
Water quality was evaluated by employing a Colilert-18 kit (IDEXX Laboratories, Inc., Westbrook, Maine, USA) and Enterolert-18 kit (IDEXX Laboratories, Inc.,Westbrook, Maine, USA) according to the manufacturer's instructions to identify the amount of coliform bacteria, *Escherichia coli*, and enterococci, which served as indicator organisms. Results were stated as most probable number (MPN) in 100 mL. In addition, heterotrophic bacterial plate count (HPC) was determined by culture-dependent methods on Reasoner's 2A (R2A) agar (Difco 218263, DeMoines, USA). Water samples were serially diluted in 0.85% NaCl and spread on the medium using a spiral plater (WASP2, Don Whitley Scientific Limited, Shipley, UK). Plates were incubated at 25°C for 72 hours and colony-forming units (CFU) were enumerated by employing a template and the WASP 2 user manual (Don Whitley Scientific Limited, West Yorkshire, UK). Results are expressed as CFU mL^{-1}.
Soil and fertilizer samples, seedlings, and harvested plants were also quantified using culture-dependent methods. Bacterial groups identified and the medium applied, with the corresponding incubation temperature and time, are shown in Table 3-2. For the HPC determination, suspensions were spread onto agar plates using a spiral plater (WASP2, Don Whitley Scientific Limited, Shipley, UK), whereas all

other semi-selective media were inoculated manually using sterile glass beads to spread aliquots of 100 µL. The lower detection limit was 1.7 log CFU g^{-1} fresh weight.

All plating was performed in duplicate under sterile conditions.

Table 3-2. Semi-selective media and supplements and incubation conditions used for microbial analysis of leaves, manure and soil samples**.**

Medium		**Supplement**	**Selective for**	**Incubation temperature [°C]**	**Incubation time [h]**
0.1 x Tryptic soy agar	Difco 236950	1.2% Bacto agar Difco 214010	Heterotrophic bacterial plate count	25	72
Crystal-violet neutral-red bile glucose agar	Merck 1.10275.0500	-	*Enterobacteriaceae*	37	18
Salmonella Chromogenic agar base	Oxoid CM1007	Salmonella selective supplement Oxoid SR0194E	*Salmonella* spp.	37	24
Brilliance *E. coli*/coliform selective medium	Oxoid CM1046	-	Coliform bacteria, *E. coli*	37	24
Bile aesculin agar	LAB LAB207	-	*Enterococcus* spp.	37	48
Harlequin Listeria chromogenic agar	LAB HAL010	HAL010 Listeria selective diagnostic supplement with cycloheximide, LAB X010; Polymyxin B, ceftazidime LAB X072	*Listeria* spp., *L. monocytogenes*	37	24

Statistical analyses

Growth curves on the basis of plant fresh weight were calculated in relation to the temperature sum using Minitab version 16 (Minitab Inc., USA).

Microbiological data (viable counts) obtained from plate counts were log-transformed [$x' = \log(x+1)$]. Different treatments and crops were compared by employing multivariate ANOVA followed by pair-wise comparison (t-test) using the Tukey method ($\alpha = 0.05$) employing SAS software, version 9.4 (SAS Institute Inc., USA).

Minitab version 16 (Minitab Inc., USA) was used for calculating Pearson correlations among microbial groups and between microbial groups and weather data (air temperature and precipitation)/yield gain. It

was also used to perform principal component analysis (PCA), including microbial loads on leaves, air temperature, and precipitation. Air temperature and precipitation in the field were specified as the average daily mean value five days before sampling events.
Counts for *E. coli* and *L. monocytogenes* were near the lower detection level (>20 CFU per plate for non-diluted suspensions) and were therefore not subjected to statistical evaluation.

Results

Plant assessment

Growth curves for plant fresh weight (mean weight per plant) as a function of the temperature sum are displayed in Figure 3-1. Curves showing the weight of iceberg lettuce exhibited a short lag phase and then a slow rise. The average weight at the final sampling of iceberg lettuce (485 g in 2013 and 486 g in 2014) was considerably below the asymptote. The inflexion point, indicating the maximum growth rate, was at 708 degree-days (°Cd) in 2013 and 753°Cd in 2014, i.e., near the final sampling which took place at 799°Cd and 844°Cd. The course of Swiss chard and wild rocket growth deviated from this pattern. After a long lag phase, there was a steep increase in fresh weight of Swiss chard and wild rocket, almost meeting the estimated final weight at the last sampling. The average fresh weight of Swiss chard at the last harvest was 367 g in 2013. In 2014, the final weight of Swiss chard was not determined, and the growth curve was therefore extrapolated to the temperature sum of 844°Cd. Due to a shorter vegetative phase of wild rocket compared with iceberg lettuce and Swiss chard, the final sampling took place at a temperature sum of 482°Cd in 2013 and 638°Cd in 2014. The average weight was 86 g (2013) and 96 g (2014). The highest growth rate for Swiss chard and wild rocket was reached earlier than in iceberg lettuce, for Swiss chard at a temperature sum of 423°Cd and 448°Cd and for wild rocket at 375°Cd and 264°Cd in 2013 and 2014, respectively.

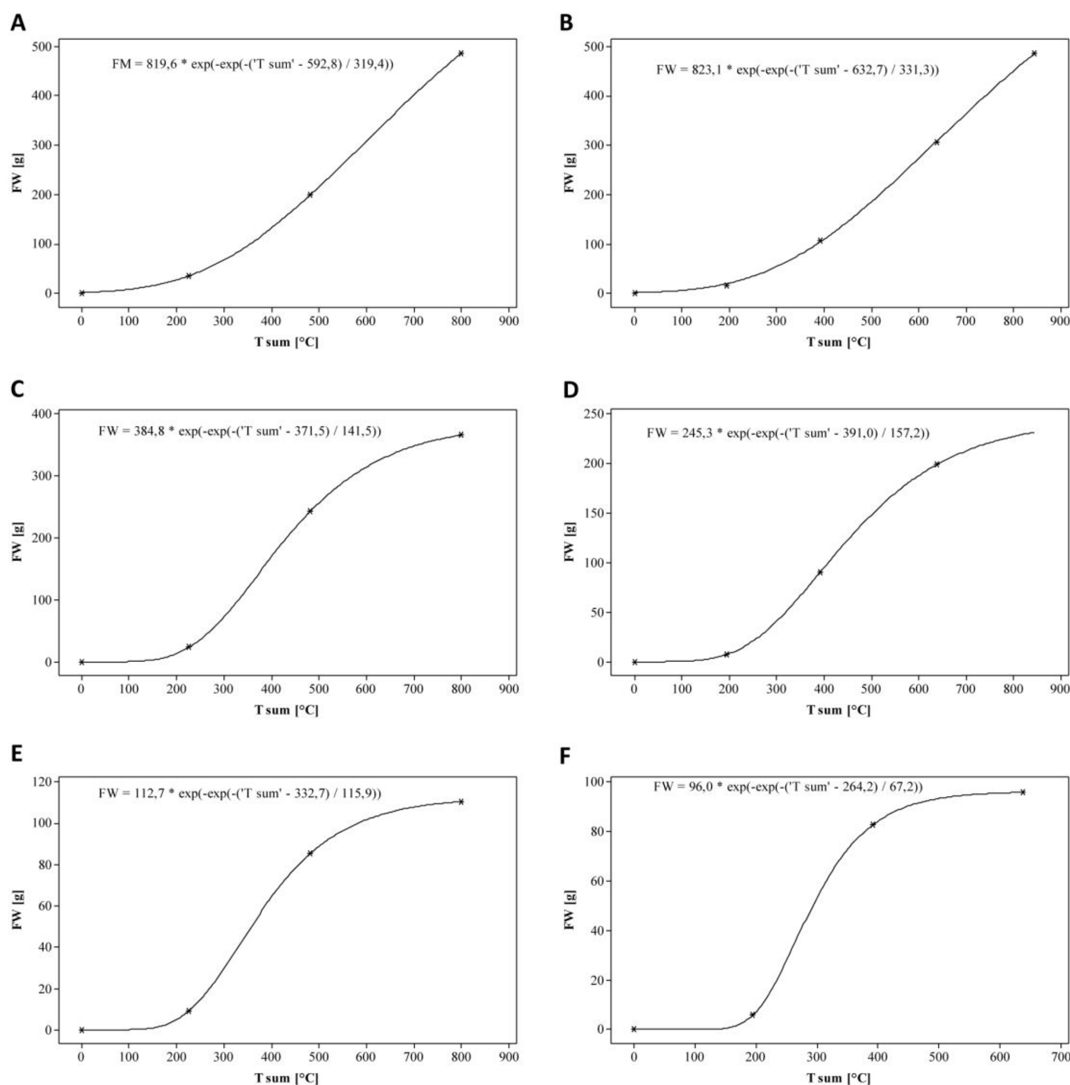

Figure 3-1. Fresh weight (FW) [g] as a function of temperature sum (T sum, degree-days) [°Cd] for: field-grown iceberg lettuce (2013. A and 2014: B), Swiss chard (2013: C and 2014: D), and wild rocket (2013: E and 2014: F). No data were available for the last sampling of Swiss chard in 2014 and the curve was thus extrapolated to a temperature sum of 844°Cd, when the final sampling took place. Due to a shorter vegetative phase of wild rocket, the final sampling in 2014 took place at 638°C. Fast growth in 2013 resulted in fewer sampling occasions and an additional sampling (generative phase) for wild rocket was performed to obtain enough data for creating a curve.

Climate data

The weather conditions during the two growing seasons differed considerably. The temperature during the growing season in 2013 showed an ascending trend, where mean temperature between planting and the first sampling and then between sampling events climbed from 16.1°C to 18.3°C to 22.7°C and resulted in an average value of 18.9°C and a temperature sum of 814.6°Cd over the entire growing period (Figure 3-2). A different picture emerged in 2014, when the growing season started with an average temperature of 12.3°C between planting and the first sampling, which proved to be the coldest period in both years, followed by two warmer intervals with 15.1°C and 17.6°C, owing to two peaks in May and

June, and ended with a constant period averaging 14.7°C. The mean temperature over the entire growing period (14.9°C) was lower than in 2013, but resulted in a slightly higher temperature sum of 833.2°Cd due to an elongated growing season.

The experimental period in 2013 was dry and there was only one rainfall event (0.1 mm) between sampling events 1 and 2. Precipitation amounted to a total of 39.9 mm during the entire growing period, consisted of 16 rainy days ranging from 0.1 to 8.1 mm during the three-week period. In 2014, precipitation amounted to 161.9 mm and was therefore considerably higher than in 2013, partly because of the longer growing season. The 33 rainfall events recorded in 2014 ranged from 0.3 mm to 25.8 mm.

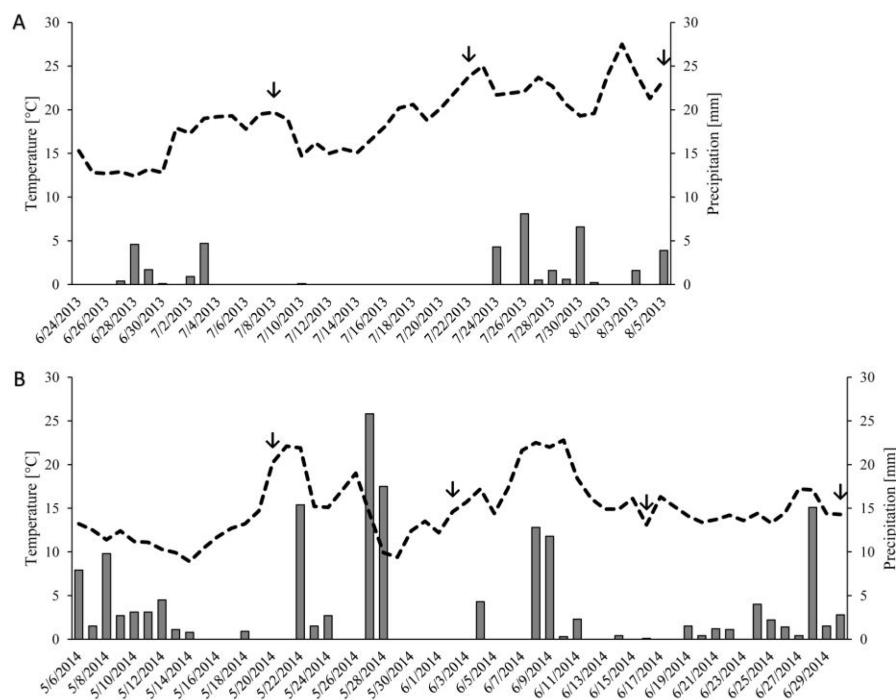

Figure 3-2. Daily mean temperature (T, broken line) and daily precipitation (P, bars) during the growing season in 2013 (A) and 2014 (B). Measurements represent the interval between planting and final harvest of the crops. Arrows indicate sampling occasions.

Microbial colonization

Heterotrophic bacterial plate count (HPC)

Mean HPC values for leaves sampled during the growing season in 2013 ranged from 5.5 to 5.9 log CFU g^{-1} fresh weight on iceberg lettuce, 6.2 to 7.0 log CFU g^{-1} fresh weight on Swiss chard, and 6.2 to 6.9 log CFU g^{-1} fresh weight on wild rocket. In 2014, the mean values were slightly higher, ranging from 5.5 to 7.0 log CFU g^{-1} fresh weight on iceberg lettuce, from 7.3 to 7.8 log CFU g^{-1} fresh weight on Swiss chard, and from 7.0 to 7.8 log CFU g^{-1} fresh weight on wild rocket. The HPC on iceberg lettuce decreased during the growing seasons, whereas it increased on wild rocket. Higher values for HPC were also found on small plants planted in 2014 (6.4±0.2 log CFU g^{-1} fresh weight compared with 5.0±0.2 log CFU g^{-1} fresh weight in 2013). Comparison of the average HPC for the three crops studied revealed

the lowest values for iceberg lettuce on all sampling occasions, all differences being statistically significant compared with Swiss chard and all but one compared with wild rocket (Figure 3-3A). Comparisons between the different treatments showed variations of less than 0.5 log CFU g^{-1} fresh weight, which were occasionally statistically significant but did not show any pattern (Table 3-3). Chicken manure (CM) showed an initial HPC of more than 8 log CFU g^{-1} and pig hair pellets (PHP) counts of around 3 log CFU g^{-1}. Differences in the bacterial load and total amount of fertilizer applied were not reflected in the HPC of soil samples. Irrespective of treatment, soil samples taken in 2014 had an average HPC of between 6.1 and 6.5 log CFU g^{-1}.

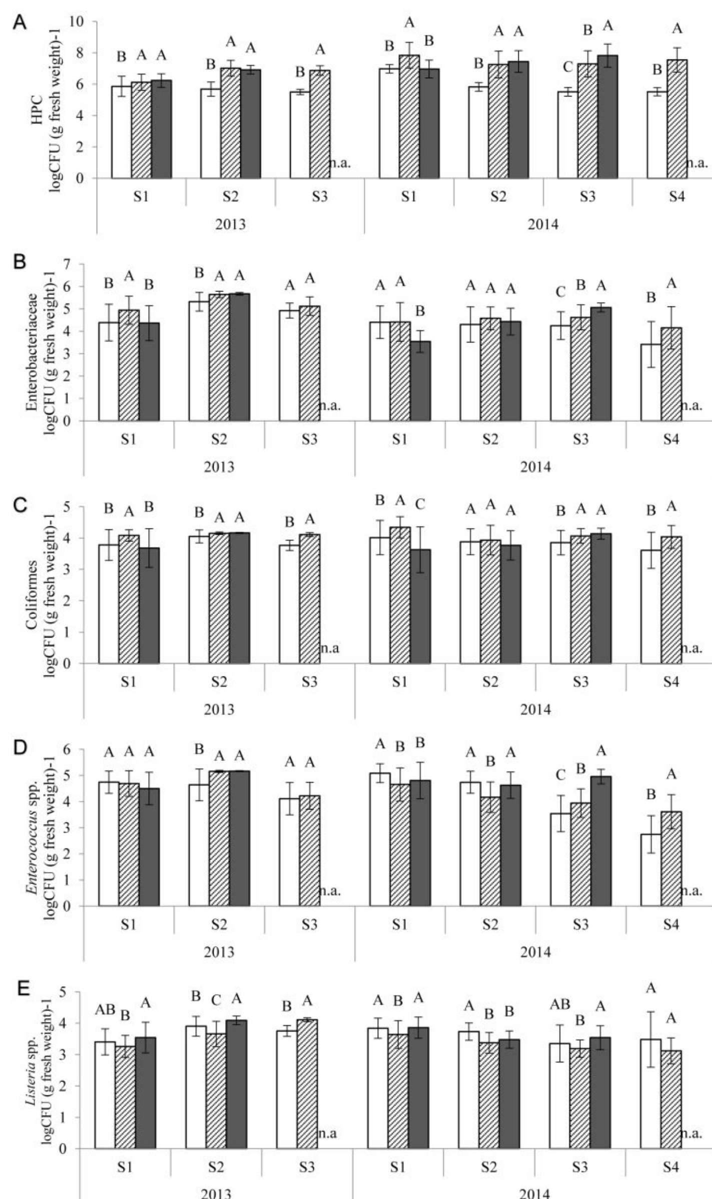

Figure 3-3. Comparison between different bacterial loads (A-E) on: iceberg lettuce, Swiss chard, and wild rocket during two growing seasons (2013 and 2104). Sampling of leaves (S1-S4) for microbial analysis with culture-dependent methods was conducted at two-week intervals. Bars for each harvest with different letters are significantly different according to ANOVA (Tukey-test, $p<0.05$). HPC = heterotrophic bacterial plate count; na = no data available. □: Iceberg lettuce, ▨: Swiss chard, ■: wild rocket

Enterobacteriaceae

Mean counts for *Enterobacteriaceae* ranged from 3.4 to 5.3 log CFU g^{-1} fresh weight for iceberg lettuce, 4.1 to 5.6 log CFU g^{-1} fresh weight for Swiss chard, and 3.5 to 5.6 log CFU g^{-1} fresh weight for wild rocket. In both 2013 and 2014, the microbial load of *Enterobacteriaceae* on wild rocket leaves increased during the growing season, by 1.3 and 1.6 log CFU g^{-1} fresh weight, respectively, from the first sampling occasion to the final sampling. Slightly higher counts (differences <0.7 log CFU g^{-1} fresh weight) on iceberg lettuce fertilized with pellets compared with chicken manure showed statistical significance for some sampling events. For other treatments and crops, no relationships were found (Table 3-3).

Escherichia coli was detected occasionally on plant leaves, irrespective of treatment. Except for some individual samples, counts were just slightly above the detection limit. In 2013, one iceberg lettuce sample (sampling event 1) and three Swiss chard and one wild rocket sample (both sampling event 2) showed counts ≥ 3 log CFU g^{-1} fresh weight. On sampling 3 in 2014, one iceberg lettuce sample and one wild rocket sample were colonized to the same extent. This amounted to seven high contamination events, which corresponded to 0.37% of all samples analyzed. The number of positive samples varied between sampling events and crops (Figure 3-4A). The incidence was highest for Swiss chard on sampling event 2 in 2013, with 50% positive samples. Seedlings of all crops tested positive for *E. coli* in 2013 (iceberg lettuce: 2.2 log g^{-1} fresh weight, Swiss chard: 2.0 log CFU g^{-1} fresh weight, wild rocket 3.2 log CFU g^{-1} fresh weight), whereas no positive sample was found in 2014.

With respect to the fertilizers, CM showed an initial *E. coli* load of 4.5 log CFU g^{-1}, but the level was below the detection limit for PHP. Analysis of the soil during the growing period in 2014 showed mean values of 1.0 to 1.5 log CFU g^{-1} for soil fertilized with manure, whereas 33 to 50% of the samples were above the detection limit. Positive samples showed contamination levels of up to 3.3 log CFU g^{-1} soil. No *E. coli* was detected in soil samples taken from plots fertilized with PHP.

Colonies presumed to be *Salmonella* were detected in high numbers on leaves (≥ 3 log CFU g^{-1} fresh weight) on almost all samples and on every sample taken at both final sampling events. As recent analysis conducted by our group indicates, that colonies showing characteristics for *Salmonella* on this medium, are *Pseudomonas*, these results are not further considered in this study (Hartmann *et al.*, 2017).

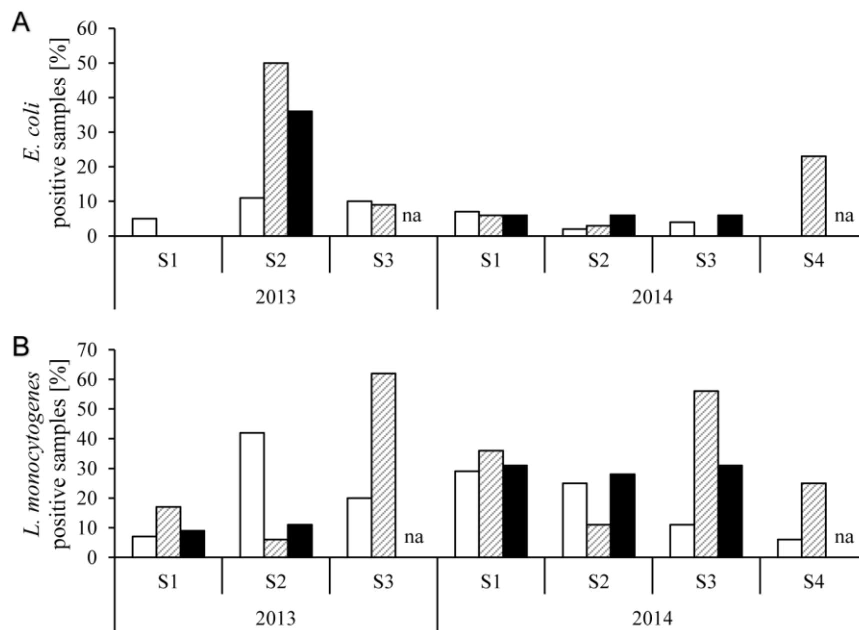

Figure 3-4. Samples testing positive for *E. coli* (A) and *L. monocytogenes* (B) [%] on iceberg lettuce, Swiss chard, and wild rocket during two growing seasons (2013 and 2014). Sampling of leaves (2013: S1-S3; 2014: S1-S4) for microbial analysis with culture-dependent methods (lower detection limit: ~1.7 log CFU g^{-1} fresh weight) was conducted at two-week intervals. na = no data available.
□: Iceberg lettuce, ▨: Swiss chard, ■: wild rocket

Coliform bacteria

The bacterial colonization with coliform bacteria ranged from 3.6 to 4.0 log CFU g^{-1} fresh weight on iceberg lettuce, 3.9 to 4.3 log CFU g^{-1} fresh weight on Swiss chard, and 3.6 to 4.2 log CFU g^{-1} fresh weight on wild rocket over both growing seasons. On wild rocket leaves, an increasing trend during the course of crop growth was observed. Occurrence of coliform bacteria was higher on Swiss chard than on iceberg lettuce, with significant differences at all samplings except the second in 2014 (Figure 3-3C). No consistent differences were observed between the treatments (Table 3-3).

Enterococcus spp.

No consistent differences were seen between the --crops regarding their colonization with enterococci (Figure 3-3D). In 2013, mean values for *Enterococcus* spp. ranged from 4.1 to 4.7 log CFU g^{-1} fresh weight on iceberg lettuce, 4.2 to 4.7 log CFU g^{-1} fresh weight on Swiss chard, and 4.5 to 5.2 log CFU g^{-1} fresh weight on wild rocket. In 2014, mean values were between 2.7 and 5.1 log CFU g^{-1} fresh weight on iceberg lettuce, 3.6 and 4.7 log CFU g^{-1} fresh weight on Swiss chard, and 4.6 and 5.0 log CFU g^{-1} fresh weight on wild rocket, showing a considerably higher range in 2014 for iceberg lettuce and for Swiss chard. Both tended to have a decreasing load of *Enterococcus* spp. during the growing season in 2014, a trend which was also seen for iceberg lettuce in 2013. Initial *Enterococcus* spp. load on seedlings was 4.5 log CFU g^{-1} fresh weight for all crops in 2013, while in 2014 it was 5.1 log CFU g^{-1} fresh weight

on iceberg lettuce seedlings and 5.2 log CFU g^{-1} fresh weight on Swiss chard, and slightly higher on wild rocket seedlings.

Differences between treatments were only very rarely statistically significant and no pattern was observed (Table 3-3).

Listeria spp.

Mean values of *Listeria* spp. determined for the different sampling events ranged between 3.4 and 3.9 log CFU g^{-1} fresh weight on iceberg lettuce, 3.1 and 4.1 log CFU g^{-1} fresh weight on Swiss chard and 3.4 and 4.1 log CFU g^{-1} fresh weight on wild rocket. Comparisons between the crops showed lower values for Swiss chard than wild rocket, a difference which was statistically significant except for one sampling occasion (Figure 3-3E). Iceberg lettuce fertilized with PHP had occasionally higher counts than plants fertilized with CM, while covering the soil with mulch tended to decrease the colonization of Swiss chard leaves. No other effects of treatments were evident regarding the microbial load of *Listeria* spp. (Table 3-3).

Listeria monocytogenes was detected occasionally on leaves, irrespective of treatment, with counts slightly above the detection limit. The highest incidence was found for Swiss chard, which had 62% positive samples (final sampling in 2013). The highest incidence on iceberg lettuce and wild rocket was 42 and 31%, respectively (Figure 3-4B). None of the seedlings tested positive for *L. monocytogenes*.

Table 3-3. Comparison between different bacterial loads [log CFU g^{-1}] on iceberg lettuce (IB), Swiss chard (SC), and wild rocket (WR) during two growing seasons (2013 and 2014) in different treatments. Sampling of leaves (S1-S4) for microbial analysis with culture-dependent methods was conducted at two-week intervals. Values for each treatment, crop, and harvest marked with different letters are significant different according to ANOVA (Tukey-test, $p<0.05$).

			2013								2014										
			S1			S2			S3		S1			S2			S3			S4	
			IB	WR	SC	IB	WR	SC	IB	SC	IB	WR	SC	IB	WR	SC	IB	WR	SC	IB	SC
TSA	Fertilizer	PHP	6.0 A	6.2 A	6.2 A	5.7 A	7.0 A	6.9 A	5.5 A	6.9 A	7.1 A	7.1 A	7.9 A	5.9 A	7.7 A	7.3 A	5.6 A	7.9 A	7.5 A	5.4 A	7.7 A
		CM	5.7 A	6.2 A	6.1 A	5.8 A	6.9 A	7.1 A	5.5 A	6.9 A	6.9 B	6.9 A	7.8 A	6.0 A	7.2 B	7.2 A	5.5 A	7.7 A	7.1 A	5.6 A	7.4 A
	Mulch	no	5.7 B	6.1 A	6.1 A	5.7 A	6.9 A	7.1 A	5.5 A	6.9 A	6.9 A	6.9 A	7.9 A	5.9 A	7.6 A	7.5 A	5.6 A	8.0 A	7.3 A	5.5 B	7.6 A
		yes	6.0 A	6.4 A	6.2 A	5.7 A	6.9 A	6.9 A	5.5 A	6.9 A	7.0 A	7.0 A	7.8 A	6.0 A	7.3 A	7.0 B	5.6 A	7.7 A	7.3 A	5.5 A	7.5 A
	$H_2$0 source	pond				5.6 A	6.9 A	6.9 A	5.6 A	7.0 A							5.6 A	7.9 A	7.1 A	5.4 A	7.3 A
		well				5.8 A	7.0 A	7.1 A	5.5 A	6.8 A							5.6 A	7.9 A	7.4 A	5.6 A	7.6 A
		tap				5.8 A	6.9 A	7.1 A	5.5 A	6.8 A							5.6 A	7.7 A	7.5 A	5.5 A	7.8 A
	irrigation	overhead				5.7 A			5.5 A								5.5 B			5.5 A	
		drip				5.7 A			5.5 A								5.7 A			5.5 A	
Enterobacteriaceae	Fertilizer	PHP	4.6 A	4.6 A	4.9 A	5.4 A	5.7 A	5.7 A	4.9 A	5.1 A	4.7 A	3.7 A	4.5 A	4.6 A	4.7 A	4.6 A	4.1 A	5.1 A	4.7 A	3.7 A	4.4 A
		CM	4.3 A	4.1 A	4.9 A	5.4 A	5.7 A	5.6 A	5.0 A	5.2 A	4.0 B	3.4 A	4.3 A	4.2 B	4.2 A	4.5 A	4.2 A	5.1 A	4.6 A	3.6 A	3.9 A
	Mulch	no	4.4 A	4.2 A	4.9 A	5.4 A	5.7 A	5.7 A	5.1 A	5.1 A	4.5 A	3.5 A	4.5 A	4.3 A	4.4 A	4.6 A	4.2 A	5.1 A	4.5 A	3.6 A	4.0 A
		yes	4.5 A	4.5 A	5.0 A	5.4 A	5.7 A	5.6 A	4.9 B	5.2 A	4.3 A	3.6 A	4.3 A	4.5 A	4.5 A	4.5 A	4.1 A	5.0 A	4.7 A	3.7 A	4.3 A
	$H_2$0 source	pond				5.3 A	5.7 A	5.6 A	5.0 A	5.2 A							4.0 A	5.1 A	4.6 A	3.4 B	4.0 A
		well				5.4 A	5.7 A	5.7 A	5.0 A	5.1 A							4.3 A	5.1 A	4.5 A	4.2 A	4.1 A
		tap				5.5 A	5.6 A	5.7 A	5.0 A	5.0 A							4.1 A	5.0 A	4.8 A	3.4 B	4.5 A
	irrigation	overhead				5.3 A			4.9 A								4.3 A			3.4 B	
		drip				5.5 A			5.0 A								4.0 A			3.9 A	
Coliform bacteria	Fertilizer	PHP	3.9 A	3.8 A	4.1 A	4.1 A	4.2 A	4.2 A	3.8 A	4.1 A	4.2 A	3.9 A	4.4 A	4.0 A	4.0 A	4.0 A	3.6 B	4.1 A	4.1 A	3.7 A	4.0 A
		CM	3.7 A	3.5 A	4.1 A	4.0 B	4.2 A	4.2 A	3.7 B	4.1 A	3.7 B	3.4 A	4.3 A	3.9 B	3.6 B	3.8 A	3.9 A	4.2 A	4.1 A	3.7 A	4.0 A
	Mulch	no	3.7 A	3.5 A	4.1 A	4.1 A	4.2 A	4.1 A	3.8 A	4.1 A	3.9 A	3.7 A	4.3 A	3.9 B	3.8 A	4.0 A	3.7 A	4.2 A	4.1 A	3.7 A	4.1 A
		yes	3.9 A	3.8 A	4.1 A	4.0 A	4.2 A	4.2 A	3.7 B	4.1 A	4.0 A	3.6 A	4.3 A	4.0 A	3.7 A	3.9 A	3.8 A	4.1 A	4.1 A	3.7 A	4.0 A
	$H_2$0 source	pond				4.1 A	4.2 A	4.2 A	3.8 A	4.1 A							3.8 A	4.2 A	4.1 A	3.6 A	4.0 A
		well				4.0 B	4.2 A	4.2 A	3.7 B	4.1 A							3.8 A	4.2 A	4.0 A	3.8 A	4.1 A
		tap				4.1 A	4.2 A	4.2 A	3.7 b	4.1 A							3.7 A	4.1 A	4.1 A	3.7 A	4.0 A
	irrigation	overhead				4.0 A			3.8 A								3.9 A			3.6 A	
		drip				4.1 A			3.8 A								3.7 A			3.8 A	
Enterococcus	Fertilizer	PHP	4.7 A	4.7 A	4.6 A	4.8 A	5.2 A	5.2 A	4.2 A	4.4 A	5.2 A	4.8 A	4.7 A	4.8 A	4.9 A	4.3 A	3.6 A	4.9 A	3.9 A	2.8 A	3.5 A
		CM	4.7 A	4.3 A	4.8 A	4.5 A	5.2 A	5.2 A	3.9 A	4.1 B	5.0 A	4.8 A	4.6 A	4.7 A	4.3 B	4.0 A	3.3 A	5.0 A	4.0 A	2.6 A	3.8 A
	Mulch	no	4.6 B	4.3 A	4.8 A	4.7 A	5.2 A	5.1 A	4.1 A	4.2 A	5.1 A	4.8 A	4.8 A	4.7 A	4.6 A	4.3 A	3.4 A	5.0 A	4.0 A	2.7 B	3.7 A
		yes	4.9 A	4.7 A	4.6 A	4.6 A	5.2 A	5.2 A	4.0 A	4.3 A	5.1 A	4.8 A	4.5 A	4.8 A	4.6 A	4.1 A	3.5 A	5.0 A	3.9 A	2.7 A	3.5 A
	$H_2$0 source	pond				4.8 A	5.2 A	5.2 A	4.2 A	4.3 A							3.4 A	5.1 A	3.8 A	2.6 A	3.4 A
		well				4.5 A	5.2 A	5.2 A	3.9 A	4.4 A							3.6 A	4.9 A	3.9 A	2.8 A	3.8 A
		tap				4.6 A	5.2 A	5.2 A	4.0 A	4.0 A							3.3 A	4.9 A	4.1 A	2.7 A	3.7 A
	irrigation	overhead				4.6 A			4.1 A								3.5 A			2.7 A	
		drip				4.7 A			4.0 A								3.4 A			2.7 A	
Listeria	Fertilizer	PHP	3.5 A	3.5 A	3.3 A	3.8 B	4.1 A	3.6 A	3.8 A	4.1 A	4.0 A	4.0 A	3.7 A	3.8 A	3.6 A	3.4 A	3.7 A	3.5 A	3.2 A	3.7 A	3.1 A
		CM	3.4 A	3.5 A	3.2 A	4.0 A	4.1 A	3.7 A	3.7 B	4.1 A	3.7 B	3.8 A	3.6 A	3.7 A	3.4 B	3.4 A	3.2 B	3.6 A	3.1 A	3.3 A	3.1 A
	Mulch	no	3.3 B	3.5 A	3.3 A	4.0 A	4.1 A	3.9 A	3.8 A	4.1 A	3.8 A	4.0 A	3.9 A	3.7 A	3.6 A	3.6 A	3.2 B	3.6 A	3.3 A	3.5 B	3.2 A
		yes	3.6 A	3.5 A	3.2 A	3.8 B	4.1 A	3.4 B	3.7 B	4.1 A	3.9 A	3.7 A	3.4 B	3.8 A	3.3 B	3.2 B	3.6 A	3.5 A	3.1 B	3.6 A	3.0 A
	$H_2$0 source	pond				3.8 A	4.1 A	3.6 A	3.8 A	4.0 A							3.5 A	3.4 A	3.2 A	3.5 B	3.1 A
		well				3.9 A	4.1 A	3.6 A	3.6 B	4.1 A							3.3 A	3.5 A	3.3 A	3.3 B	3.0 A
		tap				4.0 A	4.1 A	3.8 A	3.7 AB	4.1 A							3.4 A	3.7 A	3.1 A	3.7 A	3.3 A
	irrigation	overhead				3.9 A			3.8 A								3.4 A			3.5 A	
		drip				3.9 A			3.7 A								3.5 A			3.5 A	

Table 3-4 shows the HPC and contamination with coliform bacteria, *E. coli* and enterococci via the irrigation water. Except for the last sampling in 2014 for well water, HPC in the samples ranged around 5 log CFU mL^{-1}. Coliform bacteria were above the upper detection limit in pond water on all occasions, while well water and tap water ranged between the lower detection limit and 372.4 and 45.0 MPN mL^{-1}, respectively. *Escherichia coli* was detected in pond water on all sampling occasions, but was below the detection limit for well water and tap water. Enterococci were found in all pond water samples and ranged from the lower detection limit to 3.1 and 1011.2 MPN mL^{-1} in tap water and well water, respectively. For both water sources, negative results for coliform bacteria corresponded to negative tests for enterococci.

Table 3-4. Microbial loads in water from different sources applied to field-grown leafy vegetables in 2013 and 2014 at different sampling occasions (S). Heterotrophic plate count (HPC) was assessed as colony-forming units [log CFU mL^{-1}] and coliform bacteria, *E. coli*, and enterococci as most probable number [MPN 100 mL^{-1}].

		2013		2014	
		S2	S3	S3	S4
HPC	Pond	5.1	5.2	5.1	5.0
	Well	5.7	5.4	4.8	3.8
	Tap	5.1	5.0	5.2	5.4
Coliform bacteria	Pond	>2419.6	>2419.6	>2419.6	>2419.6
	Well	<1	112.0	372.4	93.3
	Tap	45.0	24.7	<1	<1
E. coli	Pond	248.9	195.4	533.5	93.4
	Well	<1	<1	<1	<1
	Tap	<1	<1	<1	<1
Enterococcus spp.	Pond	29.2	55.2	60.2	10.9
	Well	<1	1.5	1.0	3.1
	Tap	1011.2	1.5	<1	<1

Interactions between weather conditions and phyllosphere bacteria

For all crops, there was a negative correlation between HPC and temperature (iceberg lettuce: r = -0.45, p<0.001; Swiss chard: r = -0.37, p<0.001; wild rocket: r = -0.33, p<0.001). Precipitation was negatively

correlated with HPC on iceberg lettuce (r = -0.21, p<0.001), but positively with HPC on Swiss chard (r = 0.14, p=0.031) and wild rocket (r = 0.18, p=0.014). On all crops, *Enterobacteriaceae* correlated positively with temperature (iceberg lettuce: r = 0.34, p<0.001; Swiss chard: r = 0.44, p<0.001; wild rocket: r = 0.58, p<0.001) and negatively with precipitation (iceberg lettuce: r = -0.39, p<0.001; Swiss chard: r = -0.37, p<0.001; wild rocket: r = -0.15, p=0.048). Negative correlations were also found for precipitation and *Listeria* (iceberg lettuce: r = -0.38, p<0.001; Swiss chard: r = -0.38, p<0.001; wild rocket: r = -0.34, p<0.001), as well as enterococci (iceberg lettuce: r = -0.34, p<0.001; Swiss chard: r = -0.50, p<0.001; wild rocket: r = -0.25, p<0.001). As found for *Enterobacteriaceae*, *Listeria* and enterococci correlated positively with temperature on Swiss chard (*Listeria*: R^2 = 0.37, p<0.001; enterococci: r = 0.19, p=0.002) and wild rocket (*Listeria*: r = 0.23, p=0.002; enterococci: r = 0.16, p=0.032), while on iceberg lettuce enterococci correlated negatively with temperature (r = -0.09, p=0.036). No correlation was found between *Listeria* and temperature on iceberg lettuce. Coliform bacteria showed a weak correlation with temperature on wild rocket (r = 0.23, p<0.001) and with precipitation on Swiss chard (r = -0.26, p<0.001). Scatterplots of correlations with r > 0.4 are displayed in Figure 3-5.

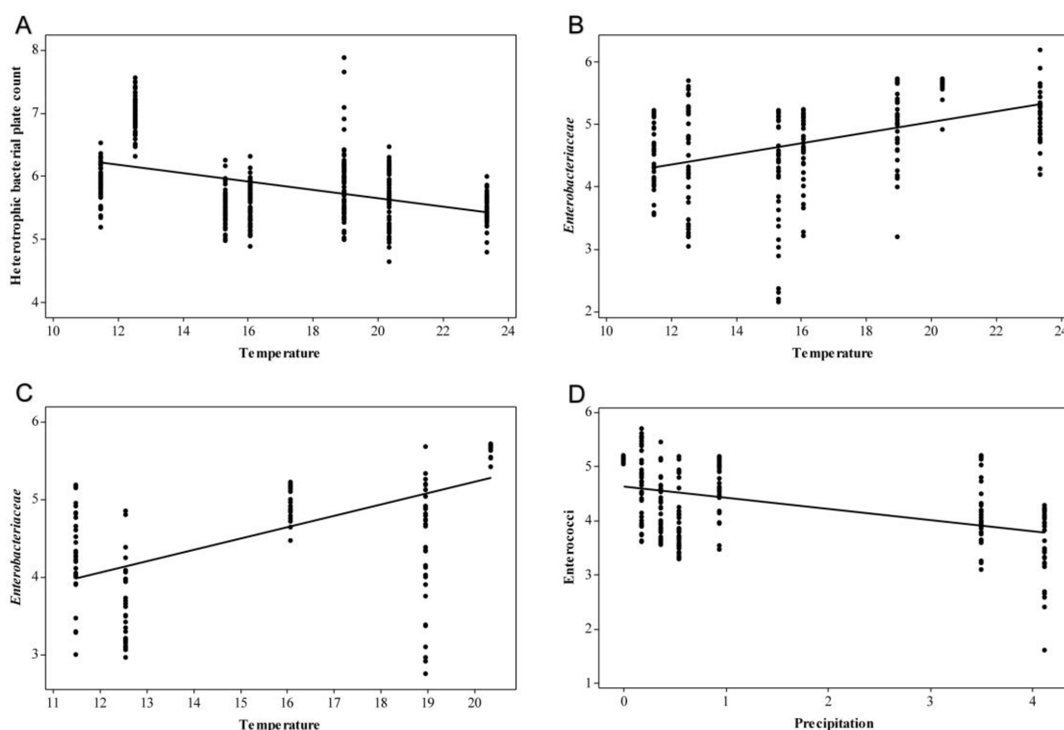

Figure 3-5. Scatterplots of bacterial counts [log CFU g^{-1}] and temperature [°C]/precipitation [mm] on leaves of iceberg lettuce (A), Swiss chard (B, D), and wild rocket (C). Bacterial loads were determined at two-week intervals and correlated to weather data averaged over five days prior to analysis.

The highest correlations between different bacterial groups were found for *Enterobacteriaceae* and coliform bacteria on iceberg lettuce and wild rocket, bacterial HPC and enterococci on iceberg lettuce, *Enterobacteriaceae* and enterococci on Swiss chard, and coliform bacteria and enterococci on wild

rocket. Scatterplots for these correlated parameters are shown in Figure 3-6. Lower, but also positive, correlations were also found for other bacterial groups, exceptions being HPC, *Enterobacteriaceae*, and *Enterococcus* spp. on Swiss chard and HPC and *Listeria* spp. on wild rocket.

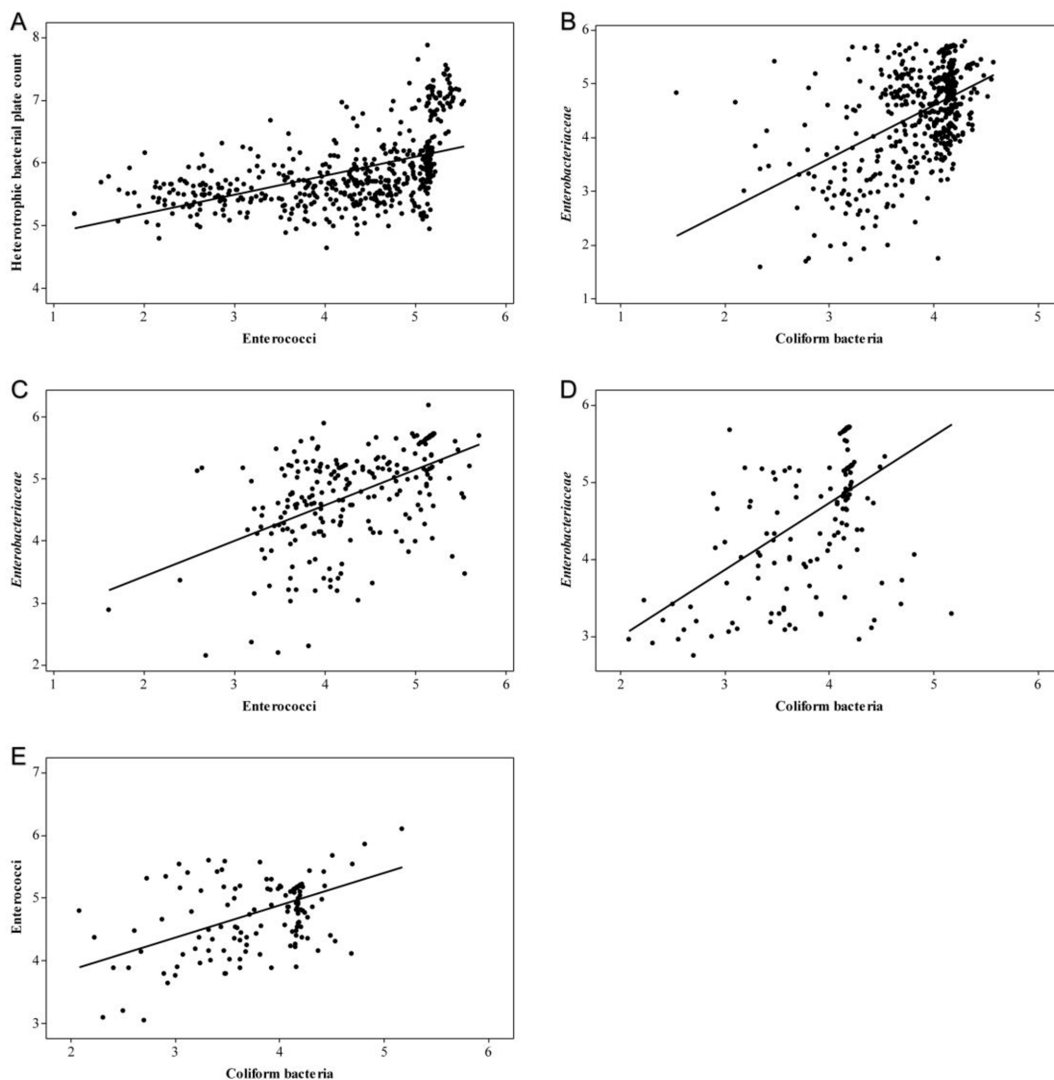

Figure 3-6. Scatterplots for bacterial counts [log CFU g^{-1}] on leaves of iceberg lettuce (A, B), Swiss chard (C), and wild rocket (D, E). Bacterial loads were determined at two-week intervals.

In PCA, the samples were clustered according to sampling occasion and differences between the seasons were apparent, but not for the different treatments (Figure 3-7). Average temperature and precipitation five days before each sampling proved to have a moderate influence on the microbial load on some sampling occasions, but failed to explain the clustering.

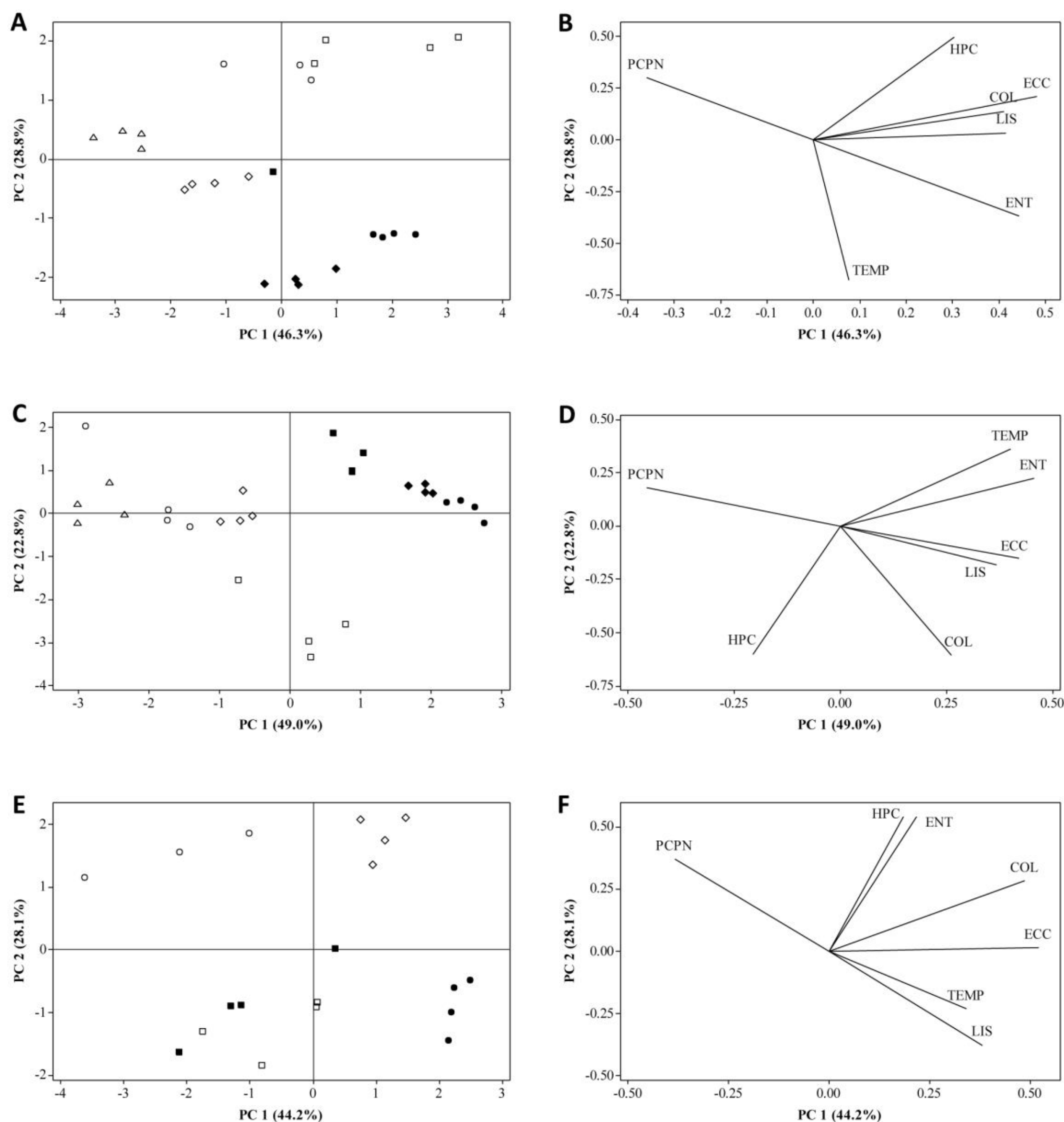

Figure 3-7. Principal component analysis based on temperature (TEMP), precipitation (PCPN), and microbial loads on leaves of iceberg lettuce (A, B), Swiss chard (C, D), and wild rocket (D, E) during cultivation. Samples were taken at two-week intervals after planting (□ = sampling 1, ○ = sampling 2, ◊ = sampling 3, Δ = sampling 4) in two growing seasons (filled symbols = 2013, open symbols = 2014). No data were obtained for wild rocket on the last sampling event in 2013 and 2014. Microbial loads [log CFU g^{-1}] were determined for heterotrophic bacterial plate count (HPC), *Enterobacteriaceae* (ENT), *Listeria* spp. (LIS), *Enterococcus* spp. (ECC), and coliform bacteria (COL) by culture-dependent methods. Temperature [°C] and precipitation [mm] values were averaged over five days before each sampling.

Nevertheless, a strong negative correlation was observed between precipitation and *Enterobacteriaceae* on iceberg lettuce, and between precipitation and *Listeria* spp. and *Enterococcus* spp. on Swiss chard and wild rocket. A negative influence of higher temperature on HPC on iceberg lettuce was found, whereas higher temperatures correlated positively with *Enterobacteriaceae* on Swiss chard and *Enterococcus* spp. on wild rocket.

Interactions between yield gain and phyllosphere bacteria

Correlations between yield gain and bacterial loads are displayed in Table 3-5. Low to moderate dependencies were found, in which all correlations were negative. Iceberg lettuce showed correlations for all groups analyzed, all below 0.300. For Swiss chard, only *Enterobacteriaceae* and enterococci were correlated with yield gain. Wild rocket showed the highest correlations for all bacterial groups analyzed except HPC, which did not show a correlation.

Table 3-5. Pearson correlation ($p<0.05$) between yield gain and bacterial groups on iceberg lettuce (IB), Swiss chard (SC), and wild rocket (WR) over two growing seasons. Bacterial loads were determined at two-week intervals and correlated to yield gained between sampling occasions. HPC = heterotrophic bacterial plate count.

	HPC	*Enterobacteri-aceae*	Coliform bacteria	*Listeria*	Enterococci
IB	-0.279	-0.135	-0.295	-0.161	-0.288
	<0.001	0.012	<0.001	0.001	<0.001
SC		-0.302			-0.368
	n.s.	<0.001	n.s.	n.s.	<0.001
WR		-0.574	-0.514	-0.283	-0.412
	n.s.	<0.001	<0.001	0.003	<0.001

Discussion

Many studies have provided evidence of possible transmission of potentially human pathogenic bacteria from highly contaminated inputs to edible parts of vegetables. However, these studies have often used artificial inoculation with attenuated pathogens in high numbers. This leads to limitations in translating the results to open-field commercial produce production, as (i) the release of genetically modified organisms into the environment is prohibited/associated with administrative problems and trials are thus mostly conducted in the greenhouse and (ii) the concentrations applied are higher than those expected in the environment. To obtain a situation closer to reality, this study was conducted with naturally contaminated inputs that were similar to materials used in organic farming.

Microbial colonization, measured on the leaf surface as HPC, was within the range (10^5-10^7 CFU g^{-1}) commonly reported for vegetables (Francis *et al.* (1999), irrespective of treatment, in 2013. Numbers in 2014 exceeded this upper level by up to 0.7 log for wild rocket and Swiss chard. These results are in agreement with HPC found in other studies for iceberg lettuce and wild rocket (Alam *et al.*, 2014;

Wießner *et al.*, 2009; Fischer-Arndt *et al.*, 2008), whereas HPC on Swiss chard was in the same range as found by Agüero *et al.* (2005), but up to log 2 higher than indicated by Ponce *et al.* (2003).

As nutrient availability is assumed to be a limiting factor in the harsh phyllosphere, HPC reflects nutrient abundance on the leaves. Conditions that promote nutrient leakage, e.g., tissue damage, not only stimulate total bacterial growth, but also enhance the persistence of human pathogens on the leaf (Brandl, 2008). Thus, HPC does not show the probability of microbial contamination, but elevated levels could indicate increased survival if contamination occurs.

For indicator organisms like *E. coli* and *L. monocytogenes*, the German authority DGHM (Deutsche Gesellschaft für Hygiene und Mikrobiologie, 2010) provides warning values that refer to minor microbial safety of food. In accordance with these, numbers of 10^3 (*E. coli*) and 10^2 (*L. monocytogenes*) CFU g^{-1} fresh weight on salads are alarming. In this study, the occasionally observed contamination with *E. coli* was below this required microbial limit except for seven samples. The lower detection limit is about the same as the warning value for *L. monocytogenes*, and all positive samples ranged around this threshold. However, the comparison was limited as only surface-attached microorganisms were determined. Furthermore, samples were analyzed directly after harvest without a preceding washing step, which is often carried out before sale and potentially reduces microbial contamination (Simons & Sanguansri, 1997). Incidental contamination with these two bacterial species has also been found even when no artificial inoculation has taken place (Wießner *et al.*, 2009; Fischer-Arndt *et al.*, 2008; Johannessen *et al.*, 2004; Sagoo *et al.*, 2001).

As expected, there were differences in microbial colonization between plant species. Plant-related properties may have resulted in lower HPC on iceberg lettuce than on the two non-head forming species, as well as the highest observed incidence of *E. coli* and *L. monocytogenes* on Swiss chard samples. Certain leaf characteristics such as topography (Hunter *et al.*, 2010; Yaron *et al.*, 2000), the number of trichomes and stomata (Leveau, 2006), and nutrient availability and wettability (Whipps *et al.*, 2008) have a great impact on phyllosphere colonization. This might not only result in different total bacterial numbers for plant species, but might also affect the composition of the microbiome (Bodenhausen *et al.*, 2014). Indigenous microorganisms can enhance or impair the establishment of alien bacteria such as *E. coli* (Klerks *et al.*, 2007), which are potentially pathogenic to humans (Bell, 2002).

While there was no evident trend for HPC on Swiss chard leaves, the HPC load increased during both growing periods for wild rocket. With longer time in the field, more bacteria have the possibility to immigrate to the leaf surface and thus establish in the phyllosphere (Kinkel, 1997). The occurrence of cracks in the cuticle with increasing leaf age releases a greater amount of nutrients, which allows more bacteria to feed on plant exudates (Kerstiens, 1996; Beattie & Lindow, 1995). This could either enhance multiplication of the microorganisms present or ease the establishment of new arrivals (Kinkel, 1997). For some bacteria, survival is also enhanced under higher microbial density (Kinkel, 1997), as they are e.g., protected from environmental influences by aggregate formation (Ölmez & Temur, 2010). Apparently, other impacts dictated the culturable microbial load on wild rocket compared with iceberg

lettuce, where HPC declined over the growing period in both years. Higher microbial loads on young lettuce leaves has also been reported by Brandl and Amundson (2008), who suggested a dependency between higher initial trichome density and higher HPC. In addition, an increasing surface to weight ratio and protection from environmental contamination after head formation can contribute to decreasing HPC on iceberg lettuce. Interestingly, the correlations found between yield gain and bacterial groups were all negative, irrespective of crop. Reasons can be the higher surface area increase of the crop compared with the multiplication rate/immigration of phyllosphere bacteria, or a dependency on nutrient availability on the leaf surface and growth rate of the plants.

The correlations between some of the bacterial groups analyzed indicate a common dependency for microbial colonization. However the correlations were not strong, suggesting that the community composition is also influenced in some other way. The culturable microbial community structure on the leaves was driven by sampling occasion. The overall weak to moderate negative and positive correlations between air temperature/precipitation and microbial loads support our hypothesis of an influence of climate conditions on the microbial dynamics on leaves. Ultraviolet irradiation has been shown to have a great influence on epiphytic microbiota and, while some bacteria have the ability to protect themselves from this hazard by pigmentation (Sundin & Jacobs, 1999) or repair of damaged DNA (Brandl, 2006), others are harmed. Thus UV irradiation can result in declining microbial loads and shifts in the community (Heaton & Jones, 2008). Direct measurements of UV irradiation were not carried out during this experiment, but could be very interesting to conduct in future trials. As higher temperatures often accompany increased sunshine, die-off due to UV irradiation can be one possible explanation for the negative correlation found between HPC and air temperature. Rainfall has the potential to wash off microorganisms from leaves and introduce others through soil splashing or collection from the atmosphere (Whipps *et al.*, 2008). It is worth noting that both non-head forming species seemed to develop similar correlations between bacteria and air temperature/precipitation, whereas the head-forming iceberg lettuce showed partly deviating trends. Differences were also seen in the growth curves, indicating that growth is a possible influencing factor for the interaction between climate and microbial colonization.

Differences between the sampling occasions were not completely explained by the weather data. Hence other factors also influence the microbial structure on leaves. These could include other climate factors, e.g., relative humidity and wind (Kinkel, 1997), but also plant-related factors like nutrient availability, moisture conditions, cuticle properties (Redford *et al.*, 2010), and leaf surface topography, which are not consistent over time. In accordance with our results, Rasche *et al.* (2006) found that growing stage had an influence on the bacterial community.

Occasionally observed trends between treatments, sometimes statistically significant, did not exceed 0.6 log CFU g^{-1} and are hence solely of technical interest. The lack of a clear effect between treatments as regards bacteria on genus and higher levels could be due to the natural occurrence of these bacteria in the soil and/or on leaves. Several genera assigned to coliform bacteria and also belonging to the

Enterobacteriaceae, e.g., *Klebsiella* spp. *Enterobacter* spp., and *Citrobacter* spp., are indigenous habitants of the phyllosphere (Tortorello, 2003). Enterococci are also frequently isolated from leaves (Švec *et al.*, 2012) and *Listeria* spp. are known for their ubiquitous occurrence and high abundance in soil (Attaran *et al.*, 2008). Additional introduction of these bacteria with manure or water did not result in higher cell counts in the soil (data not shown) or on the leaves, indicating that other factors such as nutrient abundance and climate conditions dictated the microbial equilibrium in this study.

Our findings for the different treatments are in agreement with those of other studies comparing fertilizers with differing microbial quality. Wießner *et al.* (2009) and Johannessen *et al.* (2004) evaluated field-grown lettuce for microbial quality (using culture-dependent methods) after applying manure or inorganic fertilizer to the soil. In both studies, no difference was observed in bacterial counts on lettuce. A less consistent picture has been reported regarding water quality. Holvoet *et al.* (2014) identified water quality as having a strong influence on produce safety, while Castro-Ibáñez *et al.* (2015) did not find any correlation between water quality and microbial colonization of baby leaves. Similarly, in a study by Tomás-Callejas *et al.* (2011), irrigation method did not affect the microbial load on leafy greens. The absence of visible effects in the present study can be due to multiple causes. While there were considerable differences in the microbial load with *E. coli* in the different water sources, other coliform bacteria and enterococci were at least occasionally detected, irrespective of water origin, and HPC was consistently high, indicating overall low water quality. In addition, comparably little volume was used for irrigation, suggesting that the differences in water quality could not be seen as distinct results on the plants. Moreover, soil splash/aerosol formation during rain can disperse bacteria and thus mask the effect of water quality, but also the irrigation method. In addition, a recent study by Joung *et al.* (2017) showed that bioaerosol formation caused by water droplets is minor on sandy soils (as in the present study) compared with clay. Thus, the effects of irrigation methods could be more pronounced/visible on other field sites. Beside contaminated input, feces of wild animals can also lead to contamination of produce (Beuchat & Ryu, 1997). Droppings of rabbits were observed during our experiment in the field, which could be the reason for the high contamination with *E. coli* on three samples, but also the lack of effect of the different treatments.

In conclusion, we found that crop species and climate conditions affected leaf colonization by microbes but that the microbial community on leaves was not detectably influenced by treatments, as assessed with culture-dependent methods, contradicting our starting hypothesis. However, possible effects could have been masked by other environmental factors. Thus the study shows the difficulties in addressing the complex field of phyllosphere microbiology in a field trial imposing various impacts (Wießner *et al.*, 2009).

Although no differences could be found between low and high contamination cultivation strategies, hazards coming from inputs with low microbial quality should not be neglected in primary production of ready-to-eat vegetables, as even small amounts of human pathogens that are not detectable without previous enrichment in culture methods can lead to severe disease.

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Chapter 4: Impact of the source of organic manure on persistence of *E. coli* O157:H7 *gfp*+ in rocket (*Diplotaxis tenuifolia*) and Swiss chard (*Beta vulgaris* cicla)

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Highlights

- The probability of infection with *E. coli* O157:H7 differs for baby leaf items
- Consumption of Swiss chard poses a higher risk of infection than rocket
- Organic nitrogen sources affect the risk of infection

Abstract

The influence of organic nitrogen sources on the establishment of *E. coli* O157:H7 and the occurrence of other potentially human pathogenic bacteria on baby leaf salads was evaluated. Greenhouse-grown rocket and Swiss chard were spray-inoculated with *gfp*-tagged *E. coli* O157:H7 twice a week from when their first true leaves reached a length of 2 cm until three days before harvest. Analysis of nitrogen content in leaves revealed differences between treatments. Untreated plants had the lowest values, followed by plants fertilized with pig hair pellets and chicken manure. The same pattern was seen for the growth medium at the day of harvest. The applied strain showed similar establishment (measured using culture-dependent methods) irrespective of treatment, but Swiss chard hosted significantly more *E. coli* O157:H7 than rocket. Differences in the risk of infection were found for the crops, with plants fertilized with pig hair pellets showing a slightly higher risk. No relationship was found for total nitrogen content in leaves and colonization with *E. coli* O157:H7 *gfp*+. Colonies showing matching

characteristics for *Listeria* spp. and *Salmonella* spp. were detected in a range from below the detection limit to 4 log CFU g^{-1} fresh weight on rocket, and from 3.5 to 5.5 log CFU g^{-1} fresh weight and 1 to 5 log CFU g^{-1} fresh weight, respectively, on Swiss chard. However, presumptive *Listeria* spp. and *Salmonella* spp. could not be confirmed by sequencing of the 16S rRNA gene.

Keywords: culture-dependent methods, *Enterobacteriaceae*, food safety, human pathogenic bacteria, organic fertilizers, phylogenetic analysis, risk assessment

Introduction

The surface of vegetables is highly colonized with microorganisms, bacteria being the most abundant, followed by fungi and yeasts. However, archaea, protozoa, and nematodes can also be found in the phyllosphere (Whipps *et al.*, 2008). On leafy vegetables, bacteria belonging to the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroides* show the highest occurrence, based on culture-independent analysis (Alsanius *et al.*, 2017c; Rastogi *et al.*, 2012). On family level, the *Pseudomonaceae* and *Enterobacteriaceae* account for the highest numbers (Lopez-Velasco *et al.*, 2011; Hunter *et al.*, 2010). In general, these colonizers are indifferent or have even been claimed to promote human health (Berg *et al.*, 2014). However, increasing outbreaks linked to contaminated fresh produce in recent years have led to a focus on the risk caused by produce eaten raw (Warriner *et al.*, 2009). Particular concerns are human pathogenic *Escherichia coli* and *Salmonella* spp., due to their frequent implication in outbreaks, and *Listeria monocytogenes*, owing its high case fatality rate (lethality) (Carrasco *et al.*, 2010; Brandl, 2006). Among human pathogenic *E. coli*, serotype O157:H7 is most often identified as the causative agent in disease outbreaks (Dikici *et al.*, 2015), but other strains, e.g., belonging to the serogroups O26, O103, O104, O111, and O145, also have the ability to infect humans (European Food Safety Authority (EFSA), 2011; Karmali *et al.*, 2003). Clinical disease has been observed following exposure at low doses of less than 100 bacteria (Rheinbaben, 2011) and can lead to diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Agbodaze, 1999). Infection with *Salmonella* spp. typically causes gastroenteritis and septicemia (Trevejo *et al.*, 2003). An exposure level of 10^5 to 10^{10} cells is needed to give a tangible probability of infection (Kothary & Babu, 2001). Listeriosis can manifest in meningitis, sepsis, and miscarriage in pregnant women (Farber & Peterkin, 1991) and mortality rates during an outbreak can be as high as 40% (Carrasco *et al.*, 2010). In healthy people, the *Listeria* numbers in exposures associated with clinical listeriosis are reported to be high, while for susceptible groups, i.e., people with immunosuppression, even small numbers can lead to infection (Todd & Notermans, 2011; Franciosa *et al.*, 2005).

Human pathogenic bacteria are not part of the normal flora of the phyllosphere and thus have to be introduced to the plant environment (Chitarra *et al.*, 2014). Transmission can occur via several routes, including pre- and post-harvest processes (Taban & Halkman, 2011). Critical points for transmission of human pathogenic bacteria during cultivation are associated with application of contaminated manure or irrigation water (Islam *et al.*, 2004a), use of plant material of low microbial quality (Jablasone *et al.*, 2005), and insufficient field worker hygiene (Brackett, 1999). Moreover, there are hazards arising from contaminated runoff water (Kirby *et al.*, 2003) and airborne transmission (Berry *et al.*, 2015) from nearby farms and through wild animals visiting crop fields (Beuchat, 2006). A risk assessment evaluating the use of manure in terms of transmission of human pathogens to vegetables (Koller, 2011) concluded that pre-treatment of manure and a time lag between application and cultivation are needed to reduce the risk of contamination of vegetables. However, no previous risk assessment has quantified the risk posed by fertilizers with differing nutrient availability.

The establishment of an alien bacterium in the phyllosphere can be influenced by the existing microbiome and by the level of available nutrients (Oliveira *et al.*, 2012; Monier & Lindow, 2005). Pedraza *et al.* (2009) showed that urea application results in a shift in the leaf endophytic microbiome in rice, while Huber and Watson (1974) demonstrated that the form of fertilizer nitrogen used has an impact on leakage quantity and nutrient composition of leaf exudates. In addition, the growth medium has an indirect influence on the phylloplane by altering the soil microbiome (Andrews, 1992). These findings raise the question of whether organic nitrogen fertilizer applied to the growth medium of vegetable crops has an impact on the establishment of human pathogenic bacteria, other than its potential of functioning as a carrier.

The aim of this study was thus to evaluate the influence of organic nitrogen source on establishment of artificially introduced (via spray irrigation) *E. coli* O157:H7 *gfp+* on rocket and Swiss chard baby leaves. Native occurrence of *Salmonella* spp. and *Listeria* spp. was also analyzed with respect to the fertilizer. A risk assessment of the probability of infection when eating the contaminated leafy greens was conducted. Furthermore, dominant bacteria grown on a culture medium selective for *Enterobacteriaceae* were identified. Previous studies have revealed that phylloepiphytic *Enterobacteriaceae* spp. can have an impact on the growth of *E. coli* O157:H7 on leafy greens (Lopez-Velasco *et al.*, 2012; Cooley *et al.*, 2006).

The hypotheses tested were that: (i) food pathogens, such as *Salmonella* and *Listeria*, and (ii) the number of *E. coli* O157:H7 *gfp+* introduced are affected by the nitrogen source and (iii) that the infection risk is higher for crops fertilized with non-treated organic nitrogen sources than with heat-treated organic nitrogen sources.

Materials and Methods

Inoculum preparation

An *Escherichia coli* O157:H7 strain, obtained from the Swedish Institute for Communicable Disease Control, Solna, Sweden (registry no E81186), was used in the experiment. This strain expresses the *eae* gene but lacks virulence factors verotoxin-1 and -2 and is labeled with a plasmid coding for ampicillin resistance and for green fluorescent protein (*gfp*), which is emitted in the presence of arabinose when exposed to UV light (Alam *et al.*, 2014; El-Mogy & Alsanius, 2012).

The *E. coli* O157:H7 *gfp+* was cultured from a cryo-culture on lysogenic broth (LB) agar supplemented with 0.2% arabinose and 100 µg mL^{-1} ampicillin at 37 °C. A single fluorescent colony was picked and cultivated in 30 mL LB agar plus 100 µg mL^{-1} ampicillin on a rotary shaker at 180 rpm and 37 °C for 18 hours. Cells were pelleted by centrifugation of the suspension at 3000 xg and 4 °C for 45 min, and washed once with 0.85% sterile NaCl. Cells were suspended in 0.85% NaCl to a density of 9.7 log CFU mL^{-1} (optical density (OD_{620}) = 1.0). This stock suspension was diluted with 0.085% NaCl to a final density of 6 log CFU mL^{-1} for plant inoculation.

Greenhouse experiment

Swiss chard (*Beta vulgaris* subsp. *cicla,* SCR 107, AdvanSeed) and rocket (*Diplotaxis tenuifolia*, Tricia, Enzo) were grown in the greenhouse with a target humidity of 70% and the temperature set to 21°C. Peat based growing medium, consisting of a bottom layer (4.5 cm, K-jord, Hasselfors Garden AB, Örebro, Sweden), and a top layer (1.5 cm, S-jord; Hasselfors Garden AB, Örebro, Sweden) was filled in trays (0.52 m x 0.42 m x 0.09 m) with the addition of chicken manure (CM) or pig hair pellets (PHP) in the amount of 81 kg ha^{-1} available nitrogen. The control plots did not receive any additional fertilizer. When the first true leaves reached a size of two cm, the plants were spray-inoculated with an *E. coli* O157:H7 *gfp+* suspension in an amount of 5 mm every three days until three days before harvest.
Six replicates for each treatment, defined as separate trays, were harvested six weeks after sowing, and brought directly to the laboratory for further analysis.

Nitrogen analysis

The residual nitrogen content in three independent replicates of the fertilizers used and in the growth medium, including the root system, was assessed with respect to mineralized nitrogen (NH_4^+, NO_3^-) and total nitrogen content by Eurofins, Kristianstad, Sweden. Growth medium samples were collected for each tray and kept at -20 °C until analysis.
Leaves (100 g fresh weight) used for determination of nitrogen content were dried in a drying oven at 70 °C. Analysis of total nitrogen content was conducted by Eurofins, Kristianstad, Sweden. Nitrate (NO_3^-) content was measured on fresh plant material according to Method no. G-287-02 Rev. 5 (Multitest MT7B / MT8B).

Microbial analysis

Aliquots of 25 g of the harvested leaves were weighed into sterile filter bags, 50 mL of TRIS buffer (0.1 M, pH 5.6) were added, and epiphytic microorganisms from the leaf surface were abraded using a stomacher (Smasher, Chemunex, Bruz, France) at normal speed for 30 s. The suspension was serially diluted with 0.85% NaCl and plated in triplicate on semi-selective medium. The media used and the conditions applied are described in Table 4-1. The results are expressed as log colony-forming units (CFU) per gram fresh weight (FW).

Table 4-1. Semi-selective media and supplements, and incubation conditions used in culture-dependent analysis.

Medium		Supplement	Selective for	Incubation temperature [°C]	Incubation time [h]
0.1 x Tryptic soy agar	Difco 236950	1.2% Bacto agar, Difco 214010	Heterotrophic bacterial plate count	25	72
Crystal-violet neutral-red bile glucose agar	Merck 1.10275.0500	-	*Enterobacteriaceae*	37	18
Lysogenic broth agar	LB, L3022, solidified with 15% BactoAgar, DIFCO 214010	0.2% ampicillin; 100 µg * mL^{-1} arabinose	*E. coli* O157:H7 *gfp+*[1]	37	18
Salmonella Chromogenic agar base	Oxoid CM1007	Salmonella selective supplement, Oxoid SR0194E	*Salmonella* spp.	37	24
Harlequin Listeria chromogenic agar	LAB HAL010	HAL010 Listeria selective diagnostic supplement with cyclohexamide LAB X010; Polymyxin B, ceftazidime LAB X072	*Listeria* spp., *L. monocytogenes*	37	24

[1]Viable counts enumerated under UV-light

Identification of dominant Enterobacteriaceae

After termination of incubation and colony enumeration of violet red bile dextrose agar (VRBD) plates, five solitary colonies were randomly selected from each of the plates and pure-cultured on full-strength tryptic soy agar (TSA) for 24 h at 37 °C to characterize dominant *Enterobacteriaceae* in the phyllosphere. Pure-cultured strains were kept as cryo-cultures in sterile freezing medium (0.05 M K_2HPO_4, 0.001 M KH_2PO_4, 0.002 M trisodium citrate, 0.001 M $MgSO_4$ x $7H_2O$, 12% glycerol) at -80 °C before 16S rDNA sequencing.

Dual culture test

The selected isolates from VRBD were tested for an inhibiting effect on the applied *E. coli* O157:H7 *gfp+* strain *in vitro*. For this purpose, the isolated strains and *E. coli* O157:H7 *gfp+* were propagated overnight on full-strength TSA from cryo-cultures at 37 °C. Single colonies of the isolates and *E. coli* O157:H7 *gfp+* were streaked on another full-strength TSA plate in orthogonal lines and again incubated overnight at 37 °C. Inhibition was expressed by a distance between the target strain and the isolate.

Verification of Listeria spp. and Salmonella

The second run of the greenhouse experiment with rocket was used to specify colonies which were presumptive *Salmonella* spp. or *Listeria* spp. Up to five colonies per replicate showing the characteristics

of one of the genera were randomly picked and transferred to full-strength TSA. After overnight incubation at 37 °C, a single colony was picked and recultured under the same conditions. Pure strains were propagated in 8 mL of full-strength tryptic soy broth (TSB) (37 °C, o/n) and the suspensions were centrifuged at 2500 xg and 4 °C for 20 min. The bacterial pellets were washed in 0.85% NaCl and stored as cryo cultures in 1 mL freezing medium at -80 °C until use for 16s rRNA gene sequencing.

16S DNA sequencing of isolated bacterial cells

DNA extraction of *Enterobacteriaceae* and presumptive *Salmonella* and *Listeria*

Cryo-preserved isolates were recultured on full-strength TSA. After overnight incubation at 37 °C, a single colony was transferred to 5 mL TSB and incubated under the same conditions. The suspension was then centrifuged at 3000 xg and 4 °C for 10 min. The pellet was washed with 5 mL autoclaved 0.85% NaCl through repeated centrifugation and resuspended in 1 mL autoclaved, deionized water and boiled in a water bath for 2-4 min. The suspension was centrifuged at 13.000 xg for 10 min and about 750 µL of the supernatant were transferred to a sterile tube. The extracted DNA was stored in a refrigerator (4 °C) until further use.

For polymerase chain reaction (PCR), the 16S rRNA genes were amplified by applying universal forward and reverse primers ENV1 (5′-AGA GTT TGA TII TGG CTC AG-3′) and ENV2 (5′-CGG ITA CCT TGT TAC GAC TT-3′), respectively. The master mix contained 0.5 µL of each primer, 0.156 µL Taq-polymerase, 2.5 µL Taq-buffer, 0.5 µL deoxyribonucleotide triosphosphate (dNTP), 1.5 µL $MgCl_2$ and 2 µL of the extracted DNA sample. To achieve a final reaction volume of 25 µL, sterile ultrapure water was added. The PCR was initiated at 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 45 s, 72 °C for 2 min and a final annealing temperature of 72 °C for 7 min.

The PCR products were separated on a 1.5% agarose Tris-borate-EDTA-(TBE-) buffer gel. A portion of 2.0 µL of each sample or 2.5 µL of marker (O'GeneRuler 100 bp Plus DNA Ladder, Thermo Scientific, Braunschweig, Germany) was loaded on the gel together with 5.0 µL 6x DNA Loading Dye (Thermo scientific, Braunschweig, Germany). Electrophoresis was run in TBE buffer at 100 V. Amplification of the DNA was confirmed by staining the gel with intercalating dye GelRed (VWR International, Stockholm, Sweden) and visualizing the bands under UV-light. Samples with a confirmed size of 1500 bp were sequenced by Eurofins MWG Operon (Ebersberg, Germany), using primer ENV1. Sequences isolated from VRBD that appeared to be *E. coli* were grown on LB supplemented with arabinose and ampicillin to differentiate between the applied *E. coli* O157:H7 gfp+ and other strains. Isolates growing under ampicillin and showing green fluorescence under arabinose were considered to be the applied strain. Other isolates were tested for the presence of the *eae* gene by applying PCR with *eae* specific primers eae A-F (5'-GAC CCG GCA CAA GCA TAA GC-3') and eae A-R (5'-CCA CCT GCA GCA ACA AGA GG-3') under the following conditions: 95 °C for 3 min, (95 °C for 1 min, 60 °C for 2 min, 72 °C for 1.5 min) x 30, 72 °C for 7 min, 4 °C. Electrophoresis of the amplified DNA was run as described above, with lengths of 384 bp indicating the presence of the *eae* gene. Negative results were confirmed twice.

Risk assessment

The associated risks of the numerical findings of pathogens obtained by culture-dependent analysis were calculated. Models were selected based on the criteria of being simple, parsimonious, and firmly grounded in biological reasoning (Teunis & Havelaar, 2000). A portion size of 30 g was assumed throughout. The *E. coli* O157:H7 risk was estimated using a Beta-Poisson model developed by Teunis *et al.* (2004) and refined by Strachan *et al.* (2005).

Statistics

The experiment was organized as a two-factorial design, with the first factor being two different crops and the second factor different fertilizer applications. The trials were repeated twice, with six independent replicates in each run. Nitrate content in leaves was conducted in run 2 for rocket. The treatments were compared in respect of their nitrogen content and data obtained from culture-dependent analysis by employing one-way ANOVA followed by pair-wise comparison (t-test) using the Tukey method ($\alpha = 0.05$). Minitab version 17 was used.
Sequences of isolated strains were edited using BioEdit (version 7.2.5) to obtain sections with clear signals and therefore reliable sequences. These sections were uploaded on Ribosomal Database, release 11 (RDP, Michigan State University, East Lansing, USA) for phylogenetic comparison with bacterial type strains. Best matches from bacterial strains isolated from VRBD were used to build a phylogenetic tree. All sequences were aligned using ClustralX2 (version 2.1) and bootstrapping was done by employing RAxML (version 8). For tree visualization, TreeGraph (version 2.4.0_456 beta) was utilized.

Results

Nitrogen content in leaves

Total nitrogen content was lowest in leaves that did not receive any fertilizer treatment (rocket: 3.4 mg g^{-1} FW, Swiss chard: 4.1 mg g^{-1} FW), followed by PHP-treated plants (rocket: 5.7 mg g^{-1} FW, Swiss chard: 5.8 mg g^{-1} FW) and CM-treated plants (rocket: 7.8 mg g^{-1} FW, Swiss chard: 6.1 mg g^{-1} FW). Apart from those between PHP- and CM-fertilized Swiss chard, the differences were statistically significant.
Analysis of the nitrate content in rocket leaves, conducted in one run with six replicate samples, showed the same pattern, with mean values of 0.6 mg g^{-1} FW for untreated plants, 4.5 mg g^{-1} FW for PHP-fertilized plants, and 6.9 mg g^{-1} FW for CM-fertilized plants, all differences being statistically significant.

Nitrogen content in growth medium

Nitrogen content in the fertilizers is shown in Table 4-2. Proportions relative to dry matter (DM) content were clearly higher in pig hair pellets than in chicken manure.

Table 4-2. Total nitrogen (N_{tot}), nitrate (NO_3^-) and ammonium (NH_4^+) content in organic fertilizers (pig hair pellets: PHP, chicken manure: CM) applied to rocket and Swiss chard. DM = dry matter.

		Rocket	**Swiss chard**
$\mathbf{N_{tot}}$ [mg 100 g^{-1} DM]	PHP	14267	14300
	CM	3000	2810
$\mathbf{NO_3^-}$ [mg 100g^{-1} DM]	PHP	0.63	0.07
	CM	1.75	0.12
$\mathbf{NH_4^+}$ [mg 100g^{-1} DM]	PHP	152	14
	CM	676	634

Residual content of total nitrogen and nitrate and ammonia in the growth medium for rocket and Swiss chard are shown in Figure 4-1. As for nitrogen content in the leaves, the value was lowest in the control treatments (rocket medium: 498 mg 100g^{-1} DM, Swiss chard medium: 720 mg 100g^{-1} DM), followed by PHP treatment (rocket medium: 593 mg 100g^{-1} DM, Swiss chard medium: 847 mg 100g^{-1} DM) and CM treatment (rocket medium: 720 mg 100g^{-1} DM, Swiss chard medium: 1049 mg 100g^{-1} DM). These differences were statistically significant.

A similar pattern was observed for residual nitrate content. With concentrations of 0.6 and 0.3 mg 100g^{-1} DM, respectively, rocket and Swiss chard control treatments showed the lowest value. Values for PHP-fertilized medium were slightly higher, with 1.6 mg 100g^{-1} DM for rocket and 1.7 mg 100g^{-1} DM for Swiss chard. The highest nitrate accumulation was found in CM-fertilized medium, with 81.3 and 83.7 mg 100g^{-1} DM in rocket and Swiss chard, respectively. Other values between the two runs were on the same level, but total nitrate residual content in rocket growth medium for run 1 (131.8 mg 100g^{-1} DM) was considerably higher than in run 2 (30.9 mg 100g^{-1} DM).

For ammonia, no differences were found in the growth medium of the rocket plots. The highest content was found in CM-fertilized medium (1.4 mg 100g^{-1} DM), followed by PHP-fertilized medium (1.3 mg 100g^{-1} DM) and the control treatment (0.6 mg 100g^{-1} DM). In contrast, significant differences were found for the residual ammonia content in the growth medium of the treatments with Swiss chard in PHP-fertilized plots (1.7 mg 100g^{-1} DM) and a lower residual content in medium supplemented with CM (1.1 mg 100g^{-1} DM). The lowest residual ammonia content was again noted in the control treatment (0.4 mg 100g^{-1} DM).

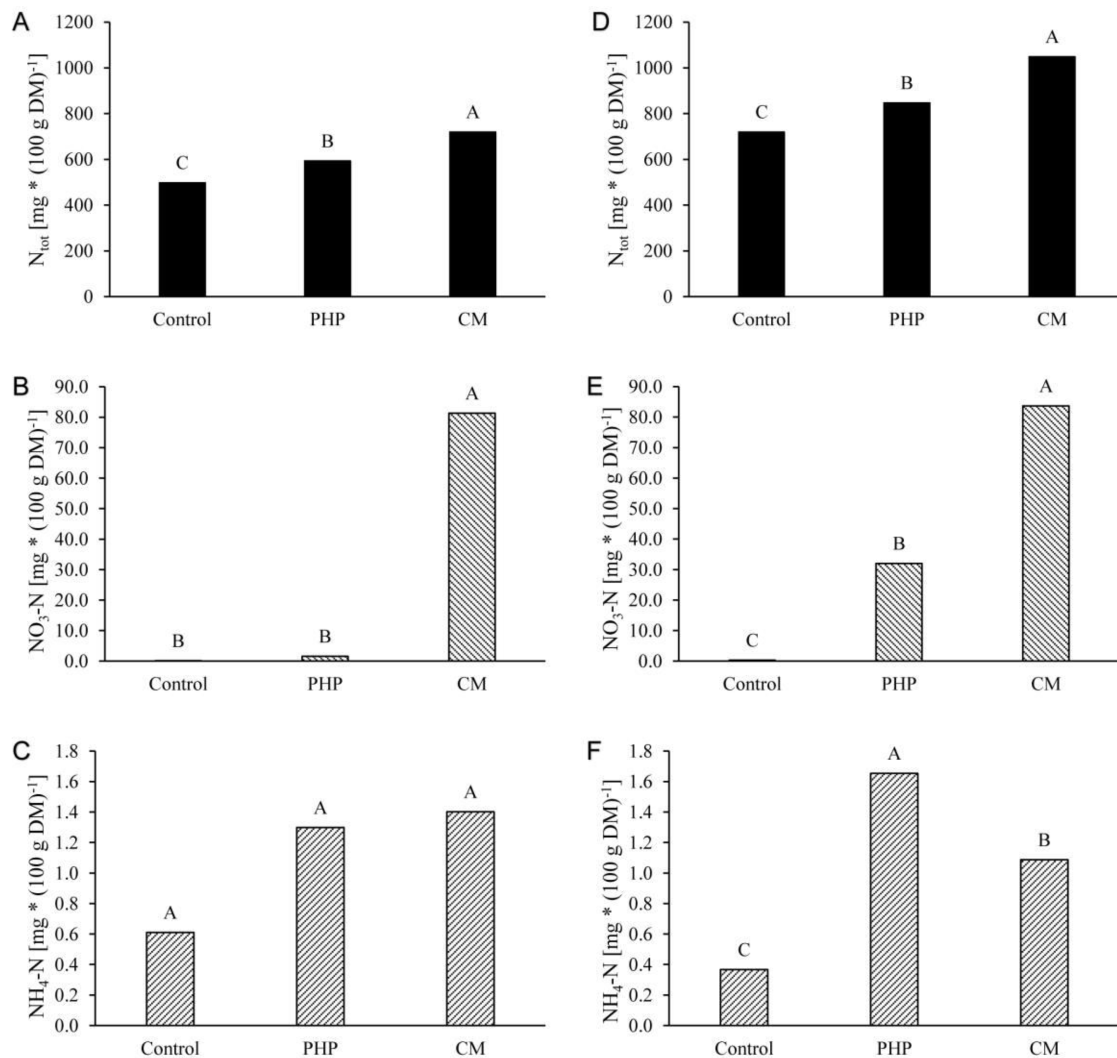

Figure 4-1. Comparison of total nitrogen content (N_{tot}), nitrate (NO_3-N) and ammonia (NH_4-N) on the day of harvest in growth medium of rocket baby leaves (A, B, C) and Swiss chard baby leaves (D, E, F) for different organic fertilizer treatments. Growth medium was supplemented with pig hair pellets (PHP) or chicken manure (CM) to an amount of 81 kg readily available N ha^{-1}, while the control did not receive any fertilizer. Bars within diagrams marked with different letters are significantly different according to ANOVA (Tukey-test, $p<0.05$).

Comparisons of growth medium for the two crops revealed significantly higher total nitrogen residual contents for Swiss chard than rocket. For nitrate the mean was also higher for Swiss chard, while for ammonia it was slightly higher for rocket. These differences were not significant.

Microbial colonization

Numbers of heterotrophic bacterial plate count, *E. coli* O157:H7 *gfp*+, and *Enterobacteriaceae* as obtained from culture-dependent methods are displayed in Figure 4-2. The average load of the heterotrophic bacterial plate count was highest on leaves from plants fertilized with PHP (5.1 log CFU g^{-1}), followed by plants treated with CM (4.9 log CFU g^{-1}), and unfertilized plants (4.7 log CFU g^{-1}). For Swiss chard, the heterotrophic bacterial plate count was significantly higher for the control treatment (7.0 log CFU g^{-1}) and PHP-fertilized plants (6.9 Log CFU g^{-1}) than for CM-fertilized plants (6.4 log

CFU g^{-1}). For *E. coli* O157:H7 *gfp+*, no statistically significant differences were found between fertilizer treatments in either crop. However, PHP treatments resulted in the highest colonization rate on leaves of rocket (3.9 log CFU g^{-1}) and Swiss chard (5.4 log CFU g^{-1}). On unfertilized and CM-fertilized rocket leaves, loads of *E. coli* O157:H7 *gfp+* averaged 3.4 log CFU g^{-1}. Swiss chard leaves fertilized with CM showed an average value of 5.2 log CFU g^{-1} and unfertilized leaves 5.1 log CFU g^{-1}. The loads of *Enterobacteriaceae* matched the colonization with *E. coli* O157:H7 *gfp+*, with CM-fertilized plants again showing the highest loads (3.6 and 6.0 log CFU g^{-1} for rocket and Swiss chard, respectively). On rocket leaves, values of 3.5 log CFU g^{-1} and 3.4 log CFU g^{-1} were obtained for CM-treated plants and control plants, respectively. Loads averaged 5.8 log CFU g^{-1} on CM-treated leaves and 5.9 log CFU g^{-1} on untreated leaves for Swiss chard. In general, Swiss chard harbored significantly higher bacterial loads in growth medium than rocket.

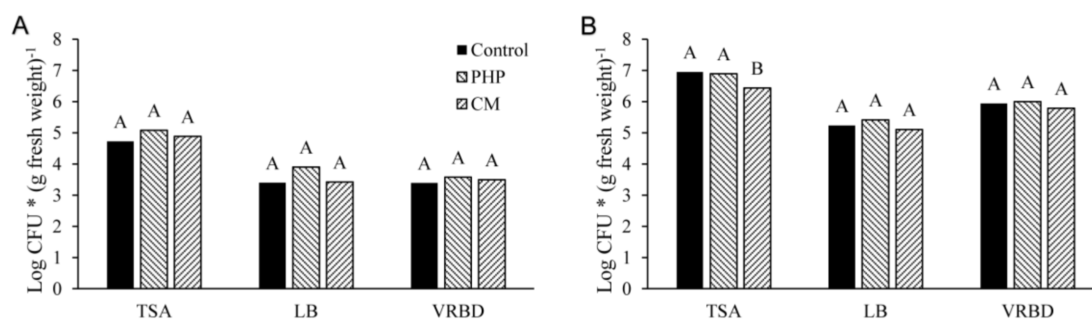

Figure 4-2. Comparison of bacterial loads (TSA: Heterotrophic bacterial plate count, LB: *E. coli* O157:H7 gfp+, VRBD: *Enterobacteriaceae*) on rocket baby leaves (A) and Swiss chard baby leaves (B) for different organic fertilizer treatments. Plants received pig hair pellets (PHP) or chicken manure (CM) to an amount of 81 kg readily available N ha^{-1}, while the control did not receive any fertilizer. Bars for each medium with different letters are significantly different according to ANOVA (Tukey-test, $p<0.05$).

Bacteria showing matching characteristics for *Salmonella* spp. and *Listeria* spp. were identified on the chromogenic nutrient agar. None of the colonies showed corresponding characteristics for *L. monocytogenes*. For *Salmonella* spp., the numbers ranged between the lower detection limit and 4 log CFU g^{-1} on rocket leaves and between 1 and 5 log CFU g^{-1} on Swiss chard leaves. Further identification by sequencing revealed the colonies not to be *Salmonella*, but belonging to the genus *Pseudomonas*. *Listeria* spp. also ranged from below the lower detection limit to 4 log CFU g^{-1} on rocket leaves. For Swiss chard, loads between 3.5 and 5.5 log CFU g^{-1} were found. Further identification suggested that the colonies belong to the genera *Microbacterium*, *Bacillus* and *Paenibacillus*, but not *Listeria*.

Characterization of bacteria grown on media selective for Enterobacteriaceae

Further identification of colonies showing specific characteristics for *Enterobacteriaceae* on VRBD was conducted and phylogenetic trees were drawn for the two crops (Figures 4-3 and 4-4). Considerable

differences were found between the crops. Strains isolated from Swiss chard leaves predominantly comprised bacteria belonging to the *Enterobacteriaceae*, while strains obtained from rocket leaves were affiliated with *Pseudomonas*, which was not the target phylogenetic family.

A phylogenetic tree containing 27 isolates, with length 345 to 507 bp, obtained from wild rocket leaves is shown in Figure 4-3. A majority of 15 colonies isolated from VRBD grouped together with *Enterobacter* species, at which six were obtained from plants fertilized with PHP and nine from plants fertilized with CM. Another isolate recovered from PHP-fertilized plants showed similarity to *Lelliottia*, completing the branch of *Enterobacteriaceae*. A second major group of isolates matched with different type strains belonging to the genus *Pseudomonas*. This group included all four isolates from unfertilized plants, as well as two and one from PHP- and CM-fertilized plants, respectively. Another cluster containing type strains of *Pseudomonas hibiscicola* and *Stenotrophomonas maltophilia* grouped with an isolate from a CM-fertilized plant. Three isolates clustered with *Acinetobacter*, one originating from CM-fertilized and one from PHP-fertilized plants.

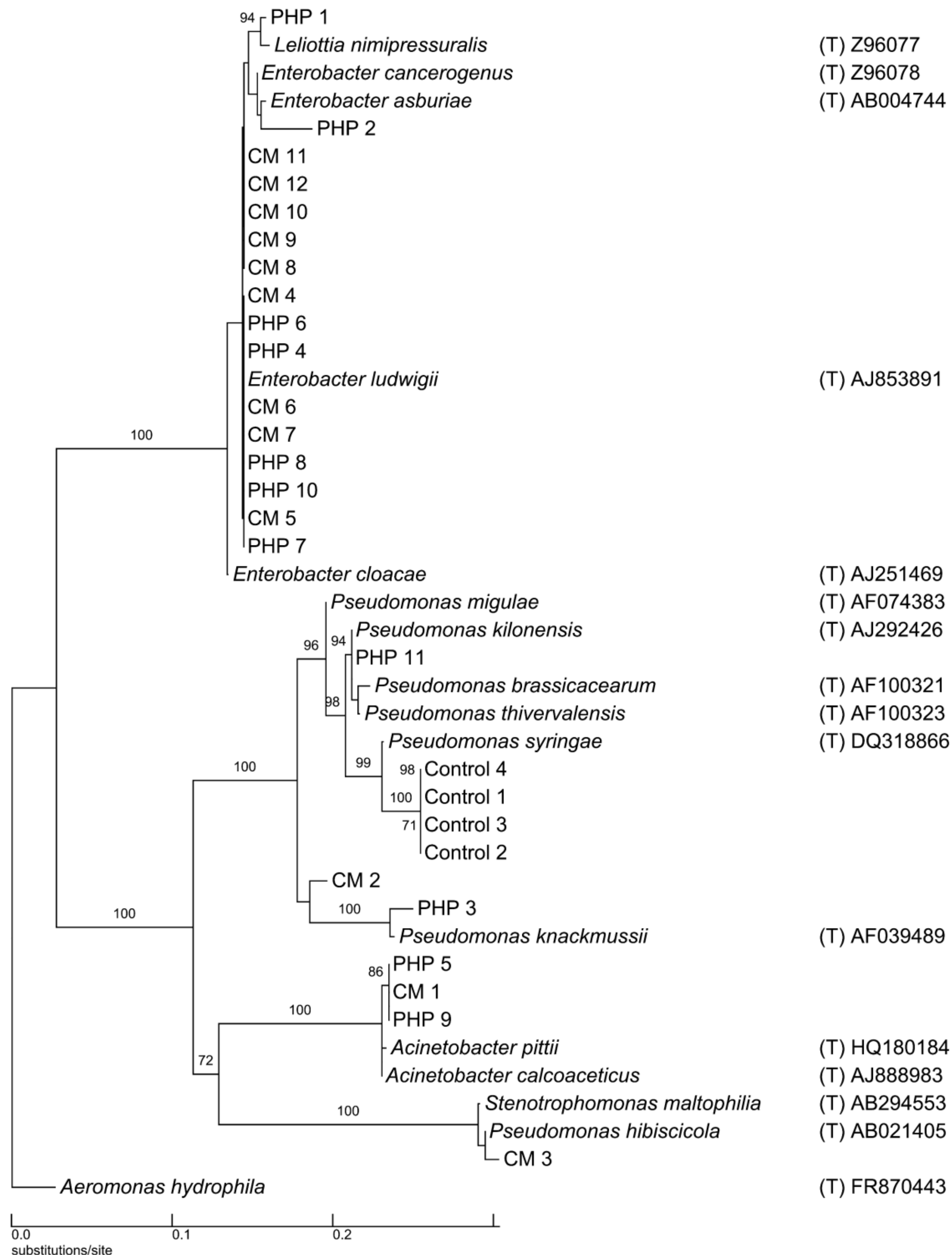

Figure 4-3. Phylogenetic tree based on analysis of 16S rRNA gene sequences retrieved from isolates from the leaf surface of rocket cultured in the greenhouse and fertilized with chicken manure (CM) or pig hair pellets (PHP). Control treatments did not receive any fertilizer. The isolates were collected from nutrient agar for selective for *Enterobacteriaceae* (violet red bile dextrose agar).

With respect to Swiss chard, all but one of the colonies isolated matched with type strains belonging to the family *Enterobacteriaceae*. A phylogenetic tree including 29 isolates with length 353 to 541 bp shows the different groupings (Figure 4-4). Two major groups, containing *Pantoea* or *Enterobacter* species, respectively, were defined. Seven, two, and five isolates extracted from unfertilized plants,

PHP-treated, and CM-treated plants were represented in the *Pantoea* cluster. The *Enterobacter* cluster comprised three isolates from unfertilized and nine from CM-fertilized plants. One colony isolated from a plant fertilized with CM clustered together with *Citrobacter*. Another single colony, isolated from an unfertilized plant, showed sequence patterns similar to *E. coli*, but could not be grown on supplemented LB and was *eae* gene negative. An isolate obtained from an unfertilized plant grouped with *Achromobacter spanius*, a species belonging to the family *Alcaligenceae*.

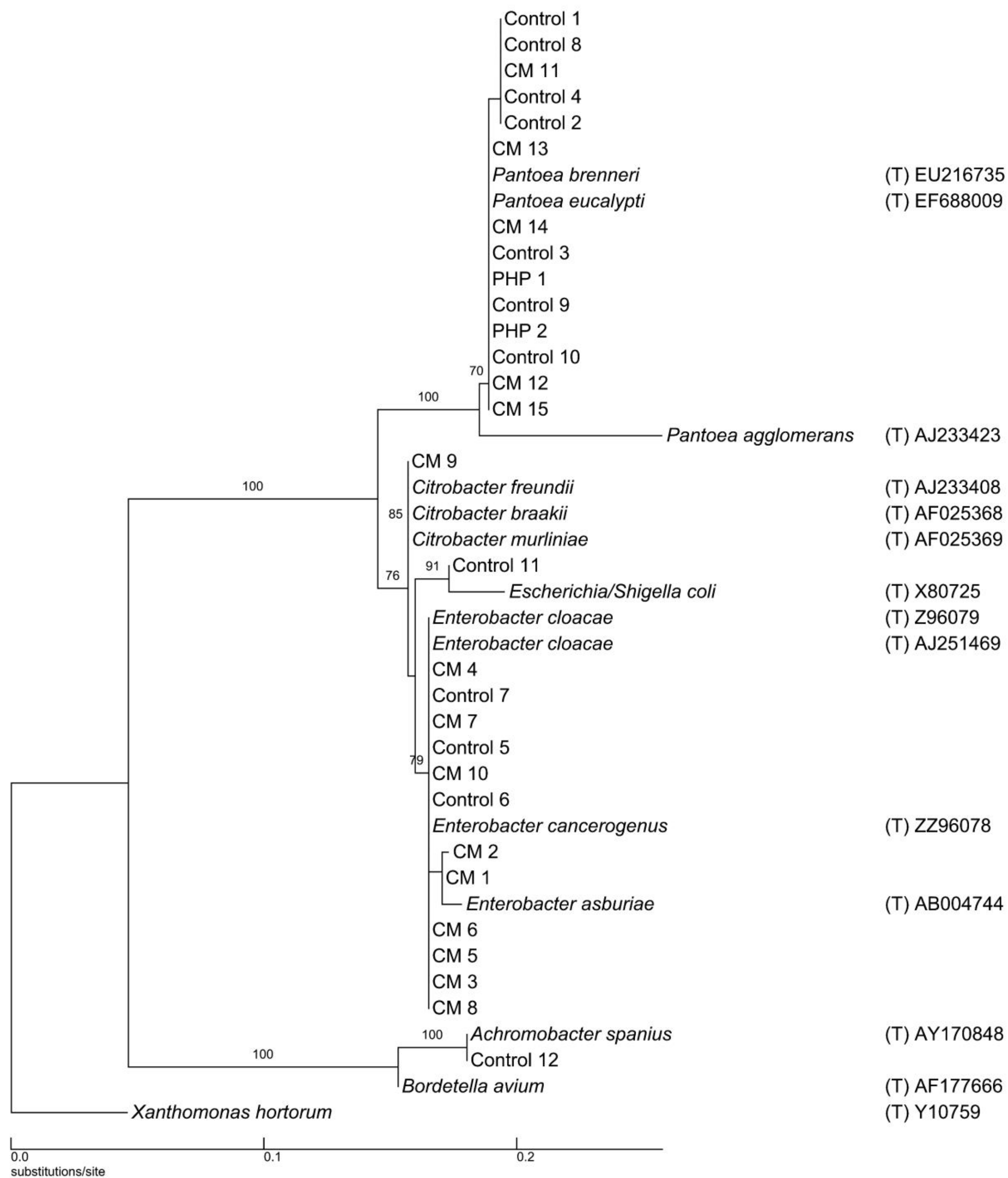

Figure 4-4. Phylogenetic tree based on analysis of 16S rRNA gene sequences retrieved from isolates from the leaf surface of Swiss chard cultured in the greenhouse and fertilized with chicken manure (CM) or pig hair pellets (PHP). Control treatments did not receive any fertilizer. The isolates were collected from nutrient agar selective for *Enterobacteriaceae* (violet red bile dextrose agar).

No direct inhibition of the isolated *Enterobacteriaceae* strains from rocket and Swiss chard leaves on the applied *E. coli* O157:H7 *gfp*+ was detected *in vitro*.

Interactions between nitrogen content in leaves and microbial colonization

Principal component analysis including culture-dependent and leaf-nitrogen assessments separated between the two crops rocket and Swiss chard (principal component (PC) 1), while PC 2 discriminated the different fertilizer regimes (Figure 4-5). PC 1 was mainly driven by the crop and the bacterial loads on the leaves, while the treatment and the total nitrogen content in leaves were decisive for PC 2.

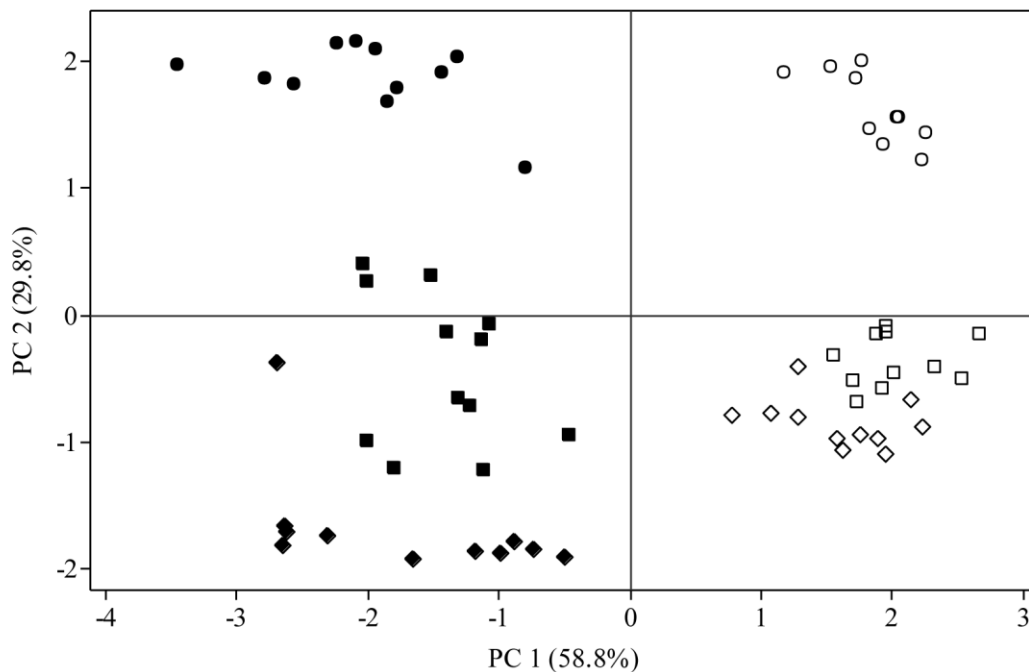

Figure 4-5. Principal component (PC) analysis based on nitrogen content on the day of harvest in leaves and microbial loads on leaves (determined by culture-dependent methods for heterotrophic bacterial plate count, *E. coli* O157:H7 gfp+, *Enterobacteriaceae*, presumptive *Salmonella* spp., and presumptive *Listeria* spp.) of baby leaf rocket (filled symbols) and Swiss chard (open symbols) exposed to different fertilizer regimes: ○ = control, □ = pig hair pellets, ◊ = chicken manure).

Correlations between the bacterial groups analyzed were strong and highly significant ($p<0.001$). For the heterotrophic bacterial plate count and *Enterobacteriaceae*, Pearson correlation coefficient (r) was 0.800 and for heterotrophic bacterial plate count and *E. coli* O157:H7 *gfp*+ it was 0.749. Between *Enterobacteriaceae* and *E. coli* O157:H7 *gfp*+ the highest correlation had a Pearson correlation coefficient of 0.930.

Correlations between nitrogen and nitrate contents in rocket leaves were also pronounced, with a Pearson correlation coefficient of 0.957 ($p<0.001$).

No interactions between nitrogen content in leaves and bacterial colonization were found (Figure 4-6).

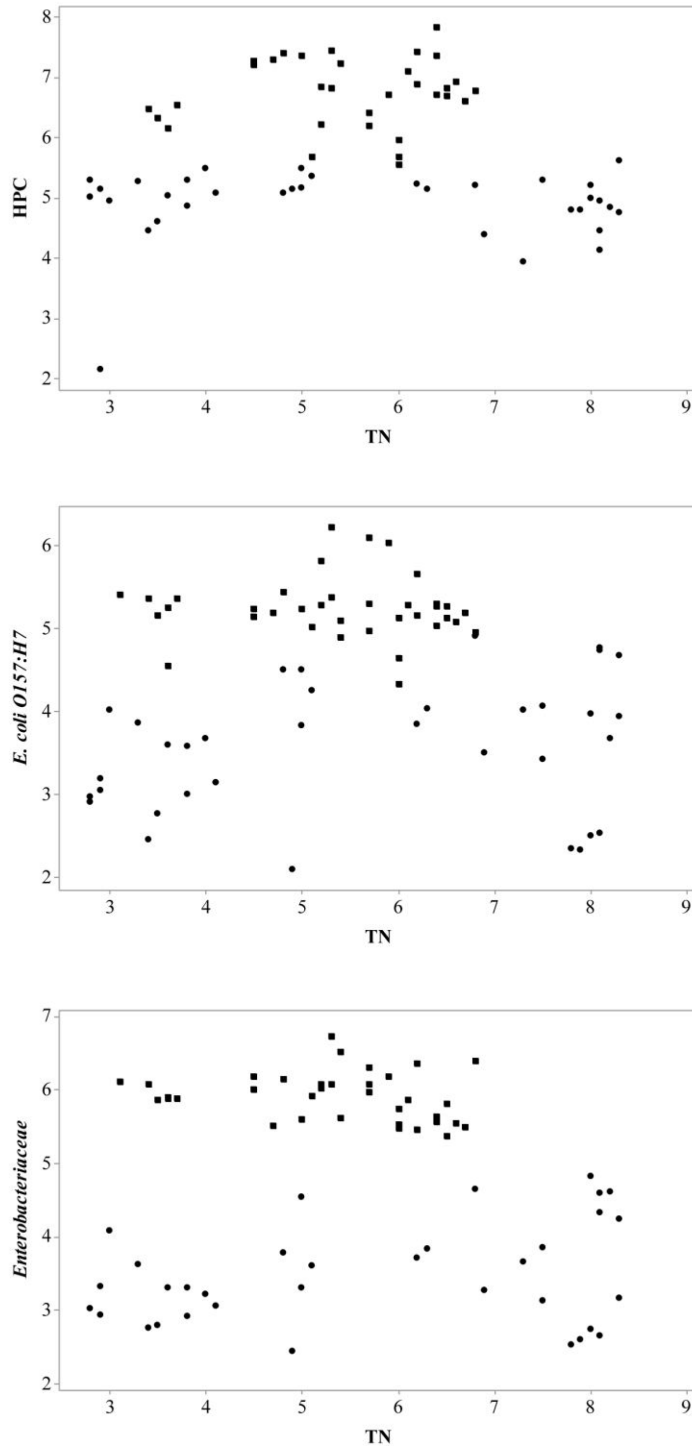

Figure 4-6. Scatterplots for total nitrogen (TN) content in Swiss chard (■) and rocket (●) leaves [mg g^{-1} fresh weight (FW)] and epiphytic bacteria [log CFU g^{-1} FW]. Plants were spray-inoculated with *E. coli* O157:H7. HPC: heterotrophic bacterial plate count.

Risk assessment

The results of the risk assessment are shown in Table 4-3. There were clear differences in bacterial numbers and associated risks between rocket and Swiss chard. For five out of the six experiments (plant

species-fertilizer), the numbers on leaves fertilized with PHP were higher, but not dramatically higher. The risk of infection was slightly different.

Table 4-3. Risk measured as probability of infection with *E. coli* O157:H7 assuming a 30 g portion of rocket and Swiss chard is consumed. Crops were fertilized with pig hair pellets (PHP) or chicken manure (CM), or left untreated (control). Leaves were spray-inoculated with *E. coli* O157:H7. Risks are given as averages.

	Risk (probability of infection) per portion 30 g salad	
	Rocket	**Swiss chard**
Control	0.60	1
PHP	0.63	1
CM	0.60	1

Discussion

The use of non-sanitized manure as fertilizer can carry a high risk of microbial contamination of fresh produce, while animal products that are heat-treated before application are assumed to carry a lower risk (Möller & Schultheiss, 2014). However, the risk assessment conducted within this study showed that not only the carrier risk, but also the interactions on other levels, must be considered in food hazard analysis. The numbers of *E. coli* O157:H7 bacteria were very high and, given that the dose response curves were shifted leftwards, no dramatic differences in risk were observed. However, in the case of lower numbers of *E. coli* O157:H7 inoculated or naturally contaminating the leaves, even these small differences could have an impact. Repeating this experiment using lower numbers of *E. coli* O157:H7 bacteria contaminating the vegetable leaves could be interesting. To our knowledge, this is the first study to address the risk posed by fertilizers of animal origin other than acting as a potential carrier of human pathogens. The study also analyzed the impact of manure as a nitrogen source on establishment of *E. coli* O157:H7 in the phyllosphere.

Nitrogen is the limiting factor for biomass gain in crops (Lawlor *et al.*, 2001). Nitrogen is taken up by plants as nitrate or ammonia, the former dominating. Fertilizer application was calculated based on readily available nitrogen and mineralization rate and thus the initial proportions of ammonia and nitrate present in the fertilizers did not correspond to the proportions found in the growth medium at harvest. This might be due to slow mineralization, or to promotion of root growth and nitrogen fixation in root biomass. Separate analysis of the root biomass and the residual growth medium in further studies can provide insights into the differences in the fertilizers used. As the cropping units in the greenhouse were non-leaching containers and water was supplied upon demand, the higher amounts of nitrate in the fertilizers than in growth medium and roots point to high uptake and metabolism of this easily available

nutrient. Differences in the time of mineralization lead to varying amounts of readily available nitrogen for the crops at a specific growth stage, even if the sum of supplied nitrogen in total is comparable (Laber, 2013). As the requirement for nitrogen varies during the course of plant development (Feller *et al.*, 2001), the supply might be (sub)optimal depending on the fertilizer used. Point sample analyses of the available nitrogen content at the point of harvest revealed differences in the supply, indicating unequal conditions for the plants depending on the treatment. Differences between rocket and Swiss chard can be a consequence of different nutrient requirements and/or different cultivation periods. In addition, El-Sharkawi (2012) found that the performance of microbial nitrogen turnover, and thus the nitrogen availability and uptake by plants (rice), was influenced by the applied nitrogen source in growth medium. These factors can explain the differences found in leaf nitrogen and nitrate content in the present study.

The nitrate incorporated by the plants has to be reduced to ammonia before it is converted to glutamate and aspartate (Lam *et al.*, 1996). These amino acids are the precursors for other amino acids and thus for the metabolism of essential proteins, including enzymes, hormones, and chlorophyll (Snoeijers *et al.*, 2000). High uptake of nitrate results in accumulation in leaves (Yusof *et al.*, 2016), which is also reflected in the leaf exudates (1-2 µg N g^{-1} lettuce leaf) on which the phyllosphere microbiota feed (Brandl & Amundson, 2008). In the plant environment, the abundance of nutrients is the limiting factor for bacterial growth. Depending on the C/N ratio in leaf exudates, the microbial community can be either nitrogen- or carbon-deprived (Wilson & Lindow, 1994). Research conducted by Brandl and Amundson (2008) suggests that nitrogen availability defines the establishment of *E. coli* O157:H7 on (young) lettuce leaves. In this study, significant differences in nitrogen and nitrate contents of the leaves were not found for *E. coli* O157:H7 gfp+ colonization of the leaves, indicating that nitrogen was not the limiting factor for establishment.

Beside nitrogen mineralization, the fertilizers used may also have other impacts on plant growth due to other nutrients present. Chicken manure is rich in nitrogen and phosphorus, while PHP is high in nitrogen and sulphur, but low in potassium and phosphorus (Möller & Schultheiß, 2014). These differences might not only affect plant development, but also influence microbial colonization in the phyllosphere. Alsanius *et al.* (2017c) found that the content of macro- and micronutrients (e.g., P, K, Mg, Ca, S, Na, Mn, Fe, Cu, Zn) in the leaves of baby rocket and Swiss chard had an impact on colonization by artificially introduced *E. coli* O157:H7.

Beside nutrient abundance, the indigenous microbiota can have an impact on the establishment of immigrant microorganisms in the phyllosphere (Rastogi *et al.*, 2013; Lopez-Velasco *et al.*, 2011). These interactions can be either promotive, inhibitory, or indifferent. Commensalism between microorganisms on the leaf surface can be expressed, (i) in making nutrients available through the degradation of plant tissue or an available source and (ii) by altering the microenvironment, making it more suitable (Deering *et al.*, 2012; Cooley *et al.*, 2006). Negative effects have been well-studied for biocontrol agents and include competition for nutrients and/or space, siderophore production, and antibiosis (Whipps, 2001).

Studies investigating the effect of strains inhabiting leafy vegetables on *E. coli* O157:H7 have found evidence of interactions for several strains with the human pathogenic bacterium. For example, Cooley *et al.* (2006) found that *Wausteria paucula* promotes survival of *E. coli* O157:H7 on lettuce seedlings, while *Enterobacter asburiae* inhibits its establishment. *Enterobacter cloaceae* also had a negative effect on *E. coli* O157:H7 when co-inoculated on lettuce seeds (Jablasone *et al.*, 2005). Lopez-Velasco *et al.* (2012) analyzed the phyllosphere of spinach and found bacteria belonging to the genus *Enterobacteriaceae* with an inhibiting effect on *E. coli* O157:H7, e.g., *Erwinia perscina* and *Pantoea agglomerans*, while species belonging to *Pseudomonas* spp. and *Bacillus* spp. also had a negative effect. In the present study, some of the strains isolated from a culture medium selective for *Enterobacteriaceae* showed high conformity with bacteria that have been shown to inhibit *E. coli* O157:H7 growth. However, this could not be confirmed by our analysis *in vitro*, for different reasons. For example, nutrient agar does not reflect conditions on plants, e.g., by providing more space and nutrients. Thus, the absence of *in vitro* effects does not necessarily mean that there is no competition in the phyllosphere. Moreover, only the 16S rRNA gene, and not the whole genome, was studied, so assignment to individual species is associated with high uncertainty and the strains identified might lack significant genes that play a role in the interaction.

Various studies have provided evidence that human pathogenic bacteria are able to colonize edible parts of leafy vegetables when introduced to/with the growth medium. Islam et al. (2004a; 2004b) showed that *E. coli* O157:H7 and *S. enterica* serovar Typhimurium are able to persist in soil when inoculated together with manure before planting. The potentially pathogenic bacteria in that study were transmitted from the contaminated growth medium to leaves of lettuce and parsley, and persisted in the phyllosphere for more than two months. It has also been shown that *L. monocytogenes* can invade alfalfa crops when inoculated into the soil, with sewage sludge being the vector (Al-Ghazali & Al-Azawi, 1990). In agreement with these results, we found when employing culture-dependent methods that *Listeria* spp., but not *L. monocytogenes*, and *Salmonella* spp., colonized baby leaves irrespective of treatment. However, further identification revealed the strains not to be the presumed bacteria, illustrating the need to confirm culture-dependent results. This is particularly the case if bacteria are isolated from a harsh environment, where conditions might force them to express atypical patterns to survive. The identification of an isolate from growth medium selective for *Enterobacteriaceae* that appeared to be *E. coli* (but not the applied strain) shows the potential of food pathogens to colonize leafy greens, even if not artificially introduced. Contrary to our hypothesis, the bacterium was found on leaves of plants that were not treated with organic fertilizer (control treatment).

Conclusion

It should be borne in mind that this study required the use of animal waste products, in which the composition of ingredients may differ depending on the batch. Furthermore, other substances that may also affect the target factor cannot be controlled. Thus, a limiting approach in comparing and interpreting

results is needed when organic fertilizers are considered. The organic fertilizers applied resulted in different residual nitrogen content in the growth medium at harvest and different nitrogen content in the leaves. However, establishment of *E. coli* O157:H7 *gfp+* on leaves was not affected by the treatment according to ANOVA. Similarly, other studies have found that a high risk treatment does not enhance the colonization of leafy greens with potentially human pathogenic bacteria (Hartmann *et al.*, submitted). Nevertheless, our risk assessment suggested that the treatments tested affected the infection dose of *E. coli* O157:H7 on baby leaves, and that the impact of human pathogen establishment as determined by the nitrogen source needs further attention. Treatments were grouped in principal component analysis according to the leaf parameters assessed, while there was also a clear separation between rocket and Swiss chard. Differences in the indigenous microbiota, here assessed for *Enterobacteriaceae*, can have an impact on the establishment of human pathogens in the phyllosphere. As found for establishment of *E. coli* O157:H7 in the phyllosphere, differences in naturally abundant *Enterobacteriaceae* were more pronounced between crops than between treatments. None of the presumptive *Salmonella* spp. or *Listeria* spp. analyzed was confirmed and thus results obtained with culture-dependent methods should be regarded with caution.

Acknowledgements

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Chapter 5: Impact of organic fertilizers on the microbial phyllosphere structure and prevalence of *E. coli* O157:H7 *gfp*+ in rocket (*Diplotaxis tenuifolia*) and Swiss chard (*Beta vulgaris* cicla)

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Highlights

- A low risk organic fertilizer does not prevent extensive establishment of *E. coli* O157:H7 *gfp+*
- The crop and nutrient regime decide on the prevalence of *E. coli* O157:H7 *gfp+*
- The crop rather than organic fertilizer regime dictates the bacterial phyllosphere community structure
- Leaf nutrient content and plant physiological parameters corroborated with the microbial phyllosphere community affect the occurrence of *E. coli* O157:H7 *gfp+*

Abstract

To mitigate the dispersal of human pathogens in organically grown ready-to-eat vegetables, low risk strategies are often recommended. Also, a negative impact of microbial biodiversity on the survival of human pathogens has been postulated. In the present study, we used the low-high risk scenario approach investigated the survival of the spray-inoculated *gfp*-tagged *E. coli* O157:H7 in non-fertilized (control), pig hair pellet (PHP, low risk scenario) and raw chicken manure (CM, high risk scenario) fertilized rocket and Swiss chard under greenhouse conditions. Two independent experiments with six replicates in each were conducted. Interactions between the occurrence of the inoculated strain as well as heterotrophic bacterial plate counts (HPC), *Enterobacteriaceae* and culturable fungi (viable counts, log CFU g^{-1}) was related to plant physiological and agronomic parameters, especially leaf nutrient content as well as bacterial phyllosphere biome as assessed using Illumina Mi-Seq. The nutrient treatments affected biomass production and leaf size in both plant species, with highest biomass and leaf area in

CM amended plants. Principal component analysis on the basis on viable counts, physiological and agronomic factors separated between the two experiments and fertilization strategies. Plant species dictated the occurrence of inoculated strain as well as the bacterial relative abundance. Total species richness (Chao1) deviated between the plant species but not between the treatments. Magnesium, manganese and iron explained 70.4% ($p<0.001$) of the variance in *E. coli* O157:H7 *gfp+* when leaf nutrient contents of both crops were considered. Agronomic and plant physiological factors predicting viable counts of *E. coli* O157:H7 *gfp+* differed between the two crops. Manganese and for rocket also iron and boron correlated to the relative abundance of decisive bacterial families.

Keywords: food safety, greenhouse, leaf nutrient content, leafy vegetables, metagenomics, viable count

Introduction

During recent years, outbreaks of foodborne diseases have increasingly been reported from fruits and vegetables (Warriner & Namvar, 2010). Especially commodities consumed raw without heating are at risk (Yaron, 2014). In this context, organic farming systems display at least theoretically higher risks as the boundary between animal husbandry and production of plant food is closer than in conventional/integrated cropping systems. Domestic animals may play an active role within the cropping system (Dorais & Alsanius, 2015; Möller & Schultheiss, 2014; Pell, 1997); furthermore, animal waste products, such as manure and slaughter house waste products are used as sources for maintenance and management of soil fertility (Xie *et al.*, 2014; Beuchat, 2006).
Common sources of transmission of foodborne pathogens during primary production are irrigation water, organic manure, domestic and wild animals, management practices as well as cross contamination by humans during management, harvest and within the processing line (Pachepsky *et al.*, 2011; Beuchat, 2006; Brackett, 1999). To minimize food hazards, the transmission cycle needs to be interrupted (Nicholson *et al.*, 2005). For organic production of vegetables, it is recommended to use composted manure (Shepherd *et al.*, 2007) and to keep an interval of six months between the date of manure application and sowing (Köpke *et al.*, 2007). Also, the high risk organic fertilizers may be replaced by low risk ones, e.g. feather or hair meal pellets, vinasse (Möller & Schultheiss, 2014). Composting is often claimed to be sufficient to kill human pathogens in compost if the process is conducted properly. But the kind of organism and its tolerance to temperature of both vegetative and survival structures must be considered (Termorshuizen & Alsanius, 2016; Franke-Whittle & Insam, 2013). Organic low risk fertilizers are often exposed to conditions during processing (i.e. heat, high pressure, hydrolysis) that inactivate potentially hazardous organisms. However, administration of organic low risk fertilizers is not a warranty for hazard avoidance, as foodborne pathogens may be imported by other routes of transmission. In the present study, we investigated the effect of organic low and high risk fertilizers on the survival/occurrence of *E. coli* O157:H7 *gfp+* on two leafy vegetable crops (rocket, Swiss chard) and the community structure of different fertilizing regimes under greenhouse conditions. We hypothesized that a high risk fertilization scenario promotes the occurrence of *E. coli* O157:H7 *gfp+* when introduced through a secondary route of transmission.

Material and methods

Inoculum preparation

The *E. coli* O157:H7 strain used in the experiments was a biochemical strain (verotox-1 and -2 negative, eae-positive, obtained from the Swedish Institute for Communicable Disease Control, Solna Sweden, registry no. E81186), which was *gfp*-tagged. The *gfp*-tagged strain was induced to fluoresce when grown on lysogenic broth (LB, L3022-1kg, Sigma, Stockholm, Sweden) broth or agar supplemented with 100 µg ml^{-1} ampicillin and 0.1% L-arabinose. The strain was precultured from a cryo-culture (stored at -80 °C) on LB agar solidified with 1.5% Bacto agar (DIFCO 214010, DeMoines, USA) for 18 h at 37 °C.

Single colonies were transferred to sterile Erlenmeyer flasks containing 30 ml LB broth and shaken at 180 rpm (Minispin rotary shaker, VWR International AB, Stockholm, Sweden) at 37°C (Friocell™ FC III incubator, MMM group GmbH, München, Germany) for 18 h. Cells were harvested by centrifugation (Avanti™ J-20 centrifuge, Beckman-Coulter Corporation, Brea CA, USA, and Minispin™ microfuge, Eppendorf GmbH, Hamburg, Germany) at 3000 rpm and 4 °C for 45 min. Pelleted cells were washed once with sterile NaCl (0.85%) and cell density was adjusted to an optical density at 620 nm (OD_{620}) of 1.0, corresponding to log 9.7 CFU ml^{-1} (Expert 96™ spectrophotometer, AsysHiTech, Eugendorf, Austria). For preparation of the plant sprays, stock solutions with a final density of 10^8 CFU ml^{-1} were prepared using 0.85% NaCl. The inoculation solutions for the plant sprays were prepared in triplicates by transferring aliquots of 20 ml of the stock solution to 180 ml 0.085% NaCl which results in a final density of 10^6 CFU ml^{-1} corresponding to Log 2.9 CFU ml^{-1} when added as the usual irrigation dose of 5 mm.

Greenhouse experiment

To mimic treatments implying low and high food hazards, pig hair pellets (PHP) and fresh chicken manure (CM) were used as organic nitrogen fertilizers. Doses of nitrogen supplied by the organic fertilizers were calculated on the basis of average nitrogen content (PHP: 14 %; CM: 1 %) and mineralization rate (PHP: 50 %; CM: 30 %) and were then incorporated into peat-based growing medium (K-jord, Hasselfors Garden AB, Örebro, Sweden). This conditioned commercial growing medium contains 1600 mg/l total N and has been fertilized with 140 g N/m^3 substrate. Plain K-jord was used for the untreated control and no additional fertilizers were added. Metalaxyl-M/Thiram/Thiophanate-methyl baited (Seminis, Oxnard CA, USA) wild rocket (*Diplotaxis tenuifolia* L. cv. Tricia; Enza) and Swiss chard (*Beta vulgaris cicla* cv. SCR 107; AvanSeed) seeds were sown at a density of 400 (wild rocket) and 500 (Swiss chard) seeds per tray in trays (0.52 m x 0.42 m x 0.09 m) filled with two layers of growing medium (a 4.5 cm bottom layer of the fertilized peat-based growing medium (K-jord, Hasselfors Garden AB, Örebro, Sweden), and a 1.5 cm top layer of peat-based growing medium (S-jord; Hasselfors Garden AB, Örebro, Sweden). The trays were placed in the experimental greenhouse at the Department of Biosystems and Technology, SLU, Sweden. The temperature set-point during the entire growing period was 21 °C and relative humidity was kept at 70%. Plants were harvested at harvest stage BBCH code 49 for rocket and at stage BBCH code 38 for Swiss chard (in practice after 35-42 days) when they had reached marketable size.

Inoculation with *E. coli* O157:H7 *gfp+* was conducted when the first true leaves had reached a leaf length of 2 cm until three days before harvest. The treatment was repeated every three days, resulting in three and five treatments for Swiss chard and rocket, respectively. All inoculations were performed in a greenhouse inoculation chamber, approved for experiments with genetically modified microorganisms (REK 2011/1072; ID202100-2817v28), maintaining the same environmental conditions. Each of the trays were top-irrigated (5 L m^{-2}) and inoculated with 25 ml of the *E. coli* suspensions.

Plants were harvested tray-wise 1.5 cm above the growing medium 72 h after the last inoculation, with six independent replicates per treatment and experiment, transferred to sterile plastic bags and immediately brought to the laboratory for analysis.

Analysis

Plant parameters

Four days before harvest, leaf temperature and chlorophyll fluorescence were measured. Leaf temperature measurement (Flir IX, Flir systems inc., Wilsonville OR, USA) was done traywise at 07^{00} before the plants were exposed to direct sunlight. Scanned data were separated into minimum, maximum and average temperature per tray. For chlorophyll fluorescence, cuvettes were fixed on one representative leaf per tray and the target leaf area exposed to darkness for 20 min before measurement of the minimum chlorophyll fluorescence (F_0). The maximum chlorophyll fluorescence (F_m) was measured after a saturating pulse (7800 µmol m^{-2} s^{-1} PPFD; Red LED light, peak 630 nm) (PAM-2500 fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The maximum quantum yield of PSII (F_v/F_m) was calculated as $((F_m - F_0)/F_m)$.
Total yield per tray was monitored directly after harvest. Twenty randomly selected leaves were selected for leaf area (LI-3100 Area meter, LI-COR Inc., Lincoln, USA) and fresh as well as dry weight assessment. Dry weight was measured after lyophilization. Dry matter content was calculated. Leaves (100 g fresh weight) used for determination of nutrient content were dried in a drying oven at 70 °C. Leaf quality was evaluated with respect to nutrient content in the dry matter (total nitrogen, TN; P; K; Mg; Ca; Na; S; Mn; Fe; Cu; Zn; B). Nutrient analysis was conducted by Eurofins, Kristianstad, Sweden.

Microbial colonization

The microbiota associated with the canopy was analyzed by culture-dependent (viable count) and culture-independent methods (Illumina Mi-Seq).

Culture dependent methods:

For viable count assessment, 25 g of leaves per tray were filled into filter bags (Separator 400, 180mm*300 mm*70 µm; Grade products Ltd, Coalville, UK), supplemented with 50 ml of TRIS buffer (0.01 molar; TRIS-HCl, 1.08219.100, Merck KGaA, Darmstadt, Germany, pH 5.63) and microorganisms associated to the leaf surface extracted by stomacher treatment (30 s; normal mode; Smasher, Chemunex, Bruz, France). Aliquots of 1 ml of the suspension in the filtered phase were used for serial dilution in sterile 0.85 NaCl before inoculation (100 µl) on semi-selective media. The introduced *E. coli* O157:H7 *gfp+* was selectively reisolated on lysogenic broth (LB) agar supplemented with 100 µg ml^{-1} ampicillin and 0.2% L-arabinose. To assess the general culturable microbiota, 0.1x tryptic soy agar (TSA; DIFCO 236950, USA; 4 g of TSA solidified with 1.2% Bacto agar) and O.5x maltextract agar (0.5 x MA; 10 g Malt extract DIFCO 218630, Sparks, MD, USA; 20 g Bacto Agar; ad 1000 ml distilled H_2O) as well as violet red bile dextrose agar (VRBD; Merck 1.10275.0500, Darmstadt,

Germany) were used for total aerobic bacterial counts and fungal counts as well as *Enterobacteriaceae*, respectively. The incubation conditions are presented in Table 5-1.

Table 5-1. Incubation conditions (temperature, °C; length, h) as well as targeted groups of organisms for the different semi-selective media (TSA: Tryptic Soy Agar; MA: Maltextract Agar; LB: Lysogenic Broth; VRBD: Violet-Red Bile Dextrose Agar).

Medium	**Incubation**		**Targeted groups of organisms**
	Temperature [°C]	Length [h]	
0.1x TSA	25	72	Aerobic heterotrophic bacterial counts (heterotrophic plate counts, HPC)
0.5x MA	25	168	Aerobic heterotrophic fungal counts
LB supplemented with arabinose and ampicillin	37	18	Introduced *E. coli* O157:H7 *gfp+* read under UV light (fluorescent colonies)
VRBD	37	18	*Enterobacteriaceae*

The remaining extraction suspension was centrifuged at 3000 xg for 45 min, the supernatant discarded and the pellet washed by repeated resuspension in 6 ml and 1 ml of 0.85% NaCl before storing in 1 ml of sterile freezing medium (4.28 mM K_2HPO_4, 1.31 mM KH_2PO_4, 1.82 mM Na-citrate, 0.87 mM $MgSO_4$ x $7H_2O$, 1.48 mM glycerol (98%,(Fåk *et al.*, 2012)) at -80 °C.

Microbial community structure:

Harvest of the phyllosphere bacteria:

Another 10 g of fresh leaves were weight into sterile filter bags and stomached together with 40 ml TRIS-buffer at normal speed for 2 minutes. 20 ml of the suspension were pipetted into a sterile tube and centrifuged at 5000 xg and 4 °C for 15 minutes. The gained pellet was dissolved in 2 ml phosphate buffered saline solution (PBS), the suspension distributed equally in two microreaction vessels and centrifuged at 6000 xg for 10 minutes. The supernatant was discarded and the pellet stored at -80 °C until further use.

DNA extraction:

The pellets were thawed in 1 ml of PBS buffer and vortexed until the pellets were dissolved. Aliquots of 200 µl were taken from each sample for DNA extraction. The DNA extraction was performed using the King Fisher Duo DNA extraction robot (Thermo Fisher Scientific Oy, Vantaa, Finland) and the King

Fisher Cell and Tissue DNA Kit (Product number: 97030196, Thermo Fisher Scientific Oy, Vantaa, Finland) according to the manufacturer's instructions.

DNA amplification:

The extracted DNA was amplified performing a polymerase chain reaction with primers 799F (5′-AAC MGG ATT AGA TAC CCK G-3′) and 1492R (5′-TAC GGH TAC CTT GTT ACG ACT T-3′). The master mix contained 0.156 µL Taq-polymerase, 2.5 µL Taq-buffer, 0.5 µL of each primer and deoxyribonucleotide triosphosphate (dNTP), 1.5 µL $MgCl_2$ and 2 µL of the extracted DNA sample. Sterile, ultrapure water was added to the mix to a finale volume of 25 µL. A polymerase chain reaction was conducted as described by Sun *et al.* (2008). Confirmation of the target DNA size of approximate 735 bp was conducted by gel electrophoresis as done by Hartmann *et al.* (2017).

Sequencing:

In total, there were 96 samples in two sequencing libraries with 300 bp paired-end reads (Illumina MiSeq V3). All Illumina data were analyzed by the bioinformatics service of LGC Genomics, Berlin, Germany using QIIME 1.8.0. The Illumina reads were demultiplexed using Illumina CASAVA data analysis software and clipped. Chimera with the uchime algorithm were eliminated. Reads with a final length <100 bases were discarded. For taxonomical classification of the 16S rRNA sample pool, representative sequences were aligned with reference sequences against the Silva reference classification and sequences from other domains of life were removed. Operational taxonomic units (OTUs) were picked by clustering sequences at the 97% identity level.

Calculation and statistical analyses

The experiment was based on a two-factorial design with two crops (factor 1) and three fertilizer regimes (factor 2). All the trials were repeated twice with six independent replicates per trial. The results from viable counts, expressed as mean ± SD after log transformation (Angle *et al.*, 1996). Relative abundances of different taxa were calculated based on metagenomic data from 3201 reads per sample and taxa >0.01 were considered. The core microbiome was selected on the basis of 16000 reads per sample. Biodiversity was estimated on genus level using PAST 3.0 (PAleontological Statistics; https://folk.uio.no/ohammer/past/). Interactions were assessed calculated using stepwise regression with forward selection and backward elimination using α=0.15 to enter or remove. All data was analyzed using a general linear model (crop; treatment; crop*treatment) using the "run" as a covariate followed by Tukey B test ($p<0.05$). Minitab vers 17.2.1 (www.minitab.com) was used for calculation of correlations, ANOVA, regressions and principal component analysis.

Results

Plant parameters

The fertilizer regimes affected plant performance significantly with respect to total yield per m^2, leaf area (Table 5-2) as well as to leaf fresh and dry weight (data not shown) where the highest values were

obtained for CM supplied plants and lowest ones for the non-fertilized control. Similar trends were found in both crops. The dry matter content was highest for the non-fertilized controls and lowest for the CM supplied plants whereas the ones fertilized with PHP took an intermediate position and significant differences were obtained for all three treatments. Maximum, minimum and average leaf temperature deviated between the two crops and significant differences were stated between the fertilized trays and the non-fertilized ones. No differences were found in chlorophyll fluorescence within the crops. Dry matter content of the different macro- and micronutrients in Swiss chard and rocket leaves was affected by the treatments and significant differences were obtained for N, P, K, S, Zn and B when both crops were considered. However, apart from total nitrogen content (TN), significant differences between crops were obtained. Table 5-3 displays the dry matter content of macro- and micronutrients in Swiss chard and rocket for the three treatments.

Table 5-2. Performance (fresh biomass, g m^{-2}; dry matter content, leaf area, cm^2 $leaf^{-1}$; average leaf temperature T_{av}, °C; minimum leaf temperature $T_{min,}$ °C) of Swiss chard and rocket exposed to a low risk and high risk fertilization scenario involving pig hair pellets (PHP, low risk) and raw chicken manure (CM, high risk). Plants in the control plots did not receive any additional fertilizers apart from the basis fertilizers in the growing medium. Leaf temperature values display average values for each tray.

	Swiss chard						**Rocket**					
	Control		**PHP**		**CM**		**Control**		**PHP**		**CM**	
Biomass	1297.26	B	1902.79	A	1969.76	A	832.04	C	1588.40	B	2161.29	A
Dry matter content	6.83	A	6.02	B	5.10	C	10.26	A	7.94	B	6.33	C
Leaf area	9.96	C	12.06	B	15.92	A	6.17	C	9.49	B	12.77	A
T_{av}	17.43	B	18.05	A	18.17	A	16.88	A	16.36	B	16.62	AB
T_{min}	17.07	B	17.61	A	17.71	A	15.96	A	15.31	B	15.56	AB

Table 5-3. Macro- (% dry leaf weight) and micronutrient (mg (kg leaf dry weight)$^{-1}$) content in leaves of greenhouse grown Swiss chard and rocket, fertilized either with pig hair pellets (PHP) or fresh chicken manure (CM). The control treatment was not supplied with any additional fertilizer. Values followed within the same line, followed by different letters are significantly different according to ANOVA followed by Tukey test ($p<0.05$). N=12.

	Swiss chard						**Rocket**					
	Control		**PHP**		**CM**		**Control**		**PHP**		**CM**	
Macronutrients												
Total nitrogen	4.14	C	5.83	B	6.13	B	3.42	C	5.6	B	7.77	A
Phosphorus	1.33	A	0.94	B	0.81	C	0.68	D	0.70	D	0.88	BC
Potassium	7.15	BC	6.23	C	10.18	A	4.99	D	4.67	D	7.34	B
Magnesium	0.99	B	1.36	A	0.89	B	0.32	C	0.42	C	0.45	C
Calcium	2.20	C	2.58	BC	1.67	D	2.73	B	3.40	A	3.74	A
Sulfur	0.53	C	0.45	C	0.41	C	2.21	A	2.08	A	1.83	B
Sodium	1.80	B	1.73	B	2.38	A	0.21	D	0.36	CD	0.42	C
Micronutrients												
Manganese	327.50	A	332.50	A	326.67	A	180.00	B	157.50	B	170.00	B
Iron	102.92	A	83.50	ABC	97.08	AB	64.75	C	73.25	BC	70.17	C

Copper	12.78	AB	13.63	AB	16.75	A	6.76	B	7.23	B	6.68	B
Zinc	75.25	A	65.50	B	75.83	A	55.25	C	56.75	C	72.92	AB
Boron	29.33	B	27.67	B	34.17	A	28.83	B	27.17	B	34.17	A

Leaf colonization

Leaf colonization of culturable heterotrophic bacteria (HPC) and fungi as well as the inoculated strain and *Enterobacteriaceae* are displayed in Table 5-4. CM treated Swiss chard harbored significantly less HPC as compared to PHP treated plants and the control. No differences were stated with respect to HPC between the treatments for rocket, but HPC were significantly higher on Swiss chard than rocket leaves. Fungal counts were one magnitude higher on Swiss chard as compared to rocket leaves. No differences between the treatments were stated on Swiss chard, but culturable fungi were less slightly, but significantly less frequent on rocket leaves amended with PHP fertilizer than the non-fertilized control. Although Swiss chard had been sprayed less times with *E. coli* O157:H7 *gfp*+ than the rocket plants, counts of the inoculated strain were more than one magnitude higher on Swiss chard than on rocket leaves. No statistically significant differences were found between the treatments, but PHP treated plants showed a trend towards higher colonization. *E. coli* O157:H7 *gfp*+ accounted for the majority of the culturable *Enterobacteriaceae*. No differences were found with respect to fertilizer strategy within the crops with respect to *Enterobacteriaceae*, but followed the same pattern as for other cultured microbial groups between crops.

Table 5-4. Microbial colonization (Log CFU g^{-1}) of Swiss chard and rocket leaves grown in the presence of *E. coli* O157:H7 *gfp*+ under greenhouse conditions. Organic fertilizers were supplied to the growing medium during sowing bed preparation, before sowing and the fertilization strategies displayed a high risk (fresh chicken manure, CM) and a low risk scenario (pig hair pellets, PHP) and a non-fertilized control plot. *E. coli* O157:H7 *gfp*+ was sprayed two times a week with an interval of 3 and 4 days, respectively, with three and five inoculations for Swiss chard and rocket. The experiment was repeated twice with six individual replicates per trial. (n=12).

	Swiss chard						**Rocket**					
	Control		**PHP**		**CM**		**Control**		**PHP**		**CM**	
0.1x TSA	6.92	A	6.90	A	6.45	B	4.73	A	5.07	A	4.88	A
MA	4.61	A	4.48	A	4.36	A	3.03	A	2.71	B	2.78	AB
LB	5.23	A	5.42	A	5.11	A	3.40	B	3.90	A	3.42	B
VRBD	5.94	A	5.99	A	5.78	A	3.39	A	3.58	A	3.49	A

Principal component analysis resulted in very similar pictures for the two crops when all plant parameters and data for culturable microorganisms were involved (Figure 5-1). PC 1 discriminated

between plants exposed to CM and the non-fertilized control whereas PC 2 discriminated between the two runs. No distinct picture was found for PHP treated rocket or Swiss chard. In both crops these plants clustered in the first run to the unfertilized control. In the second run, PHP treated rocket plants clustered with CM treated ones, whereas PHP treated Swiss chard plants took an intermediate position. Despite the similarities in pattern, different parameters dictated the outcome; for rocket, leaf total nitrogen and magnesium content had the strongest load on PC 1 whereas the leaf area (PC 1) as well as leaf temperature (PC 2) were decisive in Swiss chard.

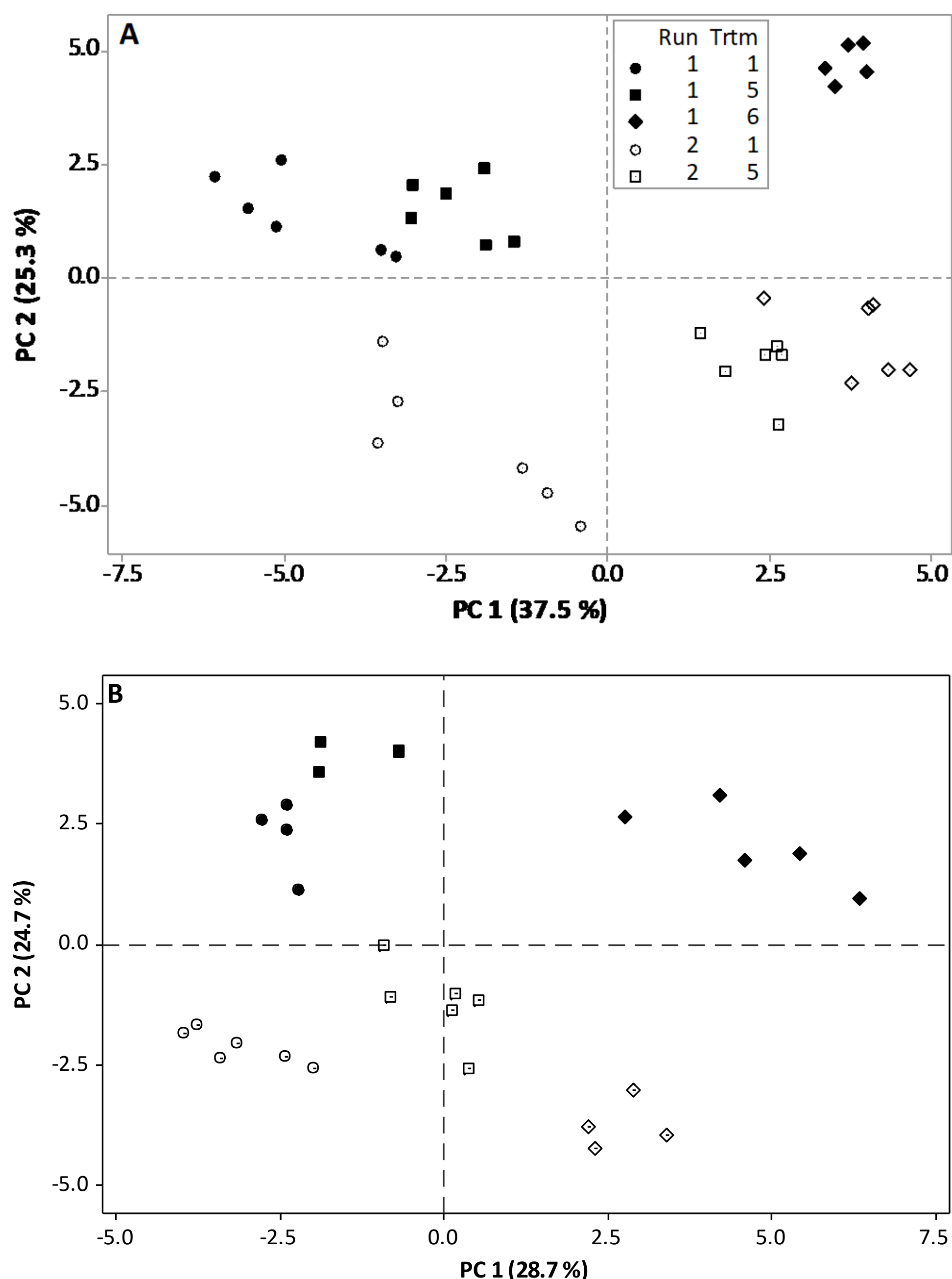

Figure 5-1. Principal component analysis of all plant parameters (yield m^{-2}; leaf fresh and dry weight, dry matter content; leaf area; minimum, maximum and average leaf temperature; chlorophyll fluorescence; leaf macro- and micronutrient content) and log values of enumerated microbial groups on semi selective media (culturable heterotrophic bacteria; culturable fungi; *Enterobacteriaceae*;

inoculated *E. coli* O157:H7 *gfp+*; intestinal enterococci). Greenhouse grown rocket (A) and Swiss chard (B) plants were subjected to three fertilizer strategies (no additional fertilizer (●); pig hair pellets (■); fresh chicken manure (♦)). Filled and open symbols denote the first and second experiment, respectively.

Culture-independent analysis

The number of reads was in average similar for the two crops and the different treatments (Swiss Chard: 15011 reads; rocket: 16251 reads (Table 5-5)). Replicates with reads <3274 were excluded from the further calculations.

Table 5-5. Numbers of 16S rDNA sequences (excluding chloroplast and mitochondria reads) in the phyllosphere samples from Swiss chard and rocket exposed to different fertilizer regimes accounting for a low and high risk scenario (Control=no additional fertilizer supplied; organic fertilizers: PHP=pig hair pellets, CM=fresh chicken manure).

		Swiss Chard Replicate						Rocket Replicate					
Treatment	**Run**	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
Control	1	16963	18220	3275	17074	18460	7539	18772	17465	18347	17204	17059	17607
	2	14259	16784	10079	16714	11155	15249	560	12289	12184	18158	18323	18091
PHP	1	15498	15114	16679	12468	12934	17704	18136	18009	4453	16583	17456	17963
	2	16997	9654	16073	16871	16601	15713	18738	18891	19289	19016	11079	12000
CM	1	18170	12887	16415	13500	18682	12543	16702	15348	18615	17284	16702	18751
	2	16833	16809	16667	17404	16838	15566	18402	17779	18515	13721	16437	19136

Relative abundance of phyla differed between the two crops (Figure 5-2). Twelve bacterial phyla were represented >1% in the phyllosphere of rocket, as opposed to the less diverse phyllosphere community structure of Swiss chard. In both crops, *Proteobacteria* was the dominant bacterial phylum, contributing by 86%-96% and 96-97% to the bacterial phyllosphere community of rocket and Swiss chard. The community structure on phylum level was not affected by the fertilizer regime in Swiss chard whereas significant differences were found between CM and PHP fertilized rocket leaves, with respect to *Proteobacteria*. Despite low relative abundances of the phyla *Clamydiae* and *Gemmatimonadetes* in the rocket phyllosphere of all three treatments, significant differences were stated between the non-fertilized plants and the fertilized ones. No discrimination on genus level between the treatments could be done when genera of these phyla were considered. No differences in biodiversity, calculated on genus level, were found between the treatments within the crops. However, the total species richness, estimated using Chao1, differed significantly between the bacterial phyllosphere biota associated with rocket and Swiss chard (Table 5-6).

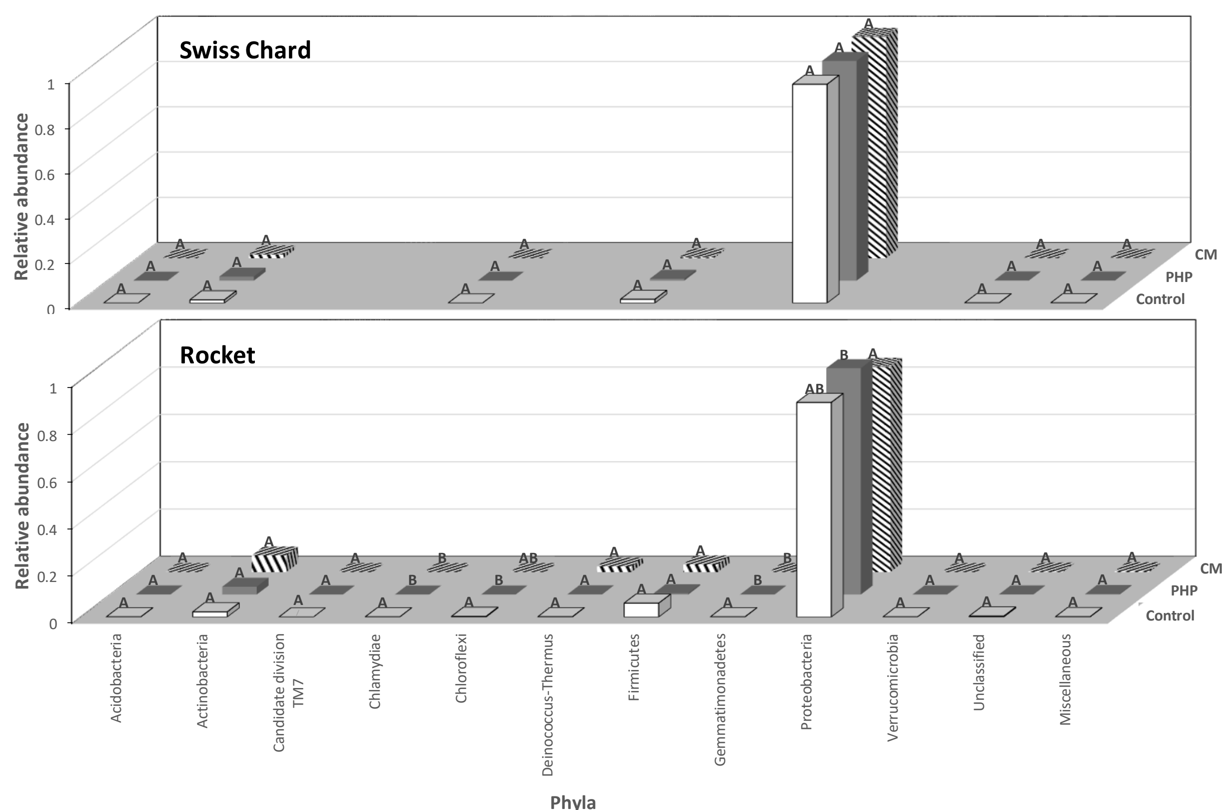

Figure 5-2. Relative abundance of bacterial phyla on greenhouse grown Swiss chard (A) and rocket (B) leaves which had been spray inoculated with *E. coli* O157:H7 *gfp+*. The Swiss chard and rocket crops were sown in growing medium and exposed to different fertilizer strategies mimicking a high (CM: fresh chicken manure) or low (PHP: pig hair pellets) risk scenario for transmission of enteric diseases (Control: no additional fertilizer).

Table 5-6. Biodiversity of the bacterial phyllosphere biome of greenhouse grown Swiss chard and rocket estimated on genus level by Shannon-H and Chao1. The crops were fertilized with either pig hair pellets (PHP) or fresh chicken manure (CM). The control did not receive any additional fertilization. (N=12). Values within the same column followed by different letters are significantly different according to Anova followed by Tukey-test (p<0.05).

		Shannon-H	Chao1
Swiss chard	Control	1.9 A	114.1 A
	PHP	1.9 A	127.0 A
	CM	1.7 A	124.5 A
Rocket	Control	1.7 A	30.7 B
	PHP	1.7 A	28.3 B
	CM	1.9 A	32.1 B

Among *Proteobacteria*, *Gammaproteobacteria* was the most dominant class in both crops and the orders *Enterbacteriales*, *Pseudomonadales* and *Xanthomonadales* were predominant (Figure 5-3). Differences were found between crops with respect to *Rhodobacterales* and *Nitrosomonadales*, both of them less abundant than 1 % in the Swiss chard phyllosphere, *Rhodospirillales*, *Methylophilales*, *Myxococcales*, *Enterobacteriales*, *Xanthamonadales*, unclassified *Proteobacteria* as well as miscellaneous

Proteobacteria. Significantly higher abundances of *Myxococcales* and *Legionellales* were found in the phyllospheres of the non-fertilized controls as opposed to the two treatments with organic fertilizers when both crops were included in the analysis. Also, *Nitrosomonadales* and *Caulobacteriales* were significantly more frequent in the untreated control than in the phyllospheres of PHP treated crops and CM treated crops, respectively. *Enterobacteriales* were significantly more abundant in the phyllospheres of CM than PHP treated plants. Vice versa, there was a trend towards higher abundances of *Pseudomonadales* and *Burkholderiales* in the phyllospheres of crops treated with PHP as opposed to CM, however, these differences were only significant when Fisher test was used. The *Enterobacteriaceae* genera, *Cedecea*, *Erwinia*, *Escherichia-Shigella* as well as *Pantoea* were hosted by both crops. Furthermore, also the genera *Buttiauxella*, *Klebsiella*, *Leminorella*, *Morganella*, *Providencia*, *Raoultella*, *Tatumella*, *Trabulsiella* were represented in the Swiss chard phyllosphere. Unclassified *Enterobacteriaceae* accounted for the majority of genus within *Enterobacteriaceae* in Swiss chard (62-64 %) whereas 9-12 % of the reads belonged to the genus *Escherichia-Shigella* which corresponded to 3.5-5.8 % of the leaf microbiome's bacterial reads. Other prominent *Enterobacteriaceae* genera within the Swiss chard phyllosphere were *Pantoea* (11-22 %) and *Erwinia* (2-9 %). *Cedecea* accounted for approximate 4 % of the *Enterobacteriaceae* genera in the CM treated Swiss chard leaves, but considerably less abundant in the two other treatments. No significant differences between treatments were stated. The picture differed for the rocket phyllosphere, where 23-25 % of the *Enterobacteriaceae* reads (corresponding to 13-15 % of the leaf microbiome's bacterial reads) accounted for the genus *Escherichia-Shigella.* The majority of the *Enterobacteriaceae* reads aligned to unclassified *Enterobacteriaceae* (66-73 %), whereas a low percentage of reads within *Enterobacteriaceae* accounted for genera *Pantoea* and *Erwinia*.

Apart from *Escherichia-Shigella*, the genera *Arthrobacter*, *Rhizobium*, *Sphingomonas*, *Pseudomonas* as well as *Stenotrophomonas* were constituents of the bacterial core biome of the both phyllospheres. *Curtobacterium*, *Paenibacillus*, *Caulobacter*, *Massila*, *Methylophilus*, *Erwinia* and *Pantoea* were only present in the core biome of Swiss chard leaves, whereas *Microbacterium*, *Methylobacterium*, *Delftia* and *Acinetobacter* were represented only in the one of rocket leaves.

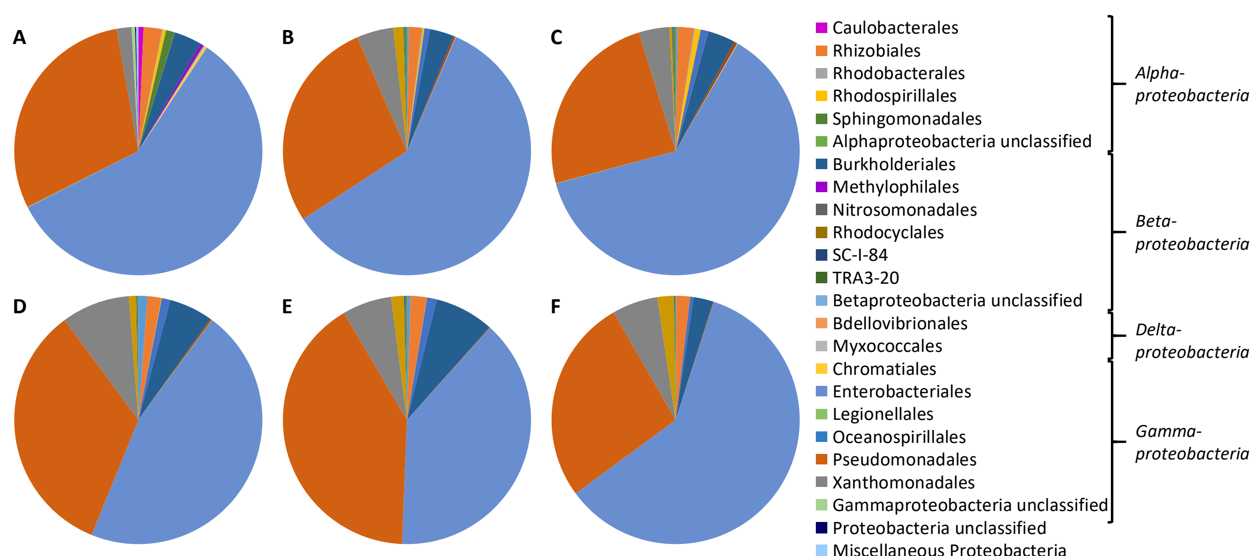

Figure 5-3. Relative abundance of bacterial orders within *Proteobacteria* on rocket (A-C) and Swiss chard (D-F) leaves that had been spray-inoculated by *E. coli* O157:H7 *gfp+*. The crops were grown under greenhouse conditions in growing medium without additional fertilizer (A, D) or fertilized with different organic fertilizers mimicking a low (B, E: pig hair pellets) or a high (C, F: fresh chicken manure) risk scenario for transmission of enteric diseases.

Interactions between plant parameters and E. coli O157:H7 gfp+

When nutrient contents of both crops were related to the occurrence of the inoculated strain, Mg, Mn and Fe explained 70.4% ($p<0.001$) of its variance, whereas a low leaf temperature was positively and leaf dry weight and dry matter content were negatively correlated to the occurrence of the inoculated strain. On the basis of both crops and all measured plant parameters, ten factors (content of Mg, Mn, Fe, TN, Na, K; yield; F_0) explained the variance of the inoculant by 81.1% ($p<0.001$) (eq. 1).

Log *E. coli* O157:H7 *gfp+* = 4.18 + 0.874 Mg + 0.00052 Mn + 0.00347 Fe - 0.00454 yield + 0.309 TN + 1.01 Na - 0.197 K - 0.000374 F_0 (eq. 1)

With Mn = mg Mn (kg leaf dry matter)$^{-1}$; Fe = mg Fe (kg leaf dry matter)$^{-1}$; TN = mg total N (mg leaf dry matter)$^{-1}$; Na = mg Na (kg leaf dry matter)$^{-1}$; K = mg K (kg leaf dry matter)$^{-1}$; F_0 = minimum chlorophyll fluorescence.

However, differences occurred when only single crops were considered. Among all assessed plant parameters, the leaf Fe and B content as well as the log values of *Enterobacteriaceae* as enumerated on VRBD and the log values of culturable fungi counted on MA were decisive for the prevalence of *E. coli* O157:H7 *gfp+* on rocket leaves. These four factors explained 86% ($p<0.001$) of the variance of the inoculated strain (eq. 2).

Log *E. coli* O157:H7 *gfp+* = - 0.643 + 0.955 VRBD - 0.0621 B + 0.0215 Fe + 0.427 MA (eq.2)

With VRBD = log value of *Enterobacteriaceae* enumerated on VRBD; B = mg B (kg leaf dry matter)$^{-1}$; Fe = mg Fe (kg leaf dry matter)$^{-1}$; MA = log value of culturable fungi as enumerated on MA

In contrast, variations in the inoculated strain could only be explained to a minor extent (R^2=25.3%; p=0.008) by plant parameters and log values of culturable microorganisms based on the following equation (eq. 3).

Log *E. coli* O157:H7 *gfp+* = 3.12 + 0.472 VRBD - 0.00914 Zn (eq.3)

With LB = log value of *E. coli* O157:H7 *gfp+*; VRBD = log value of *Enterobacteriaceae* enumerated on VRBD; Zn = mg Zn (kg leaf dry matter)$^{-1}$.

Interactions between plant parameters and the microbial community structure

On rocket, few interactions were found between nutrient dry matter content and the microbial community structure on phylum level, *Actinobacteria* correlated positively to the TN content (R^2=36.1%; p=0.033) and Ca content (R^2=35.6%; p=0.036), *Chlamydiae* and *Gemmatimonadetes* to S (R^2=42.8%; p=0.001; R^2=39.7%; p=0.018) and Mn (R^2=55.0%; p=0.001; R^2=42.6%; p=0.011) contents in the leaf dry matter. In general, the relative abundance of families was only occasionally dictated by the general nutrient content in the plant dry matter, among these *Propionibacteriaceae*, unclassified families of *Actinobacteria, Brucellaceae, Rhodobacteraceae* and *Solimonadaceae* (Table 5-7). Micronutrients, especially Mn, but also B and Fe, were strong predictors for the relative abundance of families on rocket.

On Swiss chard leaves, interactions between plant parameters were mainly found for *Actinobacteria* which were positively correlated to leaf content of TN (R^2= 36 %, p=0.031), K (R^2=34.4%, p=0.004), Mn (R^2=69.2%, $p<0.001$) and B (R^2= 37%, p=0.026) and negatively correlated to the leaf dry weight (R^2= -44.5 %, p=0.007) and dry matter content (R^2=-43.2%, p=0.008), leaf temperature (maximum temperature: R^2=-51%, p=0.001; average temperature: R^2=-48.4%, p=0.003; minimum temperature: R^2=47.8%, p=0.003). Likewise, the phylum *Proteobacteria* showed several interactions with plant parameters among these (leaf dry weight: R^2=39.7%, p=0.016; temperature: maximum temperature: R^2=41.3, p=0.012, average temperature: R^2=40.1, p=0.015, minimum temperature: R^2=40 %, p=0.016; Cu; R^2=40.3 %; p=0.015; F_0: R^2=-45.2 %, p=0.006). The general nutrient content in the plant content was correlated with the abundance of four *Actinobacteria* families, among these *Nocardiaceae*, *Brevibacteriaceae*, *Micrococcaceae* as well as an unclassified *Micrococcales*-family. Also on Swiss chard, Mn was correlated to the abundance of eighteen families, whereas B and Fe did not play a similar decisive role as on rocket.

Table 5-7. Pearson correlations between leaf dry matter nutrient content and relative abundance of bacterial families, inhabiting the phyllosphere of rocket. The rocket plants had been fertilized with either organic fertilizers (raw chicken manure or pig hair pellets or remained without additional fertilization). Probabilities are displayed.

Phylum	Class	Order	Family	TN	P	K	Mg	Ca	S	Na	Mn	Fe	Cu	Zn	B
Actinobacteria	Actinobacteria	Coryne-	Coryne-	0.436	0.263	0.133	0.512	0.460	0.074	0.286	0.221	0.553	0.385	0.305	0.181
		bacteriales	bacteriaceae	0.009	0.127	0.447	0.002	0.005	0.671	0.096	0.202	0.001	0.022	0.075	0.298
Actinobacteria	Actinobacteria	Micrococcales	Derma-	0.308	0.203	0.062	0.397	0.480	0.157	0.044	0.384	0.445	0.449	0.272	0.241
			cocacceae	0.072	0.243	0.725	0.018	0.004	0.367	0.803	0.023	0.007	0.007	0.115	0.162
Actinobacteria	Actinobacteria	Micrococcales	unclassified	0.358	0.295	0.249	0.345	0.376	-0.248	0.329	0.005	0.032	0.056	0.263	0.064
				0.035	0.085	0.150	0.042	0.026	0.151	0.053	0.975	0.857	0.749	0.127	0.715
Actinobacteria	Actinobacteria	Propionibacteria	Propionibacte	0.341	0.452	0.324	0.416	0.458	0.087	0.151	0.443	0.573	0.362	0.420	0.464
		les	ria	0.045	0.006	0.058	0.013	0.006	0.620	0.387	0.008	<0.001	0.032	0.012	0.005
Actinobacteria	Actinobacteria	unclassified	unclassified	0.308	0.333	0.343	0.352	0.336	0.018	0.226	0.326	0.365	0.241	0.349	0.464
				0.072	0.050	0.043	0.038	0.049	0.920	0.191	0.056	0.031	0.163	0.040	0.005
Firmicutes	Clostridia	Clostridiales	Family XI	0.439	0.510	0.460	0.282	0.209	-0.244	0.317	-0.076	0.069	-0.063	0.506	0.253
				0.008	0.002	0.005	0.100	0.227	0.158	0.064	0.666	0.692	0.72	0.002	0.143
Proteobacteria	Alpha-	Rhizobiales	Brucellaceae	0.379	0.440	0.452	0.261	0.228	-0.174	0.269	0.199	0.198	0.053	0.484	0.437
	proteobacteria			0.025	0.008	0.006	0.131	0.187	0.316	0.118	0.251	0.255	0.763	0.003	0.009
Proteobacteria	Alfa-	Rhodo-	Rhodo-	0.416	0.283	0.234	0.418	0.408	0.048	0.108	0.254	0.498	0.248	0.405	0.338
	proteobacteria	bacteriales	bacteriaceae	0.013	0.099	0.177	0.013	0.015	0.784	0.536	0.140	0.002	0.151	0.016	0.047
Proteobacteria	Beta-	Burk-	Burk-	0.276	0.396	0.384	0.171	0.242	0.136	0.054	0.445	0.189	0.010	0.402	0.422
	proteobacteria	holderiales	holderiaceae	0.108	0.019	0.023	0.325	0.161	0.437	0.758	0.007	0.276	0.957	0.017	0.012
Proteobacteria	Gamma-	Xantho-	Soli-	0.463	0.351	0.376	0.467	0.420	-0.009	0.170	0.430	0.444	0.443	0.384	0.440
	proteobacteria	monadales	monadaceae	0.005	0.039	0.026	0.005	0.012	0.958	0.330	0.010	0.008	0.009	0.023	0.008

Interactions between plant parameters, microbial community structure and the presence of E. coli O157:H7 gfp+

Principal component analysis on the basis of the analyzed parameters clustered crops but also treatments (Figure 5-4). No interactions between the prevalence of the inoculated strain of *E. coli* O157:H7 *gfp+* and the *Escherichia-Shigella* group on genus level were found on neither rocket nor Swiss chard. A set of 22 microbial genera and five plant parameters explained the variation (R^2=99.6 %; p<0.001) of the inoculated strain on rocket. The plant leaf content of Fe as well as average leaf temperature and maximum fluorescence value F_m after exposure to a light flash were positively correlated which assessed minimal temperature and leaf dry weight were negatively correlated among plant factors. Within the framework of the performed treatments, amongst positive predictors the bacterial genera *Acinetobacter*, *Stenotrophomonas*, *Arthrobacter*, *Rhizobacterium*, *Rhizomicrobium*, *Bacillus* were found and whereas *Sphingomonas*, *Pantoea*, *Pseudomonas*, *Novosphingobium*, *Rhizobium*, *Lactobacillus*, *Brevundimonas*, *Photobacterium*, and *Hyphomicrobium* were found amongst negative predictors for the inoculated *E. coli* strain.

On Swiss chard, a set of 28 factors explained 99.8% (p<0.001) of the variance in log values of the inoculated *E. coli* O157:H7 *gfp+* strain, among these three plant parameters. Amongst genera present in the Swiss chard phyllosphere, the relative abundance of *Desulfitobacterium*, *Kaistia*, *Paracoccus*, *Sphingobium*, *Klebsiella*, *Burkholderia*, (positively) and *Bryobacter*, *Erwinia*, *Chthoniobacter*, *Aquabacterium*, *Phenylobacterium*, *Rhodococcus*, *Rheinheimera*, *Acidovorax*, *Mesorhizobium*, supported by leaf calcium content predicted the occurrence of the inoculated strain.

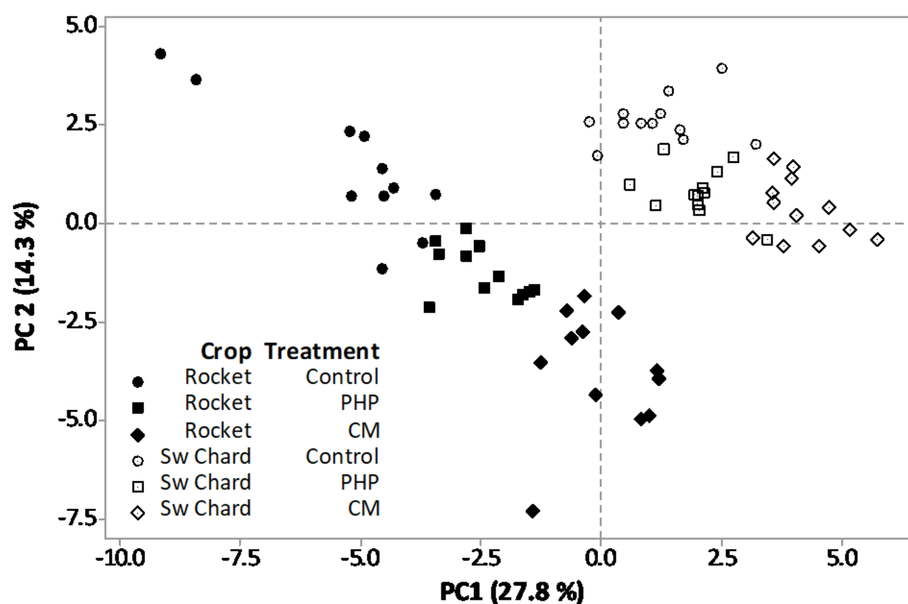

Figure 5-4. Principal component analysis of all plant parameters (yield m^{-2}; leaf fresh and dry weight, dry matter content; leaf area; minimum, maximum and average leaf temperature; chlorophyll fluorescence; leaf macro- and micronutrient content) and relative abundance of bacterial phyla present in greenhouse grown rocket and Swiss chard leaves. The plants were subjected to three fertilizer strategies (Control: no additional fertilizer; PHP: pig hair pellets; CM: fresh chicken manure).

Discussion

The prevalence of foodborne pathogens on plants exposed to different sources of organic fertilizers has been studied previously by Islam *et al.* (2004a) and (2005). In their studies, a steady decline was monitored over a period of approximately half a year (parsley: 177 days; carrots: 168 days; lettuce: 77 days; onions: 74 days). Within the final report of PathOrganic, Sessitsch (2011) appointed critical control points for field vegetable production in relation to organic fertilizers. However, the prevalence of *E. coli* O157:H7 has not previously been studied within the framework of an organic low- and high risk scenario when a secondary route of transmission was considered. Our manipulation corresponds to irrigation water as an environmental contamination source. Our findings contradict our hypothesis that a high risk fertilization scenario will support the occurrence of *E. coli* O157:H7 *gfp*+ on leafy vegetables. Moreover, our results indicate, that crop specific factors and the bacterial phyllosphere microbiome must be considered. Thanks to the monitoring of parameters describing plant performance and metagenomics analysis of the bacterial phyllosphere biome, this publication adds novel insights to phyllosphere microbe interactions, to colonization of *E. coli* O157:H7 and to measures for prevention of transmission of foodborne pathogens in organic production of leafy vegetables.

As a consequence of high inoculation densities in the spray solution and repeated inoculations, *E. coli* O157:H7 *gfp*+ established well in both crops and all three treatments. Despite two more sprays in rocket due to its slower development, the Log CFU of the inoculated strain was lower in rocket than in Swiss chard. The higher relative abundance of the genus *Escherichia-Shigella* in rocket as opposed to Swiss chard indicates that the spray treatment was successful, but that the inoculant did not remain as viable or culturable on rocket as on Swiss chard. This means that plant specific conditions determine the prevalence of microorganisms immigrating to the phyllosphere.

The plant leaf and crop stand as a habitat of microorganisms is a well-established fact and likewise their interactions between biotic and abiotic factors (Whipps *et al.*, 2008; Lindow & Brandl, 2003). The present results support previous findings that the microbial community structure of leaves differs between species (Redford *et al.*, 2010; Kinkel *et al.*, 2000), but also between cultivars (Hunter *et al.*, 2010). Our results indicated that the impact of the crop is superior to the one of the applied fertilization regimes. In contrast to most community structures displayed by Redford *et al.* (2010), the phyllosphere community of Swiss chard and rocket was dominated by *Proteobacteria*. This might be due to the different technique used for characterization of the bacterial phyllosphere biome and/or to the extraction method to harvest the microbiome or a consequence of environmental effects prevailing under greenhouse conditions.

Also abiotic factors shape the community structure, among these light spectrum (Kadivar & Stapelton, 2003), relative humidity, water availability, nutrient availability, temperature and their dynamics (Bulgarelli *et al.*, 2013; Rasche *et al.*, 2006; Kinkel, 1997). Also the crop stand alters the microclimatic and light conditions (e.g. humidity, temperature, shade). The external conditions in greenhouses deviate from the ones under natural conditions; the greenhouse covering material shields the crop from UV-

irradation (Krizek *et al.*, 2005) which leads to higher nitrate content in leaves (Voipio & Autio, 1995). Furthermore, alterations in humidity and diurnal temperature changes are less expressed. Under optimal maintenance conditions, greenhouse grown crops are not exposed to unintentional drought or nutrient deficiencies.

In the present study, we attempted to restrict the analysis to epiphytic colonizers. We found interactions between log values of *E. coli* O157:H7 *gfp+* and quantities of most plant nutrients detected in the leaf dry matter. In the present study, we did not quantify the content of the amount of readily available nutrients or nutrients present in leaf rinsates. Most likely, the nutrients' function within the plant metabolism rather than their availability to the epiphytic phyllosphere colonizers is therefore of interest. Magnesium, iron and manganese are important factors for photosynthetic activity (Marschner, 1995). Apart from its role as the central atom in the chlorophyll molecule, Mg is involved in enzymes mediating phosphorylation. The close positive correlation of Mg and Fe to the log value of the inoculant might indicate that the crops' photosynthetic activity affects the survival of *E. coli* O157:H7 *gfp+*. A positive impact of high manganese plant content on soluble carbohydrates in leaves was described by Mulder and Gerretsen (1952) and Vielemeyer *et al.* (1969). The strong interaction between prevalence of culturable *E. coli* O157:H7 *gfp+* and photosynthesis is also displayed by the negative correlation with F_0, the minimum fluorescence in the absence of light. These findings also bind to previous studies on nutrient availability in the phyllosphere, emphasizing the role of sugars on leaf colonization (Mercier & Lindow, 2000), their heterogenous availability pattern (Leveau & Lindow, 2001) and persistence of *E. coli* O157:H7 on leaves in relation to sugar (Aruscavage *et al.*, 2010).

The distinct pattern regarding interactions between occurrence of *E. coli* O157:H7 *gfp+* and plant nutrients and plant performance parameters became much more complex when considering the bacterial biome on genus level. On the basis of the present study, no specific bacterial predictors supporting the occurrence or survival of *E. coli* O157:H7 *gfp+* could be appointed.

The different nutrient regimes resulted in differences of plant nutrient content but also plant performance. Addition of organic fertilizers was calculated on the basis of nitrogen content and nitrogen mineralization rate. However, chicken manure contains apart from nitrogen and other macro- and micronutrients also considerable amounts of sodium and chloride; meanwhile, pig hair pellets provide sulfur, iron and zinc (Möller & Schultheiss, 2014). Sodium is not an essential element for rocket and Swiss chard. The positive interaction between Na and the log value of *E. coli* O157:H7 *gfp+* and the negative interaction between K and the inoculated strain might be an artefact and a consequence of competition between the two ions. This interaction needs to be further observed in studies with less cation imbalanced fertilizer regime. For optimal nutrient uptake, all essential nutrients need to be available in proportion to crop demands (Marschner, 1995). Both crops were in a vegetative stage requiring high amounts of nitrogen. The lower performance of PHP supplied treatments might be due to restricted availability of essential mineral elements. Plant performance and physiognomy also shapes

the microclimate as well as interception of light in the crop stand. Also, differences in plant physiognomy may affect the interception of the spray inoculated strain.
Therefore, more detailed investigations to the impact of fertilizers on the colonization of *E. coli* O157:H7 need to be done to confirm the described interactions.

Conclusions

On the basis of the present data we conclude that

- Hazard analysis and appointment of critical control point must also account for secondary transmission routes and a low risk fertilizer does not necessarily reduce the risk for it.
- The crop and the nutrient regime are essential predictors for the occurrence of *E. coli* O157:H7 *gfp+*
- Factors promoting photosynthetic activity also favor the occurrence of *E. coli* O157:H7 *gfp+* on leaves of Swiss chard and rocket
- The crop rather than the fertilizer regime governs the bacterial phyllosphere biome
- The impact of nutrient regime on the prevalence of *E. coli* O157:H7 *gfp+* on leafy vegetables needs more detailed attention in future studies.

Supplementary material

Table 5-8. Pearson correlations between leaf dry matter content of manganese (Mn), iron (Fe) and boron (B) and the relative abundance of bacterial families, inhabiting the phyllosphere of rocket. The rocket plants had been fertilized with either organic fertilizers (raw chicken manure or pig hair pellets or remained without additional fertilization). Probabilities are displayed.

Phylum	Class	Order	Family	Mn	Fe	B
Acidobacteria	*Acidobacteria*	Subgroup 3	SJA-149	0.5	0.293	0.369
				0.002	0.088	0.029
Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*	*Iamiaceae*	0.614	0.266	0.385
				0	0.123	0.022
Actinobacteria	*Actinobacteria*	*Corynebacteriales*	*Corynebacteriace*	0.221	0.553	0.181
				0.202	0.001	0.298
Actinobacteria	*Actinobacteria*	*Corynebacteriales*	*Mycobacteriaceae*	0.457	0.2	0.407
				0.006	0.25	0.015
Actinobacteria	*Actinobacteria*	*Corynebacteriales*	*Nocardiaceae*	0.371	0.446	0.466
				0.028	0.007	0.005
Actinobacteria	*Actinobacteria*	*Frankiales*	*Sporichthyaceae*	0.683	0.233	0.404
				0	0.177	0.016
Actinobacteria	*Actinobacteria*	*Micrococcales*	*Dermacoccaceae*	0.384	0.445	0.241
				0.023	0.007	0.162
Actinobacteria	*Actinobacteria*	*Micrococcales*	*Intrasporangiace*	0.324	0.53	0.348
				0.058	0.001	0.041
Actinobacteria	*Actinobacteria*	*Propionibacteriales*	*Nocardioidaceae*	0.49	0.358	0.426
				0.003	0.035	0.011
Actinobacteria	*Actinobacteria*	*Propionibacteriales*	*Propionibacteria*	0.443	0.573	0.464
				0.008	0	0.005
Actinobacteria	*Actinobacteria*	*Pseudonocardiales*	*Pseudonocardiace*	0.717	0.328	0.471
				0	0.055	0.004
Actinobacteria	*Actinobacteria*	unclassified	unclassified	0.326	0.365	0.36
				0.056	0.031	0.034
Actinobacteria	*Thermoleophilia*	*Gaiellales*	*Gaiellaceae*	0.153	0.421	-0.008
				0.382	0.012	0.962
Actinobacteria	*Thermoleophilia*	*Solirubrobacterales*	480-2	0.69	0.446	0.402
				0	0.007	0.017
Actinobacteria	*Thermoleophilia*	*Solirubrobacterales*	unclassified	0.364	0.23	0.288
				0.032	0.183	0.094
Actinobacteria	*Thermoleophilia*	unclassified	unclassified	0.424	0.335	0.298
				0.011	0.049	0.082
Chlamydiae	*Chlamydiae*	*Chlamydiales*	*Parachlamydiacea*	0.664	0.182	0.388
				0	0.296	0.021
Chloroflexi	S085	unclassified	unclassified	0.393	0.108	0.187
				0.02	0.537	0.282
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	-0.418	-0.268	-0.195
				0.012	0.119	0.262
Firmicutes	*Bacilli*	*Lactobacillales*	*Aerococcaceae*	0.273	0.362	0.201
				0.112	0.033	0.246
Firmicutes	*Bacilli*	*Lactobacillales*	*Enterococcaceae*	0.556	0.413	0.373
				0.001	0.014	0.027
Firmicutes	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	0.487	0.179	0.243
				0.003	0.304	0.16
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	0.206	0.358	0.264
				0.234	0.035	0.126
Gemmatimonadetes	*Gemmatimonadetes*	*AT425-EubC11* terrestrial group	unclassified	0.731	0.287	0.451
				0	0.094	0.007
Gemmatimonadetes	*Gemmatimonadetes*	*Gemmatimonadales*	*Gemmatimonadacea*	0.431	0.266	0.282
				0.01	0.122	0.101

Phylum	Class	Order	Family	Mn	Fe	B
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Bradyrhizobiacea*	0.548	0.31	0.372
				0.001	0.07	0.028
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Brucellaceae*	0.199	0.198	0.437
				0.251	0.255	0.009
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiacea*	0.486	0.414	0.319
				0.003	0.013	0.062
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Phyllobacteriace*	0.603	0.454	0.41
				0	0.006	0.014
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiaceae*	0.521	0.47	0.494
				0.001	0.004	0.003
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiales* Incertae sedis	0.478	0.263	0.307
				0.004	0.127	0.073
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Xanthobacteracea*	0.428	0.338	0.251
				0.01	0.047	0.146
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	unclassified	0.405	0.192	0.375
				0.016	0.268	0.026
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	0.254	0.498	0.338
				0.14	0.002	0.047
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	JG37-AG-20	0.396	-0.032	0.232
				0.019	0.853	0.181
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillales* Incertae sedis	0.417	0.122	0.211
				0.013	0.485	0.225
Proteobacteria	*Alphaproteobacteria*	*Rickettsiales*	*Rickettsiales* Incertae sedis	0.451	0.057	0.248
				0.007	0.743	0.151
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	0.445	0.189	0.422
				0.007	0.276	0.012
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	0.614	0.299	0.353
				0	0.081	0.038
Proteobacteria	*Betaproteobacteria*	*Methylophilales*	*Methylophilaceae*	0.389	0.321	0.365
				0.021	0.06	0.031
Proteobacteria	*Betaproteobacteria*	*Neisseriales*	*Neisseriaceae*	0.46	0.271	0.176
				0.005	0.116	0.313
Proteobacteria	*Betaproteobacteria*	*Nitrosomonadales*	*Nitrosomonadacea*	0.344	0.065	0.21
				0.043	0.712	0.227
Proteobacteria	*Deltaproteobacteria*	*Myxococcales*	*Cystobacteraceae*	0.623	0.252	0.399
				0	0.145	0.018
Proteobacteria	*Deltaproteobacteria*	*Myxococcales*	unclassified	0.756	0.256	0.411
				0	0.138	0.014
Proteobacteria	*Deltaproteobacteria*	unclassified	unclassified	0.619	0.389	0.367
				0	0.021	0.03
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriace*	0.314	0.51	0.411
				0.066	0.002	0.014
Proteobacteria	*Gammaproteobacteria*	*Legionellales*	*Coxiellaceae*	0.447	0.207	0.24
				0.007	0.232	0.165
Proteobacteria	*Gammaproteobacteria*	*Legionellales*	*Legionellaceae*	0.485	0.247	0.39
				0.003	0.152	0.021
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	-0.336	-0.266	-0.402
				0.048	0.123	0.017
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	-0.117	-0.352	-0.303
				0.503	0.038	0.076
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Solimonadaceae*	0.43	0.444	0.44
				0.01	0.008	0.008
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	-0.361	-0.318	-0.316
				0.033	0.063	0.064
Verrucomicrobia	OPB35_soil_group	unclassified	unclassified	0.409	0.162	0.217
				0.015	0.351	0.21

Chapter 6: Internalisation of *Escherichia coli* O157:H7 *gfp*+ in rocket and Swiss chard baby leaves as affected by abiotic and biotic damage

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Significance and impact of the study

Contamination of leafy vegetables with *E. coli* O157:H7 is a growing problem, as reported outbreaks are increasing. However, establishment of this human pathogen in the phyllosphere is not completely understood. Using laser scanning confocal microscopy, we demonstrated that *E. coli* O157:H7 *gfp*+ can invade plant tissue of Swiss chard and wild rocket leaves and that the bacterium is more sensitive to surface sanitization of Swiss chard leaves. Damage to leaf tissue promoted leaf invasion, but the nature of the damage (abiotic or biotic) and plant species had an impact.

Abstract

Internalization of human pathogens in edible parts of vegetables eaten raw is a major concern, since once internalized they are protected from sanitizing treatments. In this study, we examined invasion of *gfp*-labelled *E. coli* O157:H7 into intact and biotically (infection with *Xanthomonas campestris*/*Pseudomonas syringae*) and abiotically (grating with silicon carbide) damaged leaves of wild rocket (*Diplotaxis tenuifolia*) and Swiss chard (*Beta vulgaris* subsp. *cicla*) using laser scanning confocal microscopy. Bacterial cells were found in internal locations of the tissue, irrespective of tissue health status. Contaminated leaf sections of biotically and abiotically damaged wild rocket leaves showed higher susceptibility to microbial invasion, while the pathogen was internalized in greater

numbers into intact Swiss chard leaf sections when abiotically, but not biotically, damaged. The greatest differences were observed between the plant species; after surface sanitization, *E. coli* O157:H7 was still detected in wild rocket leaves, but not in Swiss chard leaves.

Keywords: Agriculture, EHEC, Food safety, *E. coli*, microbial contamination

Introduction

Although it is not a member of the indigenous microbiota of the phyllosphere, *E. coli* O157:H7 has increasingly been linked to outbreaks originating from consumption of leafy vegetables (Chitarra *et al.*, 2014; Beuchat, 2002). Leaves are known to be a harsh environment for bacteria due to low moisture content, ultraviolet (UV) irradiation, temperature fluctuations and limited and unevenly distributed nutrient supply (Monier & Lindow, 2005). Nevertheless, it has been shown that *E. coli* O157:H7 can persist in the phyllosphere (Mootian *et al.*, 2009). A particular concern is its ability to internalize plant tissue and thus gain protection from environmental influences and from washing and sanitizing processes during the production chain for vegetables eaten raw (Yaron, 2014).

As *E. coli* is not capable of breaking down pectin, a major constituent of plant cell walls (Teplitski *et al.*, 2012), it is reliant on existing loopholes when invading leaves (Deering *et al.*, 2012). Stomata have been identified as a preferred site for bacteria to persist on leaves (Mariano & McCarter, 1993) and have also been reported to function as a portal into the tissue (Takeuchi & Frank, 2001). Beside natural openings, cracks in the cuticle and more pronounced injuries to the leaf surface, of abiotic or biotic origin, can act as possible invasion routes for human pathogens (Deering *et al.*, 2012). Mechanical (abiotic) damage can occur at preharvest, during processing or at postharvest (Brandl & Amundson, 2008; Kays, 1999), while biotic damage is caused by plant pathogens (Park *et al.*, 2004; Koike *et al.*, 2003). *Xanthomonas campestris* pv. *campestris* is a vascular plant pathogen that causes black rot in *Brassica* species, e.g. wild rocket. It persists in the mesophyll and induces symptoms such as chlorosis and necrosis (Park *et al.*, 2004). In Swiss chard and other beets, bacterial infections caused by *Pseudomonas syringae* pv. *aptata* can result in leaf spot (Koike *et al.*, 2003). Infection with plant pathogens represents a possible invasion route for human pathogens into plant tissue (Beuchat, 2006).

Damaged plant tissue offers an entry point for bacteria to invade the plant, but also involves nutrient release (Beuchat, 2002). As nutrients are the limiting factor for survival in the phyllosphere (Brandl & Amundson, 2008), injured plant parts are favoured by microorganisms (Brandl & Amundson, 2008). It has been shown that bacteria preferably accumulate on cut edges of lettuce leaves and that accumulation of *E. coli* O157:H7 cells can occur in necrotic leaf tissue (Brandl & Amundson, 2008). Good nutrient availability results in higher survival rates of human pathogens on leaf surfaces (Aruscavage *et al.*, 2008). Moreover, a study by Solomon *et al.* (2002) found more bacteria invading a leaf on applying higher inoculum concentrations. Hence damage to leaf tissue has the potential to enhance internalization of human pathogenic bacteria in several ways.

The aim of this study was to examine internalization of *gfp*-labelled *E. coli* O157:H7 in intact and abiotically and biotically damaged leaves of Swiss chard and wild rocket by means of laser scanning confocal microscopy. Our starting hypothesis was that invasion by this human pathogen occurs to a greater extent in damaged compared with intact leaves.

Material and methods

Plant material and treatments

Swiss chard (*Beta vulgaris* subsp. *cicla,* SCR 107, AdvanSeed) and wild rocket (*Diplotaxis tenuifolia*, Tricia, Enzo) were grown in 10 L K-soil (bottom layer) and 3 L S-soil (top layer) in the greenhouse at an average temperature of 21°C and about 70% relative humidity. Additional lightning ensured a minimum photoperiod of 12 hours. Plants were manually irrigated and fertilized with Axan (27% N + 9% SO_3; Yara, Oslo, Norway) at an available amount of 81 kg N ha^{-1} when the first true leaves reached a length of 2 cm (week two after sowing). Leaves of 6-7 cm were inoculated with a plant pathogen (*Pseudomonas syringae* pv. *aptata* for Swiss chard and *Xanthomonas campestris* pv. *campestris* for wild rocket) by directly injecting the bacterial suspension into the leaf with the help of a syringe (disposable hypodermic needle, size 18, B. Braun, Melsungen, Germany). Symptoms appeared after 5-7 days. Mechanical damage was inflicted by grating leaves with silicon carbide (CAS: 409-21-2, Aldrich Chemistry, St. Louis, USA). Examples of intact and damaged leaves are displayed in Figure 6-3.

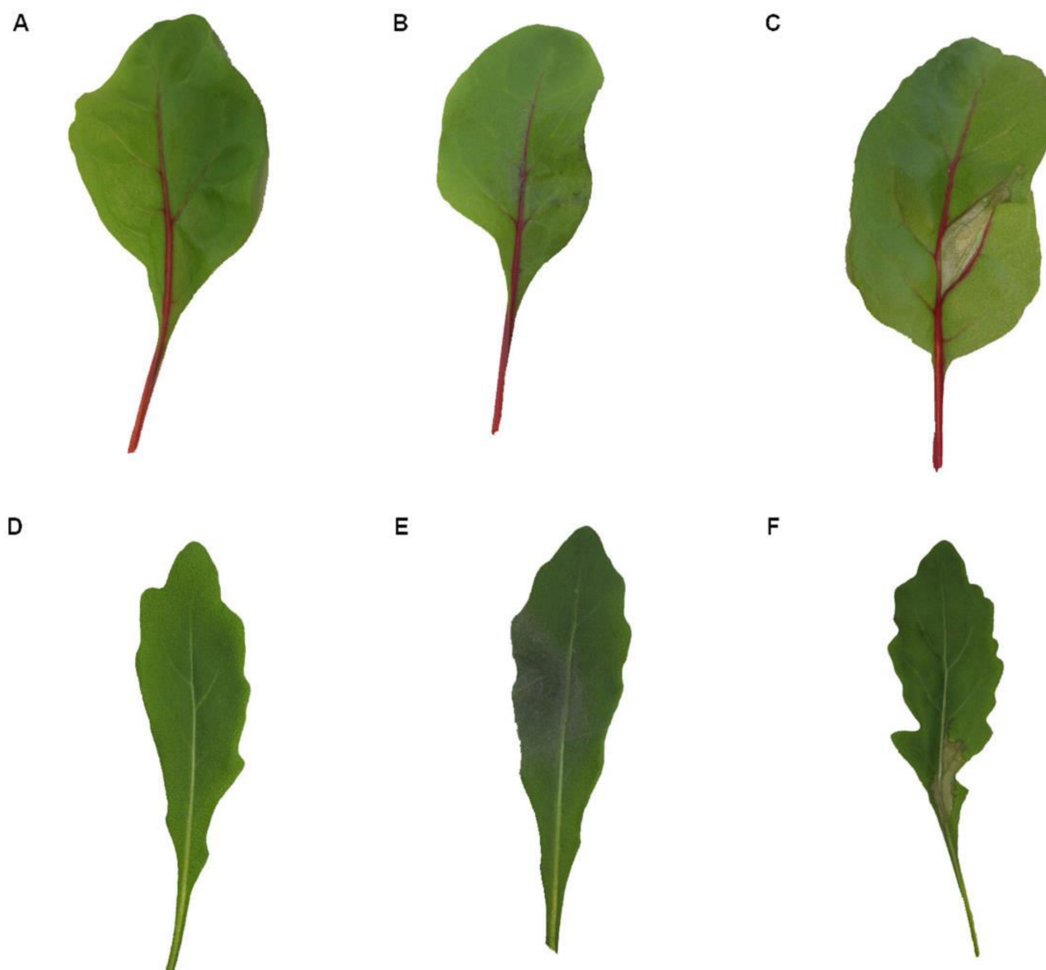

Figure 6-1. Leaves of Swiss chard (A-C) and wild rocket (D-F). A and D show intact leaves; B and E show leaves abiotically immediately after damage by silicon carbide; and C and F show biotic damage caused by *Pseudomonas syringae* pv. *aptata* and *Xanthomonas campestris* pv. *campestris*, respectively.

Microorganisms

Xanthomonas campestris pv. *campestris* (B027/24-R-92 G) possessing rifampicin resistance and *Pseudomonas syringae* pv. *aptata* (DSM-50252) with spontaneously induced rifampicin resistance were cultivated in tryptic soy broth (TSB). For this purpose, a single colony grown on tryptic soya agar (TSA) was picked and inoculated in 30 mL TSB with 100 µg (*X. campestris*) or 50 µg (*P. syringae*) rifampicin per mL, and incubated shaking (200 rpm) at room temperature for 1-2 days. The suspension was then centrifuged at 3000g and 4°C for 30 min and the pellet was washed in 10 mL of 0.85% NaCl and centrifuged at 3000g and 4°C for 15 min. The pellet was then re-suspended in 0.085% NaCl until approximately 10^7 CFU mL^{-1} were reached. A spectrophotometer (Expert 96™, Asys HiTech, Eugendorf, Austria) was used to determine the cell density (OD_{620}).

Escherichia coli O157:H7 (strain E81186) expressing *eae* but lacking virulence factors verotoxin-1 and -2 and labelled with a plasmid coding for ampicillin resistance and green fluorescent protein (*gfp*), which is emitted in the presence of arabinose (El-Mogy & Alsanius, 2012), was precultured on Lysogeny (LB) agar. A single *E. coli* O157:H7 colony was inoculated onto 30 mL LB broth enriched with 100 µg mL^{-1} ampicillin and 0.2% arabinose and incubated at 37°C overnight. The suspension was centrifuged at 3000g and 4°C for 45 min and washed in 0.85% NaCl, as already described for the two plant pathogens. The pellet was dissolved in 0.085% NaCl to an approximate optical density (OD_{620}) corresponding to 10^{10} CFU mL^{-1}, using a spectrophotometer (Expert 96™, Asys HiTech, Eugendorf, Austria). Wild rocket and Swiss chard plants with leaves that were still intact, with leaves showing symptoms of biotic damage and with leaves just after being subjected to abiotic damage were spray-inoculated with the *E. coli* O157:H7 suspension (100 mL m^{-2}).

Analysis

Contaminated intact leaves and leaves with abiotic and biotic damage were harvested one day after inoculation with *E. coli* O157:H7. The leaves were then surface-sanitized by washing in 0.25% sodium hypochlorite for about 20 s. Surplus solution was washed off with sterilized, deionised water. As no bacteria could be found on Swiss chard after surface sanitization, unsanitized contaminated leaves were prepared for microscopy. Small portions (approx. 0.5 cm x 0.5 cm) of intact and mechanically and biologically damaged sites on leaves were cut out and placed individually in the cavity of a microscopic slide (REF 13 200 02 Marienfeld, Germany) with phosphate buffered saline (PBS) + 50% glycerol and a cover slip. For visualization of fluorescent *E. coli* O157:H7 in the phyllosphere, a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany) equipped with a 40x, 1.3 oil-immersion DIC objective was used. Chlorophyll (and vasculature) autofluorescence and *gfp*-expressing *E. coli* O157:H7 were detected after exciting with an argon (488 nm) and helium-neon (543 nm) laser and passage through a band pass (505-530 nm) and a long pass (560 nm) filter.

Re-isolation

To confirm that the emerging symptoms on crop leaves were caused by inoculation with the two test plant pathogens, bacteria were isolated from injured leaves. This was done by harvesting a leaf showing symptoms and dipping it in 70% ethanol to kill microorganisms residing on the surface. After rinsing the disinfected leaf, an injured piece was cut out and shaken in 5 mL 0.85% NaCl at 200 rpm and room temperature for 30 min. Then 100 µL of the suspension were plated on TSA amended with rifampicin (100 µg mL^{-1} for *X. campestris* pv. *campestris* and 50 µg mL^{-1} for *P. syringae* pv. *aptata*) and incubated at 25°C.

Inhibition test

To exclude an inhibitory effect of the plant pathogens, through production of antimicrobial agents, on the *E. coli* O157:H7 test strain, an *in vitro* inhibition test was performed. For this purpose, a single colony of *X. campestris* pv. *campestris* or *P. syringae* pv. *aptata* was stroked on TSA in one line. A single colony of *E. coli* O157:H7 was then applied on the same plate in an orthogonal line just touching the other line in its centre. The plates were incubated overnight at 25°C. The distance between the two bacterial lines was taken as an indicator of inhibition. No inhibitory effect of the plant pathogens on *E. coli* O157:H7 was observed.

Results and Discussion

In intact Swiss chard leaves, surface sanitization before preparation for microscopy resulted in bacterial cell numbers that were too low to be visualized, despite several hours of prospecting. Hence the images displayed in Figure 6-1 A-C represent leaves without any previous sanitization treatment. In intact leaves, *E. coli* O157:H7 was found at depths down to 35 µm. The bacterial cells were located in intercellular spaces and vacuoles.

In abiotically damaged Swiss chard leaves, the bacterium was mainly found to be present as microcolonies in cavities, with bacterial cells found at depths down to 40 µm. In biotically damaged leaves, *E. coli* O157:H7 cells were found in microcolonies, mainly along intercellular cavities, at depths down to 45 µm, i.e. the greatest penetration depth observed in Swiss chard leaves.

In contrast to Swiss chard leaves, wild rocket leaves continued to harbor bacterial cells after surface sanitization (Figures 2D-F). On intact wild rocket leaves, single *E. coli* O157:H7 cells were most likely to be found next to stomata. The bacteria were below the surface and reached about 15 µm deep into the leaf tissue.

In abiotically damaged leaves, dense colonies located in cavities were seen. These colonies were found at depths down to 20 µm in the plant tissue. In biotically damaged wild rocket leaf tissue, *E. coli* O157:H7 was able to invade to depths of 25 µm and bacterial cells were found in both intercellular and intracellular locations.

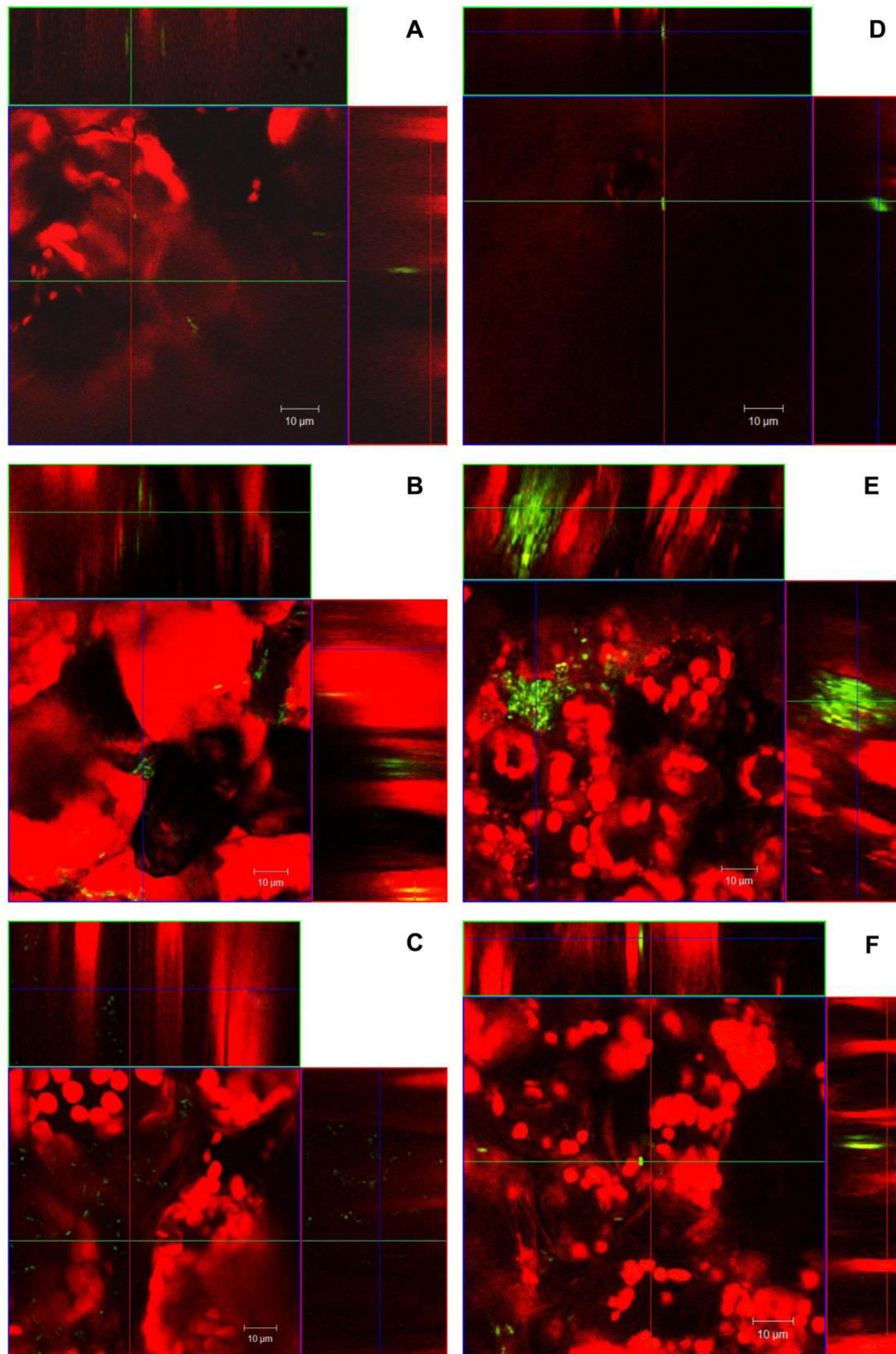

Figure 6-2. Laser scanning confocal microscope images of intact (A, D), abiotically damaged (B, E) and biotically damaged (C, F) leaf tissue of Swiss chard (A, B, C) and wild rocket (D, E, F) contaminated with *E. coli* O157:H7 cells (green fluorescence). Wild rocket leaves were surface-sanitized (20 s in 25% sodium hypochlorite), while Swiss chard leaves were not sanitized before microscopy. The images show an xy section (90 x 90 µm) with corresponding cross-sections to the red and green lines.

Several previous studies have found evidence that *E. coli* O157:H7 is able to invade edible parts of leafy greens. For example, in a study using laser scanning confocal microscopy, Solomon *et al.* (2002) detected this pathogen at depths down to 45 µm below the leaf surface in lettuce plants fertilized with contaminated manure or irrigated with contaminated water. Another study was able to detect *E. coli* O157:H7 in lettuce leaves using culture-dependent methods, but only after previous enrichment (Mootian *et al.*, 2009). Plant species dependency of the ability of this human pathogen to internalize in plant tissue was also demonstrated by Chitarra *et al.* (2014), who found that *E. coli* O157:H7 was able to invade rocket and various salad plants but not basil, which they attributed to antimicrobial agents in the latter inhibiting the pathogen. The viable cells we observed within leaf tissue of unsanitized Swiss chard in the present study appeared to be located in cavities, but were apparently not protected from the sanitizing agent due to surface characteristics of the chard leaf. Swiss chard leaves are covered with a waxier surface and express fewer trichomes than rocket leaves and this protracts wetting of the cuticle, so sanitization treatment is more efficient (Keeratipibul *et al.*, 2011). The plant leaf cuticle consists of aliphatic hydrocarbons, ketones, esters, fatty alcohols, fatty acids and aldehydes (Naumoska & Vovk, 2015) and its composition varies with plant species and leaf age (Holloway, 1970). Differences in these surface substituents may result in varying hydrophobicity of the leaf (Holloway, 1970). Thus analysis of cuticle wax can help predict the efficacy of a sanitizing agent on a particular plant species. No such data are currently available for baby wild rocket and Swiss chard. Takeuchi and Frank (2001) demonstrated a considerable impact of leaf surface structures on protection of human pathogens from sanitizing agents, e.g. they found a higher survival rate of bacteria that internalized deeper into the tissue of lettuce leaves.

Tissue damage to leaves results in enhanced water availability and release of nutrients (León *et al.*, 2001), offering bacteria a more favorable niche in the otherwise harsh environment of the phyllosphere. However, plants respond by activation of various genes in order to invoke wound healing and to mobilize defense mechanisms, resulting in metabolic changes upon wounding (León *et al.*, 2001). Beside the upregulation of ethylene, jasmonic acid and abscisic acid pathways, antimicrobial secondary metabolites like flavonoids, alkaloids and phenylpropanoid are increasingly produced (Cheong *et al.*, 2002). Furthermore, reactive oxygen species, encompassing hydrogen peroxide (H_2O_2) and superoxide (O_2^-), are synthesized to defeat bacteria (Maffei *et al.*, 2006; Orozco-Cardenas & Ryan, 1999). Whether a human pathogen can establish at a wound site and benefit from the enhanced water and nutrient availability depends upon its capacity to cope with the physiochemical conditions (Kyle *et al.*, 2010). Our hypothesis that bacteria invasion occurs to a greater extent in damaged compared with intact leaves was partly supported by the results obtained. For instance, we observed *E. coli* O157:H7 *gfp*+ invading damaged and intact leaf tissue, but accumulation of bacterial cells could only be observed in damaged tissue (Figure 6-2), indicating more favorable conditions for bacterial survival and/or multiplication. Several other studies support the observation that tissue damage promotes microbial establishment in

the phyllosphere. Aruscavage *et al.* (2008) compared the persistence of *E. coli* O157:H7 on abiotically or biotically damaged and intact lettuce leaves and found that the numbers of bacterial cells decreased more rapidly on the intact leaves. Han *et al.* (2000) compared the effect of surface sanitization on uninjured and injured green pepper and found that the treatment was less effective on the latter. Kyle *et al.* (2010) revealed upregulation of genes in *E. coli* O157:H7 when exposed to physiochemical conditions present in wounded lettuce leaves, resulting in enhanced antimicrobial resistance and withstanding of oxidative stress. The expression of these factors not only affects the defense against plant metabolites, but also improves the resistance to sanitizing agents (Kyle *et al.*, 2010).

Interestingly, biotically damaged Swiss chard leaves were more susceptible to invasion than abiotically damaged leaves, while the opposite was found for wild rocket leaves, although no inhibitory effect on the *E. coli* O157:H7 test strain was observed *in vitro* for either plant pathogen used. In future studies, the effect of other microorganisms on *E. coli* O157:H7 establishment in leaves should be considered, since competition for e.g. the same nutrient source might be enhanced in the phyllosphere.

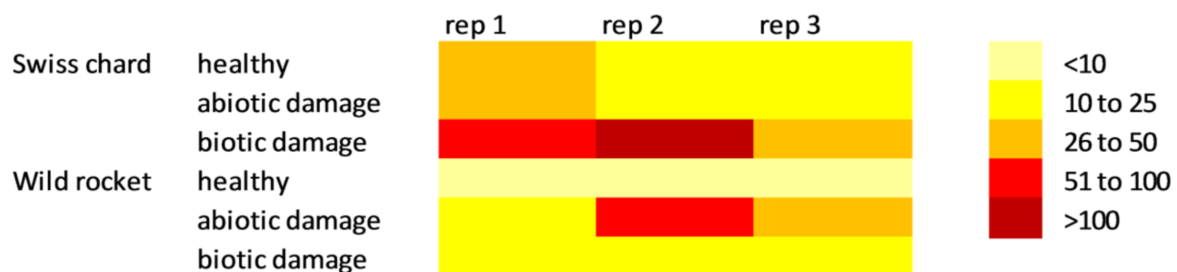

Figure 6-3. Number of *gfp*-tagged E. coli O157:H7 cells that invaded into 90 x 90 µm2 portions of leaf tissue as determined by laser scanning confocal microscopy. Wild rocket leaves were surface-sanitized (20 s in 25% sodium hypochlorite), while Swiss chard leaves were not sanitized before microscopy. Numbers are not representative of the entire leaf, as contaminated spots were selected.

The high inoculum levels of *E. coli* O157:H7 used in this study are unlikely to occur during normal commercial plant production. Nevertheless, bacteria often encounter the phyllosphere in particle-bound form and thus the concentration per droplet is realistic (Alam *et al.*, 2014; Stephens *et al.*, 2009). The possibility of *E. coli* O157:H7 invading edible leaf tissue, and thus being protected from the washing procedure, poses a considerable health threat, as even a small number of viable cells is sufficient to cause infection in humans (Rheinbaben, 2011). In conclusion, plant tissue damage may increase invasion of the leaf by human pathogens and the bacterial cell densities that accumulate in individual cavities may be high enough to induce disease in humans consuming the produce. However, the origin of the injury (biotic, abiotic) and the plant species involved are decisive parameters for internalization. Trichomes expressed by wild rocket leaves might preserve bacteria from surface sanitation, while the waxy surface of Swiss chard was not protective.

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Chapter 7: General discussion

Impacts on phyllosphere colonization

Human pathogenic bacteria are no common colonizers of the phyllosphere (Chitarra *et al.*, 2014). Their immigration and establishment in this biotope is significantly influenced by its habitants (Rastogi *et al.*, 2013) and surveying this microbial community and the identification of impacts on its equilibrium can as well help understand the dynamics of human pathogens on leaves. This thesis thus deals with phyllosphere microbiology and the establishment of *E. coli* O157:H7 on leafy greens. Heterotrophic bacterial plate counts determined in this thesis on vegetable leaves grown in the open field as well as the greenhouse were within the common range of 10^5 to 10^7 CFU (g fresh weight)$^{-1}$ as reported by Francis *et al.* (1999). These deviations might be viewed from the perspective of environmental conditions prevailing at the growing site (in- or outdoor), the crop (species, cultivar) and the crop management.

Climatic factors

The community of phyllosphere bacteria is shaped by climatic factors (Bulgarelli *et al.*, 2013; Rasche *et al.*, 2006; Kinkel, 1997). Light quality affects the microbial phyllosphere community structure (Alsanius et al., 2017). Natural light implies UV irradiation, which is harmful to several bacteria, while others are less susceptible (Brandl, 2006; Sundin & Jacobs, 1999). Also, sunshine often accompanies high temperatures that impair or enhance multiplication, depending on the bacteria and the temperature level (De Bonis & Ruocco, 2016). Rainfall implies cooling and has the potential to introduce microorganisms to the phyllosphere through soil splash and wash off other ones (Whipps *et al.*, 2008). Thus, climate factors influence the leaf microbiota qualitatively and quantitatively. Accordingly, we found temperature and precipitation to impact phyllosphere bacteria as determined by culture-dependent methods (Chapter 3). Yet, these factors cannot explain all variations between the sampling incidents, what can be due to (I) other climatic factors, e.g. humidity, that also impact the microclimate on the leaves (O'brien & Lindow, 1989) and (II) the impact of the growing stage of the crop on the leaf microbiota (Rasche *et al.*, 2006).

Beside the different impacts posed by these factors, climatic conditions are highly fluctuating and the phyllosphere thus represents a harsh environment for its habitants (Thompson *et al.*, 1993). By contrast, in a greenhouse fluctuations of environmental factors like temperature, humidity and rain can be controlled to a certain extend and are hence less pronounced than in an open field. Further, the natural light is filtered through greenhouse coverage and thus the incoming spectrum of the light is altered. Especially UV-irradiation, which is harmful to a couple of microorganisms (Heaton & Jones, 2008), is less abundant and hence survival can be enhanced. Nonetheless, we found 1-2 Log higher heterotrophic bacterial plate counts on field grown as compared to greenhouse cultivated wild rocket and Swiss chard leaves (Chapters 3, 4, 5).

Differences were seen between the occurrence of *E. coli* applied through high risk treatments in the field (Chapter 3) and *E. coli* O157:H7 *gfp+* applied in the greenhouse (Chapter 4, 5). The establishment of *E. coli* on field grown leafy vegetables was only occasionally observed and not attributable to high risk treatments, although high risk input factors (chicken manure, pond water) were considerably contaminated with *E. coli* and differences were evident for soil sampled from plots relating to their fertilization. Nevertheless, absolute contamination was higher for greenhouse grown plants as compared to crops grown in the open field. The fact that the establishment of *E. coli* O157:H7 *gfp+* was improved in the greenhouse and through the application of higher contaminated input material can just partly explain the inconsistencies between the trials. While Islam *et al.* (2004a) found *E. coli* O157:H7 to establish on field grown leafy greens, the amount of exposure with *E. coli* for high risk treatments in our open field trail was comparable with a study by Mootian *et al.* (2009). Hence also intrinsic factors of different *E. coli* strains could have an influence on the persistence on leaves. Comparison of the genome from pathogenic *E. coli* O157:H7 and the non-pathogenic strain K-12 revealed *E. coli* O157:H7 to have considerably more horizontally transferred DNA and additional pathways for transport and synthesis of some carbon hydrates (Hayashi *et al.*, 2001). Durso *et al.* (2004) compared commensal *E. coli* strains and *E. coli* O157:H7 strains and found the latter to produce more inhibitory substances and to have a higher antimicrobial resistance. Accordingly, some of the changes and additions in the genome of pathogenic *E. coli* could turn out to be useful tools in struggling the harsh environment in the phyllosphere, where nutrients and thus the competition with other microorganisms count as the limiting factor for bacterial survival.

Crops

Differences between crops were seen for the absolute numbers of the heterotrophic bacterial plate count (Chapter 3, 4, 5) as well as the course during cultivation (Chapter 3). With progressing leaf age, cracks in the cuticle release more nutrients and more bacteria can feed from these exudates. This could either enhance microbial multiplication of already present bacteria or ease the establishment of new arrivals (Kinkel, 1997). In accordance, the heterotrophic bacterial plate count increased during the growing periods for wild rocket, but contradictory, the microbial load on iceberg lettuce was decreasing. Brandl and Amundson (2008) suggested a higher abundance of trichomes on young iceberg lettuce leaves as compared to older leaves to play a role in nutrient availability. Like in this study, they found higher microbial loads on immature as compared to older iceberg lettuce leaves. No trend was visible for numbers of heterotrophic bacterial plate counts on Swiss chard, likewise a study conducted by Thompson *et al.* (1993), who suggested climate conditions to have a greater influence on bacterial numbers than leaf characteristics.

General differences found between crops could be owed to differing leaf characteristics such as topography, number of trichomes and stomata, which also impact the wettability and thus the availability of water (Hunter *et al.*, 2010; Leveau, 2006; Yaron *et al.*, 2000). In addition, head formation, like typical for iceberg lettuce, leads to weak boundary layer effects, which shape the microclimate on leaves (Zhang

et al., 2016). But not only different crops vary in their characteristics, also cultivars within one species can have distinctive attributes which can result in specific microbial colonization (Hunter *et al.*, 2010). In addition to incomparable climatic conditions (see previous section), different cultivars might have contributed to a discrepancy between open field and greenhouse cultivated crops. Also, this might apply in a different manner depending on the crop, which could explain why wild rocket showed higher variations in comparison to Swiss chard. In accordance, also analyzed seeds varied in dependence of crop and cultivar (Chapter 2).

Plate count, as used in the field trial (Chapter 3), presents a suitable method to determine viable bacterial cells of a community (Hugenholtz & Pace, 1996). Yet, culturable microorganisms only display a small portion of the phyllosphere microbiome. Culture-independent methods target the entirety of microorganisms, but also dead and therewith inactive cells (Josephson *et al.*, 1993). Thus, the combination of culture-dependent and -independent methods is appropriate to get a full picture of leaf colonization (Alsanius *et al.*, 2017a). Beside culture-dependent methods, metagenomics analysis was conducted targeting the 16S rRNA gene in the greenhouse trial. Likewise the culture-dependent analysis, differences between crops were more pronounced as compared to the treatments. Looking at the core microbiome, Swiss chard and wild rocket baby leaves share common genera, but differ in others (Chapter 5), confirming crop specific characteristics to have an influence on the phyllosphere microbiome. Interestingly, *Pantoea* was found to belong to the core microbiome of Swiss chard but not rocket baby leaves (Chapter 5), while simultaneously species belonging to *Pantoea* were isolated from a nutrient agar selective for *Enterobacteriaceae* (VRBD) only from bacterial suspensions gained from Swiss chard baby leaves (Chapter 4). These findings are an example for the complementary character of the different methods applied in this thesis.

Treatments

In all conducted trials, no consistent differences were found for the applied cultivation methods. In addition, mean values for the different applied treatments in the field trial did not exceed an interval of 0.6 Log CFU (g fresh weight)$^{-1}$ and are thus, even if occasionally statistically significant, not relevant for everyday practice. The fact that the few statistically significant differences were not consistent and seemed to be rather random could be owed to the natural occurrence of the considered bacterial genera and families on leaves and/or their surrounding environment. Interestingly, additional introduction of *Enterobacteriaceae*, *Listeria* spp. enterococci to the soil with manure did not result in a higher abundance, neither in the soil (data not shown) nor on the leaves, indicating that (i) the microbial equilibrium is dictated by other factors, (ii) these bacteria were not able to compete with the present microbiota and (iii) could not cope the changing conditions. This is supported by the findings that contamination of iceberg lettuce seeds with *L. monocytogenes* (Chapter 2) did not result in an elevated level on the leaves as compared to Swiss chard and wild rocket (Chapter 3). However, only the epiphytic colonization was analyzed in this trial and bacterial cells might have persisted endophytically (Warriner *et al.*, 2003a). Also, *Listeria* spp. was enhanced on field grown iceberg lettuce and greenhouse cultivated

wild rocket when fertilized with pig hair pellets as compared to chicken manure, although the latter harbored considerably more of these bacteria, compared to pig hair pellets and the pure soil (data not shown).

Positive tested samples for both, *E. coli* and *L. monocytogenes*, were among all treatments and crops and not elevated for manure applied lots or iceberg lettuce seeds, respectively, indicating additional contamination routes that have not been considered in this study. To determine if some of the found indicator species were transmitted from input means to the leaves, the use of DNA based identification methods should be considered for further research. The comparison of genome sequences, for instance, can show the phylogenetic distance and thus the likelihood of direct ancestry from isolates from input material to isolates gained from leaves.

Unfortunately, further identification of the great number of *Salmonella*-characteristic bacteria identified on field grown salad was not planned and thus not conducted (Chapter 3). Analysis of selected colonies presumptive to be *Salmonella* from greenhouse cultivated crops, however, uncovered them to be mainly *Pseudomonas* (Chapter 4) – a genus which should be inhibited on the chosen medium due to the addition of cefsulodin (Rambach, 1990). Resistance to this antibiotic in *Pseudomonas* is often mediated by a plasmid, encoding a gene for ß-lactamase (Livermore & Yang, 1989). Besides, bacteria encode multidrug efflux pumps on their chromosomes that are expressed dependent on the surrounding environment. Interestingly, the highest incidents are found in microorganisms associated with soil occupied by plants. The main aim of these kinds of efflux pumps is considered to affect the behavior of the bacteria in the environment, but, as a side effect, they promote antibiotic resistance (Martinez *et al.*, 2009).

Applying PCA (different plant parameters, microbial colonization of leaves), greenhouse grown wild rocket (Chapter 4, 5) and Swiss chard (Chapter 4) was grouped according to the different treatments, showing the impact of the fertilizer regime on leaf characteristics and the microbial community. However, this clustering could not be observed for field grown crops. Here, a defined grouping according to the sampling incident was seen, suggesting that possible impacts of the treatments on the microbial community were masked by other factors (see previous sections).

Isolation of microorganisms from leaves (Chapter 4) that show similarity to facultative pathogenic bacteria, e.g. *Enterobacter cloacae*, *Enterobacter cancerogenus*, *Bordetella avium* or *Escherichia coli*, illustrate the possibility for some health threatening bacteria to establish in the phyllosphere.

Epi- and endophytic colonization of vegetable leaves

Nutrient abundance counts as the limiting factor for bacterial growth in the phyllosphere (Kinkel, 1997) and thus it seems likely, that also the establishment of human pathogenic bacteria on leafy vegetables is affected by the availability of nutrients. Accordingly, surplus nutrients, provided through tissue damage, resulted in a better establishment of *E. coli* O157:H7 endophytically (Chapter 6). A study by Brandl and Amundson (2008) suggests nitrogen to be the confining force for the persistence of *E. coli* O157:H7 on

young lettuce. This could not be supported by the results obtained within the framework of this thesis, where the abundance of *E. coli* O157:H7 on baby leaves was not influenced by the leaf nitrogen content. Although nitrogen content of leaf tissue seems to be mirrored in the leaf exudates (Brandl & Amundson, 2008), the direct extrapolation has to be regarded with care, as exterior influences might affect the composition. Unfortunately, the carbon content of the leaf tissue was not determined in this study, why a possibly carbon dependent establishment of *E. coli* O157:H7 (Wilson & Lindow, 1994) cannot be verified.

Viable counts of *E. coli* O157:H7 were correlated to leaf dry weight content of micronutrients (Mn and Fe) and for Mg, indicating the impact of nutrients - other than nitrogen or carbon - on phyllosphere colonization of *E. coli* O157:H7. Yet, this influence might be indirectly, as the named nutrients play an important role for photosynthesis (Marschner & Rimmington, 1988). Thus, plant performance is likely to be the decisive factor. Although a dependency between some micronutrients and the establishment of *E. coli* O157:H7 was found, the number between treatments did not alter significantly. These findings are thus interesting for further research studies, but not significant for the choice of the organic fertilizer regime.

As shown in the field trial (Chapter 3) and by others (Rasche *et al.*, 2006), the composition of phyllosphere microbiota is dynamic over time/growing stages. It has been shown, that species and families might have an impact on the establishment of human pathogens in the phyllosphere (Lopez-Velasco *et al.*, 2012). Thus, the time of introduction of a human pathogen might play a role for its establishment. But not only individual groups, also the microbial network structure might impact the colonization of *E. coli* in a plants environment (Cardinale *et al.*, 2014). Successful invasion of plant pathogens is influenced by the microbial diversity (Balint-Kurti *et al.*, 2010), and the invasion of *E. coli* O157:H7 is negatively impacted by a higher diversity of microbiota in soil (van Elsas *et al.*, 2012). In contrast, in this thesis, rocket baby leaves revealed to have a lower diversity of phyllosphere microbiota as compared to Swiss chard (Chapter 5), while more *E. coli* O157:H7 cells were harvested from Swiss chard baby leaves. At first sight inconsistent results for the occurrence of *E. coli* O157:H7 are also seen, when considering the greenhouse (Chapter 4, 5) and the internalization trial (Chapter 6). In contrast to the application of culture-dependent methods, looking at the invasion under the confocal microscope, the bacterium persisted considerably better in rocket. This might be explained by its potentially stronger attachment to rocket leaves and thereby less successful removal. As a non-destructive method was used to bring the phyllosphere bacteria in suspension, strongly attached bacteria might not have been captured by the applied culture-dependent method.

Conclusion and future perspective

Phyllosphere microbiology is a complex field, influenced by an abundance of different factors. Several of these factors are discussed in this thesis, including climate conditions, cultivation methods and crop specific characteristics. Even small shifts in the surrounding environment can have a great impact on

the bacterial habitants of the leaf surface and the predominating influence might change. Thus, studies addressing microbial communities on plants must cope with unsteadiness. Although various studies (Rastogi *et al.*, 2013; Fischer-Arndt *et al.*, 2010; Wilson & Lindow, 1994), including this thesis, demonstrated the impact of different factors on microbial colonization in the phyllosphere, the predominant factor/interactions under different conditions remains to be elucidated.

Only few studies (Fischer-Arndt *et al.*, 2010; Ailes *et al.*, 2008) cope with abiotic factors with respect to dynamics in phyllosphere microbiota. Future trials should take also factors like UV-irradiation, wind, soil properties in addition to air temperature and precipitation (Chapter 3) into account.

While several studies, that found differences in the establishment of human pathogens on vegetable species, assumed antimicrobial substances to be the causal influence (Chitarra *et al.*, 2014; Oussalah *et al.*, 2007), we show, that also other crop specific characteristics should be taken into account. These include microclimate, leaf topography and plant/leaf nutrition status. In this context, it should be stressed, that low and high risk scenarios should not only concentrate on the route of transmission, but also on factors influencing the persistence of human pathogens in the phyllosphere.

The combination of different microbial assessment methods turned out to be an appropriate tool for phyllosphere microbial analysis. The employment of different methods resulted in complementary as well as contradictory results, which turned out to be helpful with respect to (i) verification, (ii) falsification, (iii) interpretation of results and (iv) evaluation of the used method. The development, improvement and reduced costs for sequencing methods have led to a new view on phyllosphere colonization in recent years. As this trend is still ongoing, appropriate tools future research will be available, that help disentangle the complex field of phyllosphere microbiology.

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