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ORIGINAL ARTICLE

Differential interaction of *Salmonella enterica* serovars with lettuce cultivars and plant-microbe factors influencing the colonization efficiency

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The availability of knowledge of the route of infection and critical plant and microbe factors influencing the colonization efficiency of plants by human pathogenic bacteria is essential for the design of preventive strategies to maintain safe food. This research describes the differential interaction of human pathogenic *Salmonella enterica* with commercially available lettuce cultivars. The prevalence and degree of endophytic colonization of axenically grown lettuce by the *S. enterica* serovars revealed a significant serovar–cultivar interaction for the degree of colonization (*S. enterica* CFUs per g leaf), but not for the prevalence. The evaluated *S. enterica* serovars were each able to colonize soil-grown lettuce epiphytically, but only *S. enterica* serovar Dublin was able to colonize the plants also endophytically. The number of *S. enterica* CFU per g of lettuce was negatively correlated to the species richness of the surface sterilized lettuce cultivars. A negative trend was observed for cultivars Cancan and Nelly, but not for cultivar Tamburo. Chemotaxis experiments revealed that *S. enterica* serovars actively move toward root exudates of lettuce cultivar Tamburo. Subsequent micro-array analysis identified genes of *S. enterica* serovar Typhimurium that were activated by the root exudates of cultivar Tamburo. A sugar-like carbon source was correlated with chemotaxis, while also pathogenicity-related genes were induced in presence of the root exudates. The latter revealed that *S. enterica* is conditioned for host cell attachment during chemotaxis by these root exudates. Finally, a tentative route of infection is described that includes plant-microbe factors, herewith enabling further design of preventive strategies.
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Introduction

Salmonella enterica subspecies *enterica* (designated as *S.* enterica) are some of the most commonly known bacterial pathogens which cause human illness. Often the disease is associated with the consumption of contaminated foods like pork or poultry meat and eggs or egg products. Since recently, many human pathogenic organisms have been recognized to exist on plant root or leaf surfaces (Lyytikainen *et al.*, 2004; Brandl, 2006), and even inside plant tissues (Kutter *et al.*, 2006; Rosenblueth and Martínez-Romero, 2006). For example, outbreaks of Salmonellosis have increasingly been traced back to contaminated fresh produce (Viswanathan and Kaur, 2001; Sivapalasingam *et al.*, 2004). For greenhouse grown produce, enteric pathogens are mainly introduced as a result of bad hygiene (Beuchat and Ryu, 1997). In the field however, contamination of vegetable crops may occur via soil amended with manure from agricultural animals which are known reservoirs for *Salmonellae* (Viswanathan and Kaur, 2001; Natvig *et al.*, 2002). Both manure and irrigation water contribute significantly to the spread of human pathogens onto fields and the crops growing there (Natvig *et al.*, 2002; Solomon *et al.*, 2002; Islam *et al.*, 2004).

In recent years, it became evident that serovars of *S. enterica* are not only able to attach to and

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proliferate on the surface of plant tissues (Zenkteler *et al*., 1997; Solomon *et al*., 2002) but can also colonize plant tissues internally (Kutter *et al*., 2006). For example, gfp-tagged strains of *S. enterica* colonized the interior of tomato plants when grown hydroponically (Guo *et al*., 2001; Guo *et al*., 2002) and various *S. enterica* serovars were able to colonize *Medicago sativa* and other leguminous plants endophytically and epiphytically (Dong *et al*., 2003; Wang *et al*. 2006). Also, an avirulent strain of *S. enterica* serovar Typhimurium colonized carrots and radishes which were grown on a field treated with *S. enterica* serovar Typhimurium-contaminated composted manure or irrigation water (Islam *et al*., 2004). Just recently, *S. enterica* serovar Typhimurium LT2 and DT104h were found to endophytically colonize barley sprouts during growth in an axenic system (Kutter *et al*., 2006), and various enterobacteria, including *S. enterica*, were found to be natural endophytes of *Conzattia multiflora* (Wang *et al*., 2006).

Typically, plant defense responses upon colonization by these pathogens were observed, for example during colonization of *Medicago truncatula* by *S. enterica* serovar Typhimurium which resulted in the induction of salicylic acid—dependent and—independent plant defenses (Iniguez *et al*., 2005). The induction of salicylic acid plant defense pathway (salicylic acid resistance) appeared correlated to the expression of bacterial genes for TTSS-SPI effector proteins, whereas the presence of flagella only induced the SA-independent plant defense (induced systemic resistance). A recent study involved the molecular response of axenically grown lettuce to colonization by *S. enterica* serovar Dublin, from which a differential expression was indicated of various virulence and pathogenicity-related genes of lettuce, over time (Klerks *et al*., 2007).

From these studies it is evident that human pathogens like *S. enterica* are able to colonize fresh produce endophytically and epiphytically and interact at a molecular level with the host plant. Some previous research has been described investigating the conditions required for plant colonization by human pathogens (Rosenblueth and Martínez-Romero, 2006; Toth *et al*., 2006). Concerning the bacterial genes that are required for attachment to plant roots, some genes have been identified to be crucial for attachment (Barak *et al*., 2005). However, fundamental ecological questions concerning the route of infection of lettuce by *S. enterica* and ecological factors influencing the colonization efficiency have not yet been described. Such data are of main importance to understand the mechanism of transmission, which in its turn is required to define preventive actions to reduce or even eliminate the risk of food contamination originating from agricultural systems.

This research studied plant and microbial factors that influence the colonization efficiency of a set of epidemiologically important human pathogenic *S. enterica* serovars in association with commercially important lettuce. The effect of differences in cultivars and *S. enterica* serovars on colonization efficiency was investigated with respect to prevalence and degree of colonization. The role of the natural endophytic microflora in determining plant susceptibility was assessed by performing correlation analyses between the Shannon index (H) or the species richness and the number of *S. enterica* CFU per g lettuce. Finally, the contribution of root exudates to colonization efficiency of lettuce by *S. enterica* serovars was tested and bacterial genes that were induced over time in the presence of root exudates were identified by micro-array analysis.

Materials and methods

Plant material and bacterial strains

Liquid cultures of *S. enterica* serovar Dublin, *S. enterica* serovar Typhimurium, *S. enterica* serovar Enteritidis, *S. enterica* serovar Newport and *S. enterica* serovar Montevideo, were kindly provided by Dr H Aarts (RIKILT, The Netherlands) after overnight growth at 30 °C in tryptic soy broth. The cultures were maintained by plating on selective Hektoen enteric agar (Biotec Laboratories Ltd, Ipswich, Suffolk, UK) and were increased by overnight enrichment at 37 °C in buffered-peptone water (BPW).

Seeds of commercially available lettuce cultivar Tamburo, Nelly and Cancan were kindly provided by Mr Raats (Nickerson-Zwaan, The Netherlands). The seeds were sterilized in a solution of 1% sodium hypochloride and 0.01% Tween-20, and then rinsed in water (twice) for 1 min each. Subsequently, the seeds were air-dried for 1 h and stored.

Association of Salmonella *serovars with lettuce Tamburo grown on* Salmonella*-contaminated soil*

To determine if *S. enterica* serovars are able to colonize lettuce seedlings via the roots under seminatural conditions, lettuce seeds (cultivar Tamburo) were planted on *S. enterica*-contaminated manure-amended soil in a greenhouse. Fresh manure was collected from a Dutch organic dairy farm. Soil was collected from a field (60 kg of top layer of 20 cm) from the organic experimental farm the Droevendaal (Wageningen, the Netherlands). The soil consisted of 89% sand, 8% silt, 3% clay, a total nitrate (N) and carbon (C) of 2135 and 22400 mg per kg, 11% moisture and had a pH of 7.14. The manure contained 28.7% acid detergent fiber, 40.3% neutral detergent fiber, a total dissolved organic N and C of 740 mg per kg and 8167, 220 mg per kg ammonium, 8.14 mg per kg nitrate and had a pH of 6.8. Both substrates tested negative for presence of *S. enterica*, which was determined by plating directly on selective Hektoen enteric agar and by testing the total DNA extracts from 10 ml BPW enrichments of three random samples of 1 g of each substrate using

real-time PCR analysis (Klerks *et al.*, 2004). The manure was first inoculated with *S. enterica* serovar Typhimurium, *S. enterica* serovar Enteritidis or *enterica* serovar *S.* Dublin (10^8 CFUs per g fresh weight) and mixed thoroughly before addition to soil at a ratio of 1:10 fresh weight. The final *S. enterica* cell density was 10^7 CFU per g mixture. In total, 74 pots of 50 ml of each contaminated soil–manure mixture were prepared per *S. enterica* serovar. The negative control pots (74) consisted of non-*S. enterica*-inoculated manure–soil mixture. One lettuce seed was added to each pot. The seeds were covered with soil after planting. All 296 pots were placed on saucers in a greenhouse with 16 h of artificial light at 18 °C and 80% humidity, and twice a day plants were watered carefully on the saucers to avoid splashing. After 6 weeks, each plant was harvested by cutting the plant at the stem just above the soil, weighed and thoroughly rinsed once in 30 ml of sterilized water prior to analysis. Each wash fraction was centrifuged (5 min at 6000 *g*), the pellet was re-suspended in 100 μl of BPW and 40 μl was plated on Hektoen enteric agar, in duplicate. Next, the plants of each treatment were randomly divided in two sets. Each plant of the first set of plants was ground in 1 ml of BPW. From the second set of plants each shoot was surface disinfected in 70% ethanol and washed twice in sterile water prior to grinding in 1 ml of BPW (Klerks *et al.*, 2007). Of each suspension with ground plant material 40 μl was plated on Hektoen enteric agar, in duplicate.

Differential colonization of lettuce cultivars with S. enterica *serovars*

To evaluate whether the endophytic colonization efficiency of lettuce by *S. enterica* is dependent on the lettuce cultivar or the *S. enterica* serovar, three lettuce cultivars were tested with five *S. enterica* serovars for the degree of endophytic colonization. First, sterile seeds of three cultivars Cancan, Tamburo and Nelly, were sprouted in sterile 0.5% Hoagland's water agar (Sigma Aldrich, Irvine, UK) in 15 ml glass tubes and incubated for 2 weeks at 21 °C with light/dark intervals of 12 h in a closed container with glass cover and placed in a growth chamber. Next, 45 seedlings of each lettuce cultivar were inoculated with five *S. enterica* serovars (*S. enterica* serovar Typhimurium, *S. enterica* serovar Enteritidis, *S. enterica* serovar Dublin, *S. enterica* serovar Montevideo and *S. enterica* serovar Newport), resulting in nine replicates per combination in three blocks. A Fresh BPW culture of each *S. enterica* serovar was 100-fold diluted in sterile Hoagland's solution and 10 μl (10^7 CFU ml^{-1}) was carefully pipetted into the agar close to the roots of each lettuce seedling. Contact with leaves was avoided. After 7 days of incubation the shoots were harvested by removing the roots at the transition region. The shoot tissue of each plant was weighed and subsequently surface disinfected by washing for 10 s in 70% ethanol and rinsing twice with sterile water (Klerks *et al.*, 2007). Then, leaf tissue was thoroughly ground in cold BPW. For molecular analyses 100 μl of suspension was used to extract DNA using the Plant DNeasy DNA extraction kit (Qiagen, Westburg, Leusden, The Netherlands). A dilution series was prepared (non-diluted, 20 × and 200 × diluted) from the ground tissue suspension and 40 μl of each dilution series was plated in duplicate on *S. enterica*-selective Hektoen enteric agar and incubated overnight at 37 °C. The colonies grown from the dilution series were counted and a random selection of *S. enterica*-like colonies and other colonies (68 in total) from each of the cultivar–serovar combinations were enriched by growing overnight in BPW at 37 °C. The bacteria were pelleted from these enrichment cultures by centrifuging 500 μl at 6000 *g* for 5 min. Next, the DNA was extracted from the pellet according to the protocol of the microbiological DNA extraction kit (MoBio Laboratories, Solana Beach, CA, USA). The purified DNA was eluted in 100 μl of elution buffer and stored at −20 °C until further use.

Detection and identification of S. enterica

S. enterica serovars were isolated from surface disinfected lettuce seedlings by dilution plating on selective Hektoen enteric agar. Surface disinfection of lettuce plants was performed as mentioned above. Total DNA was extracted from surface-disinfected lettuce tissue that was ground in a BPW suspension. For DNA extraction the Plant DNeasy DNA extraction kit was applied on 100 μl of suspension that was added to 400 μl of lysis buffer supplied with the kit. Further treatment was according to the supplied protocol of the DNA extraction kit (Qiagen). The purified DNA was eluted in 200 μl of elution buffer and stored at −20 °C. DNA was isolated as mentioned above, followed by Taqman PCR amplification using primers and probe sequences that were described in Klerks *et al.*, 2004.

Relationship between Salmonella *colonization and the natural endophytic microbial community*

To investigate a possible relationship between endophytic colonization by *S. enterica* and natural endophytic microbial communities, the diversity index, Shannon index (*H*) (Shannon and Weaver, 1963) and the species richness of natural endophytic bacteria were determined for different lettuce cultivars. Denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993) was performed using the DNA extracts from three lettuce plants with high endophytic *S. enterica* populations and one *S. enterica*-negative plant of each serovar–cultivar combination from the previous experiment. These DNA samples (60 in total), and additional control DNA samples (DNA of each *S. enterica* serovar) were first subjected to 16S rDNA PCR using primers

directed to bacterial ribosomal DNA that excluded amplification mitochondria and chloroplasts 16S ribosomal DNA sequences (Chelius and Triplett, 2001). The PCR mix (final volume 24 µl) consisted of 200 µM dNTP, 3.75 mM $MgCl_2$, 1 × Stoffel buffer (Applied Biosystems, Foster City, CA, USA), 0.4 µM primer799F (Chelius and Triplett, 2001), 0.4 µM primer1492R (Lane, 1991), 0.05% bovine serum albumin and 0.1 U Amplitaq Stoffel polymerase. To each reaction tube 1 µl of the total DNA extract was added before PCR amplification was started. The PCR program was set at an initial incubation of 3 min at 95 °C, followed by 30 cycles of 20 s at 94 °C, 40 s at 53 °C and 40 s at 72 °C. The reaction was stopped after 7 min incubation at 72 °C.

The primary PCR was followed by a second PCR using the primers U968 (Engelen *et al.*, 1995) and R1378 (Heuer and Smalla, 1997); the latter contained a strong 5′-GC-clamp required for DGGE analysis. The second PCR mix (total volume of 49 µl) consisted of 200 µM dNTP, 1 × SuperTaq buffer (Applied Biosystems), 0.4 µM primerU968, 0.4 µM primer R1378 and 1 U SuperTaq polymerase. To each reaction mix 1 µl of primary PCR product was added and the second PCR was started with an initial incubation of 4 min at 94 °C. This was followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C. The PCR was stopped after 10 min incubation at 72 °C followed by 5 min incubation at 10 °C.

Of each second PCR product 20 µl was mixed with 10 µl of loading buffer and applied to the gradient gel. The 6% (w/v) polyacrylamide gels in 0.53 TAE buffer (20 mM Tris-acetate (pH 7.4), 10 mM sodium acetate, 0.5 mM disodium ethylenediamine tetraacetic acid) contained a linear denaturing gradient of 45–65% of urea and formamide. The gels were run for 15 h at 60 °C and 100 V. After electrophoresis, the gels were stained for 30 min with SYBR Gold I nucleic acid gel stain (Molecular Probes Europe, Leiden, the Netherlands) and bands were visualized using a Docugel V system apparatus with ultraviolet light (Biozym, Landgraaf, The Netherlands).

Response of Salmonella *serovars to root exudates of lettuce Tamburo*

To determine if root exudates affect the movement of *S. enterica* serovars, the occurrence of chemotaxis was studied. First, lettuce Tamburo plants were grown in Hoagland's agar (0.5%) for 4 weeks under axenic conditions. From each plant 1 g of agar was collected that contained root exudates. To remove the organic-soluble compounds from water-soluble compounds, 1 ml of water and 1 ml of ethyl acetate was added to the agar and ground using mortar and pestle. The suspension was centrifuged at max speed (8000 *g*) for 5 min. The upper layer (water-phase (WP)) was transferred to a new tube, 1 ml of fresh ethyl acetate was added and mixed thoroughly. After centrifugation the WP was transferred to a new collection tube and stored at −20 °C until further use. The final water-soluble exudate concentration was half the initial concentration in agar, as the final volume was similar to the volume of agar used for extraction of exudates.

For the chemotaxis experiments (modification of Adler, 1966), micro-capillaries (volume of 50 µl, diameter of 1 mm) were filled with 0.2% of Hoagland's agar, including 0.5% of the metabolism marker 2,3,5-triphenyl tetrazolium chloride to measure bacterial movement. In two separate experiments the WP of three lettuce plants (50 µl of WP per plant) were each tested in duplicate for chemotaxis of *S. enterica* serovar Typhimurium, *S. enterica* serovar Enteritidis, *S. enterica* serovar Dublin and water (in total 12 tubes per serovar). Of each *S. enterica* serovar 50 µl of 10^7 CFU ml^{-1} of phosphate buffer was added to 0.5 ml tubes. Phosphate buffer was used since it lacks any substrate, but retains the viability of the bacterial cells. One end of a capillary was positioned horizontally inside a 0.5-ml tube (also horizontal and sealed with parafilm) to allow contact with the bacterial suspension or control solution. The other end of the capillary was placed in another 0.5 ml tube containing the WP sample or negative control (water, Hoagland's solution or phosphate buffer), carefully covered with parafilm to prevent evaporation. The capillaries were incubated horizontally overnight at 37°C prior to observation of chemotaxis by color transition inside the capillaries.

Gene expression of S. enterica *serovar Typhimurium in the presence of root exudates*

To investigate the molecular response of *S. enterica* serovars to the presence of root exudates, gene expression of *S. enterica* serovar Typhimurium exposed to root exudates was analyzed over time. First, fresh enriched bacterial cultures of *S. enterica* serovar Typhimurium were prepared, centrifuged, washed twice with phosphate buffer and finally re-suspended in phosphate buffer. Four tubes were prepared containing 900 µl bacterial suspension. One tube contained the WP of plant exudates of one chemotaxis-inducing plant (cultivar Tamburo) and one tube contained a non-chemotaxis-inducing WP of another Tamburo plant, whereas the control tubes contained either 0.1% sucrose or water. The suspensions were then incubated at 37 °C in a heating block. At different time intervals 100 µl of each bacterial suspension was collected and immediately transferred to 350 µl of lysis buffer of the RNeasy RNA extraction kit. The time series consisted of 0, 10, 20 min postinoculation. Next, total RNA was extracted from 100 µl of the bacterial suspensions using the RNeasy RNA extraction kit (Qiagen). RNA was eluted in 100 µl of RNase-free water and stored at −80 °C until further use.

From the time series cDNA was prepared to allow gene expression analysis using a thematic

micro-array of *S. enterica* serovar Typhimurium designed to specifically detect virulence, growth and stress-related genes (Hermans, 2007). Each sample for cDNA synthesis was subjected to amino-allyl-dUTP labeling for subsequent Cy3 and Cy5 labeling, according to the protocol described by Hermans (2007). After cDNA synthesis and Cy3 or Cy5 labeling, the cDNA was precipitated according to general sodium acetate/ethanol precipitation. After drying of the pellet the cDNA was resuspended (in duplicate) in filter sterilized hybridization buffer (0.2% sodium dodecyl sulfate, 5 × Denhardt's solution, 5 × sodium saline citrate, 0.5 × formamide and 0.25 μg salmon sperm). Next, the samples were incubated for 10 min at 65 °C. Finally, for each cDNA sample the Cy3 reference labeled fractions of each sample were combined and mixed separately with each Cy5-labeled cDNA fractions at a 1:1 ratio. The cDNA samples were boiled prior to application to the micro-array. Specific hybridization was analyzed using the ScanArray 3000 confocal laser scanner (GSI Lumonics, Kanata, ON, Canada), measuring the fluorescence of each spot for Cy3 and Cy5 and four background areas around each spot. After calculating the signal-to-noise ratio of each spot (Hermans, 2007), the data were corrected for inter-chip and intra-chip variations and a specific labeling as described by Hermans (2007). The corrected micro-array expression profile of each gene was compared between the treatments (two root exudates, 0.1% sucrose and water).

Statistical analysis

χ^2 Analysis was performed to assess the difference between the number of emerged lettuce plants that were grown on *S. enterica*-contaminated manure-amended soil, and the plants grown on non-contaminated manure-amended soil. A similar analysis (χ^2 test) was performed to determine the difference between the *S. enterica* serovars with respect to the number of colonized plants.

To determine the effect of lettuce cultivar or *S. enterica* serovar on the prevalence of *S. enterica* CFU on/in lettuce seedlings a nonparametric test (Kruskall–Wallis) was performed. The interaction was determined by a χ^2 test on the basis of the number of positive plants (prevalence). To determine if there was an interaction between cultivar and serovar with respect to the degree of endophytic colonization of lettuce plants, univariate analysis of variance (including the interaction term cultivar* serovar) was performed on the number of CFU per g of fresh tissue.

The DGGE banding patterns were analyzed using Gelcompar II software (version 1.61; Applied Maths, Woluwe, Belgium) to allow comparison of the gels. Each gel contained four marker lanes for reference purposes and background corrections were performed prior to identification of bands with settings of 5% significance threshold. Correspondence of bands between different samples was performed with 1% dynamic range settings. The Shannon index of diversity (*H*) and species richness were calculated (Van Diepeningen *et al*., 2005) and correlated with the degree of endophytic colonization by *S. enterica* serovars ($\log \mathrm{CFU\,g^{-1}}$), using Pearson's correlation analysis (SPSS). In addition, the $\log \mathrm{CFU\,g^{-1}}$ was regressed on both the diversity parameters.

To determine the significance of the root exudate treatment relative to the controls, χ^2 analysis was performed on 54 capillary tubes which were positive or negative for chemotaxis of *S. enterica* serovars to root exudates. In total, 18 of these 54 tubes were controls without exudates (water, Hoagland's solutions or phosphate buffer). Relative attractiveness of exudates to different *S. enterica* serovars was also compared with a χ^2 test.

Results

Colonization by S. enterica *serovars of lettuce Tamburo grown on manure-amended soil*

Of the 74 seeds planted per *S. enterica* serovar, 70% lettuce plants emerged compared to 85% of the non-inoculated control plants. Of the emerged plants, 16 out of 56 were colonized with *S. enterica* serovar Dublin, 8 out of 48 with *S. enterica* serovar Enteritidis and 14 out of 53 with *S. enterica* serovar Typhimurium. The number of emerged plants was significantly different between the non-inoculated plants and the *S. enterica*-inoculated plants ($\chi^2 = 9.231$; $P = 0.026$). The number of colonized plants did not differ significantly among serovars ($\chi^2 = 2.056$; $P = 0.358$). The control plants were all negative for *S. enterica*. For *S. enterica* serovar Dublin 3 out of 28 plants were also positive for endophytic colonization after surface disinfection. No endophytic colonization was observed for surface-disinfected plants grown on soil contaminated with *S. enterica* serovar Enteritidis or *S. enterica* serovar Typhimurium. The endophytic colonization of lettuce plants grown on *S. enterica* serovar Dublin-inoculated manure-amended soil was only 164 CFUs per g lettuce for *S. enterica* serovar Dublin if averaged over all tested plants (Table 1).

Table 1 Mean of endophytic *Salmonella* CFU per serovar in association with lettuce Tamburo grown on manure-amended soil or Hoagland's agar, 6 weeks after planting of lettuce seeds

	Manure-amended soil	*Hoagland's agar*
	Mean CFU per g	*Mean CFU per g*
S. Dublin	164	40 800
S. Typhimurium	0	18 739
S. Enteritidis	0	8506

Abbreviation: CFU, colony forming unit.

Endophytic colonization of lettuce cultivars by S. enterica *serovars on Hoagland's agar*

The number of endophytically colonized plants (prevalence) was significantly affected by the *S. enterica* serovar ($P=0.024$) but not by the lettuce cultivar ($P=0.727$), as determined by the nonparametric Kruskall–Wallis test. The percentages of lettuce plants endophytically colonized were 59%, 85%, 93%, 85% and 89% for *S. enterica* serovars Dublin, Enteritidis, Montevideo, Newport and Typhimurium, respectively. There was no significant interaction between cultivar and serovar with respect to prevalence of *S. enterica* CFU in lettuce seedlings ($\chi^2=3.11$; $P=0.215$).

Univariate analysis of variance indicated that there was a significant interaction between *S. enterica* serovar and cultivar with respect to the degree of endophytic colonization (CFU per g leaf) ($P=0.047$). This suggested a difference in colonization pattern of a specific lettuce cultivar by the five *S. enterica* serovars, in particular *S. enterica* serovar Typhimurium colonized cultivar Nelly more easily than the other cultivars (Figure 1). The overall lettuce cultivar effect on internal colonization (CFU per g leaf) was not significant ($P=0.116$),

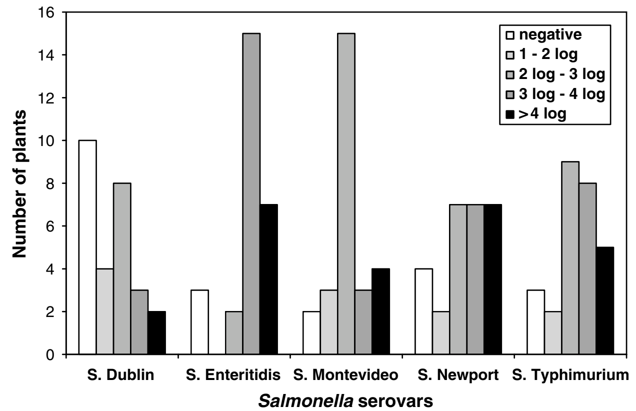

Figure 2 Number of lettuce seedlings in different classes of degree of endophytic *Salmonella* colonization for each *Salmonella* serovar was tested.

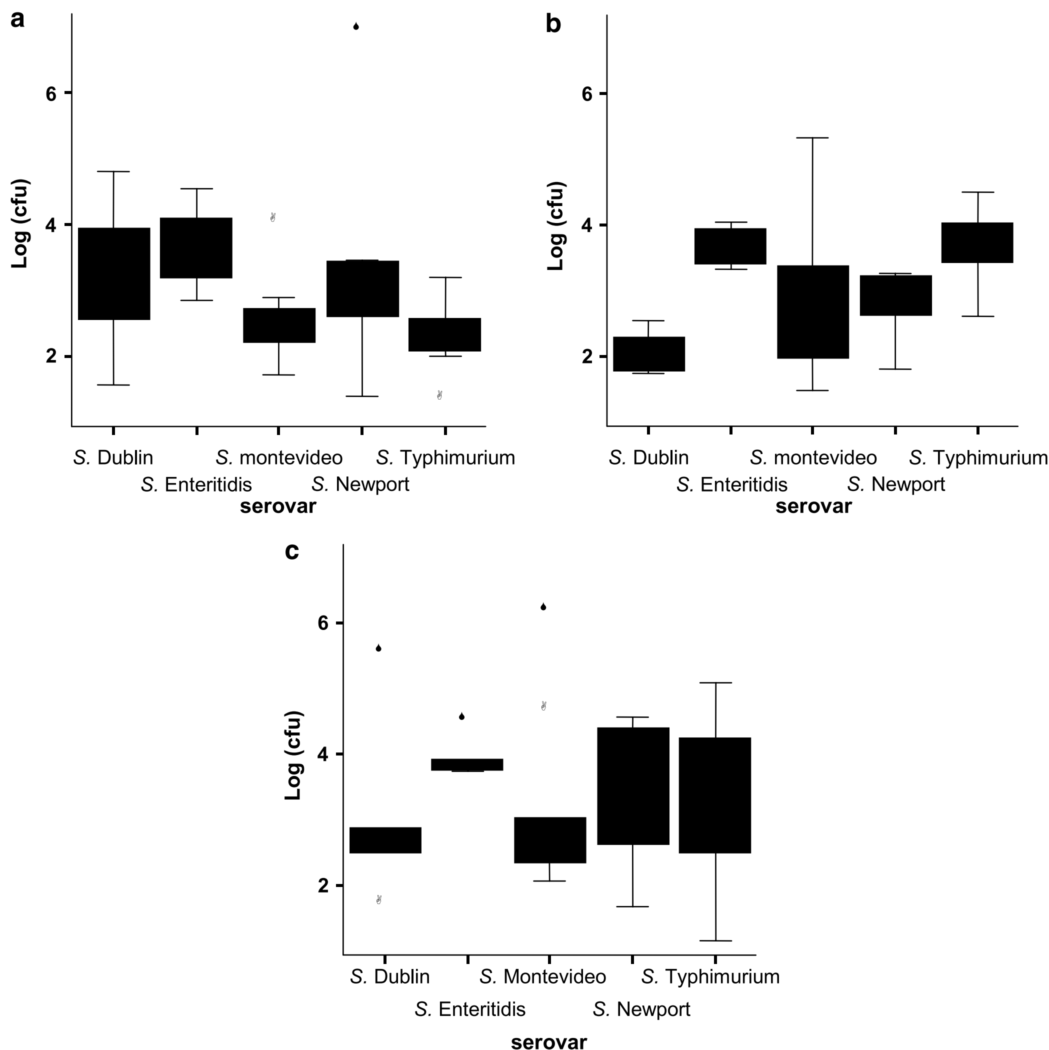

Figure 1 Box plot of the degree of colonization per serovar *Salmonella* Dublin, S. Enteritidis, S. Montevideo, S. Newport and S. Typhimurium for each cultivar Cancan (**a**), Nelly (**b**) and Tamburo (**c**) separately. CFU, colony forming unit.

while the *S. enterica* colonization was significantly affected by *S. enterica* serovar ($P = 0.004$) (Figure 2). The endophytic colonization from inoculated Hoagland's agar was highest for *S. enterica* serovar Dublin and much lower for *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis. Although this trend between the *S. enterica* serovars tested on Hoagland's agar is similar to the trend obtained from the *S. enterica* serovars tested on manure-amended soil, the level of colonization was much higher in Hoagland's agar (Table 1).

Natural endophytic microbial communities of S. enterica*-colonized lettuce cultivars*

Correlation analysis of the natural endophytic species richness based on number of DGGE bands versus the degree of endophytic colonization (log *S. enterica* CFU per g fresh weight) indicated a significant negative correlation ($r = -0.31$ and $P = 0.04$). However, analyses of the cultivars separately did not result in significant correlations (Cancan, $r = -0.508$ with $P = 0.064$; Nelly, $r = -0.389$ with $P = 0.151$; Tamburo, $r = 0.039$ with $P = 0.889$) although a negative trend was observed with Cancan and Nelly, but not with Tamburo. Correlation analysis of the Shannon index of diversity (H), on the basis of the number and relative intensity of the bands on a sample lane, versus the degree of endophytic colonization (log *S. enterica* CFU per g) was not significant ($r = -0.276$; $P = 0.066$).

Response of S. enterica *to lettuce root exudates*

The root exudates of three Tamburo plants resulted in metabolic activity (red coloring) inside the micro-capillaries of *S. enterica* serovar Typhimurium (8 out of 12 positive), *S. enterica* serovar Dublin (5 out of 12) and *S. enterica* serovar Enteritidis (0 out of 12, that is no activity observed) (Figure 3). Each control tube (treatments without exudates was negative for color transition (18 out of 18), which indicated that no random, passive movement due to diffusion occurred in these capillaries. A χ^2 test on the total number of positive samples (movement inside tube) between the control tubes and the tubes containing root exudates showed a significant difference ($\chi^2 = 8.56$; $P = 0.05$). There were also significant differences among the *S. enterica* serovars with respect to number of positive reactions in response to the root exudates ($\chi^2 = 11.8$; $P = 0.01$). There was no differences between *S. enterica* serovar Typhimurium and *S. enterica* serovar Dublin ($\chi^2 = 1.5$; $P = 0.53$), but there were differences between *S. enterica* serovar Dublin and *S. enterica* serovar Enteritidis: ($\chi^2 = 6.3$; $P = 0.05$) and between *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis ($\chi^2 = 12$; $P = 0.01$).

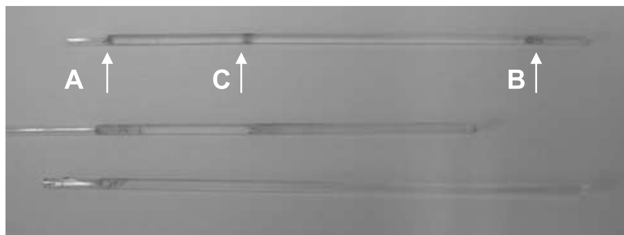

Figure 3 Chemotaxis of *Salmonella* serovars in microcapillary tubes with root exudates or control solutions traced by reaction with TTC. The left side of a microcapillary tube was placed in a suspension of *Salmonella* (or water as control) present in a 0.5 ml eppendorf tube (A). The right end of the micro-capillary tube (B) was inserted into another 0.5 ml tube containing either the water fraction of root exudates or control solution (phosphate buffer, Hoagland's solution or water). Movement of *Salmonella* serovars was visualized by tetrazolium (red color). The upper micro-capillary tube was positive for movement of S. Typhimurium toward lettuce root exudates (C). The middle tube indicated movement of S. Dublin to root exudates. The bottom micro-capillary was negative for chemotaxis, having *Salmonella* inoculated on the left end and a control solution on the right end of the capillary. TTC, 2,3,5-triphenyl tetrazolium chloride.

Gene-expression of S. enterica *serovar Typhimurium in response to lettuce root exudates*

To test the response of *S. enterica* serovars upon exposure to root exudates, micro-array analysis was performed on the time series of *S. enterica* serovar Typhimurium (0, 10 and 20 min postinoculation) of each treatment. After normalization (according to Hermans, 2007), most of the genes were equally expressed between the different treatments. However, some genes did show differential expression levels in time between the treatments (chemotaxis-inducing root exudate, a non-inducing root exudate, 0.1% sucrose and water) (Table 2). The differentially expressed genes (due to chemotaxis-inducing root exudates) appeared either associated with pathogenicity or pointed toward a relationship with a sugar-like carbon source.

The genes related to a sugar-like carbon source were *Ots*A (trehalose-6-phosphate synthase), which utilizes glucose-6-phosphate as substrate (Giζver *et al.*, 1988), *Uhp*C (hexose phosphate utilization protein), which is a sensor for external glucose-6-phosphate) (Schwφppe *et al.*, 2003), *Met*E (methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase), a vitamin B_{12}-independent enzyme (Urbanowski and Stauffer, 1989), *Dsr*A (putative anti-silencer RNA), a regulator of transcription to express *Rcs*A promoter, which in its turn is responsible for capsular polysaccharide synthesis (Sledjeski and Gottesman, 1995), *Rse*A (sigma-E factor regulatory protein), which is involved in the storage of sigma which is released during stress (Ades *et al.*, 1999), *Ssa*H and *Ssa*M (putative effector proteins), both regulators of secretion of the type III secretion system (Lee *et al.*, 2000) and *Spa*O, involved in surface presentation of antigens, secretory proteins. Other genes that were differentially expressed were related to specific limiting factors like iron (gene *Sit*D) or anaerobic respiration (*Ttr*A).

Table 2 Corrected gene expression levels from micro-array analysis of *S.* Typhimurium incubated with water, 0.1% sucrose, a chemotaxis-inducing root exudate and a non-chemotaxis-inducing root exudate, in time

Spot labels	water			0.1% sucrose			Chemotaxis inducing exudate			Chemotaxis non-inducing exudate			Gene function
	0	10	20	0	10	20	0	10	20	0	10	20	
UHPC													sensor for external glucose-6-phosphate
UHPC													
SPAO									++				surface presentation of antigens; secretory proteins
SPAO									++				
RSEA-O									++				storage of sigma which is released during stress
RSEA-O									++				
RSEC-O									++				storage of sigma which is released during stress
RSEC-O									++				
RELA									++				
RELA									++				
PROP		+							+				catabolite repression, and induced by growth
PROP		+							+				
PIPA-O								+			+		
PIPA-O								+			+		
OTSA									++				trehalose-phosphate synthase which utilized glucose-6-phosphate as substrate
OTSA									++				
GST													glutathionine S-transferase
GST													
HSLU													ATPase component of HslUV protease
HSLU													
TTRA													tetrathionate respiratory electron acceptor
TTRA													
SSAM									++				regulator of secretion, type3 secretion
SSAM									++				
SSAH									++				regulator of secretion, type3 secretion
SSAH									++				
SITD	++	++	++	++	++	++	++	++	++	++	++	++	iron transport system, under iron-limiting conditions there is induction of the gene
SITD	++	++	++	++	++	++	++	++	++	++	++	++	
METE		++	++		+				++		+	++	METE
METE		++	++		+				++		+	++	
DSRA							+	+	++				regulator of transcription to express rcsA promoter (responsible for capsular polysaccharide synthesis)
DSRA							+	+	++				
rpoD-o1													sigmaD factor of RNA polymerase during exponential growth
rpoD-o1													

The level of normalized gene-expression is indicated for each gene per treatment in time. The increase (white) of normalized gene-expression level in time and the decrease in gene-expression (black) are presented as compared to the average gene-expression level (marked gray with hatching). The genes are indicated by their abbreviation (spot labels), including a short gene description (gene function).

Discussion

Several human pathogenic organisms have been recognized to exist on plant root or leaf surfaces (Lyytikainen *et al.*, 2004; Brandl, 2006), and even inside plant roots (Kutter *et al.*, 2006; Rosenblueth and Martínez-Romero, 2006). However, only few studies have investigated the physiological or molecular interaction between human pathogenic bacteria and plants (Plotnikova *et al.*, 2000; Iniguez *et al.*, 2005; Prithiviraj *et al.*, 2005; Kutter *et al.*, 2006; Klerks *et al.*, 2007). Even fewer studies have been described concerning the conditions required for plant colonization by these pathogens (Toth *et al.*, 2006, Brandl, 2006). To develop preventive strategies it is important to elucidate the critical points of plant colonization by the pathogen. This research presents plant and microbial factors that

influence the (endophytic) colonization efficiency of human pathogenic *S. enterica* serovars in association with lettuce.

In this study *S. enterica* serovars colonized lettuce endophytically as was shown before (Cooley *et al.*, 2003; Klerks *et al.*, 2007). However, we showed for the first time a differential interaction between *S. enterica* serovars and plant cultivars, besides serovar-dependent host susceptibility with respect to the degree of colonization. This result points to differences in susceptibility of the cultivars, but also differences between the *S. enterica* serovars with respect to colonization of lettuce seedlings. However, the difference between the serovars in apparent lettuce colonization might be partially determined by the survival of the different *S. enterica* serovars in manure-amended soil. *S. enterica* serovars were found to remain viable for at least several months even though a decline was observed (as expected) (Holley *et al.*, 2006). Especially, in the first weeks after inoculation of *S. enterica* into the manure prior mixing with soil, no significant difference in survival rate (that is decline in viable cell numbers) between *S. enterica* serovars is expected. Of course an effect of the survival rate cannot be completely excluded, but it will only have a minor influence on the colonization efficiency compared to other factors like chemotaxis.

The observed differential *S. enterica* serovar-dependent host susceptibility suggested the presence of host-adapted serovars but also more resistant cultivars. Also, with respect to prevalence and degree of colonization, a large difference was present between soil-grown plants and axenically grown plants. This effect is mainly attributed to the absence/presence of rhizosphere bacteria. Since no bacteria are present that colonize the roots in an axenic system, the roots are easily accessible for the inoculated *Salmonellae*. The rhizosphere of roots grown in soil however are known to contain many different soil bacteria that colonize the roots already at the sprouting stage (Yang and Crowley, 2000), herewith protecting the roots with a shield of indigenous soil bacteria (Cooley *et al.*, 2003; Berg *et al.*, 2005). Moreover, the steep gradient of root-exuded compounds in soil is continually modulated by the indigenous soil microflora. The *Salmonellae* have to compete with these environmental bacteria to establish in the rhizosphere (Gagliardi and Karns, 2000; Cooley *et al.*, 2003; Ibekwe *et al.*, 2006), which leads to lower *S. enterica* cell densities close to the roots. This suggests that the colonization efficiency is strongly dependent on accessibility of the plant roots to the *S. enterica* serovars.

Next to being exposed to plant defenses, *S. enterica* serovars also have to compete with the plant natural endophytic microflora for a certain niche with carbon sources (Leveau and Lindow, 2000). Natural endophytes mainly reside at the intercellular spaces between the plant cells and in the vascular systems of the plant. However, also *S. enterica* has a preference for these regions, implying a direct interaction between the endophytical *Salmonellae* and the natural endophytic microflora. Indeed, the species richness of the natural endophytic microbial community of lettuce cultivars was negatively correlated to the number of endophytic *S. enterica* CFUs per g shoot tissue. When tested for each cultivar separately cultivar Cancan and Nelly showed a negative trend, but not cultivar Tamburo. This suggests that the microflora of Cancan and Nelly were more antagonistic to *S. enterica*, or at least limiting the endophytic colonization by *S. enterica* serovars compared to cultivar Tamburo. Thus, the natural endophytic microflora appeared to contribute significantly to the level of susceptibility of the lettuce cultivar for *S. enterica* serovars. Of course, measuring the complexity of the microbial community is inherently biased since only the most abundant species (maximum of approximately 100) are visualized by DGGE. Therefore, the use of the Shannon index with DGGE data can only be valid for quantitative analysis if the relative difference between treatments is tested (Shannon and Weaver, 1949). Thus, a less abundant species might have a higher impact on the colonization efficiency of *S. enterica* serovars than the visualized species from DGGE analysis.

In this study, a thematic micro-array was used (Hermans, 2007), thus not all expressed genes of *S. enterica* serovar Typhimurium were detected. Even though this micro-array covered the most important functional groups (that is, virulence, stress, metabolism and quorum sensing) that are expected to play a role in chemotaxis. Indeed, it appeared that genes for general metabolism are of importance for *S. enterica* serovars to actively move to the plant roots. The active movement of *S. enterica* serovars to the roots points to a tentative route of infection that is different from previously published passive contamination due to soil splashing, irrigation, insect transmission or even passive uptake through roots (Solomon *et al.*, 2002). The chemotaxis experiment and micro-array data both confirmed the presence of an organic compound in the lettuce root exudates that is used as carbon source by the *S. enterica* serovars. Chemotaxis of *S. enterica* serovars to sugar suspensions was already described in the late 1970s (Melton *et al.*, 1978) and it is also well known that root exudates contain various (mono)-saccharides like fructose, glucose and so on (Neumann and Rφmheld, 2001). Many bacteria colonize the roots of plants or persist in the rhizosphere by using these plant root exudates as carbon source (Curl and Truelove, 1986; Rediers *et al.*, 2003). Thus, it is highly plausible that sugar-like compounds in the root exudates are responsible for the active movement of *S. enterica* serovars to the roots.

Interestingly, in the presence of root exudates and observed chemotaxis, also pathogenicity genes of *S. enterica* serovar Typhimurium were induced that

are crucial for root attachment and subsequent colonization (Barak *et al*., 2005). For example, *Ssa*H and *Ssa*M are regulators of the type III secretion system and *Spa*O is involved in the surface presentation of antigens that is secretory proteins. The observed induction of *Dsr*A implies the activation of processes that allow attachment, since it is involved in the synthesis of capsular polysaccharides, an important factor in the attachment to host cell surfaces (Eriksson de Rezende *et al*., 2005). Up to date it was not known that pathogenicity-related genes are already induced during chemotaxis toward the roots of plants. The findings of this study suggest that *S. enterica* serovars are conditioned for attachment to the plant root surface after being triggered by the root exudates to actively move toward the roots. Root exudates therefore have a dualistic result, which is the induction of chemotaxis of *S. enterica* to the roots and the simultaneous conditioning of *S. enterica* cells for host cell attachment. It is thus hypothesized that the capability of bacteria to condition for (plant) host cell attachment during chemotaxis is one of the most important factors for pathogenicity or colonization efficiency. This is in line with the observation that from the same lettuce cultivar both chemotaxis-inducing root exudates and non-inducing root exudates were extracted. This can be explained by the simple fact that most probably a difference in available root exudate concentration was obtained. Indeed plant colonization efficiency is dependent on the concentration or availability of the root exudates in the rhizosphere. This observed concentration-dependent chemotaxis should be investigated further to determine the relationship between exudate concentration and chemotaxis-dependent colonization efficiency.

Typically, amino acids could also play an important role during chemotaxis or survival close to roots, as the *Met*E gene, induced in the presence of mono-cysteine, was also upregulated (Urbanowski and Stauffer, 1989). Further analysis and identification of these organic compounds by high performance liquid chromatography separation and subsequent gas-chromatography mass spectrometry or liquid chromatography mass spectrometry might be of great interest to determine which compounds are responsible for attracting *S. enterica* serovars to the roots. In combination with gene-expression profile determination by reverse transcriptase (RT)-PCR this eventually might lead to identify marker genes for chemotaxis.

Tentative route of infection

To develop preventive strategies for infection of lettuce plants by *S. enterica* it is important to elucidate the critical points of plant colonization by the pathogen and to define a tentative route of infection. Basically, *S. enterica* serovars that are applied with manure onto or into the soil need to overcome several barriers to finally colonize the lettuce plant systemically. The impact of these barriers on the colonization efficiency is dependent on the serovar, the cultivar and the plant environment. Initially, the *S. enterica* serovars are triggered by sugar-like root exudates and due to chemotaxis the bacteria move actively toward the roots, meanwhile being conditioned for host cell attachment (this paper). The ability of *S. enterica* serovars to produce flagella and highly sensitive sensors for chemotaxis or quorum sensing compounds contributes largely to the rate of their movement (Melton *et al*., 1978). In close proximity to the roots, the attachment-conditioned *Salmonellae* compete with the rhizosphere bacteria to gain intimate access to the roots. The rhizosphere is recognized to serve as reservoir for human pathogenic bacteria (Berg *et al*., 2005), in which the availability of anaerobic and aerobic respiration pathways might enable *S. enterica* cells to compete with rhizosphere bacteria for nutrients. The *Salmonellae* efficiently attach to the roots, which is primarily dependent on the presence of using curli and lipopolysaccharides (Barak *et al*., 2005). In general the colonization of the plant roots by bacteria depends on the presence of flagella, the O-antigen of lipopolysaccharides, the growth rate and the ability to grow on root exudates (Lugtenberg and Dekkers, 1999). Upon root colonization the *Salmonellae* form a biofilm on the roots at natural openings or wounds, but also at the intercellular spaces between epidermal cells (Klerks *et al*., 2007). This suggests a preference for the intercellular spaces and not the plant cell itself.

Upon cell disruption or presentation of membrane-bound pathogenicity factors like flagella (Iniguez *et al*., 2005), the plant responds in a hypersensitive manner with subsequent triggering of induced systemic resistance. As soon as the type III secretion system translocates effector proteins into the host cells, the plant may respond by inducing the salicylic acid defense pathway (salicylic acid resistance). To what extent the plant responds by induced systemic resistance; and salicylic acid resistance depends on its ability and efficiency to recognize bacterial pathogenicity factors, degrading action and/or host cell penetration, which for each plant cultivar and species is different. Finally, endophytic spread follows that of plant pathogens by first infecting the vascular parenchyma and then the invasion of the xylem to allow systemic infection (Klerks *et al*., 2007).

Frequent intimate interaction between human pathogens and plants may make different plant species susceptible to adjusted strains and become secondary hosts. Eventually, this could lead to a higher prevalence of *S. enterica* serovars inside produce so that these cannot be removed by sanitation procedures. Even though, it should be stressed that, although the number of Salmonellosis outbreaks associated with consumption of produce is increasing, the risk is still relatively low compared

to that of Salmonellosis associated with poultry or eggs. Even so, the potential presence of human pathogens inside lettuce should be taken into consideration as an emerging risk for human health.

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